

Enzymes in Biomass Conversion

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Enzymes in Biomass Conversion

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Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing **ADVANCES IN CHEMISTRY SERIES** except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

ENZYMES, WITHOUT QUESTION, hold great potential for the industrial-scale processing of a wide variety of biologically derived materials. The advantages of enzymes over chemicals in biomass processing include higher specificity in the targeted conversions, resulting in the decreased generation of side products; the ability to carry out novel conversions; and the potential avoidance of producing toxic, difficult to degrade, or environmentally damaging side products. Currently, the effective utilization of enzymes in many new industrial applications is only a possibility. Successful application will require increased knowledge about the range of enzymes available, their characteristics, where improvements in the enzymes are needed and possible, and how best to utilize the enzymes.

Enzymes in Biomass Conversion features chapters written by many of the leading international experts from universities, government research laboratories, and enzyme-producing industries. The chapters cover the enzymes of current potential importance to large-scale commercial bioconversion processes. They describe our state of knowledge about enzymes in specific applications, preferred methods for enzyme production, characteristics of the individual enzymes, and recommendations for future research.

Chapters 1 through 6 outline our understanding of the enzymes necessary or potentially useful for biomass conversion. Included are chapters on fuels and chemical feedstock production, pulp and paper processing, waste processing and degradation, food processing, and specific classes of alkali or thermostable enzymes.

Chapters 7 through 13 outline methods for producing and recovering enzymes. Included are chapters on cloning enzymes, producing enzymes in industrial-scale liquid fermenters, producing complete synergistic sets of lignocellulose-degrading enzymes by solid-substrate fermentation, large-scale enzyme recovery and downstream processing, and enzyme immobilization and cross-linking.

Chapters 14 through 38 feature in-depth information on specific enzyme classes such as ligninases and associated oxidative enzymes; β -D-glucanases (cellulases and laminarinases); α -D-glucanases and related enzymes; hemicellulases, including xylanases, arabinanases, mannanases, and galactanases; and the necessary accessory enzymes for biomass degradation (i.e., pectinases, chitinases, glucose-xylose isomerase, and proteinases).

During the compilation of this book, the editors were reminded of the tremendous variety of hydrolytic enzymes available in nature. The process of natural selection, driven by competitive pressure in many often obscure ecological niches, has yielded biological catalysis specific for the wide range of chemical compounds produced by living systems. Biochemists working in the emerging field of artificial enzyme design must not overlook nature's own "enzyme engineers" for new inspiration.

Acknowledgments

We are grateful to the Cellulose, Paper, and Textile Division of the ACS for supplying the forum for this work and to the division and Novo Nordisk Industries for partial funding of the symposium.

We wish to thank Ross D. Brown, Jr., for sharing a timely memorial treatise on the life and contributions of Kendal W. King. The late Dr. King explored avenues in cellulase biochemistry in the early years of the discipline and was indeed a key worker in the field. We also wish to thank Thomas W. Jeffries and Douglas E. Eveleigh for organizing a special reception and recognition for Mary Mandels and Elwyn T. Reese. These workers were truly pioneers in the field of modern cellulase biochemistry, establishing many of the principles central today. Our thanks also go to Helena L. Chum of the Solar Energy Research Institute, Golden, Colorado, for administrative assistance in organizing the symposium, and Thomas M. Wood of the Rowett Research Institute, Aberdeen, Scotland, for timely external reviews. Our editors at ACS Books were Cheryl Shanks and Maureen Rouhi. Their efficient processing of the manuscripts, publishing experience, and insight into our challenges were very much appreciated.

Finally, we appreciate and admire our authors' research and contributions to the symposium and book, and their assistance in reviewing the many chapters. We wish them well and hope that this book will promote their own endeavors while assisting their many colleagues in this rapidly expanding field.

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Chapter 1

Enzymes for Fuels and Chemical Feedstocks

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The increase in the price of petroleum feedstocks has created opportunities for the development of combined biological and chemical processes that will produce liquid fuels and chemicals from alternate feedstocks such as biomass, coal, and gas. Near-term opportunities exist in the bioconversion of cellulosic materials contained in urban, agricultural, and forestry wastes. This large reservoir of raw materials can be augmented in the future by short-rotation woody and herbaceous crops as well as by specialty crops. Enzymatic conversions will play an increasing role in future biochemical processes because enzymes achieve relatively high catalytic activities and high selectivity, and they have a low impact on the environment. The wide-scale industrial application of enzymes will require development of enzymes with long-term stability and high activity under different use-conditions. Such a broad spectrum of requirements will be difficult to satisfy by a single enzyme for each transformation, but can be supplied by families of related enzymes isolated from microorganisms adapted to diverse environments and improved by site-directed mutagenesis and chemical modification.

Biopolymers of a carbohydrate and polyphenolic nature represent the largest reservoir of organic carbon fixed annually by plants (1). This pool of raw material can be obtained at very reasonable costs, comparable to the cost of major fossil feedstocks (Figure 1). The biopolymers (in aggregate termed biomass) have many uses, mainly in food, feed, fiber, and as materials of construction. Starch has a unique position among polymeric carbohydrates because it is digestible by man and livestock. The largest part of the world's agricultural production is thus devoted to the production of starchy crops (i.e., grains and tubers) from which only starch-containing organs are utilized and the rest of the plant is usually wasted. Figure 2 illustrates the ranges of agricultural and other residues generated annually in the United States over the past decade (2-4). The annual production of the basic chemical feedstocks from which practically all other organic chemicals are derived, by one route or another, is included in the same figure for comparison (5). These data show that many segments of the annual biomass pool easily exceed the total annual requirements for basic petrochemicals. However, the data in Figures 1 and 2 can be misleading. Biomass feedstocks are highly oxygenated, whereas fossil raw materials are mainly pure hydrocarbons. All processes for the conversion of carbohydrates to hydrocarbon-type materials thus suffer a penalty of high

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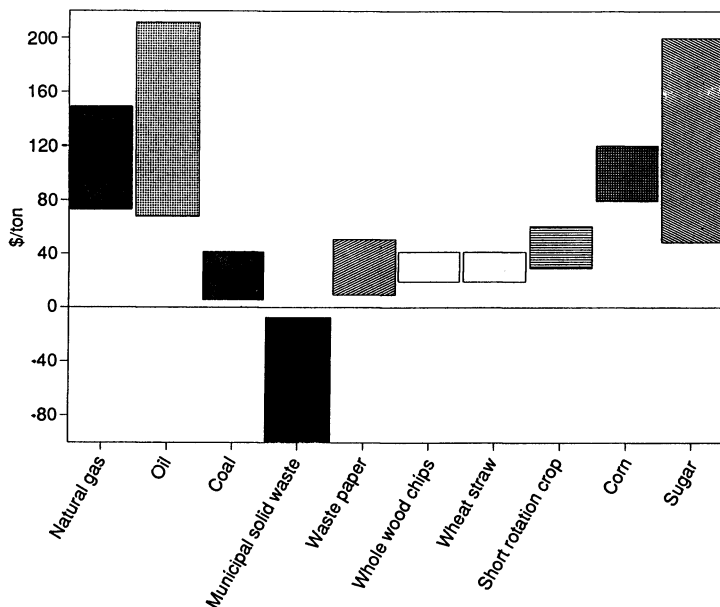


Figure 1. Approximate ranges of the major chemical and fuel feedstock costs in the United States between 1980 and 1989 (projected price only).

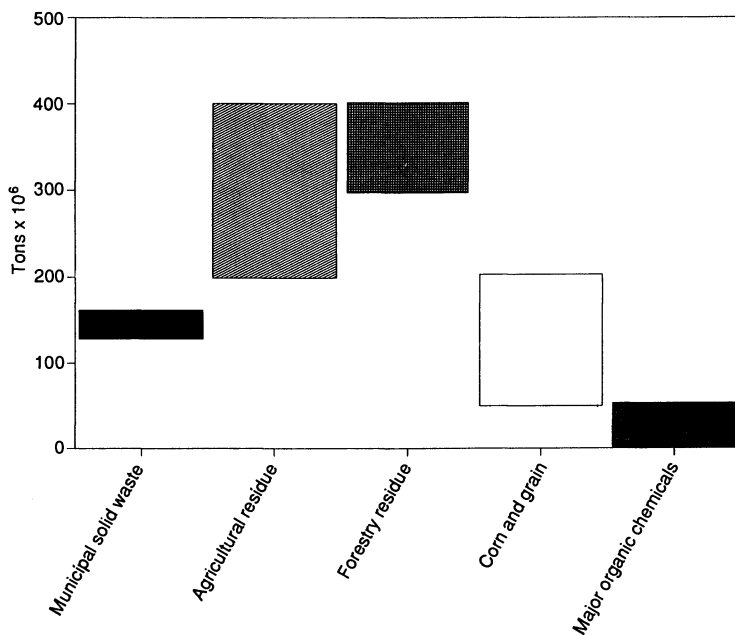


Figure 2. Approximate ranges of U.S. annual production of major carbohydrate feedstocks and organic chemical feedstocks in the 1980s. The data for organic chemicals are plotted only for 1988.

weight losses, with the exception of short chain organic acids, where high weight yields are possible (6). The oxygen content effects are easily shown when various feedstocks are compared on an energy content basis (Figure 2) rather than a weight basis. The differences in fuel costs become much smaller than was evident in Figure 1. Figure 3 illustrates the discount of solid fuels, versus gaseous and especially liquid fuels, which receive a premium for their exclusive dominance in the transportation sector. Figure 3 further shows that lignocellulosic biomass was competitively priced as a solid fuel over the past decade and as a result, its fuel use via combustion has increased (2). Such direct use for heat generation does not allow "valorization" of other useful properties which are contained in the spectrum of biomass resources. The bioconversion of biomass and generation of profits necessary for any viable commercial enterprise should concentrate on conversion into higher value products in chemical, food, feed, and liquid fuel industries where higher priced products are marketed at the present time. Some of these higher valued products can then be combusted, recycled, or converted to other uses such as production of transportation fuels or chemicals. Multiple recycle of original raw materials will decrease pressure on finite natural resources, such as land, fresh water, and mineral fertilizers. The lowest cost fuel currently available at a negative cost is municipal solid waste (Figure 1), which is a collection of discarded products from the public and various industries.

Biopolymers

The major components of terrestrial plants are two families of carbohydrates - cellulose and hemicelluloses. Cellulose fibers account for approximately 40% to 50% of the total dry weight of stems, roots, and leaves (7). Cellulose fibers are embedded in the matrix of hemicelluloses and phenolic polymers, commonly identified as lignin-carbohydrate complexes (LCCs)(8). There is evidence for covalent ether, and possibly ester, bonds between hemicellulose and lignin chains. Covalent crosslinking renders both hemicelluloses and lignin insoluble in nonreactive chemical solvents. The extraction of LCC components requires relatively mild alkaline cleavage in case of grasses and hardwoods; however, harsh oxidative pretreatments are required for extraction of hemicelluloses from softwoods (9-11). These treatments destroy the structure of lignin. Alternate pulping processes for extraction of lignin from softwoods hydrolyze (i.e., bisulfite pulping) or destroy (i.e., Kraft pulping) carbohydrates and modify lignin in the same process (12).

Starch, a widespread storage polymer in the plant kingdom, is widely available because of intensive cultivation of starch-producing grain and tuberous crops for human food and animal feeds. This starch is present in specialized tissues in the form of granules that can be separated by a steeping-milling-sedimentation sequence from grains and by a similar process from tubers (13). Grains are a favorable source of industrial starch, because they have a low moisture content and are therefore storable for long periods of time. An underutilized storage polymer in plant tubers is the polyfructosan, inulin, which is not digestible by humans but which can provide the sweet monomer, fructose (14). Other industrially useful polymeric carbohydrates are pectins (15), which are present in the tissues of fruits, tubers, and immature plants and are usually obtained as a byproduct of food processing. There are many other specialized carbohydrates of plant origin, but their availability is rather low, so they will not be addressed in this chapter.

Chitin, a polymer of N-acetylglucosamine, is present in large amounts in the exoskeletons of crustaceans and other arthropods and is a major marine biopolymer (16). Its availability is, however, limited to byproducts from seafood industries (16).

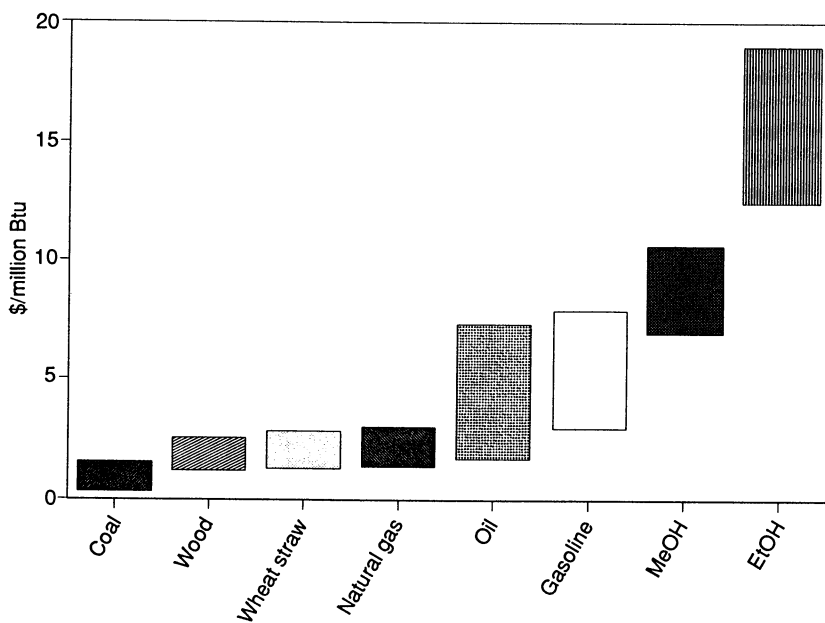


Figure 3. Ranges of relative fuels costs in dollars/million BTU in the United States in the 1980s.

Summary of Major Uses

Because mankind, like other nonphotosynthetic organisms, developed survival strategies based on the exploitation of photosynthetic plants, there are many traditional uses for biopolymers that are unlikely to be replaced in a foreseeable future. The largest use of cultivated plants is as human food, as animal feed, and in fiber production, with a relatively tiny acreage devoted to specialty crops for spices, herbs, drugs, and textile fibers (17).

With the exception of starch, many biopolymers cannot provide nutrition for humans and other omnivorous animals. In human foods biopolymers are used as additives that can improve texture, viscosity, fiber content, and other properties of prepared foods, without providing direct nutritional values. Examples of such utilization are the addition of pectins, agar, and other gums to foods to achieve thickening and gelling effects. Another example of potential large-scale utilization of cell wall biopolymers is the dramatic improvement in the texture and rising of breads prepared from corn and other starches by the addition of xylans (18).

Many biopolymers have very large utilization in traditionally chemical areas, such as fiber, film, and adhesive production, where they often successfully compete with more modern petrochemical products. Paper pulp utilization, for example, exceeds the usage of any other polymer (4,5). Cotton fiber has also maintained a significant position in the textile industry over the past ten years. Two biopolymers, cellulose and chitin, were endowed by nature with high tensile strength (7,16,19). They are linear molecules of high molecular weight that have a strong interchain bonding through multiple repetitive hydrogen bonds. Both polymers are also highly crystalline. The linear component of starch, amylose, also shows a high tensile strength in the dry state (13). Amylose is unfortunately a minor (<30%) component of natural starches and its separation from the branched amylopectins makes it too expensive for commercial use (13). The most valuable source of amylose is starch from high amylose mutants of corn, which have an amylose content as high as 80% to 82% (13).

Other abundant carbohydrates, such as hemicelluloses and pectin, are usually highly branched and thus not very suitable for fiber and film production. Hemicelluloses and some pectins are also acetylated in the native state, which makes them more resistant to enzymatic hydrolysis (20,21) and changes their solubility properties (9-11,15). Branching does not, however, preclude their utilization in such potentially large markets as thickeners and adhesives. Xylans, for example, show such a strong adhesion to cellulose fibers that they are very difficult to remove completely by both acidic and alkaline pulping processes (22).

The utilization of biopolymers from the cell walls is further complicated by their covalent bonding and contamination with lignin and other colored phenolic materials. Lignin itself, which is very light in color while in the cell wall, turns brown to black during pulping. This is mainly due to formation of quinones, free radicals, and other colored compounds (12). The dark color of isolated lignin decreases its value and precludes its use in many potential applications where light surfaces are required. These colored impurities need to be removed by oxidation/reduction steps, which add to the cost of the final polymer. An example of this is bleaching of higher grades of paper pulps, which is usually accomplished with chlorine, chlorine dioxide, and peroxy-compounds (22). This expensive and often polluting step is necessary to improve the brightness and lighten the color of high-quality paper products.

Hemicelluloses are quite difficult to extract from cell walls of softwoods (9,10) and are usually destroyed or depolymerized during the chemical pulping of these raw materials. However, other hemicelluloses, primarily xylans, can be extracted by cold, dilute sodium hydroxide from grasses and many hardwoods in very high yields (9,11). These xylans are deacetylated in an alkaline medium and are for the most part insoluble (hemicellulose A). A partially water soluble fraction (hemicellulose B) has also been

defined. Because these fractions are contaminated with colored lignin components, they may require a bleaching sequence for some applications. Xylans from hardwoods and grasses seem to represent one of the truly underutilized family of biopolymers, because they are easy to obtain but are not produced commercially anywhere in the world.

The final and perhaps most important use of plant biopolymers will involve depolymerization to oligomers and monomers, followed by microbial and chemical conversion to liquid fuels and chemicals. The gaseous fuel (i.e., biogas), which is a mixture of methane and carbon dioxide, can also be produced by fermentation of various biodegradable feedstocks using consortia of anaerobic bacteria (23). The microbial conversions of sugars to lower alcohols, ketones, volatile fatty acids, and acids of tricarboxylic acid cycle are very efficient because fermentative microorganisms can perform multiple enzymatic steps in very high yields and secrete the final product into the surrounding medium (6). With the resurgence of biotechnology and the advent of genetic engineering, novel chemicals (e.g., amino acids) and biopolymers (e.g., poly- β -hydroxybutyrate) are entering the marketplace in commercial quantities. The productivity and concentration of some so-called secondary metabolites has been improved many-fold over the past 15 years. As a result, their commercial attractiveness has increased.

In contrast to depolymerization of carbohydrates, which is a relatively straightforward hydrolytic process, the depolymerization of lignin, a family of phenylpropane polymers containing ether and carbon-carbon bonds, has been much less successful. We still have only rudimentary knowledge of how ligninolytic enzyme systems operate and the presence of resistant bonds and the reactivity of units and intermediates, which can lead to repolymerization, has prevented complete depolymerization of lignin by either chemical or enzymatic means (12,24). These problems are compounded by variations in lignin composition across tissues, the presence of reactive extractive compounds, and the change from guaiacyl to guaiacyl-syringyl lignin families thought to accompany the development of flowering plants (12).

Enzymes and Enzymatic Modifications

It is clear that many enzymes and enzymatic systems are poised to make major contributions in future food and chemical processing. Despite their real or perceived disadvantages, such as low potential stability or narrow ranges of optimal activity, some enzymes offer tangible advantages over chemical catalysts currently used to perform the same catalytic event. It is not always appreciated that many enzymes, especially when denatured by cooking, are nutritious and edible. Indeed, we ingest animal and plant enzymes to derive amino acids and other essential nutrients. The enzymes and other proteins we are unable to digest are still biodegradable by numerous other organisms with less demanding nutritional requirements than our own. In contrast, chemical catalysts currently used are generally inedible, usually nondegradable by biological means, and often contain toxic heavy metals. Toxicity of chemical catalysts precludes utilization of many systems in food processing where enzymes may find acceptable niches. The presence of heavy metals and the resistance to biodegradation will mandate extensive recycle and reprocessing of spent chemical catalysts to comply with the rapidly expanding environmental regulations and restrictions.

Along with their biodegradability, some enzymes have an additional advantage in that they do not require heavy metals, or other toxic inorganic cofactors. Polysaccharidases seem to possess this inherent advantage. An extensive search of amylases (25), pectinases, hemicellulases, and cellulases did not reveal even one enzyme that requires a potentially toxic heavy metal for its activity. Only an amino acid sequence resembling a calcium binding site was identified in some sequences of amylases (25) and cellulases (26), but it is unclear whether calcium ion is required for

binding or stability or both. This ion does not seem to be needed for catalytic activity. Chemical hydrolysis of glycosidic bonds requires catalysis by strong mineral or organic acids, which needs to be neutralized and/or recycled, and in some instances (e.g., many Lewis acids and trifluoroacetic acid) detoxified as well.

Among enzymes for carbohydrate hydrolysis and modification, the enzymes acting on starch are the most diverse and numerous (25). The trio of enzymes, amylases, glucoamylases, and glucose isomerases, from different sources and with distinct structures and properties, support a single, large enzyme-based industry -- production of food-grade corn syrup. This industry supports annual sales of over one billion dollars in the United States alone (27). This very new industry grew from practically nothing over the past 25 years. The amylase and glucoamylase enzymes also support the corn-based alcohol fuels industry, which grew in the United States over the past decade and reached an approximate annual output of 800 million gallons in 1988 (2). This success story, of course, is sharply contrasted with the struggle that many other enzyme systems have in industrial market penetrations.

The other systems for modifications and hydrolysis of biopolymers from biomass do not have the inherent chemical simplicity and long history of starch enzymology and are thus much less developed. Adoption has often been wylaid by practical deterrents. For instance, many cellulolytic, hemicellulolytic, and pectinolytic microorganisms, notably bacteria of the genus *Erwinia* and molds of the genus *Fusarium* (28), are well-known plant pathogens. While they are good sources of hydrolytic enzymes, their dissemination is controlled by the U.S. Department of Agriculture and justifiably more effort has been spent on their control than on their development as enzyme producers. Biomass-degrading microorganisms cause huge annual losses because of biodeterioration of wooden structures, cellulosic textiles, and other biomaterials. Therefore, a highly legitimate part of research efforts in the enzymology of biomass modification and conversion should be the development of selective inhibitors for hydrolytic enzymes because these inhibitors have large potential applications in crop and material protection.

A second deterrent to the development of hydrolytic enzymes for lignocellulosic biomass has been the resistance of plant cell walls to enzymatic deterioration, which has been built into plant cell walls through evolution. Some trees outlive long-living animals by a factor of 50 or so, while being fixed in the same place from birth to death. The complexity, resistance to enzymatic hydrolysis, and the physically large size of some forms of biomass lead to the very long times required for biodegradation of biomass when compared to the hydrolysis of starch (25,29). Attempts to shorten the reaction times for enzymatic hydrolysis of untreated biomass lead only to minuscule yields of hydrolytic products, both from cellulose and hemicelluloses regardless of enzyme loadings used (30). Therefore, most if not all lignocellulosic biomass has to be extensively pretreated to open the cell wall structure, break the lignin-hemicellulose complexes, and expose the cellulose fibers to enzymatic attack (31,32). This requirement contrasts sharply with starch hydrolysis, where swelling and hydration of starch granules, which can be simply accomplished by steaming or cooking in hot water, is usually all the pretreatment necessary for efficient enzymatic hydrolysis (13). Because starch chains are more open to hydration than cellulose, a possibility exists that uncooked starch granules can be enzymatically hydrolyzed while being simultaneously hydrated in the aqueous suspension (25).

The effective pretreatments for enzymatic hydrolysis of cellulose in hardwoods and agricultural residues have been developed over the past 90 years and new or improved ones are still being developed. The initial impetus for pretreatment research was the expansion of a feed base for ruminants (i.e., cattle and sheep). The concentration of pretreatment research on animal feed preparation placed an additional burden on researchers, because the product must be not only fully convertible to animal live-weight, but also nontoxic and palatable. Early feeding trials of pretreated fine sawdust to cattle produced very dismal results (33). This may be the reason, besides the

inherent resistance to many pretreatment methods, why much less effort has been spent on the development of efficient pretreatments for softwoods as compared to hardwoods and grasses.

Many physical, chemical, and biological pretreatment methods have been tested. However, the chemomechanical pretreatments that combine one or two mechanical (e.g., chipping, milling, or shredding) treatment steps with chemical treatments seem to be most efficient and cost effective at the present time. Examples of very effective pretreatments for hardwoods and grasses are high-temperature steaming with or without explosive decompression and dilute acid prehydrolysis. Yields of enzymatic hydrolysis of cellulose in the 80% to 100% range are obtainable using selected biomass substrates pretreated by these methods. Addition of sulfur dioxide to steam explosion increases even the susceptibility of cellulose in softwoods to enzymatic hydrolysis (34). An advantage of acid-catalyzed pretreatments is the retention of xylose or other hemicellulosic sugars, which are only slightly converted to furfural and other undesirable byproducts. Furthermore, the hemicelluloses are extensively hydrolyzed during these pretreatments to monomeric sugars, which are potentially fermentable by numerous microorganisms without the need for additional hydrolytic enzymes.

Fermentation of glucose and other six-carbon sugars does not present serious problems in practically all known fermentation processes, but fermentation of the five-carbon sugars, xylose and arabinose, is a challenge in ethanolic and lactic acid fermentations. Great progress has been made in the past ten years in the development of fermentation systems for the conversion of xylose to ethanol both by classical selection and modern genetic engineering techniques (35). Despite rapid progress and dramatic improvements in both the productivity and yields of xylose to ethanol fermentations, the development of enzymatic and microbial systems for the conversion of five-carbon sugars and uronic acids present in biomass hydrolysates will continue to be a challenging area of biomass research. Environmentally sound conversion processes of the future must concentrate on the conversion and valorization of all components, even minor ones, to comply with waste minimization policies pursued by the U.S. Environmental Protection Agency.

Although ample experimental evidence has been accumulated over the past ten years that cellulose fibers in properly pretreated plant cell walls can be completely hydrolyzed to sugars by potent cellulase preparations, there are numerous deficiencies in currently available cellulase enzymes. This latter point was summarized in an excellent recent article (36). The low specific activity (i.e., International Filter Paper Units (IFPU)/mg protein) of current commercial cellulase preparations leads to requirements for high cellulase enzyme loadings in weight of enzyme per weight of cellulose to be hydrolyzed. Resolution of this situation will require a significant research effort devoted to the identification of improved cellulase enzymes or a decrease in the cost and consumption of known enzymes through increased enzyme productivity, yield, resistance to end-product inhibition, specific activity, and enzyme recycle.

Development of thermophilic enzymes will help to alleviate contamination of sugar streams by unwanted microorganisms, many of which are killed at elevated temperatures. The sensitivity to end-product inhibition and long reaction times, up to many days, also characterizes important weaknesses of current enzymatic cellulose hydrolysis schemes. The high sensitivity to end-product inhibition is a well-known property of cellulase complexes from *Trichoderma reesei* (36), but long reaction times seem to be caused by the nature of the cellulosic substrate as well as by the choice of enzymes. Cellulose hydrolysis is strictly a surface reaction, aided by the splintering of cellulose fibers to smaller fibrils. Large enzyme molecules cannot penetrate into the interior of cellulose crystallites, which effectively exclude even much smaller molecules. The high surface area of cellulose fibers, uncontaminated by deposits of lignin or other non-carbohydrate materials, seems to be a first prerequisite for efficient and perhaps rapid enzymatic hydrolysis of cellulose. Xylans and other hemicelluloses cannot be considered inert, and easily hydrolyzed components of cell walls, because both they and

lignin surround cellulose fibers. Efficient removal of hemicelluloses from cellulosic pulp fibers seems to require the synergistic action of both cellulase and hemicellulase enzymes, both of which are often fortuitously present in commercial cellulase preparations.

The commercial development of xylans, which are available in agricultural residues and hardwoods, will require the development of enzymes capable of debranching xylans and performing the selective removal of unwanted components such as lignin, other hemicelluloses, and perhaps acetyl ester groups (35). Crude hemicelluloses, as currently isolated, are mixtures of components of largely unknown structure. Broader application of these biopolymers could be aided by the development of improved separation of individual biopolymers (i.e., better isolation methods) and determination of their precise structure. From a physicochemical point of view, the compositional heterogeneity and polydispersity of these fractions, as currently isolated, provides a major analytical hurdle to subsequent analysis. Enzymatic hydrolysis with selective enzymes could greatly help solve this problem in a similar way to that used currently in analysis of nucleic acids and proteins.

It is difficult to exhaustively cover the vast, exciting, and rapidly developing field of enzymology of plant biopolymers in a short introductory review. However, the chapters that follow this introduction will deal in depth with various aspects of enzymology of plant polymers. Potential applications and improvements are many and through dedicated research and development efforts new industrial enzyme-based processes can become a reality in the future.

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Literature Cited

1. Hall, D. O. *Solar Energy* 1979, 22, 307-328.
2. Lynd, L. R. *Adv. Biochem. Eng./Biotechnol.* 1989, 38, 1-52.
3. Miller, D. C.; Eisenhauer, R. A. In *CRC Handbook of Processing and Utilizing Agriculture*; Wolff, I. A., Ed; CRC Press: Boca Raton, FL, 1982; Vol. 2, p. 691.
4. *Pulp and Paper*; Conrad, M.; Mikulenska, J., Eds.; Miller Freeman: San Francisco, CA, 1988; pp 6-62.
5. Anonymous, *Chem. & Eng. News* 1989, 67(25), 38-50.
6. *Primary Products of Metabolism*; Rose, A. M., Ed.; Academic Press: New York, NY, 1978; 470 pp.
7. Fengel, D.; Wegener, G. *Wood: Chemistry, Ultrastructure, Reactions*; Walter de Gruyter: New York, NY, 1984; 613 pp.
8. Koshijima, T.; Watanabe, T.; Yaku, F. In *Lignin, Properties and Materials*; Glasser, W. G.; Sarkanen, S., Eds.; American Chemical Society Symposium Series 397, American Chemical Society: Washington, DC, 1989; p. 11.
9. Timell, T. E. *Adv. Carb. Chem.* 1964, 19, 247-302.
10. Timell, T. E. *Adv. Carb. Chem.* 1965, 20, 409-483.
11. Wilkie, K. C. B. *Adv. Carb. Chem. Biochem.* 1979, 36, 215-264.
12. *Lignins: Occurrence, Formation, Structure, and Reactions*; Sarkanen, K. V.; Ludwig, C. H., Eds.; Wiley-Interscience: New York, NY, 1971; 916 pp.
13. *Starch: Chemistry and Technology*; Whistler, R. L.; Beniller, J. N.; Paschall, E. F., Eds.; Academic Press: New York, NY, 1984; 718 pp.
14. Kosaric, N.; Wicrowek, A.; Cosentina, G. P.; Duvnjak, Z. *Adv. Biochem. Eng./Biotechnol.* 1985, 32, 1-24.

15. *Chemistry and Function of Pectins*; Fishman, M. L.; Jen. J. J., Eds.; American Chemical Society Series 310, American Chemical Society: Washington, DC, 1986; 283 pp.
16. *Chitin and Chitosan: Sources, Chemistry, Biochemistry, Biochemistry, Physical Properties and Applications*; Skjak-Braek, G.; Anthousen, T.; Sanford, P., Eds.; Elsevier Applied Science: New York, NY, 1989; 835 pp.
17. Schery, R. W. *Plants for Man*, 2nd Ed., Prentice-Hall: Englewood Cliffs, NJ, 1972; 657 pp.
18. Casier, J. P. J.; De Paefe, G. M. J.; Williams, H. E. J.; Gaffings, G. J. G.; Hermans, J. L.; Nappen, H. E. *Tropical Foods: Chemistry and Nutrition*; Inglet, G. E.; Charalambous, G., Eds., Academic Press: New York, NY, 1979; Vol. 1, 279 pp.
19. Sanford, P. A.; Hutchings, G. P. In *Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications*; Yalpani, M., Ed., Elsevier Science: Amsterdam, Holland, 1987; 363 pp.
20. Grohmann, K.; Mitchell, D. J.; Himmel, M. E.; Dale, B. E.; Schroeder, H. A. *Appl. Biochem. Biotech.* 1989, 20, 45-61.
21. Mitchell, D.J.; Grohmann, K.; Himmel, M.E.; Dale, B.E.; Schroeder, H.A. *J. Wood Chem. Technol.* 1990, 10, 111-121.
22. *Pulp and Paper Manufacture*; Stephenson, J. N., Ed., McGraw-Hill: New York, NY, 1950; Vol. 1, 1043 pp.
23. Gujer, W.; Zehnder, A. J. B. *Wat. Sci. Technol.* 1983, 15, 127-168.
24. *Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications*; Kirk, K. T.; Higuchi, T.; Chang, H., Eds.; CRC Press: Boca Raton, FL, 1980; Vols. 1 and 2, 488 pp.
25. *Handbook of Amylase and Related Enzymes*; The Amylase Research Society of Japan, Eds.; Pergamon Press: New York, NY, 1988; 274 pp.
26. Beguin, P.; Millet, J.; Chavaux, S.; Yague, E.; Tomme, P.; Aubert, J. P. *Enzyme Systems for Lignocellulose Degradation*; Coughlan, M. P., Ed.; Elsevier Applied Science: New York, NY, 1989; pp 57-72.
27. Vuilleumier, S. *Sugar y Azucar* 1988, 83, 14.
28. American Type Culture Collection, Rockville, MD.
29. *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed., Academic Press: New York, NY, 1985; 679 pp.
30. Grohmann, K.; Himmel, M. E.; Rivard, C. J.; Tucker, M. P.; Baker, J. O. *Biotech. Bioeng. Symp.* 1985, 14, 137-157.
31. Millet, M. A.; Baker, A. J.; Satter, L. D. *Biotech. Bioeng. Symp.* 1976, 6, 125-153.
32. Fan, L. T.; Lee, Y. H.; Gharpuray, M. M. *Adv. Biochem. Eng.* 1982, 23, 157-187.
33. Archibald, J. G. *J. Dairy Sci.* 1926, 6, 257-271.
34. Wayman, M.; Parekh, S.; Chornet, E.; Overend, R. P. *Biotech. Lett.* 1986, 8, 749-752.
35. Jeffries, T. W. In *Yeast: Biotechnology and Biocatalysis*; Verachtert, H.; De Mot, R., Eds.; Marcel Dekker: New York, NY, 1990; pp 349-394.
36. Klyosov, A. A. In *Biochemistry and Genetics of Cellulose Degradation*; Aubert, J. P.; Beguin, P.; Millet, J., Eds.; Academic Press: New York, NY, 1988; pp 87-99.

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Chapter 2

Enzymes in Pulp and Paper Processing

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Recent enzymatic applications in the processing of wood and cellulosic fibres aim at the development of processes with reduced consumption of energy, raw materials or environmentally harmful chemicals. The substrates in these new processes include native wood as well as mechanical or chemical pulps. The access of enzymes in relatively short term treatments of undelignified substrates is limited to the surface of the fibres due to the small pore volume size within the fibres. Examples of new enzymatic processes in the pulp and paper industry include enzymatic debarking, modification of fibre properties and biobleaching. Presently, the most useful enzymes belong to the groups of hemicellulases and pectinases. The enzymology of these new processes is discussed in the light of practical results.

The main constituents of wood are cellulose, hemicellulose and lignin. Recent biotechnical applications in the pulp and paper industry have mostly been directed towards the degradation or modification of hemicelluloses and lignin, since the cellulosic fibre should retain its favourable properties. Traditionally, wood degrading enzymes have been studied with the aim of obtaining complete degradation of the substrate. In these applications, versatility of the enzyme mixture is advantageous. Examples of practical studies have been the hydrolysis of cellulosic and hemicellulosic waste residues to monomeric sugars, eg. for bioconversion by fermentation (1, 2, 3).

The most promising new approaches to using enzymes in the pulp and paper industry, however, are based either on the idea of selected hydrolysis of certain components or limited hydrolysis of several components in the fibre. This concept is apparently in accord with both the technical and economic demands of a feasible process. By limiting the extent of hydrolysis

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in the lignocellulosic material, both enzyme costs and yield losses can be minimized. Improved knowledge of the degradation mechanisms is still needed in order to understand the roles of individual enzymes in different applications and to optimize production of the desired enzymes on industrial scale.

New challenges for the development of biotechnical processes are generated by increasing concern over environmental pollution. The amounts of toxic compounds should be minimized both in effluents and in the products. Other aims are savings of energy and raw materials. New applications directed towards satisfying these demands are described in this paper.

Enzymes

The diversity of lignocellulolytic micro-organisms in nature serves as a rich source of different enzymes for applications in the pulp and paper industry. Controlled microbial processes using selected organisms, such as the biopulping process (4, 5, 6, 7, 8) can also serve as models for subsequent processes in which living cells could be replaced by isolated enzymes for controlled modification of the substrate. Many micro-organisms are known to produce enzymes capable of breaking down one or more constituents of lignocellulosic materials. Most of the commercial enzyme preparations are mixtures of more than one synergistically acting group of enzymes.

At present the hemicellulases and the pectin degrading enzymes are probably most promising from a commercial point of view, although research on the biochemistry, genetics and protein modelling of the cellulolytic systems of various organisms has progressed furthest (9, 10). The enzymes participating in the hydrolysis of xylans have been well characterized (11, 12) whereas much less is known about the mechanisms of hydrolysis of mannans, galactans or pectins in wood or pulp (13). Practical applications based on enzymatic modification and degradation of lignin by the peroxidases hitherto characterized are still quite far from realization. Laccase, however, may be more readily used for the modification of lignin even commercially. The enzymology of the degradation of lignin and carbohydrates is more extensively dealt with in other chapters of this book.

In the treatment of cellulose pulps one essential criterion for a suitable enzyme preparation is that its cellulase activity should be as low as possible, or preferably absent completely. As even extremely low cellulase activities may ruin pulp quality, *Trichoderma* enzyme preparations are unlikely to be suitable for these applications. Many bacterial and fungal enzymes with low cellulase activity have been shown to be suitable for treatment of pulps (14, 15, 16, 17). Regulation of the often synchronous production of cellulolytic and hemicellulolytic enzymes in micro-organisms is not well understood, and is further complicated by substrate cross-specificity of these enzymes. Enzymes with both endoglucanase and xylanase activity have been reported for bacteria (18, 19) and fungi (20, 21, 22). In addition to selection of strain and

production media, other strategies proposed include selective inactivation of cellulases (23, 24), bulk scale purification (25, 26, 27) and cloning of the desired activities in non-cellulolytic hosts (28, 29).

Access of the Enzymes to the Substrate

When considering the efficiency of enzymatic treatments of pulps, the critical parameter is the pore volume or pore size distribution, which determines the accessibility of the enzymes to the fibres. The interfibrillar space within the cell wall ranges between 1 and 5 nm in the wet state. In general, wood itself has a median pore size of 1.0-1.2 nm, a value which increases as a function of the decreasing yield of pulp, reaching 5-6 nm at the common yield level of 50% in the sulphite and kraft processes (30). At any given yield, the sulphite pulps have a larger mean pore size than the kraft pulps. It has also been shown that an exact correlation exists between the mean pore size and the size of lignin molecules leaving the cell wall at various stages during pulping (31).

According to the results of molecular modelling of the core protein of the cellulase CBH II from *Trichoderma reesei*, the approximate diameter of the enzyme (MW 56 kD) is 5 nm (32). Although the molecular weights of different enzymes vary considerably, the xylanases usually being the smallest, it is evident that penetration of cellulases, hemicellulases and ligninases in native wood is limited. In hydrolysis experiments, eg. with cellulose, it has been long recognized that unpretreated wood is practically indigestible by enzymes (33, 34). Using immunoelectron microscopy the enzymes of white-rot fungi have been shown to invade the wood cell walls only on superficial areas in the near proximity of the hyphae or in places where predegradation has already occurred (35, 36, 37). By contrast, a large part of the fibres in sulphite pulps was fully accessible to enzymatic attack by lignin peroxidase (38), although great differences were observed with respect to the penetrability of individual fibres in the same pulp, correlating with the morphological appearance of the fibres.

Enzymatic Debarking

Removal of the bark is the first step in all processing of wood. This step consumes substantial amounts of energy. Extensive debarking is needed for high quality mechanical and chemical pulps, because even low amounts of bark residues cause darkening of the product. In addition to its high energy demand, complete debarking leads to losses of raw material due to prolonged treatment in the mechanical drums.

The border between wood and bark is the cambium, which consists of only one layer of cells. This living cell layer produces xylem cells towards the inside of the stem and phloem cells towards the outside (Figure 1). In all the wood species studied, common characteristics of the cambium include a high content of pectins and the absence or low content of lignin (39, 40,

41). The content of pectins in the cambium cells varies between the wood species studied but may be as high as 40% of the dry weight. The content of pectic and hemicellulosic compounds is also high in the phloem (42).

Enzymes specific for the hydrolysis of the cambium and phloem layers were found to facilitate bark removal (43). A clear dependence was observed between the polygalacturonase activity in the enzyme preparation and reduced energy consumption in debarking. In addition to polygalacturonase, the enzyme mixture produced by *Aspergillus niger* also contained other pectolytic and hemicellulolytic activities. The amount of energy needed for removal of bark was found to decrease down to 20% of the reference value (Table I). In this experiment wood disks were soaked in the enzyme solution and the enzyme was diffused mainly tangentially to the border between wood and bark. This new and attractive approach for enzymatic debarking shows great potential for saving both energy and raw material. Further development of both the biotechnical and technical aspects is under way.

Table I. Effect of polygalacturonase pretreatment on the relative energy consumption during debarking of spruce *

<i>Polygalacturonase activity</i> nkat/ml	<i>Relative energy consumption</i> %
0	100
37	75
185	45
915	20

* Wood disks were soaked in the enzyme solution for 24 hours at 20 C and the energy consumption of bark removal was measured using a laboratory scale debarking equipment (43). The activity was determined as described previously (44).

Fibre Modification

Enzymatic modification of fibres aims at decreased energy consumption in the production of thermomechanical pulps and increased beatability of chemical pulps or improvement of fibre properties. In high yield mechanical pulps most of the lignin and hemicellulose remain in the pulps. According to determinations of the median pore width and immunolabelings of untreated wood, it is evident that enzymatic modifications to the composition of mechanical pulps can be achieved only on the outer surface of the fibre. This was verified when xylanases were applied to thermomechanical pulp (16). Even when using rather high enzyme dosages, only about 1% of the pulp was dissolved. When combined with an alkaline pretreatment, the

enzymatic treatment was substantially improved and the amount of energy required for refining thermomechanical pulp was decreased.

Water removal on the paper machine has been shown to improve as a result of limited hydrolysis of the fibres in recycled paper. Mixtures of cellulases and hemicellulases were found to decrease the SR-value, which describes the drainage behaviour of the fibres (45). The effect is apparently due to fibrillation or change in the composition of fine particles.

Chemical pulping processes have been designed to produce fibres with optimal properties for various purposes. High hemicellulose content of the pulp results in high yields and is generally regarded as an advantage with respect to the interfibre bonding properties in papermaking. Thus removal of hemicelluloses is not expected to lead to improvement of the bonding properties. Consistently with this, reduction in the interfibre bonding was observed after treatment of pulps with xylanases (17, 24).

The desired structural changes in the fibre which are created during beating and refining are external fibrillation and fibre swelling, which improve the flexibility and bonding ability of the fibres. The role of xylans in fibre properties was studied using xylanases from *Sporotrichum dimorphosporum* in the treatment of fully bleached spruce sulphite and birch kraft pulps. Electron microscopic examination revealed external fibrillation and good flexibility of fibres, implying internal modification (46). The water retention value, which describes fibre swelling, was considerably increased. The conclusion was that xylan was hydrolyzed in the whole, delignified cell walls. The enzymatically treated pulps were comparable with slightly beaten pulps. The beatability was generally enhanced and the energy demand was reduced about threefold (24).

The degree of polymerization (DP) of pulp treated with a cellulase-free xylanase was found to increase, apparently due to the selective removal of xylan, which has a lower DP (47). Thus, treatment of pulps with xylanases has been shown to increase their viscosities (27, 48, 49). However, even low cellulase activities in the enzyme preparations result in decreased viscosity.

Attempts to remove hemicellulose for production of dissolving pulps with very low hemicellulose contents have shown that complete enzymatic hydrolysis of hemicellulose within the pulp is difficult to achieve. The xylan content in delignified mechanical aspen pulp was reduced from approximately 20 to 10%, whereas in bleached hardwood sulphite pulp the xylan content was decreased from 4 to only 3.5% even at very high enzyme dosages (50). The complete removal of residual hemicellulose seems thus unattainable, apparently due to modification of the substrate or to structural barriers.

Biobleaching

After the pulping process the residual lignin is degraded by chlorine-based chemicals or other oxidizing agents and extracted by alkaline solutions in successive steps to increase pulp brightness. The size of the residual lignin molecules is apparently greater than the median pore size. Lignin is thought

to be linked to hemicellulose in pulps, but in addition to this hemicelluloses seem to physically restrict the passage of high molecular weight lignin out of the cell wall. Under alkaline pulping conditions, xylan is first dissolved and then reprecipitated into the fibres. It has been suggested that this type of xylan is present in the fibres in different forms; loosely bound in the pulp, crystallized onto the cellulose fibres or chemically linked to cellulose (51). Hemicellulases have been proposed to degrade xyans covering the lignin or cellulose molecules in the fibres, thus enlarging the pore sizes within the pulp. Cleavage of hemicellulose bonds near the points of attachment between lignin and hemicellulose may also facilitate the extraction of residual lignin (Figure 2). However, only limited hydrolysis of xylan has been aimed at in order to retain the high yield of the pulp and the interfibre bonding properties of xylan (14).

The utilization of enzymes in bleaching aims at decreased chlorine consumption. Lignin content, usually expressed as kappa number, was reduced in pine and birch kraft pulps by 25 and 33%, respectively, using hemicellulases (14, 52). A typical bleaching sequence consists of prebleaching ((D/C)E) and final bleaching (DED). In prebleaching the main part of chlorine is in the form of molecular chlorine, leading to the formation of complex mixtures of substituted compounds in effluents. Chlorine dioxide is used in final bleaching. The consumption of active chlorine could be reduced by 25% at the prebleaching stage or by 35% at the final bleaching stage, the latter corresponding to a saving of 10 kg of chlorine dioxide per ton of pulp. The strength properties of the fully bleached pine kraft pulp were similar to those of the reference pulp.

In the enzymatic treatments, enzyme preparations from different origins have been shown to be rather similar with respect to kappa number reduction, yield and brightness. Unpurified enzyme mixtures from strains of *Aspergillus* and *Bacillus* have mainly been studied (53). It appears clear that pure endoxylanase brings about a major part of the overall effect in the treatment (Table II).

Endoxylanase produced by a transformant strain of *Escherichia coli* has been shown to decrease the kappa number and increase the brightness of pulp in an enzymatic pretreatment followed by chemical extraction (27). Beta-xylosidase, on the other hand, had no effect in the treatment. The mannanase produced by *Bacillus subtilis* was found to be equally effective as the xylanase. The specificities of mannanases produced by two different organisms, however, differed considerably (49). Enrichment of the culture broth of *Streptomyces olivochromogenes*, containing mainly endoxylanase, with different pure enzymes or enzyme mixtures acting on side chains and other hemicelluloses, resulted only in slight increases in brightness and in kappa number reduction (53).

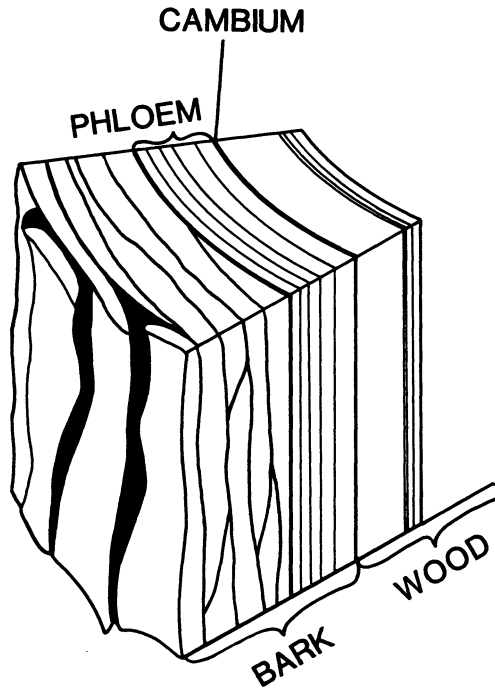


Figure 1. Outer part of a tree stem. New cells are formed in the cambium layer and differentiate either to wood or bark tissue.

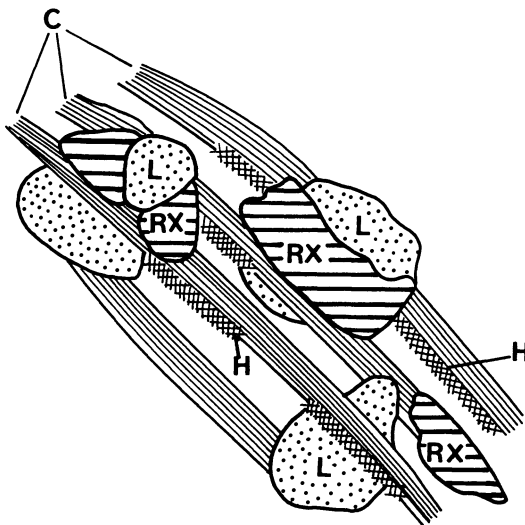


Figure 2. Hypothetical view of a fibre surface showing cellulose fibrils (C), lignin (L), hemicellulose (H) and reprecipitated xylan (RX).

Table II. Reductions of kappa numbers after peroxide delignification of enzymatically pretreated pine kraft pulps using of different enzyme preparations

<i>Organism</i>	<i>Enzyme activities</i>							<i>Kappa number reduction</i>
	XYL	β -X	MAN nkat/g	ARA	AXE	HEC	FPU U/g	
<i>Aspergillus fumigatus</i>	30	0.5	4.3	0	5.3	21.6	0.01	50
<i>A. oryzae</i>	30	3.5	8.5	2.6	0.7	14.1	0.02	51
<i>Bacillus circulans</i>	30	12.0	0	1.9	0.3	2.7	0.007	49
<i>T. reesei</i> , xyl*	30	0	0	0	0	0	0	53
<i>B. subtilis</i> , xyl*	30	0	0	0	0	0	0	48
ref. peroxide								36

* purified xylanases (unpublished). The activity determinations; xylanase (XYL), β -xylosidase (β -X), mannanase (MAN), arabinosidase (ARA), acetylxyLANesterase (AXE), cellulolytic activities (HEC, FPU) and enzymatic treatments were carried out as described previously (15, 53).

Economics of the Processes

When considering the enzymatic treatments from an economic point of view, the cost of the enzyme is clearly the critical parameter. The amounts of enzymes used in various experiments have ranged from about 2 to 50000 IU/g of pulp, the highest dosages being used in the tests for complete removal of residual xylan. Of course, different methods and substrates in the activity determinations limit the comparability of the units used. In the pulp and paper applications, the costs of using enzymes should not be essentially higher than those of the alternative, traditional methods. On the other hand the advantages of biotechnical methods, including reduced waste water purification costs and increased product safety, must also be considered.

According to our preliminary calculations on the feasibility of the enzymatic bleaching process, the economically acceptable amount of enzyme should not exceed 10 IU/g of pulp. Thus, realization of these applications may currently be confounded by economic feasibility, although improvements in the technologies involved in enzyme production and utilization are to be expected.

Literature Cited:

1. Linko, M. In *Wood and Cellulosics: Industrial Utilization, Biotechnology and Properties*; Kennedy, J.F.; Phillips, G.O.; Williams, P.A., Eds.; John Wiley & Sons, New York, 1987, pp. 231-242.
2. Blazej, A.; Biely, P. *ibid* pp. 275-282.
3. Magee, R.J.; Kosaric, N. *Adv. Biochem. Eng. Biotechnol.* 1985, 32, 61-93.
4. Eriksson, K-E.; Vallander, L. *Svensk Papperstidn.* 1982, 85, R 33.
5. Bar-Lev, S.S.; Kirk, T.K.; Chang, H-M. *Tappi* 1982, 65, 111.
6. Pilon, L.; Desrochers, M.; Jurasek, L.; Neuman, P.J. *Tappi* 1982, 65, 93.
7. Myers, G.C.; Leatham G.F.; Wegner, T.H.; Blanchette, R.A. *Tappi J.* 1988, 71, 105-108.
8. Blanchette, R.A.; Burnes, T.A.; Leatham, G.F.; Effland, M.J. *Biomass* 1988, 15, 93-101.
9. Knowles, J.; Lehtovaara, P.; Teeri, T. *Tibtech.* 1987, 5, 255-261.
10. Tomme, P.; Heriban, V.; Van Tilbeurgh, H.; Claeysens, M. In *Plant Cell Wall Polymers*; Lewis, N.G.; Paice, M.G., Eds.; ACS Symposium Series No. 399; American Chemical Society: Washington, DC, 1989, pp. 570-586.
11. Poutanen, K. *Ph. D. Thesis*, Helsinki University of Technology, Espoo, Finland, 1988.
12. Dekker, R.F.H. In *Plant Cell Wall Polymers*; Lewis, N.G.; Paice, M.G., Eds.; ACS Symposium Series No. 399; American Chemical Society: Washington, DC, 1989, pp. 619-629.
13. Shimizu, K.; Ishihara, M. *Agr. Biol. Chem.* 1983, 47, 949-955.
14. Viikari, L.; Ranua, M.; Kantelinen, A.; Sundquist, J.; Linko, M. *Proc. 3rd Int. Conf. Biotechnology in the Pulp and Paper Industry*, 1986, p 67.
15. Bailey, M.J.; Poutanen, K. *Appl. Microbiol. Biotechnol.* 1989, 30, 5-10.
16. Jeffries, T.; Lins, C.W. *Abstr. Fourth Int. Conference on Biotechnology in the Pulp and Paper Industry*, Raleigh, 1989, p 59-60.
17. Roberts, J.C.; McCarthy, A.J.; Flynn, N.J.; Broda, P. *Enz. Microb. Technol.* 1990, 12, 210-213.
18. Lee, S.F.; Forsberg, C.W.; Rattray, J.B. *Appl. Environ. Microbiol.* 1987, 53, 644-650.
19. Gilkes, N.R.; Langsford, M.L.; Kilburn, D.G.; Miller, R.C.; Warren, R.A.J. *J. Biol. Chem.* 1984, 259, 10455-59.
20. John, M.J.; Schmidt, B.; Schmidt, J. *Can. J. Biochem.* 1979, 57, 125-134.
21. Kolarova, N.; Farkas, V. *Biologia (Bratislava)*, 1983, 38, 721-5.
22. Gibson, T.S.; McCleary, B.V. *Carboh. Polym.*, 1987, 7, 225-240.
23. Barnoud, F.; Comtat, J.; Joseleau, J.P.; Mora, F.; Ruel, K. *Biotechnology in the Pulp and Paper Industry*; Third International Conference, Stockholm, June 16-19, 1986; pp 70-72.
24. Noe, P.; Chevalier, J.; Mora, F.; Comtat, J. *J. Wood Sci. Technol.* 1986, 6, 167-184.
25. Tan, L.U.L.; Yu, E.K.C.; Louis-Seize, G.W.; Saddler, J.N. *Biotechnol. Bioeng.* 1987, 30, 96-100.
26. Gibson, T.S.; McCleary, B.V. *Carbohydr. Pol.* 1987, 7, 225-240.

27. Senior, D.J.; Mayers, P.R.; Saddler, J.N. In *Plant Cell Wall Polymers*; Lewis, N.G.; Paice, M.G., Eds.; ACS Symposium Series No. 399; American Chemical Society: Washington, DC, 1989, pp 641-654.
28. Paice, M.G.; Bernier, R.; Jurasek, L. *Biotechnol. Bioeng.* 1988, 32, 235-239.
29. Bertrand, J-L.; Morosoli, R.; Sharek, F; Kluepfel, D. *Biotechnol. Bioeng.* 1989, 33, 791-799.
30. Stone, J.E.; Scallan A.M. *Pulp Pap. Mag. Can.* 1968, 6, 288-293.
31. Ahlgren, P.A.; Yean, W.Q.; Goring, D.A.I. *Tappi* 1971, 54, 737-740.
32. Rouvinen, J.; Bergfors, T.; Pettersson, G.; Knowles, J.; Jones, A. *First European Workshop on Crystallography of Biological Macromolecules*, Como, Italy, May 15-19, 1989.
33. Grethlein, H.E. *Biotechnol.* 1985, 3, 155-160.
34. Weimer, P.J.; Weston, W.M. *Biotechnol. Bioeng.* 1985, 27, 1540-1547.
35. Joseleau, J.P.; Ruel, K. In *Plant Cell Wall Polymers*; Lewis, N.G.; Paice, M.G., Eds.; ACS Symposium Series No. 399; American Chemical Society: Washington, DC, 1989, p 443-453.
36. Blanchette, R.A.; Abad, A.R.; Cease, K.R.; Farrell, R.L.; Lovrien, R.E.; Leathers, T.D. *Abstr. Fourth Int. Conference on Biotechnology in the Pulp and Paper Industry*, Raleigh, 1989, p 8-9.
37. Srebotnik, E.; Messner, K. *Abstr. Fourth Int. Conference on Biotechnology in the Pulp and Paper Industry*, Raleigh, 1989, p 125-126.
38. Srebotnik, E.; Messner, K. *J. Biotechnol.* 1990, 13, 199-210.
39. Simson, B.W.; Timell, T.E. *Cellulose Chem. Technol.* 1978, 12, 39-50.
40. Kato, K. In *Plant Carbohydrates II*; Tanner, W.; Loevus, F.A., Eds.; Springer-Verlag: Berlin, 1981; pp 29-46.
41. Dey, P.M.; Brinson, K. *Adv. Carbohydr. Chem. Biochem.* 1984, 42, 265-382.
42. Fu, Y.-L.; Timell, T.E. *Cellulose Chem. Technol.* 1972, 6, 517-519.
43. Viikari, L.; Rättö, M.; Kantelinen, A. *Finnish Patent Appl.* 896291, 1989.
44. Bailey M.J.; Pessa, E. *Enzyme Microb. Technol.* 1990, 12, 266-271.
45. Fuentes, J-L.; Robert, M. *French Patent* 8613208, 1986.
46. Mora, F.; Comtat, J.; Barnoud, F.; Pla, F.; Noe, P. *J. Wood Sci. Technol.* 1986, 6, 147-165.
47. Senior, D.J.; Mayers, P.R.; Miller, D.; Sutcliffe, R.; Tan, L.U.L.; Saddler, J.N. *Biotechnol. Lett.* 1988, 10, 907-912.
48. Puls, J.; Poutanen, K. *Enzyme Systems for Lignocellulose Degradation*; Elsevier Applied Science; London and New York, 1989, p 151
49. Clark, T.A.; McDonald, A.G.; Senior, D.J.; Mayers, P.R. *Abstr. Fourth Int. Conference on Biotechnology in the Pulp and Paper Industry*, Raleigh, 1989, pp. 39-40.
50. Paice, M.G.; Jurasek, L. *J. Wood Sci. Technol.* 1984, 4, 187-198.
51. Meller, A. *Holzforschung* 1965, 19, 118-124.
52. Viikari, L.; Ranua, M.; Kantelinen, A.; Linko, M.; Sundquist, J. *Proc. 4th Int. Congr. on Wood and Pulping Chemistry*, Paris, 1987. Vol. I, pp. 151-154.
53. Kantelinen, A.; Viikari, L.; Linko, M.; Rättö, M.; Ranua, M.; Sundquist, J. 1988 *International Pulp Bleaching Conference. Tappi Proceedings*, pp. 1-5.

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Chapter 3

Enzymes for Anaerobic Municipal Solid Waste Disposal

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It is estimated that of the 320 billion pounds of industrial and domestic waste generated in the United States each year, approximately 240 billion pounds are biodegradable (1). This waste is composed primarily of paper products, food and yard waste, and a wide array of organic byproducts. The majority of municipal solid wastes are deposited in landfills that permit decomposition through natural anaerobic processes. These anaerobic processes are complex, with a consortia of microorganisms responsible for the production of specific enzyme mixtures necessary for effective breakdown of the polymeric substrates. The uncontrolled nature of the landfill environment results in variable populations of microorganisms and/or enzyme systems, as well as sub-optimal environmental parameters, which contribute to the characteristically slow and ineffective rates of anaerobic degradation in landfills. Controlled anaerobic digestion systems may be used on a small scale to develop appropriate microbial consortia and to determine optimal nutrient levels and environmental parameters necessary for effective degradation rates in large-scale processing.

The decreasing capacity for landfill disposal of municipal solid wastes (MSW) has prompted evaluation of alternative disposal options (2). Disposal of MSW through processes that also derive energy production are of particular interest (3). However, the heterogeneous nature of MSW presents a major obstacle for conversion processes designed or optimized for more homogeneous feedstocks. Anaerobic digestion is a natural process of biological decomposition of organic matter principally to methane and carbon dioxide and occurs readily in MSW landfills. The process is exceedingly slow with biological rates dependent upon such factors as microbial populations present, ambient temperature, and moisture. In general, the advantage of anaerobic digestion is due in part to the nature of the microbial consortium, that is, degradation occurs for most all of the heterogeneous organic materials (some of which are often toxic) present in the MSW. Anaerobic digestion represents one of the possible technologies for MSW disposal that produces a useful energy product in the form of methane gas (biogas). The biogas product may be converted directly to electrical power, as a medium BTU fuel in internal combustion engines, or may be upgraded by reducing the carbon dioxide content to produce synthetic natural gas.

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MSW as a Feedstock for Biological Processes

Although the exact composition of MSW varies widely with respect to location, season and time of day, MSW consists primarily of cellulose in the form of newspaper, wood, and cardboard (4,5). Positive attributes of MSW as a feedstock include an ever increasing supply, centralized production and collection, an existing transportation system, and revenue generation for receipt of MSW materials (i.e., tipping fee). However, the composition of MSW derived from residential and industrial sources varies dramatically with respect to location and season. The major components may be approximated, however, and are compared over three decades in Table I. These data indicate that the majority of this waste is biodegradable. Furthermore, efforts to encourage source separation or use of centralized separation technologies, which serve to recycle useful materials such as glass and metals, increase the relative biodegradable fraction of the resulting processed MSW.

Table I. Materials Discarded Into the Municipal Waste Stream 1970-2000.

	1970		1986		2000	
	mill.tons/yr	%	mill.tons/yr	%	mill.tons/yr	%
Paper & paperboard	36.5	32%	50.1	36%	66.0	39%
Yard waste	32.2	21	28.3	20	32.0	19
Food waste	12.8	11	12.5	9	12.3	7
Glass	12.5	11	11.8	8	12.0	7
Metals	13.5	12	12.6	9	14.4	9
Plastics	3.0	3	10.3	7	15.6	9
Other ^a	11.0	10	15.2	11	16.5	10
Total	121.5	100	140.8	100	168.8	100

^aIncludes rubber and leather, textiles, wood, and miscellaneous inorganics fractions.

SOURCE: Adapted from reference 3.

As shown in Table I, the plastic component of MSW has increased dramatically. Past development of synthetic plastic formulations has focused on reducing the photo, chemical, and biological degradation of the plastic polymers. However, the persistence of plastics in the environment as litter, potential marine hazard, and with concern for global carbon cycling (1,6) has focused recent attention on the recycling of plastics or development of new biodegradable plastic formulations.

The anaerobic digestion process also results in a mineral-rich residue through the mineralization of the organic materials. This residue from the process is suitable for use as a soil amendment. Historically, anaerobic digestion has been used to reduce the organic content and odor of municipal sewage wastes (7). However, the application of anaerobic digestion for treating municipal sewage is not viewed as economical due to the high water content of these wastes, which result in large reactor volumes. Additionally, and possibly more important, the anaerobic digestion process exhibits slow rates of conversion of organic polymers.

Depending upon the end use for the MSW, secondary processing (following recovery of recyclables) may result in a dry, primarily paper and plastics fraction and a wet food and yard waste fraction. In this separation technology, the dry waste stream may be combusted either as is or after a densification process for handling in coal-fired boilers. The wet organic material is generally composted or anaerobically digested, resulting in a residue usable as a soil enhancer.

Landfill Disposal of MSW

The disposal of MSW through landfilling has been a common practice for centuries. However, landfills receiving MSW have been implicated in the contamination of groundwater, which occurs when liquid wastes or natural precipitation percolate through the wastes and into groundwaters (2). Organic acids generally account for 80%-90% of the contamination, however, more than 50 individual organic components have been identified in addition to toxic heavy metals (8,9). Additionally, gaseous emissions from the natural anaerobic biological processes taking place in the landfill contain methane gas. Landfills are estimated to contribute as much as $30\text{-}60 \times 10^6$ tons of methane per year to the atmosphere, which may contribute to the greenhouse effect (10).

In relation to the obvious environmental pollution potential of landfilling of MSW, few new landfills are now sited. Additionally, the U.S. Environmental Protection Agency (EPA), in a report to Congress, projected that 40% and 82% of the current landfills in operation today will be closed by the year 1993 and 2013, respectively (11). Therefore, as the standard route of disposal shrinks, new avenues for disposal must be identified and quickly commercialized to meet the disposal demand.

Landfills as Biological Reactor Systems

Although the methane gas produced in landfills may be considered an environmental pollution problem it has also been viewed as an opportunity for the recovery of significant supplemental energy (12). The recovery of landfill gas is currently a commercial technology with installations at approximately 70 sites including Great Britain, Europe, South America, Asia, Australia, and the United States (13). The U.S. EPA reports there are approximately 32,000 closed landfill sites in the United States alone (11). However, the anaerobic digestion process in landfills is often extremely slow and uncontrolled due to the nature of the *in situ* environment. Research is currently focused on improving the understanding of the key microbiological processes and the effects of environmental parameters, including moisture and temperature on the anaerobic biological degradation rate (13,14).

Anaerobic Digestion

Numerous reviews have been published detailing the level of understanding of the anaerobic digestion process and the terminal steps of methane production (15-24). The anaerobic process is far from a complete understanding and control of the process; however, the process may be simplified and described in three steps, which include hydrolysis of polymeric substrates to monomers; fermentation of monomers to organic acids, hydrogen, and carbon dioxide; and conversion of organic acids, hydrogen, and carbon dioxide to methane.

For highly polymeric solid feedstocks such as MSW, the rate-limiting step in the

overall anaerobic digestion process has been identified as the hydrolysis of polymers to soluble monomers (23,25). Several anaerobic digestion studies were conducted with the wet food/yard waste subfraction of the MSW that contains significant fractions of cellulose, hemicellulose, lignin, and protein. In these systems, hydrolysis of the polymeric substrate is dependent upon microorganisms possessing the hydrolytic capacity for the various polymers (26-28). However, when whole processed MSW is utilized (29,30) or the dry paper/cardboard subfraction (31) is utilized in anaerobic digestion processes, the major polymer is cellulose (>50% by weight)(31-34). Therefore, in an anaerobic digestion system treating a high cellulose feedstock, cellulose hydrolysis is paramount to process efficiency and yield.

Anaerobic Hydrolysis of MSW Polymers

MSW, herbaceous crops, and woody biomass share the same rate-limiting step for bioconversion processes: the hydrolysis of complex polysaccharides to fermentable sugars (32). The primary biodegradable polymer, cellulose, is often shielded by lignin, a relatively inert, polyphenylpropane, three-dimensional polymer (35-36), and by hemicelluloses (37). This complex structure dictates that natural biodegradation occurs on a time-scale of months or years, rather than hours or days. Such slow rates of polymer degradation require large retention times, reactor volumes, and thus capital costs for anaerobic digestion of MSW, woody biomass, or agricultural residues to accomplish near-complete hydrolysis (38).

Pretreatments of the polymeric feedstocks to enhance the hydrolysis of the major components such as cellulose include size reduction, thermal-chemical treatments, and specific enzyme pretreatments (32,37). Although many of the pretreatment processes increase the rate and extent of polymer hydrolysis, the cost for treatment is prohibitive based upon the revenues generated for energy production alone in anaerobic digestion (32).

Increasing the natural rate of hydrolysis of substrate polymers in the anaerobic digestion system has been demonstrated through augmentation of the normal hydrolytic populations of the microbial consortia with hydrolytic microorganisms (39), or rumen consortia (40). In single-reactor systems, the anaerobic digestion process has characteristically been controlled in preference for the fastidious acetogenic/methanogenic populations. The use of the two-phase digestion systems serves to separate the hydrolysis/acid-producing stage from the methanogenic stage (41). In this way, both processes may be optimized to environmental conditions of temperature and pH. Additionally, due to the slow growth rate of many of the methanogenic bacteria, a fixed film reactor design may be used for the methane production stage to allow microbial retention while operating with short hydraulic retention times.

Most often, the rates for feedstock destruction in anaerobic digestion systems are based upon biogas production or reduction of total solids (TS) or volatile solids (VS) added to the system. Available data for analyses conducted on the specific polymers in the anaerobic digester feed are summarized in Table II. The information indicates a rapid rate of hydrolysis for hemicellulose and lipids. The rates and extent of cellulose degradation vary dramatically and are different with respect to the MSW feedstock based on the source and processing of the paper and cardboard products (42). Rates for protein hydrolysis are particularly difficult to accurately determine due the biotransformation of feed protein into microbial biomass, which is representative of protein in the effluent of the anaerobic digestion system.

Enzymes of Anaerobic Digestion

The anaerobic biological conversion of the major polymeric components of MSW identified require appropriate microorganisms and hydrolytic enzyme systems. Extracellular hydrolytic enzymes, such as cellulases and lipases, have been shown to be effective in the post hydrolysis of anaerobic digester effluent solids (34) or pretreatment of complex organic polymers before the digestion process (48).

Table II. Average Rates of Hydrolysis of Feedstock Polymers in Mesophilic Laboratory Anaerobic Digestion Systems (43)

Polymer	Apparent hydrolysis rate K_p (d^{-1})	Reference
Cellulose	0.085	(44,45)
Hemicellulose	0.54	(44)
Lipids	0.47	(45-47)
Proteins	0.025	(44,45)

The titers of cellulase activities found in anaerobic digesters, when compared to the few other "hydrolytic environments" for which analytical data are available, are strikingly low. Table III shows such values for filter paper and carboxymethyl cellulose degrading activities. This evidence seems to indicate that the cellulose degrading enzymes in

Table III. Comparison of Cellulase Activities found in Selected Natural and Commercial Systems

System	Filter paper Activity	CMCase Activity
Digester detergent extracts ^a	0.0006 U/g dry sludge	0.15 U/g dry sludge
Moist pasture soil ^a dry soil	NA	0.006 - 0.012 U/gram
Rumen contents ^b	NA	1.4 U/gram solids
Industrial saccharifications of woody biomass ^c	20 U/g cellulose	600 U/g cellulose

^aFrom ref. 49. ^bCalculated from (Hespell, R., U.S.D.A., personal communication, 1986). ^cLoading necessary to achieve complete hydrolysis in 1 to 3 days (50).

MSW-fed digesters are operating under less than optimal enzyme titers. Table IV shows the activity optima for selected cellulase related enzymes found in actual laboratory digesters (51) and the optima for a typical commercially available cellulase preparation.

The predominant hydrolytic microorganisms in rumen and the digestive tracks of animals differ from anaerobic digestion reactors systems. In rumen systems,

Ruminococcus and *Bacteroides* predominate (52,53) where as in anaerobic reactor systems treating high-cellulose content wastes such as biomass and MSW, the predominate hydrolytic microbes are *Clostridia* (54-56). As demonstrated from the limited activity optima information available for the hydrolytic enzymes produced, the cellulase enzyme produced by *Bacteroides* more closely fits the operational parameters of normal digester systems for pH and temperature when compared to the *Clostridia* species. However, the environmental and nutrient conditions of the digestion system may not favor the growth of this organism in relationship to the condition of its more natural ecosystem of the rumen. Still, environmental selective pressures may be exerted upon the digester system to select for the growth of hydrolytic microorganisms that produce enzymes with optimum that more closely match that of the digestion system.

Table IV. Cellulase pH and Temperature Optima

System	pH optima		Temperature optima °C	
	β -D-glucosidase	CMCase	β -D-glucosidase	CMCase
Digester extracts ^a	6.5	6.6	48	55
<i>Clostridium populeti</i> ^b	NA	5.5	NA	NA
<i>Ruminococcus albus</i> ^c	NA	6.5	NA	45
<i>Bacteroides succinogenes</i> ^d	NA	7.0	NA	39
<i>Trichoderma reesei</i>	4.5-5.0 ^e	4.5-5.0 ^f	NA	50-60 ^f
Normal mesophilic digester operation	6.8-7.2		35-37°C	

^aFrom ref. 52. ^bFrom 58. ^cFrom ref. 59. ^dFrom ref. 60. ^eFrom ref. 61. ^fFrom ref. 62.

Only recently have the types, activities, and relative concentrations of these hydrolytic enzymes from MSW-fed digester systems have been examined (49,51). To monitor digester performance as a function of enzyme levels, assays were developed that accurately reflect fluxes in hydrolytic activities in digester sludge, and not simply the initial quantity of enzyme added or the weight loss of cellulose in the MSW feed (49). We report here the application of this detergent extraction methodology to the analysis of hydrolytic enzymes in sludge taken from laboratory- and large-scale anaerobic digesters-fed MSW. We also report the direct correlations between specific cellulase activities and cellulose conversions in seven bench-scale digesters.

Materials and Methods

Laboratory-Scale Reactors. Laboratory-scale systems consisted of small continuously stirred tank reactors (CSTR) with 3.5-liter working volumes and were constructed and operated as previously described (62). The digester inoculum for these systems was obtained from the anaerobic digestion of municipal sewage sludge from the Denver

Municipal Sewage Treatment Plant. The reactors were batch-fed daily an RDF (refuse derived fuel) processed MSW feedstock obtained from Future Fuels Inc. in Thief River Falls, MN. The processed MSW feed contained 52% of dry weight (DW) cellulose, 20% lignin-plastics, 2% ash, and 26% acid-detergent solubles (% DW as determined by the acid-detergent-fiber assay (63)). The MSW feedstock was added to a nutrient solution at 5% w/v for daily feeding as previously described (33).

Pilot-Scale Reactor. Anaerobic digester effluent was obtained from a non-mixed pilot-scale reactor system operated at Walt Disney World, Orlando, FL, and fed an RDF MSW/sludge blend (64). The MSW feedstock was obtained from Baltimore, MD, and the municipal sewage sludge from the Walt Disney World Complex. Fresh effluent samples were sealed under anaerobic conditions and shipped overnight in ice for analysis.

Substrates and Detergents. p-Nitrophenyl- β -D-glucoside; p-nitrophenyl- α -D-glucoside; cellulose-, amylose-, and hide powder-azure; and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. Whatman no. 1 filter paper was used as substrate for the filter paper assay, and carboxymethyl cellulose (CMC) 7LF from Hercules, Inc. was used for the CMC assays. All buffer components and salts used were reagent grade and obtained from either Sigma or Fisher Scientific.

All assays performed on digester samples were conducted in 100 mM Tris buffer pH 7.5 with substrate incubations at 37°C. Glucose-releasing assays used the same Tris buffer with 0.5% sodium azide added. Colorimetric products from enzyme assays were detected and recorded using a Milton-Roy model 601 spectrophotometer equipped with sipper and data printer.

Assays for endo-1,4- β -glucanase [EC 3.2.1.4] (i.e., CMCase) and saccharifying cellulase (i.e., international filter paper U, IFPU) activities partially followed the methods recommended in the 1987 IUPAC report (65). When even undiluted enzyme samples fail to give the required glucose yield under prescribed assay conditions, the IUPAC committee recommends a less precise method. In the current study, cellulase activities in digester extracts were so low that the CMCU could only be defined as follows: one CMC unit of activity was that amount of enzyme required to liberate one μ g glucose from CMC in 60 min.

β -D-Glucosidase was determined according to the method of Wood (66) as aryl- β -glucosidase activity by the hydrolysis of p-nitrophenyl- β -D-glucopyranoside. The concentration of p-nitrophenol (pNPOH) was determined from the extinction at A_{410} under alkaline conditions induced by the addition of 2 M Na_2CO_3 . One unit of activity was defined as that amount of enzyme that catalyzes the cleavage of 1.0 μ mol substrate per min at 37°C. Maltase activity was found with p-nitrophenyl- α -glucoside in a similar way.

General protease, α -amylase, and exoglucanase activities were estimated using hide powder-, amylose-, and cellulose-azure substrates, respectively, as described earlier (49). Here, standard curves were developed for the hydrolysis of each azure-linked substrate by standard enzymes of known activity. By this method, one cellulose-azure hydrolysis unit corresponds to one filter paper unit, one unit of hide powder-azure activity corresponds to the hydrolysis of 1.0 μ mole of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) per min, and one amylose-azure unit of activity corresponds to the hydrolysis of 1.0 μ mole of maltose from starch per 30 min.

Preparation of clarified digester sludge. Samples were removed under anaerobic conditions and moved immediately into the anaerobic chamber. Before analysis, the samples were clarified by centrifugation and filtration with 0.22- μ m Acrodisc filters. Concentrated digester supernatant was also examined, and it was prepared by ultrafiltration in the anaerobic chamber with an Amicon stirred cell ultraconcentrator and Amicon PM10 membranes.

Preparation and analysis of SDS extracts from digester sludge. The particulates from a 30-ml sample were removed by centrifugation (15,000g) at 4°C for 20 min. The particulates were washed three times with 100 mM Tris buffer pH 7.0 and resuspended in 15 ml of buffer. The extraction procedure consisted of agitating the sample with a Fisher model 346 rotator at 25°C in the presence of 0.1% SDS for 1 h. The particulate material was then removed by centrifugation at 15,000g at 4°C for 20 min, and the supernatant was used to perform the enzyme assays.

Results and Discussion

The accurate and reproducible detection of hydrolytic enzymes in active anaerobic samples has historically been problematic in the recovery of enzyme activity from the complex organic mixture, due to the high background color level of the samples that interfere with detection methodologies, and the adsorption of the enzymes on microbial or solid materials of the digestion system. Previous research (49), has identified the usefulness of dye-linked substrates to reduce interference from sludge chromophores, the strong association of hydrolytic enzyme activities with particulates, and the usefulness of detergent extraction protocols to recover active hydrolytic enzyme activities from particulates. Additionally, in experiments in which purified hydrolytic enzymes of known activity are added to digester sludge detergent extraction protocols were shown to quantitatively recover all of the activity (57). Although the actual characterization of an anaerobic digestion system may be accomplished through the determination of the microbial populations present, the direct determination of the hydrolytic enzyme activities, or hydrolytic power, may represent a more rapid assessment of the biodegradation state and potential of the system. Table V shows a comparison of the hydrolytic enzyme activities found in both bench-scale and pilot-scale digesters fed MSW.

Although many of the operational parameters of the two digester systems were similar, important differences in digester mixing design (laboratory-scale digesters were operated as continuously stirred reactors versus the Solcon reactor operated as unmixed/settling reactor) and source storage and handling of the processed MSW feedstocks used. In general, the unmixed Solcon reactor resulted in substantial variations in specific enzyme activities in digester sludge depending upon sample depth within the reactor. Significant variability was determined for all of the activities examined. However, in comparison with the laboratory-scale CSTR reactor systems (operated under optimum nutrient levels), the Solcon reactor was similar with the exception of exoglucanase and α -amylase activities.

Both digester systems exhibit extremely low levels of detectable cellulase activities (exoglucanase, endoglucanase, and β -glucosidase) when compared to industrial saccharifying processes (See Table III) in which the hydrolysis of cellulose in the feedstock is optimized with respect to enzyme loading. Therefore, the data indicate the level of improvement that may be made to attain maximum rates for cellulose hydrolysis in the anaerobic reactor system.

Figures 1 and 2 show positive correlation, although of varying degrees of goodness of fit, between the specific activities of endoglucanase, exoglucanase, and β -glucosidase activities with the cellulose composition and degradation in digester feed, respectively. These data indicate a direct relationship between the cellulose content in the feed and cellulase enzyme complex production by the digester consortia. In this system, as in many others studied, cellulose is an effective inducer of cellulase secretion (67,68).

The effects of feedstock cellulose content on cellulase enzyme activities in the digester system were examined in multiple laboratory-scale CSTR digesters operated under similar conditions with identical levels of feedstock organic loading (g VS/reactor d) but different levels of cellulose (Solka Flocc). In general, all cellulase enzyme

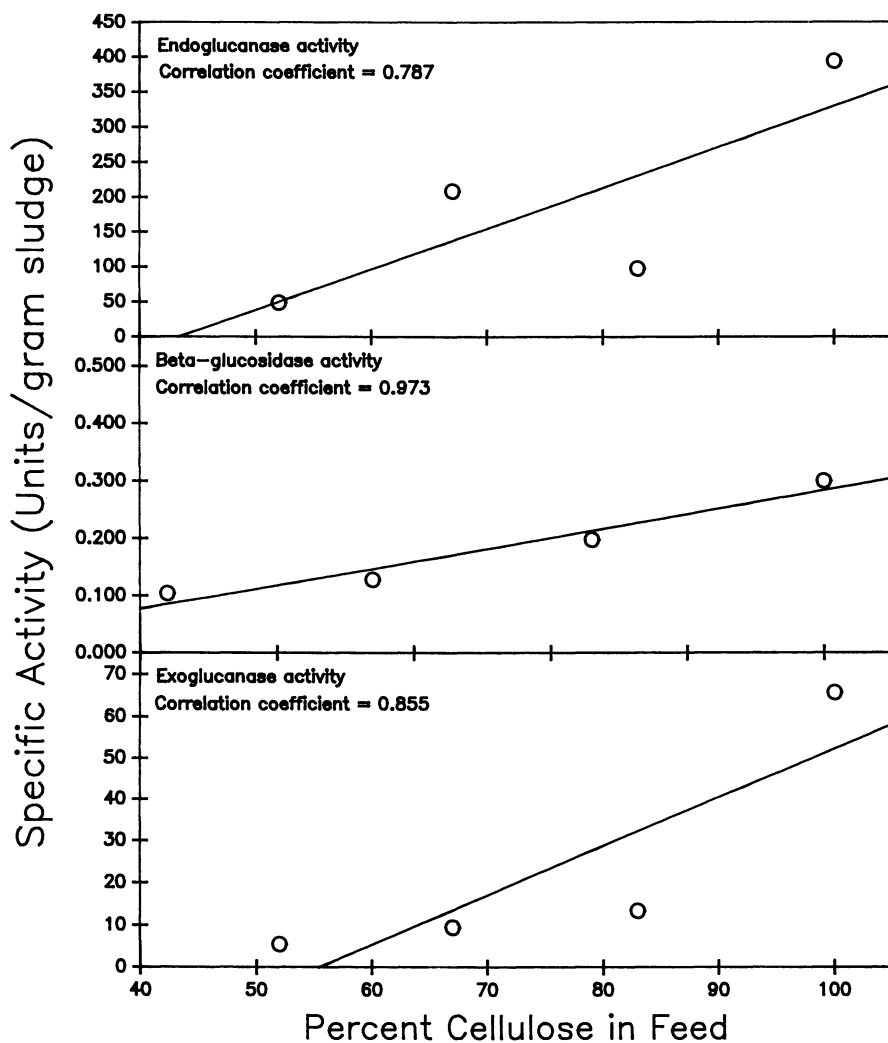


Figure 1. Comparison of the effects of feedstock cellulose content on specific cellulase enzyme activities in sludge from 4 CSTR reactors operated under similar conditions. Although the cellulose content of the feedstock was varied, the total volatile solids content for all reactors was equivalent.

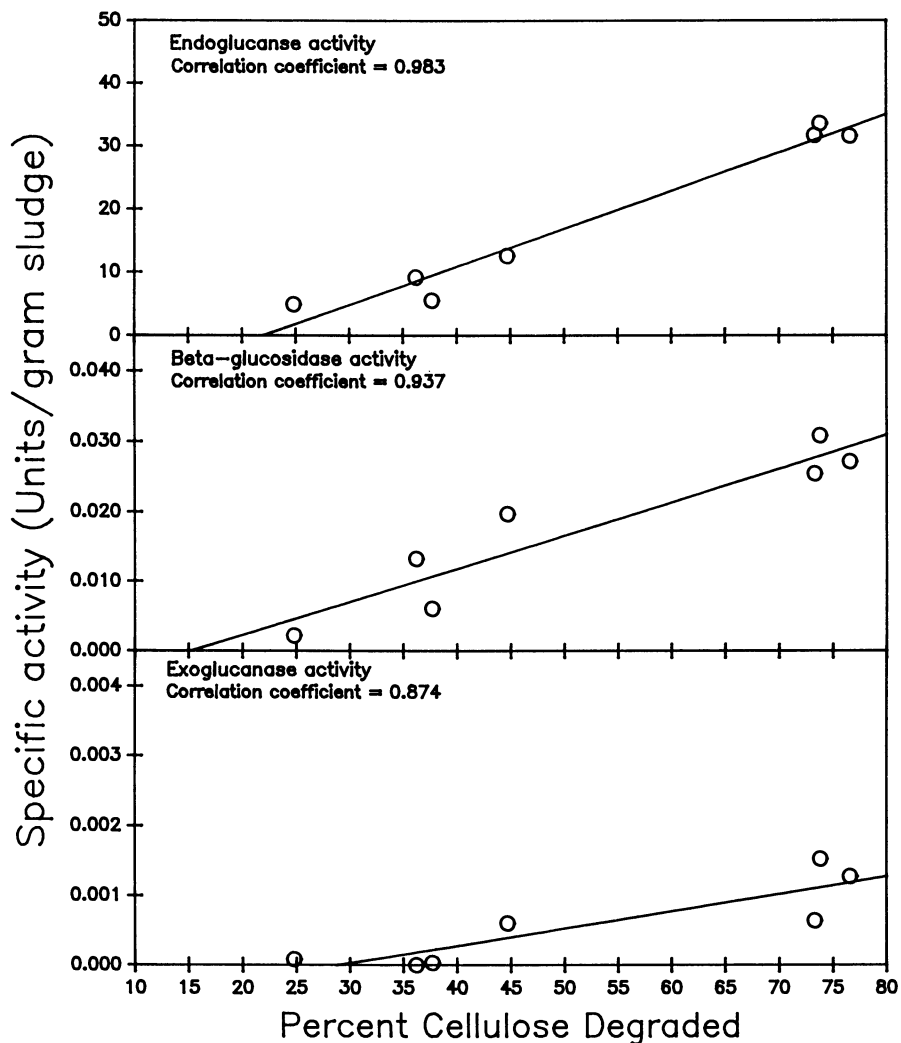


Figure 2. Comparisons of cellulose degradation of the MSW feedstock with specific cellulase enzyme activities in sludge from 7 CSTR digesters operated under different retention times and various conditions of nutrient limitation.

activities increased with increases in the cellulose content of the feedstock; however, the best correlation was determined for beta-glucosidase.

Multiple laboratory-scale CSTR digesters were also operated under similar conditions of temperature and MSW feedstock loading but differed with respect to retention time (10-30 d, ref. 69) and nutrient limitation (33). The fermentation parameters of nutrient limitation and retention time had varying effects on the

Table V. Enzyme Activity Analysis of Effluent from Laboratory-Scale and Pilot-Scale Mesophilic Anaerobic Digesters Fed Processed MSW

Activity (substrate)	Laboratory-scale ^a (U/g digester sludge)	Pilot-scale ^b (U/g digester sludge)
general protease (hide powder)	0.101 ± 0.028 S.D.	0.0238 ± 0.023 S.D.
α-amylase (amylose-azure)	0.164 ± 0.004	0.0012 ± 0.003
exoglucanase (cellulose-azure)	0.030 ± 0.003	-0-
endoglucanase (CMC)	0.198 ± 0.046	0.1070 ± 0.096
maltase (pNP-α-D-glucoside)	0.029 ± 0.002	0.0134 ± 0.006
β-D-glucosidase (pNP-β-D-glucoside)	0.150 ± 0.002	0.0410 ± 0.017

^a3.5 L continuously stirred tank reactors (CSTRs) ^bETU (Solcon), 1200-gallon digester at Disney World, FL. Units defined in Materials and Methods. Data collected from triplicate samples.

effectiveness of the overall fermentation in general, and on the hydrolysis of cellulose in specific. The comparison of cellulose degradation with resident cellulase enzyme activities as shown in Figure 2 indicates very good correlations for endoglucanase and beta-glucosidase activities. The importance of cellulase activity as a limiting factor to anaerobic digester process rates was confirmed in preliminary cellulase enzyme augmentation experiments (57). Addition of low levels (0.007 IFPU/mL sludge) of cellulase produced from the anaerobe *Clostridium populeti* resulted in significant increases (~9%) in the overall biogas production rate in digester sludge when compared to controls.

Conclusion

Although similar in some characteristics to rumen systems, little specific information is available about the characteristics of the cellulolytic enzymes produced by anaerobic bacteria found in digesters fed woody biomass or MSW. Dramatic improvements in digester performance may eventually be possible by using knowledge of the types and levels of enzyme activities, both naturally occurring or through augmentation, in anaerobic digestion systems that rely upon hydrolytic processes for overall process rates.

Current data indicate that the analysis of cellulase enzyme activities may be the best method for determining the projected cellulose conversion of the overall system, and therefore the hydrolytic power of the system under evaluation. With development, the analysis of enzyme activities may also serve as a "real time" method of monitoring the stability of the system, with radical changes in enzyme activities indicative of potential process upset.

Information concerning the effects of system operation parameters, such as temperature and pH and their effects on the optimum activity of digester-resident hydrolytic enzyme systems, may provide for the development of strategies for increased process rates.

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Literature Cited

1. Thayer, A. M. *Chem. Eng. News* 1989, 67, 7-15.
2. Rivard, C. J. *J. Environ. Health* 1989, 52, 99-100.
3. Goodman, B. J. *Overview of the Anaerobic Digestion Research Program*; SERI/SP-231-3520; Solar Energy Research Institute: Golden, CO, Jan. 1989; pp. 1-8.
4. Gibbs, D. F.; Greenhalgh, M. E. *Biotechnology, Chemical Feedstocks and Energy Utilization*; Frances Pinter: London, UK, 1983; pp 49-69.
5. Smith, F. A. Report No. SW-158; U.S. Environmental Protection Agency: Washington, DC, 1975.
6. Smock, D. *Plastics World* 1987, 45, 28-31.
7. Ramalho, R. S. *Introduction to Wastewater Treatment Processes*; 2nd ed.; Academic Press: New York, NY, 1983.
8. Albaiges, J.; Casado, F.; Ventura, F. *Water Research* 1986, 20, 1153-1159.
9. Barker, J. F.; Tessmann, J. S.; Plotz, P. E.; Reinhard, M. J. *Contam. Hydrol.* 1986, 1, 171-189.
10. Bingemer, H. G.; Crutzen, P. J. *Environment* 1987, 29, 21-22.
11. Anonymous. *Waste Age* 1988, 19, 64-66.
12. Schumacher, M. M. *Landfill Methane Recovery*; Noyes Data Corporation: Park Ridge, NY, 1983.
13. Bogner, J.; Vogt, M.; Piorkowski, R. *Landfill Gas Generation and Migration: Review of Current Research II*; SERI/SP-231-3520; Solar Energy Research Institute: Golden, CO, Jan. 1989; pp 139-163.
14. Jones, K. L.; Rees, J. F.; Grainger, J. M. *Eur. J. Appl. Microbiol. Biotechnol.* 1983, 18, 242-245.
15. Buswell, A. M.; Hatfield, W. D. *Anaerobic Fermentations*; Bulletin No. 32; State of Illinois Department of Registration and Education: Chicago, 1936.
16. McCarty, E.L. *Public Works* 1964, 95, 107-141.
17. Wolfe, R. S. *Adv. Microbial Physiol.* 1971, 6, 107-145.

18. Mah, R. A.; Ward, D. M.; Baresi, L.; Glass, T. L. *Ann. Rev. Microbiol.* 1977, 31, 309-341.
19. Zeikus, J. G. *Bacteriol. Rev.* 1977, 41, 514-541.
20. Balch, W. E.; Fox, G. E.; Magrum, L. J.; Woese, C. R.; Wolfe, R. S. *Microbiol. Rev.* 1979, 43, 260-296.
21. Bryant, M.P. *J. Animal Sci.* 1979, 48, 193-201.
22. Clausen, E. C.; Sitton, O. C.; Gaddy, J. L. *Biotechnol. Bioeng.* 1979, 21, 1209-1219.
23. Boone, D. R. *Appl. Environ. Microbiol.* 1982, 43, 57-64.
24. Daniels, L.; Sparling, R.; Sprott, G. D. *Biochim. et Biophys. Acta* 1984, 768, 113-163.
25. Noike, T.; Endo, G.; Chang, J-E.; Yaguchi, J-I.; Matsumoto, J-I. *Biotechnol. Bioeng.* 1985, 27, 1482-1489.
26. Pathe, P. P.; Alone, B. Z.; Titus, S. K.; Bhide, A. D. *Indian J. Environ. Health* 1982, 24, 8-13.
27. Cecchi, F.; Traverso, P. G.; Cescon, P. *Sci. Total Environ.* 1986, 56, 183-197.
28. Traverso, P. G.; Cecchi, F. *Biomass* 1988, 16, 97-106.
29. Pfeffer, J. T. *Energy from Biomass and Wastes X*; Institute of Gas Technology: Chicago, IL, 1986; pp 1149-1171.
30. Schmidell, W.; Craveiro, A. M.; Peres, C. S.; Hirata, Y. S.; Varella, R. F. *Water Sci. Technol.* 1986, 18, 163-176.
31. Rivard, C. J.; Vinzant, T. B.; Adney, W. S.; Grohmann, K.; Himmel, M. E. *Biomass* 1990, 23, 201-214.
32. Horton, G. L.; Rivers, D. B.; Emert, G. H. *Ind. Eng. Chem. Prod. Res. Dev.* 1980, 19, 422-429.
33. Rivard, C. J.; Vinzant, T. B.; Adney, W. S.; Grohmann, K. *J. Environ. Health* 1989, 52, 96-99.
34. Higgins, G. M.; Bullock, L. D.; Swartzbaugh, J. T. In *Fuels from Biomass and Wastes*; Klass, D. L.; Emert, G. H., Eds.; Ann Arbor Science: Ann Arbor, 1980; p. 151.
35. Sarkanen, K. V.; Ludwig, C. H. *Lignins: Occurrence, Formation, Structure and Reactions*; Wiley-Interscience: New York, NY, 1971.
36. Fengel, D.; Wegener, G. *Wood: Chemistry, Ultrastructure, Reactions*; Walter de Gruyter: Berlin, 1984.
37. Grohmann, K.; Torget, R.; Himmel, M. E. *Biotech. Bioeng. Symp.* 1985, 15, 59-80.
38. Clausen, E. C.; Gaddy, J. L. In *Fuel Gas Systems*; Wise, D. L., Ed.; CRC Press: Boca Raton, FL, 1983; pp 111-140.
39. De Macario, E. C.; Macario, A. J. L.; Rivard, C. J.; Grohmann, K. *Use of Antibody Probes to Monitor Hydrolytic Microbes in Municipal Solid Waste Anaerobic Digesters*; SERI-SP-231-3520; Solar Energy Research Institute: Golden, CO, 1989; pp 47-58.
40. Gijzen, H. J.; Zwart, K. B.; Teunissen, M. J.; Vogels, G. D. *Biotechnol. Bioeng.* 1988, 32, 749-755.
41. Ghosh, S.; Klass, D. L. *Process Biochem.* 1978, 4, 15-24.
42. Mertens, D. R.; Van Soest, P. J. Proceedings of the National ASAS Meeting; American Society of Animal Science: Champagne, IL, 1971.
43. Gujer, W.; Zehnder, A. J. B. *Wat. Sci. Tec.* 1983, 15, 127-167.
44. Ghosh, S.; Henry, M. P.; Klass, D. C. *Biotech. Bioeng. Symp.* 1980, 10, 163-187.
45. Woods, C. E.; Malina, Jr., J. F. *J. Water Pollution Control Fed.* 1965, 37, 1495-1505.
46. Heukelekian, H.; Mueller, P. *Sewage Ind. Waste* 1958, 30, 1108-1120.
47. Sawyer, C. N.; Roy, H. K. *Sewage Ind. Waste* 1955, 27, 1356-1363.
48. Higgins, G.; Swartzbaugh, J. T. In *Energy from Biomass and Wastes IX*; Klass, D.L., Ed.; Institute of Gas Technology: Chicago, IL, 1985; pp 791-810.
49. Adney, W. S.; Rivard, C. J.; Grohmann, K.; Himmel, M. E. *Biotech. and Appl. Biochem.* 1989, 11, 387-400.
50. Tatsumoto, K.; Baker, J. O.; Tucker, M. P.; Oh, K. K.; Mohagheghi, A.; Grohmann, K.; Himmel, M. E. *Appl. Biochem. Biotech.* 1988, 17, 159-174.
51. Adney, W. S.; Rivard, C. J.; Grohmann, K.; Himmel, M. E. *Biotech. Lett.* 1989, 11, 207-210.

52. Chesson, A.; Forsberg, C. W. In *The Rumen Microbial Ecosystem*; Hobson, P. N., Ed.; Elsevier: London, UK, 1988; pp 251-284.
53. Betian, H. G.; Linehan, B. A.; Bryant, M. P.; Holdeman, L. V. *Appl. Environ. Microbiol.* **1977**, *33*, 1009-1010.
54. Sleat, R.; Mah, R. A.; Robinson, R. *Appl. Environ. Microbiol.* **1984**, *48*, 88-93.
55. Sleat, R.; Mah, R. A. *Int. J. System. Bacteriol.* **1985**, *35*, 160-163.
56. Petitdemange, E.; Caillet, F.; Giallo, J.; Gaudin, C. *Int. J. System. Bacteriol.* **1984**, *34*, 155-159.
57. Himmel, M. E.; Adney, W. S.; Rivard, C. J.; Grohmann, K. *Detection of Extra-cellular Hydrolytic Enzymes in Anaerobic Digestion of MSW*; SERI/SP-231-3520; Solar Energy Research Institute: Golden, CO, 1989; pp 9-23.
58. Smith, W. R.; Yu, I.; Hungate, R. E. *J. Bacteriol.* **1973**, *114*, 729-737.
59. McGavin, M. J.; Forsberg, C. W. *Proc. Amer. Soc. Microbiol.* **1987**, K15.
60. Chirico, W. J.; Brown, R. D. *Eur. J. Biochem.* **1987**, *165*, 343-352.
61. Coughlan, M. P. In *Biotechnology and Genetic Engineering Reviews*; Russell, G.E., Ed.; Intercept: Ponteland, Newcastle upon Tyne, 1985; pp 39-109.
62. Henson, J. M.; Bordeaux, F. M.; Rivard, C. J.; Smith, P. H. *Appl. Environ. Microbiol.* **1986**, *51*, 288-292.
63. Goering, H. K.; Van Soest, P. J. *U.S. Department of Agriculture Handbook*; Number 379; U.S. Department of Agriculture: Washington, DC, 1970.
64. Chen, T. H.; Chynoweth, D. P. *Anaerobic Digestion of RDF at the Experimental Test Unit*; SERI/SP-231-3520; Solar Energy Research Institute: Golden, CO, 1989; pp 89-99.
65. Ghose, T. K. *Pure and Appl. Chem.* **1987**, *59*, 257-268.
66. Wood, T. M. *Biochem. J.* **1971**, *121*, 353-362.
67. Gong, C.S.; Tsao, G.T. *Ann. Reports Ferm. Processes* **1979**, *3*, 111-140.
68. Mandels, M. *Ann. Reports Ferm. Processes* **1982**, *5*, 35-78.
69. Vinzant, T. B.; Adney, W. S.; Grohmann, K.; Rivard, C. J. *Appl. Biochem. Biotech.* **1990**, *24/25*, 765-771.

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Chapter 4

Thermostable Saccharidases

New Sources, Uses, and Biotransformations

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Thermostable saccharidases are required for industrial processing of starch and lignocellulosic fibers. Thermophilic microbes have not been used as sources for industrial enzymes and the molecular biochemistry of enzyme thermophilicity is not understood. We have purified and characterized thermostable (70°C) saccharidases from thermoanaerobic bacteria including: β -amylase, amylopullulanase, α -glucosidase, glucose isomerase (GI), and endoxylanase. Thermostable saccharidase synthesis was regulated in *Thermoanaerobacter* to produce fructose directly from starch. The GI gene from *Thermoanaerobacter* was cloned and sequenced and site-directed mutagenesis was employed to explain enzyme catalysis and to design an enzyme active at 70°C and pH 5.5. Endoxylanase of *Thermoanaerobacter* was cloned and characterized in relation to a xylanosomal cellular organization and a source for fiber modification. Future studies on these novel saccharidases will focus on the molecular basis of thermophilicity, coordination with other hydrolases and industrial uses.

Saccharidases have a broad base for biotechnology applications (see Table I). Future industrial applications of saccharidases requires solving several problems including: finding new product uses, enhancing activity and stability, and lowering production costs in food- or environmentally-safe hosts. Our approach has been to develop thermophilic enzymes that are temperature and pH compatible with process conditions, to screen for new microbes, to clone and produce thermophilic enzymes in industrial hosts, and to enhance enzyme activity and stability by genetic design.

The major current industrial use for saccharidases is in the manufacture of high fructose corn syrup from starch. The current process requires thermostable enzymes and three processing steps because the enzymes used are not compatible at the same pH and temperature (see Figure 1). Industry is looking to improve the process by developing: 1) an improved α -amylase that works at low pH that has a low Ca^{++}

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Table I. Saccharidase Biotechnology

Applications:	Starch processing Baking and food processing Brewing and fermentation Forest products Detergents Specialty chemicals Waste treatment
Current Problems:	Limited uses Unstable biocatalysts Low efficiency and product yield High cost and safety
Approaches:	Increase thermostability and environmental compatibility Increase specificity and activity Develop thermophilic enzymes <ul style="list-style-type: none"> •screen for new microbes •clone and overexpress in industrial hosts •site-direct mutagenesis

requirement; and, 2) an improved glucose isomerase (GI) that works at acidic pH and at higher temperature (i.e., 70°C) so as to improve the final chemical equilibrium concentration of fructose.

Thermophilic enzymes are active and stable at high temperature (> 60°C) but they are generally inactive and extremely stable at low temperature (< 25°C). The molecular basis has not been elaborated to explain such thermophilicity. In general, thermophilic enzymes do not denature at high temperature and their activity is higher due to the Q₁₀ rule where a 10°C increase results in a doubling of chemical activity.

We have chosen to explore enzymes in thermophilic anaerobic bacteria, because these kinds of microorganisms were believed to have been the first forms of life on earth and have evolved under energy limited conditions that place stress on selection of enzymes with high catalytic efficiency (2). Thermoanaerobes contain a diverse array of enzymes with unique properties and their enzyme outfits now serve as models for understanding the biodegradation of polymers such as cellulose (3).

Our early studies dealt with characterization of cellulase from *Clostridium thermocellum* (4, 5), the first described thermoanaerobe. More recently, we have characterized the saccharidases in three new non-cellulolytic thermoanaerobic species (6-12). Table II compares the general properties of thermophilic saccharidases identified in *C. thermosulfurogenes* strain 4B (6), *C. thermohydrosulfuricum* strain 39E (7), and *Thermoanaerobacter* strain B6A (13). It is worth noting here that

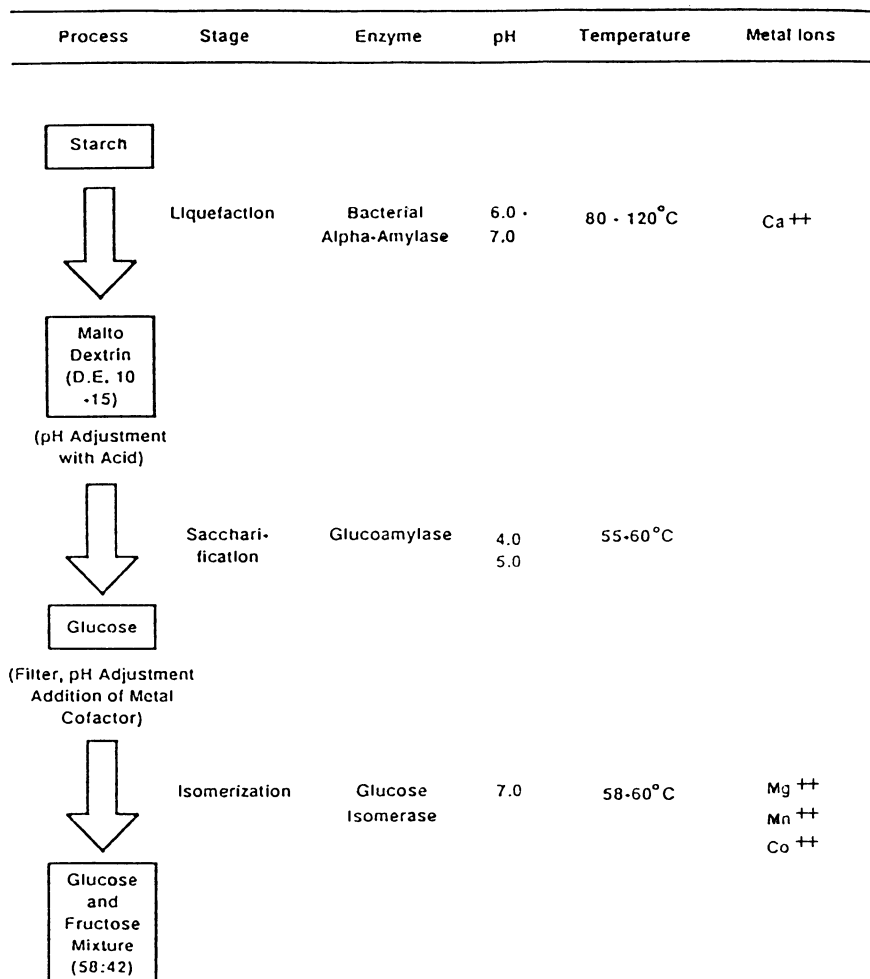


Figure 1. Schematic process biochemistry of enzymatic fructose production from starch. Reprinted with permission from ref. 1. Copyright 1990 Elsevier.

DNA/DNA hybridization studies indicate a taxonomic revision for these three species is in order. Even though these species produce spores (unpublished results) they bear no genetic similarity to *C. thermocellum*.

Table II. Novel Thermophilic Saccharidases from Thermoanaerobes

Enzyme	Optimum temperature (°C)	Optimum pH	Thermal stability (up to °C)	pH stability
<u><i>Clostridium thermosulfurogenes</i> strain 4B</u>				
β-Amylase	75	5.5 - 6.0	80	3.5 - 6.5
Glucose isomerase	80	7.0	85	5.5 - 10.0
<u><i>Clostridium thermohydrosulfuricum</i> strain 39E</u>				
Amylopullulanase	90	5.5 - 6.0	90	4.5 - 5.5
α-Glucosidase	75	4.0 - 6.0	75	5.0 - 6.0
Cyclodextrinase	65	6.0	60	5.5
Glucose isomerase	85	8.5	90	7.0 - 10.0
<u><i>Thermoanaerobacter</i> strain B6A</u>				
Amylopullulanase	75	4.5 - 5.5	70	5.0 - 6.0
α-Glucosidase	70	5.0 - 5.5	70	4.5 - 6.0
β-Galactosidase	65	6.0 - 6.5	60	5.0 - 7.0
Endoxylanase	75	5.5	70	5.0 - 7.0
β-Xylosidase	75	5.5	70	5.0 - 7.0
Cyclodextrinase	60	6.0	60	6.0
Glucose isomerase	80	7.0	85	5.5 - 10.0

This paper will review our studies on: 1) β-amylase of *C. thermosulfurogenes*; 2) α-glucosidase of *C. thermohydrosulfuricum*; 3) glucose isomerase of *C. thermosulfurogenes* and *Thermoanaerobacter* strain B6A; and, 4) endoxylanase of *Thermoanaerobacter* strain B6A. A separate paper by Saha et al., in this symposium, will report on the properties of the novel pullulanase present in these thermoanaerobes.

β-Amylase and α-Glucosidase Characterization

Table III compares the biochemical features of the β-amylase which was purified to homogeneity from *C. thermosulfurogenes* (14). The enzyme is a tetrameric glycoprotein with an apparent molecular weight of 210,000. The thermophilic β-amylase binds tightly to raw starch presumably by glycoconjugate forces and it is still active while bound to starch (15). This feature has been used for improved affinity purification of the enzyme using raw starch (16).

The β-amylase has been used to produce extremely high maltose syrups for confectionery industries and high conversion syrups used in brewing (17-19). Figure 2 shows the effect of β-amylase and pullulanase on the time course of maltose

Table III. Biochemical Features of Thermophilic β -amylase Purified from *C. thermosulfurogenes* (14)

Molecular weight	210,000
Optimum pH	6.0
Optimum temperature ($^{\circ}\text{C}$)	75
pH stability	3.5-7.0
Temperature stability ($^{\circ}\text{C}$, 1 hr)	80
Kcat (1/min)	440,000
Km for soluble starch (mg/ml)	1.68
Chemical composition	tetramer, glycoprotein
Metal ion requirement	
activity	none
stability	CA^{++}
Specific activity (U/mg protein)	4215

production from maltodextrins. To achieve extremely high maltose content (> 80%) from starch, a true debranching pullulanase that does not hydrolyze α -1,4 bonds is required to work with the β -amylase.

Table IV shows the biochemical features of thermostable α -glucosidase purified 140-fold from *C. thermohydrosulfuricum* (Saha and Zeikus, unpublished work). The enzyme has a 162,000 molecular weight and displayed an optimum temperature for activity of 75°C . Notably, the protein preparation hydrolyzed both α -1,6 and α -1,4 linkages. This enzyme appears to play an important role in starch degradation by *C. thermohydrosulfuricum* because it can hydrolyze the degradation intermediates formed by the organism's unique amylopullulanase.

Xylose-Glucose Isomerase Characterization

The regulation of glucose isomerase was studied in *Thermoanaerobacter* strain B6A (20). This species produces thermostable amylase and β -galactosidase constitutively; whereas, xylose/glucose isomerase is inducible and catabolite repressed by glucose (20). Figure 3 shows the temperature, activity, and thermostability response for these three enzymes. These thermophilic enzymes are not active at $< 30^{\circ}\text{C}$ and display optimal activity at $> 60^{\circ}\text{C}$. They are active and stable at $\geq 65^{\circ}\text{C}$ and can function together at pH 6.5 with $> 70\%$ activity for each enzyme. Because all these enzymes were produced in xylose grown cells and they displayed activities compatible at 65°C and pH 6.5, these conditions were used to develop single-step processes for production of fructose sweetener from soluble starch and maltodextrin or milk lactose (see Figure 3). Consequently, fructose sweetener production from soluble starch or milk lactose can be achieved with a single-step enzymatic process.

The xylose-glucose isomerase was purified to homogeneity from *C. thermosulfurogenes* (Lee, C.-Y and Zeikus, J. G., *Biochem. J.*, in press); and, its general

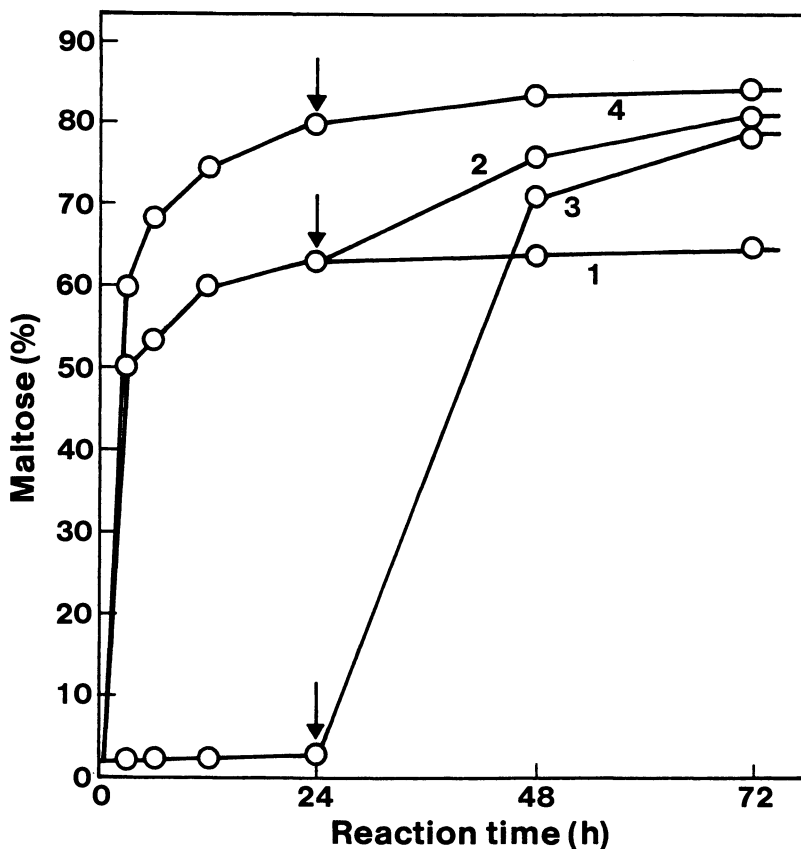


Figure 2. Time course of maltose production from maltodextrin (DE 10) by β -amylase and pullulanase: 1, β -amylase at 75°C; 2, β -amylase at 75°C for 24 h and then β -amylase and pullulanase at 60°C; 3, pullulanase at 60°C for 24 h and then β -amylases at 75°C; 4, β -amylase and pullulanase at 60°C for 24 h and then temperature raised to 75°C. Arrow indicates time of addition of second enzyme and/or changing of temperature. Enzyme used (units/g substrate); β -amylase, 200; pullulanase, 50. Reprinted with permission from ref. 18. Copyright 1989 John Wiley & Sons.

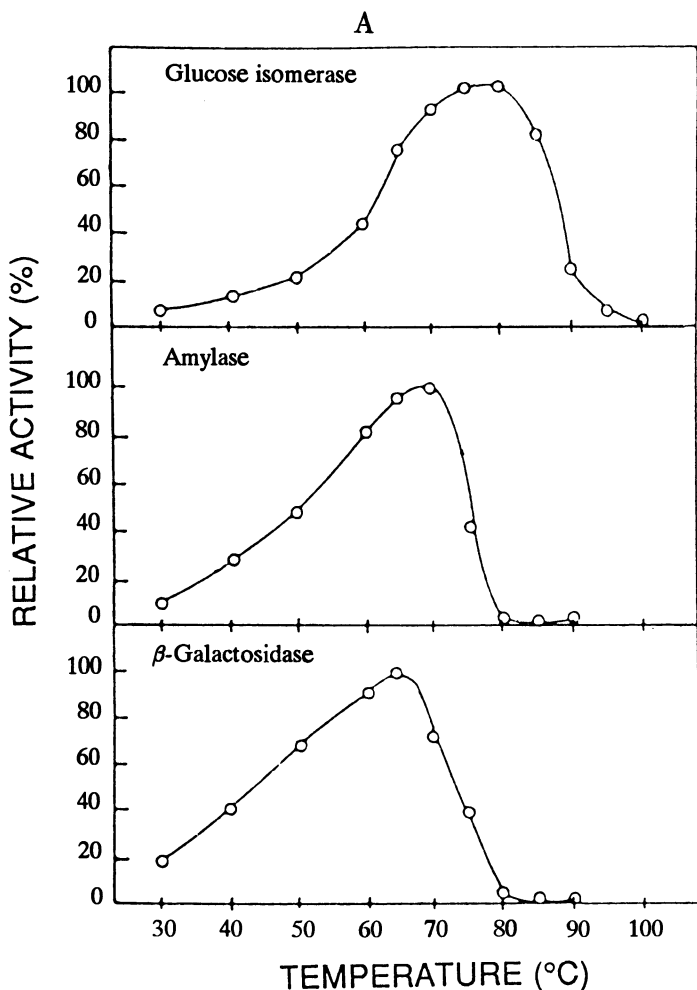


Figure 3(A). Comparison of temperature optima for activities of glucose isomerase, amylase, and β -galactosidase. Enzymes were assayed with cell extract from xylose-grown cells. A 100% activity value corresponds to 0.60, 0.58, and 0.46 U/mg for glucose isomerase, amylase, and β -galactosidase, respectively. Cell extracts in 50 mM sodium phosphate buffer (pH 7.0), 100 mM sodium acetate buffer (pH 5.5), and 100 mM sodium phosphate buffer (pH 6.0) for glucose isomerase, amylase, and β -galactosidase, respectively, were preincubated at the indicated temperatures, prior to the assay for residual enzyme activities. Reprinted with permission from ref. 20. Copyright 1990 American Society for Microbiology.

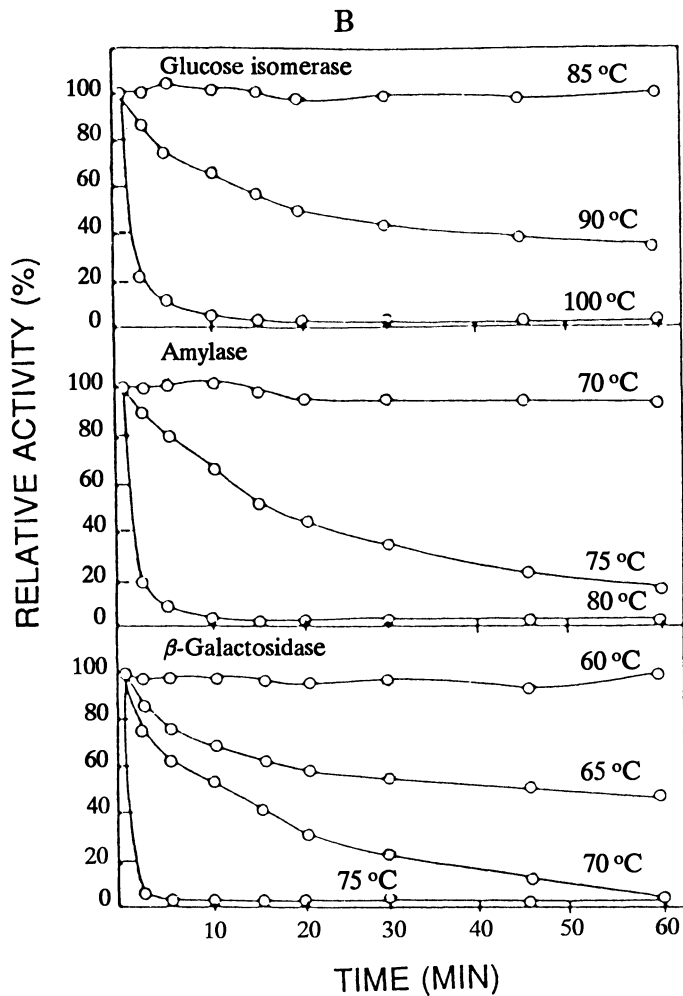


Figure 3(B). Comparison of temperature optima for stabilities of glucose isomerase, amylase, and β -galactosidase. Reprinted with permission from ref. 20. Copyright 1990 American Society for Microbiology.

Table IV. Biochemical Characteristics of Thermostable α -Glucosidase from *Clostridium thermohydrosulfuricum* 39E

Molecular weight	162,000
Optimum pH	5.0 - 5.5
Optimum temperature ($^{\circ}$ C)	75
pH stability	5.0-6.0
Km (mM)	
maltose	1.85
isomaltose	2.95
panose	1.722
maltotriose	0.58
pNPG	0.31
Metal ion requirement	
activity	none
stability	none
Half-life	
at 60 $^{\circ}$ C (h)	46
at 70 $^{\circ}$ C (min)	110
at 80 $^{\circ}$ C (min)	35
Specificity	hydrolyzes both α -1,4 and α -1,6 linkages
Inhibitor	acarbose

features are shown in Table V. The protein was a tetramer with a subunit molecular weight of 50,000. The enzyme was very thermostable and displayed an optimal activity at 80 $^{\circ}$ C. *C. thermosulfurogenes* xylose isomerase was cloned, expressed, and over-produced in both *E. coli* and food-safe *B. subtilis* (21). Heat treatment of recombinant *B. subtilis* extracts at 85 $^{\circ}$ C for 10 min enabled 90% protein purification of the thermophilic glucose isomerase. The cloned enzyme was active and stable and used to produce fructose at pH 7.0, 70 $^{\circ}$ C and with industrial saccharide concentrations (see Figure 4).

The glucose/xylose isomerase gene of *C. thermosulfurogenes* was sequenced and analyzed in relation to gene structure, function and similarity to other xylose isomerase sequences (22). Figure 5 shows that *C. thermosulfurogenes* xylose isomerase displayed higher homology to thermolabile xylose isomerase of *B. subtilis* (70%) than thermostable xylose isomerase of *Streptomyces* (24%). The putative catalytic domain of the thermoanaerobe xylose isomerase was deduced from gene sequence homologies of both *Bacillus* and *Streptomyces* xylose isomerase and indicated that His₁₀₁ was in the thermophilic enzyme active site. Table VI indicates that His₁₀₁ in the native enzyme was inhibited by diethylpyrocarbonate, but not in site-directed mutant enzymes His \rightarrow Phe₇₁ or in His \rightarrow Gln₁₀₁. Notably, the function of histidine 101 was ascertained to stabilize the transition state by hydrogen bonding and not in base-mediated ring opening for endiol formation. This biochemical evidence supports chemical/x-ray structural evidence of Collyer and Blow (23) on the

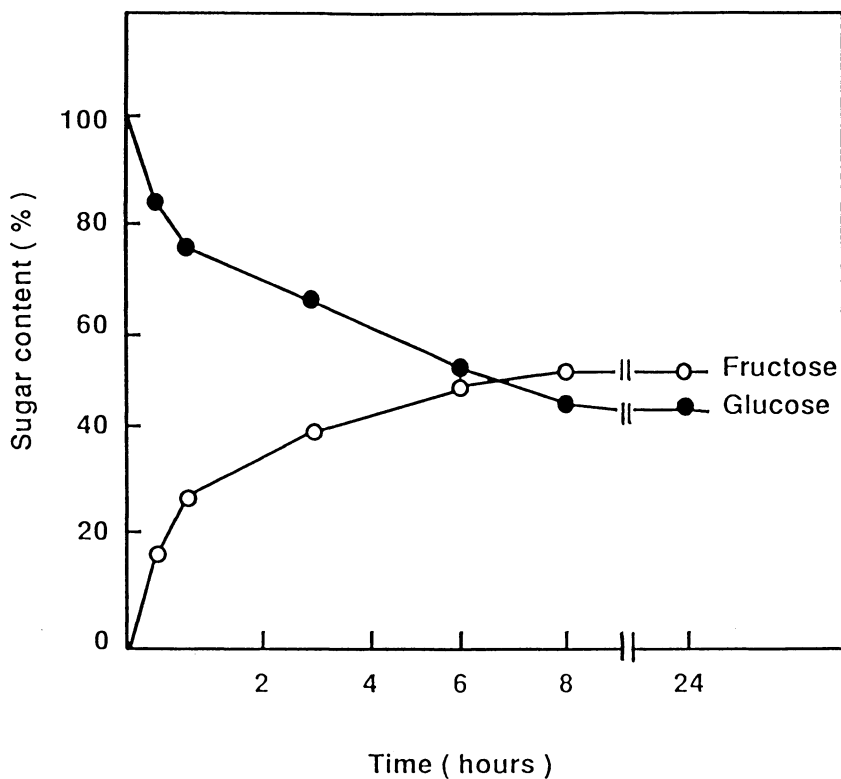


Figure 4. Time course of glucose conversion into fructose using heat-treated glucose isomerase obtained from recombinant *B. subtilis* determined at 70°C and pH 7.0 using 35% (w/w) glucose solution and 10.8 units of enzyme/g of dry substance. Reprinted with permission from ref. 21. Copyright 1990 American Society for Microbiology.

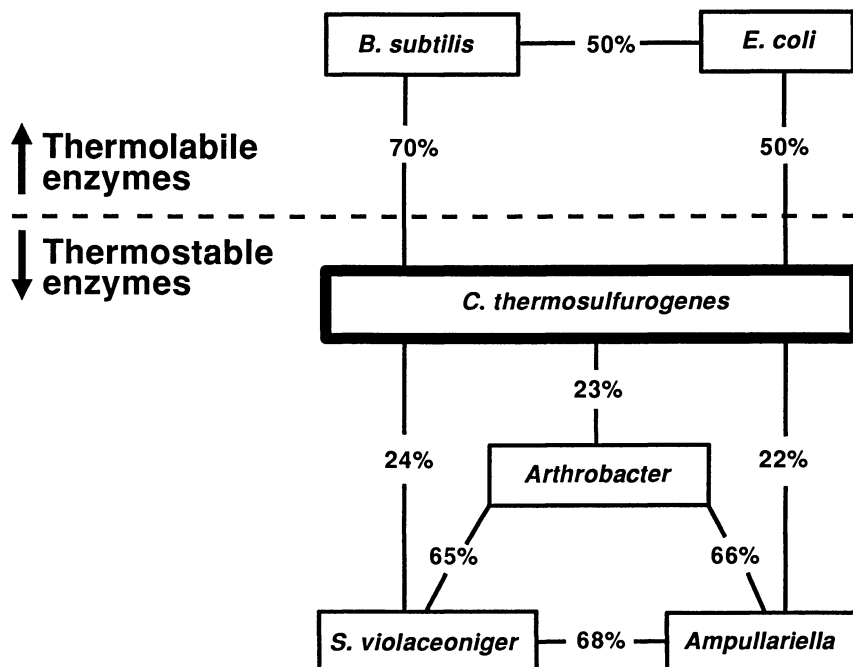


Figure 5. Summary of amino acid sequence homology between different xylose isomerases. The percent of homology was calculated by using the University of Wisconsin Genetics Computer Group, version 5, program (Devereux, I, Haeberli, P., and Smithies, O. *Nucleic Acids Res.* 12, 387-395, 1984). Reprinted with permission from ref. 22. Copyright 1990 American Society for Biochemistry and Molecular Biology.

Table V. Biochemical Characteristics of *C. thermosulfurogenes* Strain 4B Xylose/Glucose Isomerase

Property	
Molecular weight	200,000
Optimum pH	7.0
Optimum temperature (°C)	80
Temperature stability (°C, 1 hr)	85
Kcat (1/min)	1040
Isoelectric point pH	4.9
Subunit composition	tetramer
Km (mM)	
xylose	20
glucose	140
V _{max} (U/mg)	
xylose	15.7
glucose	5.2

Table VI. Effect of Diethylpyrocarbonate (DEPC) on Thermoanaerobe Glucose Isomerase Activity of the Wild-Type and Mutant Enzymes^a

Enzyme	Residual Activity (%)		
	0 mM DEPC	1 mM DEPC	10 mM DEPC
Wild-type (His ₁₀₁)	100	8	1
His → Phe ₇₁	100	10	3
His → Gln ₁₀₁	100	100	100

^aPurified enzymes (80 µg for wild-type and His-Phe₇₁ mutant enzyme and 800 µg for His-Gln₁₀₁ mutant enzyme) in 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM MgSO₄ and 0.5 mM CoCl₂ were incubated with indicated concentrations of DEPC at room temperature for 30 min. The residual glucose isomerase activity was assayed as described in Materials and Methods and expressed as percentage of specific activity found in the control without DEPC. Reprinted with permission from ref. 22. Copyright 1990 American Society for Biochemistry and Molecular Biology.

revised catalysis mechanism involving a hydride shift that is now proposed for glucose/xylose isomerase. Figure 6 shows the increased pH stability of the site-directed Gln₁₀₁ mutant over the wild-type His₁₀₁ enzyme. To date, these studies on glucose/xylose isomerase of thermoanaerobes have enabled improvement in two

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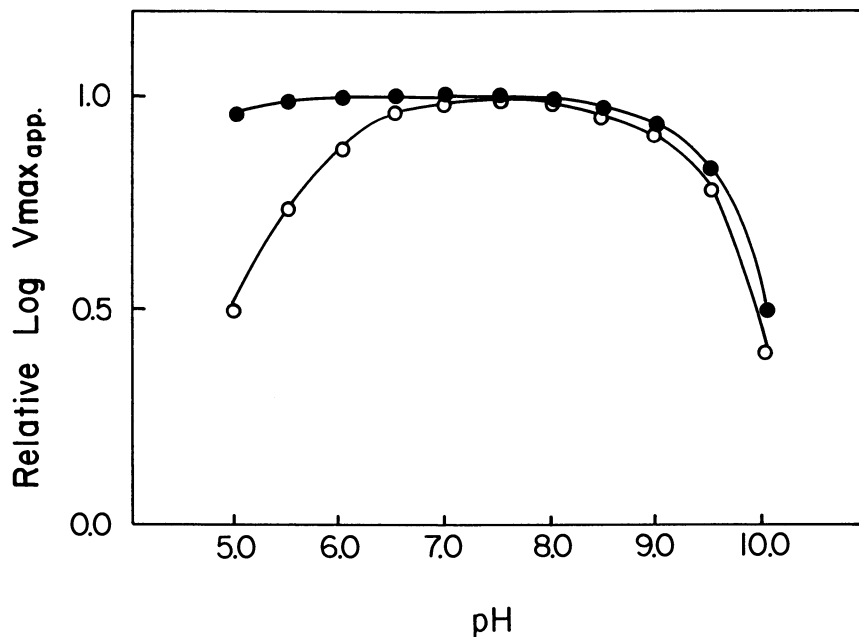


Figure 6. Plot of relative log of apparent V_{\max} versus pH for Gln₁₀₁ mutant (filled circles) and wild-type (open circles) xylose isomerases. Apparent V_{\max} values at different pH were determined from a Lineweaver-Burk plot. The scale of relative log $V_{\max,app}$ indicates the fraction of each experimental value at different pH relative to the maximal value. Both enzymes were stable under the assay conditions used. Reprinted with permission from ref. 22. Copyright 1990 American Society for Biochemistry and Molecular Biology.

industrial enzyme parameters: higher thermostability for increased fructose concentration by improved chemical equilibrium with highly thermostable glucose isomerase and higher acid stability for lower process costs and less by-product color formation with genetically engineered glucose isomerase.

Xylanase Characterization

The work to be summarized here is preliminary and will be published elsewhere (manuscripts in preparation). *Thermoanaerobacter* strain B6A produces a xylanase complex that is inducible by xylose and catabolite repressed by glucose. Noticeably, when mixed with insoluble xylan, glucose-grown cells do not bind xylan; whereas, xylose-grown cells bind very tightly. Cationized ferritin was used to identify xylanosome structures in xylose grown cells which are similar to cellulosomes observed in *C. thermocellum*. *Thermoanaerobacter* strain B6A produces thermostable cell-bound endoxylanase, which is also excreted.

A genomic library with cosmid pH79 was constructed with digested DNA from *Thermoanaerobacter* strain B6A. One xylanase clone, pXA1-H4 was obtained by screening for clear zones on Remazol brilliant blue xylan plates. This clone contained thermophilic endoxylanase activity. Heat treatment of *E. coli* HB101 containing expressed plasmid pXA1-H4 at 65°C for 30 min resulted in >50% purification of the thermophilic endoxylanase. The biochemical properties of the partially purified (90%), cloned, thermoanaerobe endoxylanase differed from the native enzyme in displaying lower thermophilicity (by 10°C) and it was not glycosylated (see Table VII). The main end products of xylan hydrolysis by the enzyme were xylose and xylobiose.

The endoxylanase gene from *Thermoanaerobacter* strain B6A was sequenced and structural interpretations were deduced from computer analysis of the amino acid sequence (see Table VIII). The endoglucanase is hydrophilic and has compact secondary structure with 30 β -turns and five potential glycosylation sites.

Potential industrial uses for thermostable xylanase include diverse food and feed manufacturing processes and pulp, paper and fiber manufacturing processes (see Table IX).

Table VII. Biochemical Properties of *Thermoanaerobacter* Strain B6A Endoxylanase Cloned in *E. coli*

Molecular weight	55,000
pH optimum	5.5
pH stability	5.0 - 6.0
Temperature optimum (°C)	60
Thermal stability (°C)	70 (< 30 min)
Metal requirements (for activity or stability)	none
Main end products from xylan hydrolysis	xylose and xylobiose

Table VIII. Structural Interpretations Deduced From Amino Acid Sequence Computer Analysis of the *Thermoanaerobacter* Strain B6A Endoxylanase Gene

-
1. The protein is hydrophilic based on overall amino acid composition.
 2. The protein contains five potential glycosylation sites.
 3. The protein is a very compact molecule with a secondary structure having > 20 β -turns.
 4. Protein thermostability may be related to glycosylation and compactness but not cysteine disulfide bonds.
 5. The enzyme is distinct from other sequenced xylanases and shows < 15% homology to others characterized including *C. thermocellum*.
-

Table IX. Potential Industrial Application Targets for Thermostable Xylanases

-
- I. Food and feed manufacture
 - A. Improved baking with high fiber materials
 - B. Clarification of fruit juices and wine
 - C. Improved nutritional and product properties of cereal fibers
 - D. Production of food thickeners
 - II. Pulp, paper, and fiber manufacture
 - A. Biobleaching of kraft pulps
 - B. Biopulping of wood
 - C. Purification of fibers for rayon manufacture
-

Direction for Future Study

We are continuing protein engineering studies on the xylose isomerase of *C. thermosulfurogenes* with the aim to: 1) redesign an enzyme via site-directed mutagenesis to be a "true" glucose isomerase (i.e., have a higher catalytic efficiency towards glucose than xylose); 2) redesign this thermophilic enzyme into a mesophilic enzyme (i.e., high activity at low temperature) via site-directed mutagenesis so as to explain the molecular basis for thermophilicity (high activity at high temperatures).

The glucose isomerase is very hydrophobic and high in alanine which may contribute to its thermophilic character.

We are continuing molecular analysis of *Thermoanaerobacter* strain B6A xylanase structure and function in relation to: 1) establishing the component enzyme basis for a xylanosomal organization; 2) to establish the putative synergistic interactions between different but potentially juxtapositioned xylanase activities; and, 3) establishing a role for glycoconjugate structure and protein secondary structure in thermophilicity. Finally, we are continuing studies on establishing process uses for thermophilic xylanases in the manufacturing of foods, feeds, paper and fibers.

Literature Cited

1. Zeikus, J. G. In *Biotechnology: Science, Education and Commercialization*; Vasil, I. K., Ed.; Elsevier Science Publishing Co., Inc.: New York, New York, 1990; pp 23-39.
2. Zeikus, J. G. *Enzyme Microb. Technol.* 1979, 1, 243-252.
3. Lamed, R.; Bayer, E.; Saha, B. C.; Zeikus, J. G. *Proc. 8th Intl. Biotech. Symp. Paris*, 1988, pp 371-383.
4. Ng, T. K.; Zeikus, J. G. *Appl. Environ. Microbiol.* 1981, 42, 231-240.
5. Ng, T. K.; Zeikus, J. G. *Biochem. J.* 1981, 199, 341-350.
6. Schink, B.; Zeikus, J. G. *J. Gen. Microbiol.* 1983, 129, 1149-1158.
7. Zeikus, J. G.; Ben-Bassat, A.; Hegge, P. W. *J. Bacteriol.* 1980, 143, 432-440.
8. Hyun, H. H.; Zeikus, J. G. *Appl. Environ. Microbiol.* 1985, 49, 1162-1167.
9. Hyun, H. H.; Zeikus, J. G. *Appl. Environ. Microbiol.* 1985, 49, 1168-1173.
10. Hyun, H. H.; Zeikus, J. G. *J. Bacteriol.* 1985, 164, 1146-1152.
11. Hyun, H. H.; Shen, G. J.; Zeikus, J. G. *J. Bacteriol.* 1985, 164, 1153-1161.
12. Hyun, H. H.; Zeikus, J. G. *J. Bacteriol.* 1985, 164, 1162-1170.
13. Weimer, P. J.; Wagner, C.; Knowlton, S.; Ng, T. K. *Arch. Microbiol.* 1984, 138, 31-36.
14. Shen, G.-J.; Saha, B. C.; Bhatnagar, L.; Lee, Y.-E.; Zeikus, J. G. *Biochem. J.* 1988, 254, 835-840.
15. Saha, B. C.; Shen, G.-J.; Zeikus, J. G. *Enzyme Microbiol. Technol.* 1987, 9, 598-601.
16. Saha, B. C.; LeCureux, L. W.; Zeikus, J. G. *Anal. Biochem.* 1988, 175, 569-572.
17. Saha, B. C.; Zeikus, J. G. *Process Biochem.* 1987, 22, 78-82.
18. Saha, B. C.; Zeikus, J. G. *Biotech. Bioeng.* 1989, 34, 299-303.
19. Saha, B. C.; Zeikus, J. G. *Enzyme Microb. Technol.* 1990, 12, 229-231.
20. Lee, C.-Y.; Saha, B. C.; Zeikus, J. G. *Appl. Environ. Microbiol.* 1990, 56, 2895-2901.
21. Lee, C.-Y.; Bhatnagar, L.; Saha, B. C.; Lee, Y.-E.; Takagi, M.; Imanaka, T.; Bagdasarian, M.; Zeikus, J. G. *Appl. Environ. Microbiol.* 1990, 56, 2638-2643.
22. Lee, C.-Y.; Bagdasarian, M.; Meng, M.; Zeikus, J. G. *J. Biol. Chem.* 1990, 265, 1362-1366.
23. Collyer, C. A.; Blow, D. M. *Proc. Natl. Acad. Sci.* 1990, 87, 1362-1366.

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Chapter 5

Mannan-Degrading Enzymes Produced by *Bacillus* Species AM-001

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An alkaliphilic *Bacillus* sp. AM-001 producing high amounts of β -mannan-degrading enzymes was isolated. Three mannanases were purified from the culture broth and characterized. A mannanase gene was cloned into *E. coli* and sequenced. Two mannanases having different C-terminal fragments were expressed from this gene in *E. coli* carrying pMAH3.

Beta-mannosidase and β -mannanase cleave the β -mannoside linkages in β -1,4-D-mannans to yield D-mannose and manno-oligosaccharides, respectively. Bacterial β -mannanases have been found in *Bacillus* (1), *Aeromonas* (2), in *Streptomyces* (3). Fungal β -mannanase have been reported by Hashimoto and Fukumoto (4), Eriksson and Winnel (5) and Civas *et al* (6). *Aspergillus niger* (7,8) and *Aeromonas* sp. (9) are known to produce β -mannosidase.

Mannans have been found in some kinds of plants such as endosperms of copra and ivory palm nuts, guar beans, locust beans, coffee beans and roots of konjak (*Amorphophallus konjac*). Most of these saccharides are used only in the food and feed processing industries. Recently, it has been reported that manno-oligosaccharides are useful as one of the best growth factors for *Bifidobacterium* sp. and *Lactobacillus* sp. Although much works has been presented on the characterization of microbial β -mannanases, little information is available about the industrial application of these enzymes.

There is no information so far available about mannan degrading enzymes of alkaliphiles. In this paper, a β -mannan degrading alkaliphile (*Bacillus* sp. AM-001) with high productivities of cell-associated β -mannosidase and three extracellular β -mannanases was isolated from soil. These three β -mannanases differed in several enzymatic properties including optimum pH, optimum temperature, pH stability, isoelectric point and molecular weight. To elucidate the genetic basis for production of multiple forms of β -mannanase by the strain, we cloned one β -mannanase gene of this strain into *Escherichia coli* using pUC19 as a vector, and characterized two different β -mannanases expressed from this gene in *E. coli*.

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Isolation of Mannan Degrading Microorganisms (10)

A small amount of soil was spread on agar plates containing 1% β -mannan from larchwood, 1% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5% sodium carbonate. The plates were incubated at 37°C for 48-72 h. Strain AM-001 with a large clear zone around the colony was selected as the enzyme producer. The isolate grew at temperatures from 20°C to 45°C, with an optimum at 37°C in the medium described above. The pH range for growth was from pH 7.5 to 11.5 with an optimum at pH 8.5 to 9.5. The bacterium was aerobic, motile and gram-variable; the rod-shaped cells (0.6-0.8 μm x 3.0-6.0 μm) had peritrichous flagella and terminal swollen sporangia containing oval spores (1-1.2 μm x 1.5-2.0 μm). The taxonomical characteristics of this alkaliphilic *Bacillus* strain were almost the same as those of *Bacillus circulans* except for the pH range for growth.

Culture Conditions for Enzyme Production

Bacillus sp. AM-001 was cultivated aerobically under various conditions, and activities of β -mannanase in the culture broth and β -mannosidase extracted from the cells treated with 0.1% Triton X-100 were monitored. Both enzymes were formed when the bacterium was grown under alkaline conditions in the presence of optimal concentrations of 0.5% Na_2CO_3 or 0.5-1.0% NaHCO_3 . Various carbohydrates were also tested and the best carbohydrate for enzyme production was konjak powder (1% w/v). The optimal cultivation temperature for enzyme production was 31°C for β -mannosidase and 37°C for β -mannanase in a production medium composed of 1% konjak powder, 0.2% yeast extract, 2% Polypeptone, 0.1% K_2HPO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5% sodium carbonate. The crude enzyme preparation showed optimum activity of β -mannosidase at pH 7.0 and 55°C and of β -mannanase at pH 9.0 and 65°C.

Purification of β -mannanase and Its Properties(11)

Three extracellular β -mannanases (M-I, M-II and M-III) were purified by ammonium sulfate precipitation (80% saturation) followed by chromatography on a DEAE-Toyopearl 650 M column (4.6 x 35 cm) equilibrated and eluted with 0.01 M phosphate buffer (pH 7.0) and by a hydroxylapatite column (1.6 x 25 cm). As shown in Fig. 1, two active fractions (Fraction 1 and 2) were detected after hydroxylapatite chromatography. Each fraction 1 and 2 was applied onto a Sephacryl S-200 column (2.6 x 90 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and eluted with the same buffer. Mannanase-I and -II were isolated from fraction 1 and mannanase-III was from fraction 2.

Polyacrylamide gel electrophoresis revealed that these three mannanases were electrophoretically homogeneous. The molecular weights estimated by SDS-PAGE were 58,500 for M-I, 59,500 for M-II and 42,000 for M-III. As shown in Fig. 2, β -mannanase M-I and M-II were most active at pH 9.0 and M-III demonstrated optimal enzyme action at pH 8.5. Each of these enzymes hydrolyzed β -1,4-mannooligosaccharides larger than mannotriose and the major components in the digest were di-, tri- and tetra-saccharides.

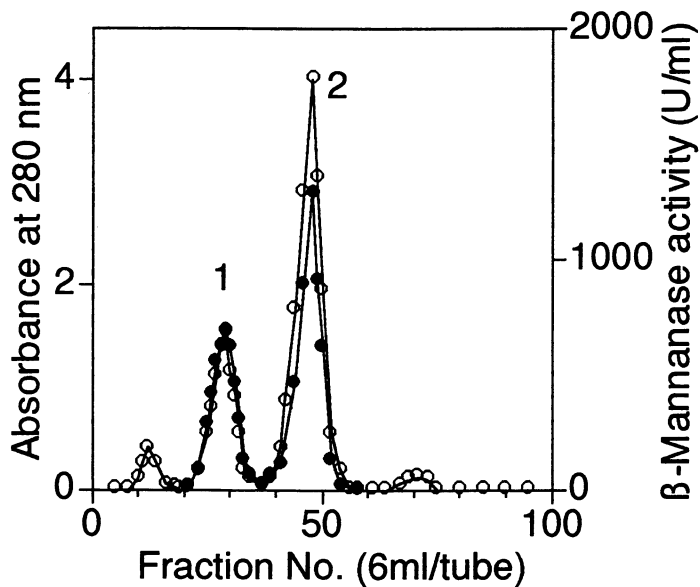


Fig. 1. Chromatography of the β -mannanases with Hydroxyapatite Column.

Symbols: (●), β -mannanase activity; (○), $A_{280\text{nm}}$

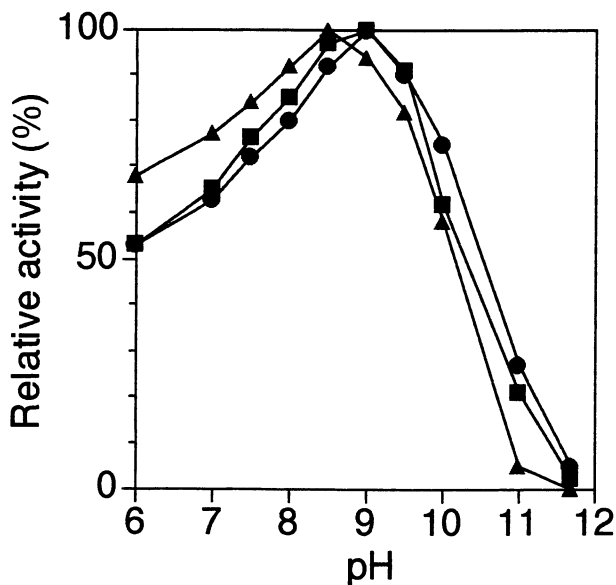


Fig. 2. Effects of pH on Enzyme Activity.

Symbols: (●), β -mannanase M-I; (■), β -mannanase M-II; (▲), β -mannanase M-III.

Purification of β -mannosidase and Its Properties(12)

The cells were cultured for 24h in the medium described above and collected by centrifugation. The β -mannosidase was extracted from the cells in the presence of 0.1% (w/v) Triton X-100. After 2 h of shaking the insoluble debris was removed by centrifugation and the supernatant fluid was used as the crude enzyme solution. The crude enzyme solution was dialyzed against 0.1 M phosphate buffer (pH 7.0) followed by DEAE-Toyopearl 650M column chromatography. The enzyme was eluted using a linear gradient of NaCl concentration from 0 to 0.5 M in the same buffer described above. The enzyme fractions were dialyzed against the same buffer and applied onto a D-mannose Sepharose 6B affinity column. The enzyme was eluted by phosphate buffer containing 5% (w/v) D-mannose and finally purified by passing through a Sephadex G-150 column. Table I summarizes the purification process used. The purified enzyme was electrophoretically homogenous and its molecular weight was estimated to be 94,000. Isoelectric focusing showed the isoelectric point to be 5.5.

The purified enzyme was most active at pH 6.0 and stable at 40°C for 30 min in the pH range of 6.5 to 8.0. The enzymatic activity was strongly inhibited by the addition of metal ions and some chemicals but not by D-mannose as shown in Table II and III. Among of the synthetic substrates so far tested, the enzyme did not hydrolyze any of them except p-nitrophenyl- β -D-mannopyranoside (Km value was 1.3 mM). The enzyme hydrolyzed β -1,4-mannooligosaccharides and the best substrate was mannotetraose. Weak transferase activity was detected after a long incubation period.

The molecular weight (94,000) was smaller than that of other microbial β -mannosidases such as *Aspergillus oryzae* (120,000-135,000) and *Tremella fuciformis* (160,000-200,000). The final product from the oligosaccharides with the enzyme was mainly D-mannose and the enzymatic activity was not inhibited by D-mannose or mannose-derivatives such as D-mannosamine, D-mannonic acid and D-mannitol. These properties of the enzyme are good for the production of D-mannose from β -mannan in the presence of the β -mannanase described above.

Molecular Cloning of Mannanase Gene (13)

As described in the previous section, this strain produced significant amounts of three extracellular β -mannanases and a cell-associated β -mannosidase. The three β -mannanases differed in several enzymatic properties including optimum pH for enzyme action, optimum temperature, pH stability, thermal stability, isoelectric point and molecular weight. To elucidate the genetic basis for production of multiple forms, we tried to clone the β -mannanase genes of this strain into *E.coli* using pUC19 as a vector.

Alkaliphilic *Bacillus* sp. AM-001 was aerobically grown to the early stationary phase at 37°C in alkaline medium (pH 9.0) containing 1% konjak mannan. Total chromosomal DNA obtained by the method of Saito and Miura(14) was digested with *Hind*III restriction enzyme. And 2 to 4 kbp DNA fraction of chromosomal DNA was collected by 1% agarose gel electrophoresis. The plasmid pUC19 was digested with *Hind*III and then dephosphorylated with bacterial alkaline phosphatase. After the digestion 1 μ g of plasmid and 5 μ g of chromosomal DNA were mixed and ligated with T4 DNA ligase for 30 min at 16°C. Ligation mixtures described above were used to transform *E. coli* JM101. Transformants having mannanase activity could be detected directly on the LB-konjak plates containing ampicillin (50 μ g/ml). Among

Table I. Purification of β -mannosidase

	<i>Total activity</i> (U)	<i>Protein</i> (mg)	<i>Specific activity</i> (U/mg)	<i>Purification</i> (fold)	<i>Yield</i> (%)
Cells	850				100
Triton X-100 extract	410	2,880	0.14	1.0	48
DEAE-Toyopearl	196	46.8	4.19	29.9	23
D-mannose-epoxy-activated Sepharose 6B	103	3.0	34.3	245	12
Sephadex G-150	62	1.6	38.8	277	7.3

Table II. Effects of Various Metal Ions on β -mannosidase Activity

<i>Metal ions</i> (1mM)	<i>Residual activity</i> (%)
None	100
Ag ⁺	0
Cd ²⁺	0
Cu ²⁺	0
Zn ²⁺	0
Hg ²⁺	0
Fe ²⁺	5
Fe ³⁺	100
Pb ²⁺	94
Co ²⁺	96
Mg ²⁺	100
Sn ²⁺	100
Mn ²⁺	100

Table III. Effects of Various Reagents

<i>Reagent</i>		<i>Residual activity</i> (%)
None	20 mM	100
D-mannosamine HCl	20 mM	100
D-mannonic acid- γ -lactone	20 mM	100
N-Acetyl glucosamine	20 mM	100
D-mannose	20 mM	100
D-mannitol	20 mM	100
Nojirimycin	0.0025%	91
Phenylmethylsulfonyl fluoride	0.1 mM	99
Monoiodoacetate	1.0 mM	98
Mercaptoethanol	1.0 mM	95
Cystein	1.0 mM	92
<i>p</i> -Chloromercuribenzoate	0.1 mM	0
N-Bromosuccinimide	1.0 mM	0
Na-dodecyl sulfate	0.1%	0

5×10^4 colonies one colony was surrounded by a large transparent halo and this colony showed β -mannanase activity in liquid cultivation using LB medium. The plasmid harbored by the transformant was designated as pMAH1. After subcloning with *Xba*I treatment, a plasmid pMAH3 containing a 2.5 kbp fragment of *Bacillus* sp. AM-001 DNA was isolated. The restriction map of this plasmid is shown in Fig. 3. The chromosomal origin of the 2.5 kbp DNA insert was confirmed by the Southern hybridization method. The pMAH3 DNA hybridized to DNA of *Bacillus* sp. A-001 but not to *E. coli* DNA. The *Xba*I-*Hind*III fragment cloned in pUC19 contained a promoter since β -mannanase activity was also seen when this fragment was inserted in the opposite orientation in pUC19.

Properties of a Mannanase Produced by *E. coli* JM101 carrying pMAH3

E. coli JM101 (pMAH3) was grown aerobically in LB broth for 24 h at 37°C. The β -mannanase activity was located mainly in the periplasmic (53%) and intracellular (43%) fractions. No induction of the enzyme could be detected in the presence or absence of mannan, such as konjak, locust bean and copra, in the growth medium. The periplasmic β -mannanase was purified by affinity chromatography and two active fractions (β -mannanase A and B) were separated. Molecular weights were 58,000 for A and 43,000 for B. As shown in Table IV, the mannanase A has enzymatic properties similar to those of β -mannanase M-I and M-II of *Bacillus* sp. AM-001, and mannanase B properties are similar to those of β -mannanase M-III. Furthermore, the Ouchterlony double diffusion test showed that these five enzymes gave fused precipitation lines. However, N-terminal amino acid sequences of the five mannanases determined by an automatic amino acid sequencer revealed that the N-terminal amino acid sequence from amino acid 1 (Asn) to 9 (Gln) of the *Bacillus* sp. AM-001 enzymes coincides with those from amino acid 4 (Asn) to 12 (Asn) of the *E. coli* JM101 (pMAH3) enzymes as shown in Fig. 4. This may reflect differences in the specificities of the signal peptidases of the two bacteria.

Nucleotide Sequence of the Mannanase Gene

Because the mannanase activity was observed in the *Xba*I-*Pst*I fragment, its nucleotide sequence was studied. The DNA sequence and the deduced amino acid sequence are shown in Fig. 4. There was a single open reading frame of 1,539 bp which encoded a polypeptide of 513 amino acids. A putative ribosome binding site, a GGAGGA sequence which highly complemented the 3' end of *B. subtilis* 16S ribosomal RNA, was observed upstream of the open reading frame. A signal peptide of 26 amino acids was cleaved during the secretion process of *E. coli*, and 29 amino acids were removed in the case of *Bacillus* sp. AM-001. As both enzyme molecules had β -mannanase activity, Ser-Glu-Ala in the N-terminal fraction is not essential for the enzyme activity. As described above, *E. coli* JM101 harboring plasmid pMAH3 produced two β -mannanases, although it showed no capacity to encode two open reading frames. To elucidate this point, C-terminal sequences of both enzymes were analyzed after tryptic digestion. The smaller enzyme (mannanase B) had a C-terminal fragment of Gly-Glu-Ile-Asp-Tyr-Gly-Gln-Ser-Asn-Pro-Ala-Thr-Val-COOH consisting of 339 amino acids. The larger was Leu-Asp-His-Val-Thr-Val-Arg-COOH consisting of 487 amino acids. Why were two protein molecules produced from one open reading frame? One possibility is processing by protease. However, analysis of the concentration of the two enzymes during cultivation of *E. coli* JM101 (pMAH3) indicated no significant change. Another possibility is that two transcripts (two

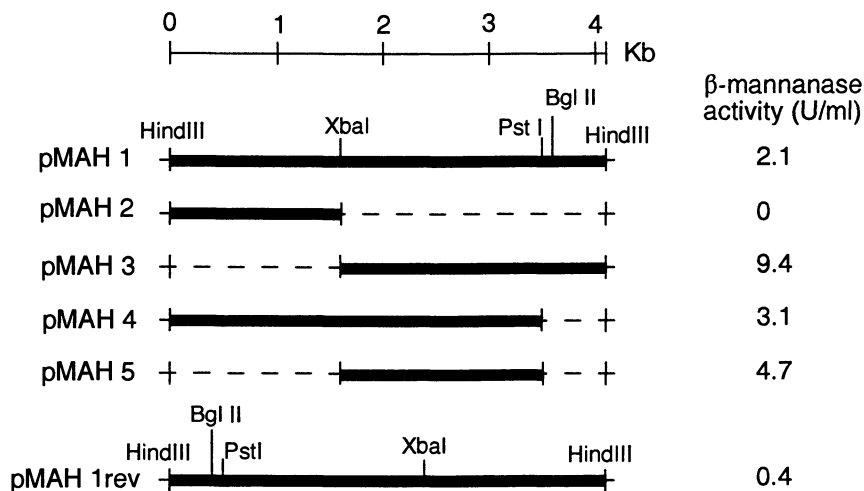


Fig. 3. Restriction Maps of pMAH1 and Its Derivatives.
Deleted areas are indicated with dotted lines.



Fig. 4. Nucleotide sequence of the XbaI-PstI fragment containing β -mannanase gene(s). Symbols: (B), N-terminal of three β -mannanases produced by alkaliphilic *Bacillus* sp. A-001; (E) N-terminal of two β -mannanases isolated from *E. Coli* carrying pMAH3; (MA), C-terminal end of the mannanase A; (MB), C-terminal end of the mannanase B. (Continued on the next page.)

550 600
 CTACAGAACAAATGGAGTGACGGTCTTTTCCGCTCTTTACATGAATGAATGGAGAATGGTCTGGTGGGGAGCAGAAGGTTACAATCAA
 LeuGlnAsnAsnGlyValThrValLeuPheArgProLeuHisGluMetAsnGlyGluTrpPheTrpTrpGlyAlaGluGlyTyrAsnGln
BclI (200)
 650 700
 TTTGATCAAACACGTCGCAATGCCTATATCAGCGCATGGAGAGATATGTATCAATATTTTACTCATGAGCGTAAGCTGAATAACCTTATT
 PheAspGlnThrArgAlaAsnAlaTyrIleSerAlaTrpArgAspMetTyrGlnTyrPheThrHisGluArgLysLeuAsnAsnLeuIle

 750 800
 TGGGTTTACTCACCTGATGTTTACAGAGATCATGTAACAAGTTACTACCCAGGAGCAAATTATGTAGATATTGTGGCTCTTGATTCTCTAC
 TrpValTyrSerProAspValTyrArgAspHisValThrSerTyrTyrProGlyAlaAsnTyrValAspIleValAlaLeuAspSerTyr

 850 900
 CATCCTGATCCACATAGCCTTACTGACCAATATAATCGAATGATCGCTTTAGATAAACCTTTTGGCTTTGTGAAATCGGTCCTCCTGAA
 HisProAspProHisSerLeuThrAspGlnTyrAsnArgMetIleAlaLeuAspLysProPheAlaPheAlaGluIleGlyProProGlu
 (300)
 950
 AGCATGGCTGGTTCCCTTGTATTATCAAATATATTTCAAGCAATTAACAAAAATATCCACGTAAGTGTCTATTTCCTAGCTTGGAAATGAT
 SerMetAlaGlySerPheAspTyrSerAsnTyrIleGlnAlaIleLysGlnLysTyrProArgThrValTyrPheLeuAlaTrpAsnAsp

 1000 1050
 AAATGGAGTCCACATAACAACAGAGGAGCATGGGATCTATTAATGATTCATGGGTTGTAATAGCGGAGAGATTGATTATGGTCAATCA
 LysTrpSerProHisAsnAsnArgGlyAlaTrpAspLeuPheAsnAspSerTrpValValAsnArgGlyGluIleAspTyrGlyGlnSer
HflI (300)
 1100 1150 1150 1200
 AATCCAGCCACTGTTCTCTAIGATTTTGA AAAACAATACGCTATCGTGGTCCGGGTGTGAATTTACGGACGGAGGACCATGGACTTCGAAT
AsnProAlaThrValLeuTyrAspPheGluAsnAsnThrLeuSerTrpSerGlyCysGluPheThrAspGlyGlyProTyrThrSerAsn
NcoI HspV
 MB 1200 1250
 GAATGGTCGGCAAAATGGTACTCAATCGTTGAAAGCAGATGTCGTTCTGGGCAATAATAGCTACCATTTCCAAAAACAGTGAATCGAAAT
 GluTrpSerAlaAsnGlyThrGlnSerLeuLysAlaAspValValLeuGlyAsnAsnSerTyrHisLeuGlnLysThrValAsnArgAsn
 (400)
 1300 1350
 CTTAGTTCATTCAAAAACCTAGAAAATTAAGTGAGCCATTCTTCGTGGGGAATGTAGGAAGTGGCATGACAGCAAGAGTTTTCGTCAA
 LeuSerSerPheLysAsnLeuGluIleLysValSerHisSerSerTrpGlyAsnValGlySerGlyMetThrAlaArgValPheValLys

 1400
 ACAGGGAAGTGGTGGAGATGGAATGCAGGTGAATTTTGTGAGTTGCAGGCAACGAACAACCCACTATCTATTGATTGACGAAAGTA
 ThrGlySerAlaTrpArgTrpAsnAlaGlyGluPheCysGlnPheAlaGlyLysArgThrThrAlaLeuSerIleAspLeuThrLysVal

 1450 1500
 AGTAATCTGCATGATGTTCCGAGAGATAGGTGTAGAGTATAAAGCACCAGCAAATAGCAACGGGAAGACGGGATTTACTTAGATCATGTG
 SerAsnLeuHisAspValArgGluIleGlyValGluTyrLysAlaProAlaAsnSerAsnGlyLysThrAlaIleTyrLeuAspHisVal
NdeI (500)
ACCGTAAGATAATACAAAAAAAGTGGTTGAAAGCGGTAAACATATCTAGCATATGATGATAGGGACTAGATAAATAAGACTGTGAGACT
ThrValArg***
 MA
AGGAGGTAAGTCATAATGAAAAAAGTCTGATCTCTTGTCTGGACTTTTATTAGCTTTCTCCATGCTATTAAATAGCCTATCTATCATTG

PstI
 ACCCCTGCAG 1720

Fig. 4 (continued). Nucleotide sequence of the XbaI-PstI fragment containing β -mannanase gene(s). Symbols: (B), N-terminal of three β -mannanases produced by alkaliphilic *Bacillus* sp. A-001; (E) N-terminal of two β -mannanases isolated from *E. Coli* carrying pMAH3; (MA), C-terminal end of the mannanase A; (MB), C-terminal end of the mannanase B.

Table IV. Properties of the Purified β -mannanases from *Bacillus* sp. AM-001 and *E. coli* carrying pMAH3

	AM-001			<i>E. coli</i> (pMAH3)	
	M-I	M-II	M-III	A	B
Optimum temp.	60	60	65	60	65
Optimum pH	9.0	9.0	8.5	9.0	8.5
Thermal stability	50	50	60	50	60
pH stability	8-9	8-9	7-9	8-9	7-9
Molecular weight	58,500	59,500	42,000	58,000	43,000

messenger RNAs) are produced from one open reading frame. We have not analyzed the m-RNA, but two transcripts may be produced as shown in other microorganisms.

Literature cited

1. Emi, S.; Fukumoto, J.; Yamamoto, T. *Agric. Biol. Chem.* **1972**, *36*, 991-1001.
2. Araki, T. *J. Fac. Agr. Kyushu Univ.* **1983**, *27*, 89-98.
3. Takahashi, R.; Kusakabe, I.; Kobayashi, H.; Murkami, K.; Maekawa, A.; Suzuki, I. *Agric. Biol. Chem.* **1984**, *48*, 2189-2195
4. Hashimoto, Y.; Fukumoto, J. *Nippon Nogeikagaku Kaishi*, **1969**, *43*, 317-322.
5. Eriksson, K. E.; Winnel, M. *Acta Chem. Scand*, **1968**, *22*, 1924-1934.
6. Civas, A.; Bverhard, D. P. L.; Petek, F. *Biochem. J.*, **1984**, *219*, 857-863.
7. Hashimoto, Y.; Fukumoto, J. *Nippon Nogeikagaku Kaishi*, **1969**, *43*, 564-569.
8. Bouquelet, S.; Spik, G.; Montreuil, J. *Biochim. Biophys. Acta*, **1978**, *552*, 521-530.
9. Araki, T.; Kitamikado, M. *J. Biochem.*, **1982**, *91*, 1181-1186.
10. Akino, T.; Nakamura, N.; Horikoshi, K. *Appl. Microbiol. Biotechnol.*, **1987**, *26*, 323-327.
11. Akino, T.; Nakamura, N.; Horikoshi, K. *Agric. Biol. Chem.*, **1988**, *52*, 773-779.
12. Akino, t.; Nakamura, N.; Horikoshi, K. *Agric. Biol. Chem.*, **1988**, *52*, 1459-1464.
13. Akino, T.; Kato, C.; Horikoshi, K. *Arch. Microbiol.*, **1989**, *152*, 10-15.
14. Saito, H.; Miura, K. *Biochim. Biophys. Acta*, **1963**, *72*, 619-626.

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Chapter 6

Proteinases and Their Inhibitors in Biotechnology

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Proteases are used in many industrial areas as well as basic research. They are classified by their mechanism of catalysis. Proteases are used in the pharmacological, food and other consumer industries to convert raw materials into a final product or to alter properties of the raw material. In biomedical research, proteases are used to study the structure of other proteins and for synthesis of peptides. The choice of a protease for an application depends in part on its specificity for peptide bonds, activity and stability. Technical advances in protein engineering have enabled alteration of these properties and allowed proteases to be used more effectively. Some easily obtained proteases can be modified so that they can substitute for proteases whose supply is limited.

For approximately a century, scientists have understood that there are proteins present in nature which are capable of enzymatically hydrolyzing the peptide bonds of other proteins. During this century a great deal of effort has gone into understanding the biochemical properties of these proteolytic enzymes and their mechanism of action. Over this period a vast amount of information has been introduced to the literature which has given an organized perspective of the these enzymes, including their physiological, biochemical and physical properties. Based on these principles, investigators in almost all fields of scientific research have introduced the use of these enzymes into their own area of study.

With the advent of recombinant DNA technologies, protein chemists have the possibility to engineer new proteins with altered biological or physical properties which better suit their particular requirements. However, this potential for protein engineering has also given rise to a practical need for a much more detailed understanding of protein folding, structure, and biophysics if the rational engineering of proteins is to be undertaken in the future.

In this chapter, we wish to present the reader with an introduction to proteolytic enzymes, and some of their current and potential uses in various fields. It is our hope that this chapter will stimulate readers to consider proteolytic enzymes for biomass conversion as well as other possible uses.

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General Background On Proteolytic Enzymes

In recent years, the nomenclature of proteolytic enzymes has been more strictly defined such that proteases/peptidases are generally considered to be those enzymes which are capable of hydrolyzing peptide bonds (1). The proteases can be subdivided further into two classes; the proteinases (equivalent to endopeptidases) and exopeptidases (2). The proteinases are peptidases which cleave peptide bonds distant from the amino or carboxy termini of the substrate whereas exopeptidases cleave peptide bonds proximal to a terminus of the substrate. Another important point of terminology when discussing proteases is the complementarity of the site(s) at which a substrate is cleaved and the substrate binding site(s) of the protease. The terminology of Berger and Schechter (3) is currently the standard of use. In this format the enzyme's substrate binding sites are denoted $S_n-S_3-S_2-S_1-S_1'-S_2'-S_3'-S_n'$ (the exact number depending upon the enzyme). These positions correspond to the $P_n-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_n'$ positions of the substrate. The site of cleavage is between the P_1-P_1' position of the substrate.

Classification of the Proteases. The classification of the proteases is based on their mechanism of catalysis (4). The four primary classes of proteases are the serine, aspartic, cysteine, and metalloproteases (5). This classification is based on the primary functional group found in the enzyme's active site. There are likely to be other proteases eventually characterized which will not precisely fit into this categorization scheme and additional categories will be needed. One example of a potential new category is the ATP-dependent proteinases (6), a group of proteinases which require ATP for activity.

Serine Proteinases. This group of proteinases is the best known because they are more numerous and better characterized than the other three groups. These proteinases are found in virtually all organisms, indicative of their importance and wide ranging proteolytic capabilities. They demonstrate broad substrate specificities with the S_n sites (amino terminal to the scissile bond) generally being more important in enzyme interaction.

The group is typically divided into two families: the subtilisin-like and chymotrypsin-like groups (7). The chymotrypsin-like group are represented by members in all organisms whereas the subtilisin-like members are found in only prokaryotes. Although both families have dissimilar primary and tertiary structures they share a common catalytic mechanism (8,9).

The mechanism of catalysis by these enzymes has been extensively investigated (for review see ref. 10). Essentially, the active site serine via its side chain hydroxyl group performs a nucleophilic attack on the carbonyl carbon of the scissile peptide bond thus forming a tetrahedral intermediate. A histidine residue in the active site serves as a general base accepting the proton from the serine residue. The acyl enzyme thus formed is broken down via a nucleophilic attack of a water molecule to complete the hydrolysis of the peptide bond.

Aspartic Proteinases. This group of proteinases is named for the aspartic acid residue in the active site. Previously, this group of enzymes was often referred to as the "acid proteases" (4). Members of this group are generally found only in eukaryotic organisms. However, clear evidence has been presented that certain viruses, most importantly the virus (HIV-1) considered to give rise to autoimmune deficiency disease (AIDS), and the polio virus, contain coding sequences for a dimeric aspartic proteinase which is involved in the

processing of the viral polypeptide (11-14). Obviously, this group of proteinases, due to their potential role in these disease states has received a great deal of renewed attention.

The mechanism of the aspartic proteinases involves two essential catalytic aspartate residues. There is some controversy in the literature as to whether the mechanism involves an acyl enzyme intermediate or an amino enzyme intermediate (4). However, there is no direct evidence for either intermediate so additional studies with inhibitors and pseudosubstrates along with crystallographic analysis will ultimately be required to resolve these questions.

Metalloproteases. This is an extremely interesting group of proteases which depend, in part, on Zn^{+2} in the active site. This group of proteases is conveniently divided into two families, one family being exopeptidases and the other endopeptidases. Examples of the exopeptidase group include carboxypeptidase A and B and angiotensin converting enzyme. The endopeptidase group includes enzymes from diverse origins such as collagenases from higher organisms and thermolysin from bacteria and hemorrhagic toxins from snake venoms (15-18). Many of these metalloproteinases have been demonstrated to play important physiological roles. One interesting example is the role certain metalloproteinases play in metastatic tumor cell migration (19,20).

It is of interest to note that all members of the endopeptidase family sequenced to date share a consensus site (-His-Glu-X-X-His-) for the binding of the zinc ion in the active site (21). Typically the metalloproteases are found with only the zinc ion bound to the consensus site; however, *in vitro* several other divalent cations can replace the zinc ion (Co^{+2} often is able to restore native enzyme activity levels)(22,23). In certain cases the metal ion has also been shown to play a structural role as well as a catalytic role (24).

The mechanisms of action of the metalloproteases are possibly somewhat different for the two families in this group. A significant amount of data has been accumulated for thermolysin, representing the endopeptidase family, which indicates that the mechanism begins by a general base catalysis with glutamate 142 initially attacking a water molecule that is polarized by the Zn^{+2} ion in the active site. In the case of carboxypeptidase (a member the exopeptidase family) there is evidence which suggests that the mechanism may follow a direct nucleophilic catalytic process with the active site glutamate (5).

Cysteine Proteinases. Cysteine proteinases are found in both eukaryotes and prokaryotes. Based upon sequence homology studies, there are at least four different subgroups of this class (25). Many of these enzymes are associated with intracellular vacuoles or lysosomes of cells. The sequestering of these enzymes is perhaps based upon their intrinsic potential to become readily oxidized to an inactive state. A very interesting subgroup of these proteins is the calcium dependent cysteine proteinases which require varying concentrations of Ca^{+2} for activity. The role which the calcium ion plays is probably an alteration of the proteinase's conformation upon binding and thus altering the activity of the enzyme. The calcium ion does not appear to play a direct role in catalysis (26,27).

The catalytic mechanism in this class is based upon similar chemical principles as the mechanism of the serine proteinases. A cysteine residue in the active site is activated by a histidine imidazolium side chain and carries out a nucleophilic attack on the carbonyl carbon of the scissile peptide bond with the complex going through an acyl intermediate transition state (28,29). Certain members of this class of enzymes have pH optima in the acidic range and

therefore these enzymes are very useful for reactions which must be carried at a low pH (30).

Inhibitors of Proteinases. A brief mention must be made regarding the inhibition of proteinases. Generally, inhibition of proteinases can be divided into two mechanisms: 1) *in vitro* chemical modification of an active site residue (or a modification of a residue distal to the active site which alters the structure of the active site); 2) *in vivo* inhibition of the protease based upon the binding to the enzyme of an endogenous protein inhibitor which may mask or inactivate the active site. It is interesting that natural inhibitors of proteinases have apparently evolved along with the proteases such that sensitive physiological control of proteases is possible.

Sources of Proteinases. More than 80 per cent of all industrial enzymes are hydrolytic in action and most are used for the depolymerization of natural substances. Almost 60 per cent of these enzymes are proteolytic, and used by the detergent, dairy and leather industries.

There are 12 categories of enzymes used in industrial processing, with 30 different activity types in common use. When it is considered that several thousand different enzymes have been identified and characterized, the future for new processing aids is promising (31).

While almost any living organism can be considered a potential source of useful enzymes, in practice a limited number of plant and animal tissues are economic sources and the greatest diversity comes from microorganisms and recombinant products.

Plant Proteinases. These include the well known proteases papain, bromelain and ficin. Most plant enzymes are available as comparatively unpurified powder extracts, although papain is notable for being available in a stabilized and purified liquid form. Prospects for increased supply of plant enzymes, in response to greater use in traditional applications or for new processes, depend on several factors. The influence of cultivation conditions, growth cycle and climate requirements make new supplies long term projects.

Animal Proteinases. These include the pancreatic trypsin and the rennets, which are produced in both ultrapure and industrial bulk qualities. Again, the prospects for large increases in supply depend on the political and agricultural policies that control the production of livestock for slaughter. Currently, these enzymes cannot meet demand on a world basis adequately, with the result that the more price sensitive users have increasing interest in microbial enzymes.

Microbial Proteinases. There are very few species of microorganism in use for industrial protease production. This is largely a limitation imposed by the desire of producing companies to have the widest possible market range for their products, which includes food processing, and the consequent high cost of obtaining approvals from regulatory authorities. This cost is substantially increased if a product is produced by an organism without a recognized history of satisfactory approvals, and whether it must be evaluated for toxicity and safety. Most industrial microbial enzymes are produced from no more than 11 fungi, 8 bacteria and 4 yeasts; producers generally seek new enzymes from among these same species (31).

Composition and Stability. With such a huge variation in potency and physical presentation, it is not sensible to present specific statements about enzyme

product composition. Those produced by extraction from plant and animal tissues will contain different substances, in addition to the active enzyme, than those from microbial fermentations.

Apart from noting the small proportion that is actually active enzyme protein, it should be said that sugar and inorganic salts are used for the stabilization of the finished product for storage and distribution, and are selected according to acceptability in the intended application. Salts and sometimes carbohydrates such as starch are used to dilute extracted enzymes to standard activity. Preservatives are generally restricted to liquid enzyme preparations and conform to regulatory information relating to the intended use and the country of destination.

Industrial enzymes are commonly used at levels of 0.1-0.5 per cent of the substrate being processed, with rare exceptions above these levels. Therefore, when the actual amount of any constituent of the preparation is evaluated as a constituent of the final processed product, it is unlikely that it will make a significant contribution in relation to other similar materials, present or introduced, in the total process.

Specificity. The generally claimed specific action of enzymes is not as sharply defined as is often expected. Proteases are broad in the range of amino acid bonds they hydrolyze and exhibit only a degree of specificity. Careful investigation of the range of bonds attacked, and testing for comparable action on the actual protein target, will enable an enzyme to be chosen that has suitable performance. Some proteases are extremely narrow in their action, for example the various cheese rennets.

Temperature Considerations. When considering enzyme processes, the general rule is that the temperature quotient is between 1.8-2.0 (The reaction rate will increase or decrease by this order for each shift of 10°C). By using high temperatures, the reaction may be of short duration and hygienic conditions may be maintained more easily. Conversely, the use of much greater heat than for thermolabile enzymes will be necessary to inactivate the enzyme at the end of the process. Alternatively, a significant shift of pH may be necessary to inactivate without a rise in temperature. Not all processes require the inactivation of the enzyme at the conclusion of the desired reaction, but is important to consider this point when designing food product applications, or to prevent subsequent further modification by residual enzyme activity.

Proteinases in the Food, Detergent and Leather Industries

Proteases are the most extensively used enzymes in the food industry, where they act to improve the quality, stability, and solubility of foods. Some of the attributes of enzymes which make them useful in industrial operations include the following: 1) They are derived from plants, animal, and microbial sources and are invariably nontoxic substances that are able catalyze specific reactions; 2) they are active at very low concentrations under mild conditions of temperature and pH where undesirable side reactions are minimized; and 3) they can be inactivated after a desired effect has been achieved. Proteases from plant, animal, and microbial sources find extensive use as food processing aids.(32) Some of the applications of proteases in the food industry are summarized in Table I.

Proteases from various sources differ in their catalytic and physical properties, and whether or not a particular enzyme would be suitable to use for a particular industrial application depends on several factors. For many uses, the

Table I. Some Uses of Enzymes in Food Industry

<i>Food</i>	<i>Purpose</i>
Cheese	Coagulate casein, flavor development during aging
Milk	Coagulate rennet puddings, prepare soybean milk
Brewing	Body, flavor, and nutrient development during fermentation, aid in filtration and clarification, chill proofing
Wine	Clarification, decrease foaming, promote malo-lactic fermentation
Protein hydrolysates	Soy sauce, fish sauce, bouillon, dehydrated soups, gravy powders, processed meats, special diets
Meats	Tenderization, recover proteins from bones
Fish	Solubilization of fish protein concentrates, recover oil or meat scraps from inedible parts
Feeds	Waste product conversion to feed, digestive aid
Baked foods	Softening action in doughs, reduce mixing time, increase extensibility, improve texture and grain loaf volume, liberate β -amylase
Cereals	Increase drying rate of proteins, improve product handling characteristics, production of miso and tofu

specificity of the protease is of paramount importance. For example, even though many proteases have the capacity to clot milk, most of them are not suitable for cheese making because their broad hydrolytic specificity culminates in lower yields, loss of fat from the curd, and development of undesirable changes in texture and flavor during aging of the cheese. The relatively narrow specificity of chymosin for hydrolysis of the phenylalanine-methionine bond of κ -casein makes it the enzyme of choice for cheese making. Other applications, such as the production of plant protein hydrolysates, are best achieved by the use of proteases with a broad specificity. Some other factors of considerable importance in the selection of an enzyme for an industrial process include pH, temperature, response to inhibitors and activators, cost and availability of the enzyme and technical service support (32,33).

It may appear much cheaper to employ a culture than add an enzyme to the food process. However, not only economic but also quality control and food safety considerations are involved in decision making in the modern commercial process. Indeed, all three elements of the choice: economy, reproducibility and process safety, presented to the manufacturer, have meant that innovations involving enzymes, which may seem fine on the drawing board, have been very tardily adopted by industry.

The lead time, for incorporation of enzymes as an adjunct in whatever form into commercial food processes, appears to be far longer than equivalent innovation lead times in non-food, or even pharmaceutical processes. The exception to this finding is that there are enzymes which play an important role in many analytical and quality control procedures in the food industry, without the use of which, for batch or continuous process monitoring, many product lines would not be possible.

The greatest variety of industrial enzymes are presently derived from microbial sources, with a lesser diversity coming from plant and animal sources (34). Enzymes derived from plant sources and which are used extensively in the food industry include papain, bromelain, ficin, and amylases. Animal enzymes of economic importance include trypsin, lipases, and gastric proteases.

In spite of the potential diversity that can be achieved by the use of microbial enzymes, very few species of microorganism are used to produce industrial enzymes. The cost of getting a microorganism approved by regulatory authorities is substantial, and few of these organisms have been stringently evaluated and accepted as safe. Proteolytic enzymes obtained from livestock offal currently do not adequately meet the demand on a world basis and the future availability of traditional enzyme sources is dependent on the political and agricultural policies that control the production of livestock for slaughter (35). Moreover, traditional animal enzyme sources have been restricted to relatively few species, namely, bovine and porcine offal, although enzymes from cold adapted fish exhibit two salient properties that make them attractive choices for certain applications: thermal instability and high molecular activity at low process temperatures. Some applications of proteinases are listed in Table I.

Further description of some of these processes can be found in the following sections.

The Dairy Industry. The first step in cheese manufacture is the coagulation of milk. Coagulation can be divided into two distinct phases, enzymatic and the non-enzymatic. In the primary enzymatic phase a proteolytic enzyme such as chymosin (rennet), or less effectively, pepsin, carries out an extremely specific and limited proteolysis, cleaving a phenylalanine-methionine bond of κ -casein, making the casein micelle metastable. In the second, non-enzymatic phase, the

milk gels owing to the influence of calcium ions. The starting material for the manufacture of cheese is normally pasteurized whole milk. Other starting milks include non-fat skimmed milk for cottage cheese, and whole milk to which cream has been added for cream cheese.

It is thought that the rennet protease attacks the casein protein on the surfaces of the sub-micelle, exposing 'sticky patches' on the micelle. The micelles aggregate together via the 'sticky patches' to produce the coagulum or gel. When the curd is collected it normally retains a small percentage of the rennet, which goes on to play a part in the ripening or curing of the curd.

Rennet is made by a saline extraction of the abomasum (fourth stomach from unweaned calves) and contains several gastric enzymes, the principal milk-clotting enzyme being an acid protease, chymosin (EC 3.4.23.4). Because of the relative scarcity of rennet, because veal consumption is decreasing at the same time that cheese consumption is decreasing, replacements have been developed from more convenient sources, the first to be discovered with a sufficiently high milk coagulating to general protease ratio being from *Mucor pusillus*.

Microbial coagulants are now useful and are responsible for about one third of all the cheese produced worldwide, but suffer from the disadvantage of being too stable and so are threatened commercially by improved methods of producing chymosin by recombinant DNA technology. The use of thermally destabilized microbial rennets results in residual enzyme levels in the milk product similar to or below those encountered when calf rennet is used (35). An unexpected benefit has been an increase on some occasions of the specificity of the microbial enzyme, making it virtually indistinguishable from the action of calf rennet. Also some microbial rennets help impart a flavor that is popular with consumers.

Given that gastric proteases from marine organisms are relatively unstable at temperatures above 30°C, it follows that it may be possible to perform the first stage of milk coagulation with these enzymes at relatively low temperatures and subsequently heat denature the coagulating enzyme during subsequent steps in the cheese making process. Recent studies have shown it is possible to prepare a satisfactory cheddar cheese with Atlantic cod pepsin as a rennet agent (32). However, when the conventional cheddar cheese process is employed, there is somewhat more loss of fat and protein to the whey fraction, indicating that additional protein degradation occurs during curd formation and the early stage of cheddaring.

Milk and Whey. Trypsin has been successfully used to solubilize water in soluble heat denatured cheese whey protein, the enzyme being recovered after the reaction is completed by a cellulose based affinity absorbent (32). The treatment of milk with trypsin also inhibits the development of an oxidized flavor on storage. Trypsin immobilized on porous glass has been used to remove such flavors, and a packed bed reactor was found to be more efficient than a bath reactor or fluidized bed. This process has also been tested using Greenland cod trypsin instead of bovine trypsin, with enzyme inactivation by pasteurization. Owing to the thermal instability of Greenland cod trypsin compared to bovine trypsin, the former enzyme can be inactivated by relatively mild heat treatments. Both trypsins were similarly effective in preventing the oxidation of lipids at concentrations greater than 0.0013%; however, only the cod trypsin, and not bovine trypsin, was completely inactivated by the pasteurization process employed.(36,37) Accordingly, there is an advantage in utilizing the trypsin from a cold-adapted fish, owing to its thermal instability, since residual enzyme would not be present to cause subsequent hydrolysis of milk proteins.

The Brewing, Fruit Juice and Wine Industries. In the brewing of beer, enzymes are used at two stages, mashing and conditioning. In mashing, proteases, pullulanase, α -amylase, and β -glucanase are added to the barley malt, which has been finely ground to increase hydration and enzyme penetration together with malt extract, cereal adjuncts, and hot water to form the mash. During mashing, starches in the barley are degraded by the enzymes to form the sweet wort.

Proteolytic enzymes are also used during beer finishing operations as chill proofing agents to ensure the long term brilliance and colloidal stability of the final product. Chill-proofing refers to the treatment of beer to avoid the formation of chill-haze during cold storage. The haze is due to the cold precipitation of protein/polyphenol and protein/tannin complexes, giving a dull cloudy appearance to the beer. Proteases prevent haze formation by partially degrading the protein necessary for haze formation. The plant enzyme papain is the most widely employed chill-proofing protease because it catalyses only a limited breakdown of the protein. A disadvantage in the use of protease for the chill-proofing is that low levels of enzyme activity may remain after pasteurization, which is supposed to inactivate the enzyme. During long periods of storage, protein breakdown may continue and generate a sharp bitter taste due to increased amounts of amino acids.

The Pharmaceutical Industry. Pepsin, trypsin and chymotrypsin have been used clinically as a digestive aid, especially when coated to protect against damage in the stomach, and also in the preparation of precooked cereals and baby foods. Proteases have also been used medically for fibrinolysis, as anti-inflammatory agents in the potentiation of drug activity, and in the treatment of cystic fibrosis, burns, ulcers and acne.

Two other very interesting applications of proteases are firstly the use of a protease to convert porcine insulin into human insulin, and secondly the use of L-aminopeptidase to produce the pharmaceutically useful substance *p*-phenylglycine by selectively hydrolyzing L-phenylglycinamide in a racemic mixture.

Other Food Industries. Aspartame is a synthetic dipeptide ester, L-asp-L-phe-OMe which is about 200 times as sweet as sucrose. It has recently been released for sale in North America and Europe by G. D. Searle. It was originally synthesized chemically and reported by Mazur *et al.* (38). Subsequent improved methods of synthesis have been developed which involve the use of metalloproteases such as thermolysin 'in reverse'. Metalloproteases are used because, unlike the more common proteases, they have no esterase activity.

There have been a number of reports of the use of enzymes in the extraction of oils from sources such as fish, rape seed, yeast, palms, and soya beans. Celluloses and pectinases are used in palm oil extraction. In soya bean and fish, much oil has been found to be associated with protein, so that addition of proteases increases the yield of oil and protein. Use of thermostable proteases is preferred, but in general the use of enzymes is limited by the minimal water contents of the various process streams. *Trichoderma uride* and *A. niger* celluloses, hemicellulases and proteases have been used to extract hydrocarbons from *Euphorbia* plants (39,40) and similar enzymes used to extract saponinogens from *Helleborus* (41).

Endogenous microbial enzymes are sometimes utilized to break down their parent cells, and thus extract valuable intracellular materials. For instance, in the production of yeast extract, cells are allowed to autolyse at about pH 5 and 55°C. Proteases are probably the most important class of enzymes involved in autolysis, although others such as glucanases, lipases and nucleases also have

some activity. Attempts have been made to increase the rate of extraction and the final yield by the addition of exogenous proteases such as papain.

A large spectrum of berries and fruits are used in the production of fruit juice products. The range of products is substantially extended by an additional (fermentation) process to produce alcohol, and transform the fruit into wine. Enzymes, some of them proteolytic, are used to increase the yield of fruit juice during extraction, and to produce the required degree of clarity for the final product.

Protein Industry; Meats and Fish. Enzymes are widely used to increase the value and availability of proteins, for instance by enabling the recovery of protein hydrolysate from otherwise scrap bones or fish. Proteases differ widely in their substrate specificity, so that combinations of different enzymes can be used to increase the degree of hydrolysis of a protein; proteases also differ in factors such as their pH optima, so that they can be used over a wide range of conditions. Although a host of microbial proteases are known, proteases derived from plant and animal sources are most widely used in industrial applications. These include papain, ficin and bromelain from plants and trypsin, pepsin and rennin from animal sources. Problems associated with protease action are bitterness due to the release of hydrophobic amino acids and difficulties in controlling the extent of reaction. Applications in the food industry enable the intrinsic functional properties of proteins such as viscosity, whipping ability and emulsifying power to be optimized by controlled proteolysis. Examples include the enzymatic modification of soy and whey proteins so as to make them functionally more suitable for food applications, brewing, hydrolysis of wheat gluten, the production of yeast extracts, the production of gelatin from collagen, and the preparation of peptones which are hydrolyzed proteins used in microbiological growth media. Proteases also prove useful in the recovery of protein from the blood, offal and bones from slaughter-houses, and from fish processing wastes. These enzymes are also used for the decolorizing of hemoglobin so as to make this abundant source of protein more acceptable.

The addition of proteases to influence the tenderization of meat is a long established practice. By far the most widely used enzyme is papain from the latex of the *Carica papaya* plant. Papain can be added prior to slaughter by injection or after slaughter. Post-slaughter addition allows greater control over the degree of tenderization achieved. The amount of time in storage is reduced, because an appreciable amount of tenderization is accomplished during cooking before the enzyme is inactivated at around 90°C. The general trend in the meat industry is toward more rapid processing (e.g. hot boning), and it is likely that the time delay required for natural conditioning will be circumvented by an increase in the application of enzyme tenderizers. The increase in home freezer storage of large meat cuts has established a market in meat tenderizers (papain) for home application.

Attempts have also been made to exploit the relatively high molecular activity of cod trypsin at low temperatures by incorporating the enzyme into herring "fermentations" that proceed at 10°C. The preparation of brine-fermented round herring (matjes) is limited to certain seasons because of the balance of digestive enzymes in the fish at this time. Other studies have indicated that proteinases are important components in matje fermentation (41).

Supplementing the fermentation of gutted herring with equivalent amount of pure bovine and Greenland cod trypsins resulted in increased solubilization of protein. While the cod enzyme was effective in solubilizing protein, the percentage of solubilized protein which was soluble in 10% trichloroacetic

(TCA) was substantially lower than the conventional matjes prepared with round herring.

The Baking Industry. Traditional methods of baking are based on the presence of endogenous enzymes which catalyze natural changes during growth, ripening and storage. They also carry out the saccharification of starch prior to fermentation and the degradation of the gluten which is a very important determinant of the rheological properties of the dough. Fungal proteases derived from *A. oryzae*, including both exo- and endo-peptidases are used because they have a low thermal stability and are inactivated during baking. The texture of the dough can thus be improved, so facilitating processing and decreasing energy inputs. Loaf volumes are substantially increased, and many other improvements are claimed (see below).

Enzymes are also employed in changing the physical viscoelastic properties of the dough to improve handling and machining, and enable wider range of bakery products to be produced. The protein content of flour is largely responsible for the viscoelastic properties of the dough, and fungal proteases are added to partially hydrolyze the protein, thus altering the viscoelastic properties. Some wheat varieties have a high protein content and give a 'hard flour', which is strong with limited elasticity. In the case of machine produced cakes and biscuits, it is necessary to have a low protein 'soft' flour with good elasticity and suitability for machine handling. Fungal proteases are normally added to both 'soft' and 'hard' flours, because in addition to improving the viscoelastic properties, the proteolysis releases amino acids, which support the growth of yeast and enhance flavor in the product.

In some applications such as biscuit manufacture a flour containing high molecular weight dextrans but with extensively degraded gluten is required in order for it to be sufficiently plastic to enable easy processing. Many flours do not naturally have this property, and possess only very low protease activities, so proteases are added to degrade the gluten. Thermostable endopeptidases obtained from *B. subtilis* are used, and they give a product with an enhanced elasticity which is important so that the dough can be spread thinly during manufacture. Use of enzymes for gluten degradation is superior to chemical methods such as bisulphite treatment.

Proteinases in the Leather Industry. Protease enzymes are used in the processing of skins and hides into leather, in particular for the removal of hair and wool, and for increasing the pliability (called bating) of leather.

Skins are soaked initially to clean them and to allow rehydration. This latter process is aided by the addition of very low concentrations of proteases. Use of pancreatic trypsin is especially favored since contaminating lipases tend to solubilize fats and gums, further improving water uptake.

The process for sheepskin is rather more gentle in order to avoid damage to top quality wool that can be spun into valuable wool yarn. Liquid protease preparations such as pancreatic extracts of trypsin and chymotrypsin are applied to the flesh side of the sheepskin. The proteases diffuse to the basal papilla of the hair follicle, and subsequent protein breakdown releases the wool hair with minimal damage (34).

Dehairing is carried out using alkaline proteases such as subtilisin in a very alkaline bath. Alkaline conditions tend to swell the hair roots, so easing the removal of the hair by allowing the proteases to selectively attack protein in the hair follicle. Other specific enzymes are used for skins from particular species.

Baking of hides involves degradation of some elastin and keratin, removal of hair residues and deswelling of collagen. A variety of proteases are used by

the industry, depending upon the type and quality of hide. The process remains a highly skilled craft requiring intuitive manipulations of conditions such as pH, temperature, enzyme concentration and incubation time if the best quality leathers are to be produced.

Proteinases in Detergents. Proteases were first compounded for use in a detergent called "Burnus" in 1919 by Rohm and Haas in Germany (42). This product, however, was not very effective and hence was not a commercial success. The 1960's saw a rapid development of the formulation of proteinases in detergents. A new enzyme isolated from *Bacillus licheniformis* by Carlsberg Laboratories and called subtilisin Carlsberg was a major factor in this renewed interest in proteinase additives in detergents. The enzyme was marketed by Novo Industries and given the commercial name "Alcalase". The new detergent product was named Biotex. Throughout the 60's detergents containing proteinase continued to grow in popularity until increasing reports of allergic reactions by workers handling the products deterred the use of the detergents. Eventually new formulation developments produced encapsulated proteinases which were dust-free and hence had decreased allergenic properties. Currently, the use of enzymes for detergent formulation accounts for approximately 25% of the total world-wide sales of enzymes (43).

Proteinases are important additives to detergents for laundry use in because they help to dissolve the typical stains found in laundry including blood, food, body secretions, and skin particles. They also help release proteinaceous dirt which can coagulate on the fiber material due to the high temperature at which the washing is often carried out (>50°C). The proteinase usually only needs to hydrolyze a few peptide bonds in the proteinaceous dirt for the protein to become soluble in the detergent milieu and be released from the fabric.

As one can imagine, the use of a biologically active proteinase under the conditions at which most laundry is washed is not to be taken for granted. The pH of most laundry detergents is in the alkaline range, usually between pH 9 and 10.5. Also certain laundry conditions have wash temperatures as high as 95°C. The detergents themselves also contain a variety of oxidizing agents, fluorescent dyes, chelating agents and cationic surfactants which all can denature the proteinase with a loss of activity. As mentioned above, since the proteinase need only hydrolyze a few peptide bonds within the protein stain for it to be released, the proteinase must only survive the conditions of the wash a short period of time. The simple approach to solve these problems is to begin the wash cycle with a pre-wash of 10 to 20 minutes at moderate temperatures (20-30°C). Fortunately, the proteinases used in modern detergent application can withstand temperatures up to 60°C for periods long enough for effective solubilization of the protein. This is particularly important for the American market where washing is generally done at temperatures starting around 50°C and then slowly cools during the wash period.

New developments in this field include the isolation of new proteinases from alkalophilic *Bacillus* species. Two commercial preparations of these enzymes are available from Nova under the trade names of Esperase and Savinase T. These enzymes have higher isoelectric points in the range of pH 11 as opposed to 9.4 for Alcalase. Because of their higher isoelectric points they are more stable at higher pH ranges which makes them particularly useful for use in liquid detergents. Recently, additional enzymes have been used in laundry detergent formulation, some of these include cellulases, amylases, and lipases (43-44).

With the current energy conscience of the consumer market and increasing use of synthetic fabrics, laundry washing at lower temperatures is becoming more

common, especially in the European market where washing is typically performed in the range between 40 to 60°C. Hence, the search for more efficient enzymes capable of high catalytic efficiency at lower temperatures is a developing area. At the other extreme a great deal of basic and applied research has, in the past few years, been directed at the modification of proteinases to increase their stability at elevated temperatures. The more interesting of these studies use site specific mutagenesis to alter the primary structure of the proteinase (typically subtilisin) such that the mutant protein has an increased temperature tolerance (45-47). Various approaches have been taken towards this end including the engineering of additional disulfide and ionic bonds in the protein (49). Unfortunately, due to an insufficient knowledge of the basic rules governing protein structure and function it is not always clear what the results of such alterations will ultimately have on the biological activity of the proteinase.

Proteases in Research

Proteases, since their discovery, have played an important role in both basic and applied research because their sometimes exquisite selectivity of peptide bond cleavage can be extremely useful in the elucidation of other proteins' structures and function. The following three sub-sections give several examples of how proteases are used in specific aspects of research.

Use of Proteases in the Structural Studies of Proteins. Some proteinases are very useful for producing limited cleavages in protein substrates due to high peptide bond selectivity. One of the major uses of proteinases has been in the primary structural studies of proteins. Due to limitations in the chemistry or technology of sequencing by Edman degradation or mass spectroscopy, the length of an amino acid sequence of a protein or peptide which can be obtained directly is limited (48-49). Hence, the use of proteinases to fragment proteins or peptides for sequence determination is quite important.

The fragmentation of a protein or peptide can be approached in either of two manners. One can perform a "limited" digestion of a substrate by a specific proteinase to yield a partial set of peptide fragments. The fragments thus produced will be based on the specificity of the enzyme, the susceptibility of those specific sites within the native structure of the protein and the conditions under which the digestion occurs. Another type of digestion is when the conditions are chosen which allow for all the potential cleavage sites in the substrate to be hydrolyzed by the proteinases. This type of digestion gives a quite different group of proteolytic fragments and requires different reaction parameters such that the substrate is denatured, higher enzyme to substrate ratios, and longer digestion times. Depending on the type of information desired, one alters the conditions of digestion to produce the appropriate digestion fragments. Additionally, the use of different proteinases in different reactions allows for the production of "overlapping" fragments which can ultimately be used to piece together the sequence of the peptide fragments into the complete sequence of the proteins (50-52).

The use of DNA sequencing as a method of generating protein sequence data (53) has proven to be extremely fast and efficient and future technical developments such as automated DNA sequencing will further expedite the production of protein sequence data (53). Nevertheless, DNA sequencing often calls for a certain amount of protein sequence information for the procedure to be most efficient. From our own experience in the amino terminal protein sequencing of over 500 samples, we find that approximately 50% of all naturally occurring proteins have amino termini which are blocked, thus precluding their

sequence analysis by typical Edman degradation. However, by generating peptide fragments of the protein and thus producing "free" amino terminus peptides, sufficient sequence data for these peptides can be obtained for the synthesis of hybridization probes or primers necessary for DNA sequencing (54).

As mentioned above, depending upon the conditions under which a protein digestion by a proteinase takes place different fragments can result. Under the conditions where only a limited number of the total potential sites are available to proteinase (low proteinase ratio to substrate, native substrate conditions, short digestion times, etc.) very interesting information can be gained on the tertiary structure of the protein. Often, native globular proteins are relatively resistant to proteolytic degradation except at sites which are not contained inside the globular protein structure. These regions are generally most often observed in the regions linking compact domains of the protein or in loops which extend out of the compact regions of structure. Thus, performing a limited digestion of a protein with the proper choice of proteinase and digestion conditions can yield information on the tertiary structure of that protein. Also depending upon the protein, information regarding the biological function of a particular domain can sometimes be obtained.

Another interesting use of proteinases in the analysis of protein structure is in the examination of conformation change of a protein upon binding a ligand, substrate, inhibitor, etc. (55) In our laboratory we have examined the change in structure of a basement membrane protein nidogen upon its binding in a 1:1 ratio with another basement membrane protein laminin. We have observed that the proteolytic susceptibility of the nidogen alone is different from that when complexed to the laminin molecule. This can be explained by one of three possibilities; either the binding to laminin physically blocks proteolytic sensitive site(s); binding to laminin alters the conformation of the nidogen such that different sites are then available for hydrolysis; or a combination of both.

Use of Proteases in Peptide Synthesis. Typically peptides are synthesized the standard solid or liquid phase methodologies (56, 57). However, both of these techniques require harsh chemical reactions which are detrimental to certain amino acids. Furthermore, in practical terms most peptide syntheses are limited to the range of 30 to 50 amino acid residues. Hence, peptide synthesis is still somewhat problematic in many cases. In certain situations, the alternative method of peptide synthesis using proteases is an attractive choice. With this form of synthesis, one can avoid the use of the noxious and hazardous chemicals used in solid or liquid phase peptide synthesis. Since the reactions are enzyme catalyzed, racemization of the peptide bond does not occur. This technique has been used with success in the synthesis and semisynthesis of several important peptides including human insulin (58,59).

As discussed above, proteases are peptide bond hydrolases and act as catalysts in this reaction. Consequently, as catalysts they also have the potential to catalyze the reverse reaction, the formation of a peptide bond. Peptide synthesis with proteases can occur via one of two routes; either in an equilibrium controlled or a kinetically controlled manner (60). In the kinetically controlled process, the enzyme acts as a transferase. The protease catalyzes the transfer of an acyl group to a nucleophile. This requires an activated substrate preferably in the form of an ester and a protected P' carboxyl group. This process occurs through an acyl covalent intermediate. Hence, for kinetically controlled reactions the enzyme must go through an acyl intermediate in its mechanism and thus only serine and cysteine proteases are of use. In equilibrium controlled synthesis, the enzyme serves only to expedite the rate at which the equilibrium is reached, however, the position of the equilibrium is unaffected by the protease.

Therefore the choice of enzyme only depends upon its specificity and not upon the particular mechanism of the protease.

The technique of using proteases in peptide synthesis has been carefully reviewed (60,61) and the technology is advanced to the state that commercially prepared kits for this type of synthesis are available. Nevertheless, due to the well defined methodological routines devised for general synthesis of peptides for laboratory use, the standard chemical approaches are likely to remain the methods of choice.

Future Developments

Two driving forces have caused an outstanding increase in the interest in proteinases in biotechnology. One is that we are increasing our understanding of the role proteinases play in nature and we have a better understanding of the biochemical and biophysical rules which govern the activities of these proteins. Secondly, with the solid development of recombinant DNA techniques which allow for the specific alteration of protein structure and hence function, a whole new area for research and exploitation of proteinases for industrial, medical, and biotechnological use has been opened. An example of how some of these new technologies are developed is described below.

Alteration of Enzyme Activity by Mutagenesis. The literature is rapidly expanding with references to proteins and enzymes whose coding DNA has been subjected to mutagenesis and then expressed giving a protein with altered properties. Unfortunately, due to our lack of complete understanding of the rules of protein structure and function the new properties do not always match those which were expected. Nevertheless, these types of experiments if carefully analyzed still add to our knowledge of protein structure. Attempts have been made to alter the substrate specificity of trypsin by altering the active site region in which the substrate binds (62). The results of these experiments have yielded surprising information on the nature of the substrate-enzyme interaction. Continuing work along these lines can be expected to give us further insights into enzyme function and thus give us a more solid foundation for future experimentation.

Synthetic Peptide Catalysts and Catalytic Antibodies

Another approach for producing proteases with specific properties which differs from the approach based upon mutagenesis of existing proteases is to synthesize peptides which contain the necessary catalytic and substrate binding groups necessary for a particular hydrolytic reaction. One can envision rather unlimited potential for altering the peptide structure to meet other biochemical demands by incorporating potential cross-linking groups, stabilizing structures etc. The approach of making peptide catalysts has been met with success by the group of Stewart. Recently they synthesized a peptide containing four helices which contains the active residues of chymotrypsin in the correct spatial arrangement such that hydrolysis of chymotrypsin substrates is observed (63). This peptide, which they have termed "chymohelizyme", has an oxyanion hole and a substrate binding pocket designed for chymotrypsin substrate esters such that hydrolysis rates of approximately 0.01 of chymotrypsin have been observed. As advances in the understanding of protein structure and function are made as well as technical advances in peptide synthesis it is likely that many more peptide catalysts will appear in the literature and will ultimately be exploited for medical and biotechnological uses.

The other approach for making specific proteases is based upon the production of catalytic antibodies. This can hypothetically be approached in three directions; catalytic groups can be positioned in the antibody combining sites; antibodies produced which recognize the transition state of hydrolytic reaction; or genetic selection of catalytic antibodies (64). The more common approach is the second and is well represented by the work of Lerner and colleagues. They have been able to generate monoclonal antibodies which when complexed with metal cofactors could cleave small peptides (65). Considering the advances made in the production of monoclonal antibodies and in the understanding of peptide bond hydrolysis one can expect future development of this procedure.

References

1. Bergmann, M.; Fruton, J.S. *J. Biol. Chem.* **1937**, *117*, 189-202.
2. Barrett, A.J.; MacDonald, J.K. *Biochem. J.* **1985**, *231*, 807.
3. Berger, A.; Schecter, I. *Phil. Trans. R. Soc. London* **1970**, *B257*, 249-264.
4. Hartley, B.D. *Annu. Rev. Biochem.* **1960**, *29*, 45-72.
5. Barrett, A.J. *Ciba Found. Symp.* **1980**, *75*, 1-13
6. Menon, A.S.; Goldberg, A.L. *J. Biol. Chem.* **1987**, *262*, 14929-14934.
7. James, M.N.G. In *Proteolysis and Physiological Regulations*; Ribbons, D.W.; Brew, K. Eds.; Academic Press: New York, N.Y., **1976**, p.125-142.
8. Young, C.L.; Barker, W.C.; Tomaselli, C.M.; Dayhoff, M.O. In *Atlas of Protein Sequence and Structure*; Dayhoff, M.O., Ed.; Natl. Biomed. Res. Found.: Washington, D.C., **1978**; pp.73-93.
9. Palthy, L. *Chem. Eng. News* **1985**, *41*, 657-663.
10. Neurath, H. *Science* **1984**, *224*, 350-357.
11. Debouck, C.; Breyer, G.B.; Gorniak, J.G.; Malinowski, J.; Meek, T.D.; Moore, M.L.; Strickler, J.E. In *Viral Proteinases as Targets for Chemotherapy* Krausslich, H.-G.; Oroszlan, S. and Wimmer, F. Eds.;
12. Hanecak, R.; Semler, B.L.; Anderson, C.W.; Wimmer, E. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3973-3977.
13. Kohl, N.E.; Emini, E.A.; Schleig, W.A.; Davis, L.J.; Heimbach, J.S.; Dixon, R.A.F.; Scholnick, E.M.; Sigal, I.S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4686.
14. McKeever, B.M.; Navia, M.A.; Fitzgerald, P.M.D., Springer, J.P.; Leu, C.-T.; Heimbach, J.C.; Herber, W.K.; Sigal, I.S.; Darke, P.L. *J. Biol. Chem.* **1989**, *264*, 1919-1921.
15. Okada, Y.; Nagase, H.; Warris, E. N. Jr. *J. Biol. Chem.* **1986**, *261*, 14245-14255.
16. Wilhelm, S.M.; Collier, I.E.; Kronberger, A.; Eisen, A.F.; Marmer, B.L.; Grant, G.A.; Bauer, E.A.; Goldberg, G.I. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6725-6729.
17. Hibbs, M.S.; Hasty, K.A.; Seyer, J.M.; Kang, A.H.; Mainardi, C.L. *J. Biol. Chem.* **1985**, *260*, 2493-2500.
18. Shannon, J.D.; Baramova, E.N.; Bjarnason, J.B.; Fox, J.W. *J. Biol. Chem.* **1989**, *264*, 11575-11583.
19. Tryggvason, K.; Hoyhtya, M.; Salo, T. *Biochim. Biophys. Acta* **1987**, *907*, 191-217.
20. Liotta, L.A.; Rao, C.N.; Wemer, U.M. *Annu. Rev. Biochem.* **1986**, *55*, 1037-57.
21. Jongeneel, C.V.; Bouvier, J.; Bairoch, A. *FEBS Lett.* **1989**, *242*, 211-214.

22. Bicknell, R.; Holmquist, B.; Lee, F.S.; Martin, M.T.; Riordan, J.F. *Biochemistry* **1987**, *26*, 7291.
23. Salvesen, G.; Nagase, H. In *Proteolytic Enzymes* Beyon, R.J.; Bond, J.S. Eds.; IRL Press: Oxford, 1989; pp 83-143.
24. Bjarnason, J.B.; Fox, J.W. *Biochemistry* **1983**, *22*, 3770-3778.
25. Barrett, A.J. In *Proteinase Inhibitors*; Barrett, A.J.; Salvesen, E. Eds; Elsevier: Oxford, 1986; p 9.
26. Barrett, A.J. In *Proteinase Inhibitors*; Barrett, A.J.; Salvesen, E. eds; Elsevier: Oxford, 1986; p 11.
27. Melloni, E.; Sparatore, B.; Salamino, F.; Michetti, M.; Pontremoli, S. *Biochem. Biophys. Res. Comm.* **1982**, *107*, 1053-1059.
28. Polgar, L.; Halsez, P. *Biochem. J.* **1982**, *207*, 1-10.
29. Asboth, B.; Stokum, E.; Khan, T.U.; Polgar L. *Biochemistry* **1985**, *24*, 606-609.
30. Barrett, A.J. *Anal. Biochem.* **1976**, *76*, 374-376.
31. Godfrey, T.; Reichelt, J. In *Industrial Enzymology*; Godfrey, T.; Reichelt, J. Eds.; MacMillan: London, **1983**.
32. Simpson, B.K.; Haard, N.F. In *Food Biotechnology*; Knorr, D. Ed., Marcell Dekker: New York, N.Y., **1987**.
33. Cheetham, P.S.J. in *Handbook of Enzyme Biotechnology*; Wiseman, A. Ed., Ellis Horwood: Chichester, West Sussex, **1985**.
34. Beckerstoff, G.F. In *Enzymes in Industry and Medicine*; Arnold: London, **1987**.
35. Stauton, R. *Food Science and Technology Today* **1989**, *2*, 180-184.
36. Olafsdottir, S.; Magnisson, S.; Bjarnason, J.B. In *Aspartic Proteinases and their Inhibitors*; Kostka, V. Ed.; Walter de Gruyler: Berlin, **1985**.
37. Asgeirsson, B.; Fox, J.W.; Bjarnason, J.B. *Eur. J. Biochem.* **1989**, *180*, 85-94.
38. Mazur, R.H.; Schlatter, J.M.; Goldkamp, A.H. *J. Am. Chem. Soc.* **1969**, *91*, 2684.
39. Weil, T.A.; Dzadzic, P.M.; Shih, C.C.J.; Price, M.L. U.S. Patent 4,338,399, 1982.
40. Issac, O. U.S. Patent 4,004,976, 1977.
41. Ritskes, T.M. *J. Fish Res. Board Can.* **1971**, *69*, 647.
42. Kalisz, H.M. In *Advances in Biochemical Engineering/Biotechnology* Fiechter, A. Ed.; Springer-Verlag: Berlin, 1988, Vol. 36; pp 28-34.
43. Layman, P.L. *Chem. Eng. News* 1986, Novo Industri Als Information Bulletin 1987, B338c-GB2500, B346c-GB1500, Novo Industri, Denmark
44. Jensen, G. Novo Industri Als Information Bullentin 1984, A-05845, Novo Industri, Denmark.
45. Pantoliano, M.W.; Ladner, R.C.; Bryan, P.N.; Rollence, M.L.; Wood, J.F.; Poulos, I.L. *Biochemistry* **1987**, *26*, 775-791.
46. Wells, J.A.; Powers, J. *J. Biol Chem.* **1986**, *261*, 6564.
47. Mitchinson, C.; Wells, J.A. *Biochemistry* **1989**, *28*, 4807-4815.
48. Hulmes, J.D.; Miedel, M.C.; Pan, Y.-C.E. In *Techniques in Protein Chemistry*; Hugli, T.E. Ed.; Academic Press: San Diego, CA, 1989; pp. 7-16.
49. Griffin, P.R.; Shabanowitz, J., Yates, J.R.; Zhu, N.Z.; Hunt, D.F. In *Techniques in Protein Chemistry*; Hugli, T.E. Ed., Academic Press: San Diego, CA, 1989; pp. 160-167.
50. Schlabach, T. D.; Colburn, J.C.; Mattaliano, R.J.; Yuen, S. In *Techniques in Protein Chemistry*; Hugli, T.E. Ed., Academic Press: San Diego, CA, 1989; pp. 497-505.
51. Shannon, J.D.; Baramova, E.N.; Bjarnason, J.B.; Fox J.W. *J. Biol Chem.* **1989**, *264*, 11575-11583.

52. Flannery, A.V.; Beyon, R.J.; Bond, J.S. In *Proteolytic Enzymes* Beynon, R.J.; Bond, J.S. Eds., IRL Press: Oxford, 1989, pp 145-161.
53. Smith, L.M.; Sanders, J.Z.; Kaiser, R.J.; Hughes, P., Dodd, C., Conell, C.R.; Heiner, C.; Kent, S.B.H.; Hood, L.E.

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Chapter 7

Subtilisin

Commercially Relevant Model for Large-Scale Enzyme Production

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Subtilisins are a class of alkaline serine proteases produced by a variety of *Bacillus* species. The primary commercial use of subtilisins is as additives in detergent formulations to aid in the removal of proteinaceous stains. Until recently, commercial availability of subtilisins was limited to those produced by certain strains of either *B.licheniformis* or *B.alcalophilus* which had undergone years of traditional strain development to enhance overall productivity. The advent of new genetic engineering techniques has given industrial enzyme producers the opportunity to commercialize enzymes from new sources or site-specific modified enzymes designed for defined applications. Strategies for the cloning and expression of the *B.amyloliquefaciens* subtilisin (BPN') will be presented.

The industrial enzyme market approaches approximately \$600 million US dollars annually. The majority of these enzymes are produced by large scale fermentation from microbial or fungal sources. Typical uses for these enzymes include food processing (pectinases), biomass conversion (cellulases) and high fructose corn syrup production (amylases, glucoamylases). In general, the enzyme market can be considered a commodity market where the value of the enzyme lies in the processing advantages and subsequent reduced production cost recognized by the user. An example of this type of enzyme would be pectinase which increases the amount of free run liquid from fruit for wine or juice production. In some cases, however, the enzyme(s) is used to produce a more value-added product from a substrate. An example of this type of enzyme system would be glucoamylase/glucose isomerase for the production of high fructose corn syrups from corn starch.

Most of the enzymes in commerce today were derived from natural isolates by the classical approaches of screening, strain selection/mutagenesis for overproduction and fermentation development. While these techniques have certainly proven useful in the past to identify and develop enzymes, there are limits to the effectiveness of this type of approach. There are two major difficulties facing the industrial microbiologist in developing future products.

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First, the ability to identify a new enzyme relies on a labor intensive screening of large numbers of natural isolates. Though it has been possible to use plate screening methods to identify potential overproducers or to develop partially selective conditions for selecting overproducers, the practice still relies heavily upon shake flask analysis of large numbers of individual isolates. While advances in robotics has eliminated some of the tedium in screening isolates, it remains a "hands-on" field of endeavor.

Second, when using a variety of natural organisms as enzyme production hosts, there must be an intensive strain and fermentation development program to increase enzyme yields. Routinely, information gathered during this process will be of little use in developing the next generation product, since a different host will most likely be employed. Additionally, the search for the new isolates is often limited, by regulatory necessity, to members of those genres which have a history of safe production (Generally Recognized As Safe or GRAS).

Why an rDNA Approach

With the advent of recombinant technology, the enzyme producer now has a tool to aid in the development of new enzyme products and in the improvement of production costs for already commercialized enzymes. The search for new enzymes does not need to be limited to GRAS organisms, since it is possible to move the enzyme from its original host into a more suitable production organism. This may prove especially important in identifying enzymes from organisms which are pathogenic or not amenable to standard fermentation practices (such as extreme thermophiles). By expanding the sources of commercial enzymes, the potential exists to identify new and exciting applications.

Recombinant DNA can be used to overcome a variety of potential problems in the industrial enzyme sector. A schematic outline of the types of problems encountered and the solutions by an rDNA program is shown in Figure 1. Basically, these problems can be divided into strict yield issues (making more of a given protein) and quality issues (making the product more useful). Because of the nature of the enzyme business, the highest return to the enzyme producer will usually come from increasing the value of the enzyme to the user.

Historically, once a new enzyme was identified, it was often a laborious effort to identify the correct conditions for small scale fermentation in order to produce enough material for laboratory scale testing. This meant a major investment in resources and equipment simply to determine if the enzyme was suitable for further analysis. Pilot scale quantities required an even greater expenditure of effort. With a recombinant approach, it is possible to rather quickly clone and express an enzyme at high enough levels to enable applications scientists to test its utility. By moving this analysis earlier into the project, decisions with regard to which candidates should be pursued can be made more effectively in terms of resources spent.

If the initial cloning and expression has shown the enzyme to be promising then a yield driven expression program can be instituted. Regardless of the host

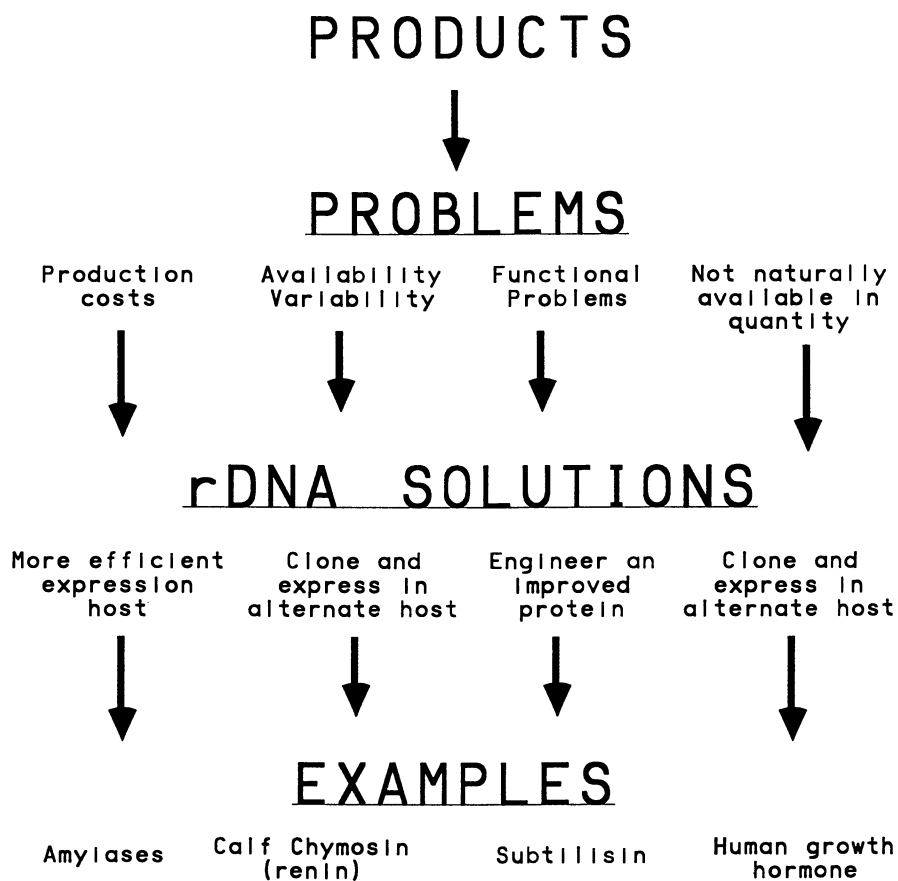


Figure 1. rDNA solutions to commercial problems in the industrial enzyme sector.

system chosen, the efforts will include placing the gene under control of a strong promoter and developing a host strain which will be compatible with a fermentation and recovery program. The main advantage of this approach is the ability to design generic host systems which are optimized for production from specific promoters. Figure 2 shows the effectiveness in utilizing recombinant DNA as compared to the classical methods for developing a series of products. As indicated in the figure, the generic host/expression system potentially shortens the time for achieving commercially viable yields of newly identified enzymes, primarily due to the elimination of a major strain improvement/fermentation development program on each new isolate.

One of the most exciting potentials of the rDNA approach lies in the ability to design enzyme activity by site-directed mutagenesis of a naturally occurring enzyme. With this approach, one is freed from the constraints of natural isolate screening in order to identify an enzyme which has properties more suitable for commercial applications. Although enzyme design may never completely eliminate the need to screen culture collections, it will certainly increase the number of candidate enzymes available for commercial use. Already, functions such as thermostability (1) and oxidative stability (2) have been engineered into subtilisin by use of these techniques.

Development of Subtilisin as a Model Enzyme

In order to prove enzyme engineering feasibility, it was important to develop a model system. One of the prime considerations for any model would be the commercial potential of the model. Table I lists the major commercial enzymes and the market size in US dollars (3). The alkaline proteases (subtilisins) are clearly the major single class of enzymes in commercial use today, representing 25% of the total enzyme market of \$600 million. The primary use of subtilisins is as additives in laundry detergents to aid in the removal of proteinaceous stains from cloth.

Table I. The Major Commercially Available Enzymes and Estimated Market Size

ENZYME	SOURCE	MARKET SIZE (millions US\$)
Alkaline protease	<u>Bacillus spp.</u>	150
Neutral protease	<u>Bacillus spp.</u>	70
Amylases	<u>Aspergillus oryzae</u>	100
	<u>Bacillus spp.</u>	
	<u>Aspergillus oryzae</u>	
Glucose isomerase	<u>Aspergillus niger</u>	45
	<u>Streptomyces spp.</u>	
Rennin	Calf stomach	60
	<u>Mucor spp.</u>	

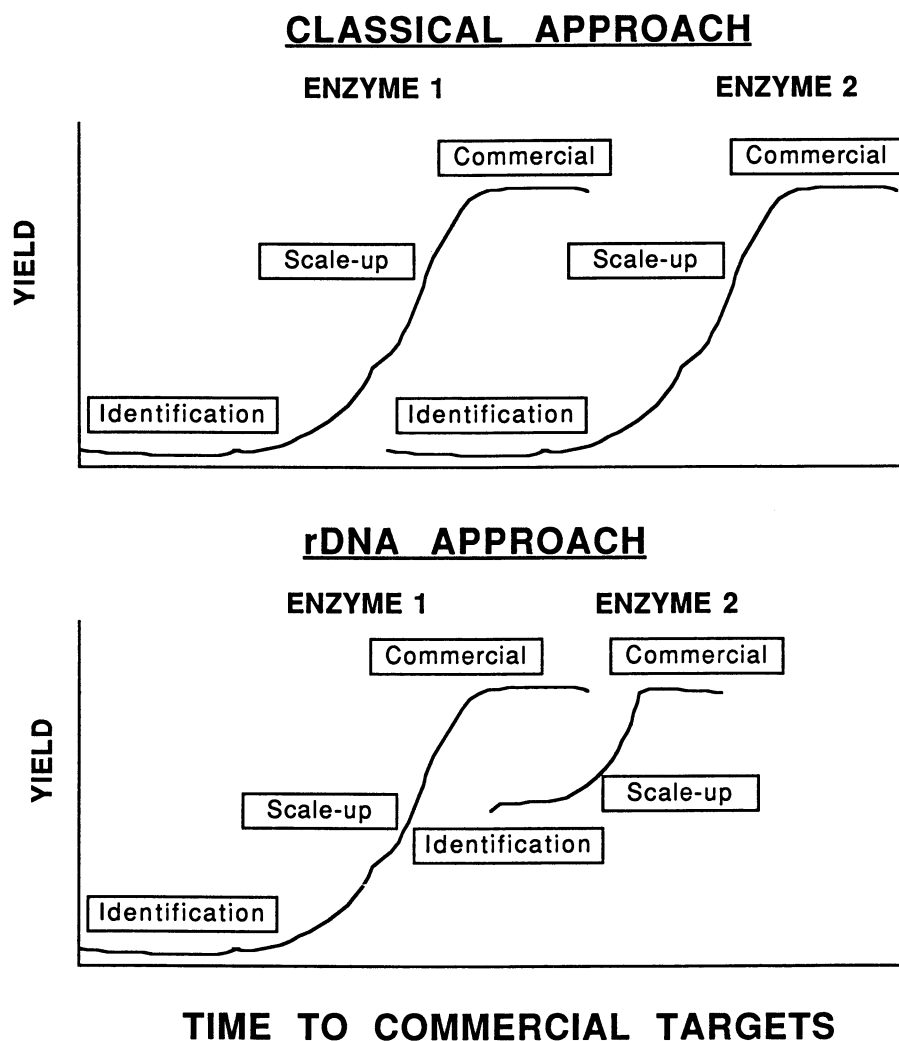


Figure 2. Efficiency of an rDNA approach to enzyme commercialization. Areas in boxes refer to the typical times required for Identification, Scale-up and Commercialization. Efficiencies are recognized in Identification of enzymes (protein engineering) and shortened Scale-up times (generic hosts).

Subtilisins are a class of related serine endo-proteases produced by members of the *Bacillus* genus. The *B.amyloliquefaciens* subtilisin (BPN') is well-characterized with regard to its DNA sequence (4), protein sequence (5), X-ray crystal structure (6) and kinetic properties (7). With this wealth of information available, BPN' was chosen as the model enzyme for our recombinant approach.

BPN' is a 275 amino acid protease with a serine in the active site. Since it is functional in an alkaline environment it has potential use in detergent applications. Our program was to change specific characteristics of BPN' to make it more effective in certain applications. Two main activities were targeted: pH range and oxidative stability (since bleaches are often components of detergents).

Using X-ray structure data, amino acids which were believed to affect the desired characteristics were identified. Using either site-directed mutagenesis or "cassette" mutagenesis (8) different amino acid substitutions were made in the subtilisin structural gene (2,9).

The BPN' variant genes were cloned onto expression vectors to produce sufficient quantities of the purified enzymes for analysis. The pH profiles of two variants compared to that of the native enzyme are shown in Figure 3. BPN' has a broad activity range from pH 7.5 to approximately pH 9.5. When the methionine which occurs at amino acid position number 222 in the native protein is replaced with a lysine (Lys-222), the pH profile shifts dramatically. The range of activity has been changed to a sharp region surrounding pH 9.5. When cysteine replaces the methionine at the same position, the optimal pH remains at approximately 8.5, but there is a small effect on the pH range with a slightly sharper dropoff in activity both above and below the optimum.

An entirely different property of subtilisin was affected by substituting leucine at the 222 location. Native BPN' is extremely sensitive to the presence of oxidation agents, showing rapid inactivation when incubated in the presence of 0.3% H₂O₂ (Figure 4). The Leu-222 variant, in contrast, was found to be totally stable under the same oxidation conditions. The data clearly show that single amino acid alterations can have dramatic effects upon the activity of the enzyme. Similarly, other changes have been shown to affect catalytic properties, substrate specificities and thermostability (1,2,9).

Development of a Host Production System

From a commercial standpoint, it is not sufficient to merely identify those variations of the naturally occurring enzymes which have properties of interest. There must be a system devised which will allow production of the variants at commercially viable yields. Since, the subtilisins are produced by members of the *Bacillus* genus, *Bacillus subtilis* was chosen as the model host system. *B.subtilis* is the most characterized member of the gram positive group of microorganisms. Genes may be transferred among individuals by both transformation and transduction. In addition, there are numerous mutants available for genetic analysis. Most importantly, however, *B.subtilis* produces a number of extracellular enzymes, including an endogenous subtilisin.

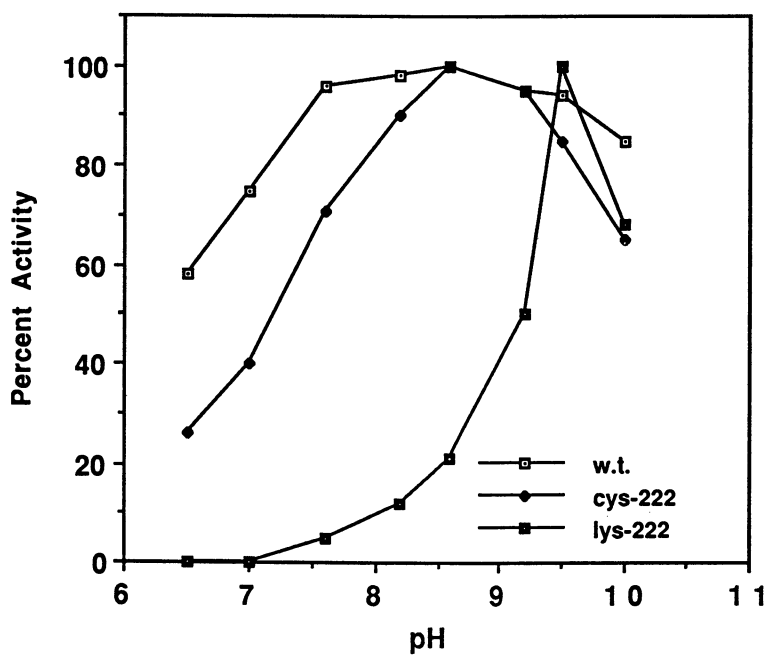


Figure 3. pH profiles of wild type and two variant subtilisins. Activity was assayed with synthetic substrates as described (2) at the indicated pH.

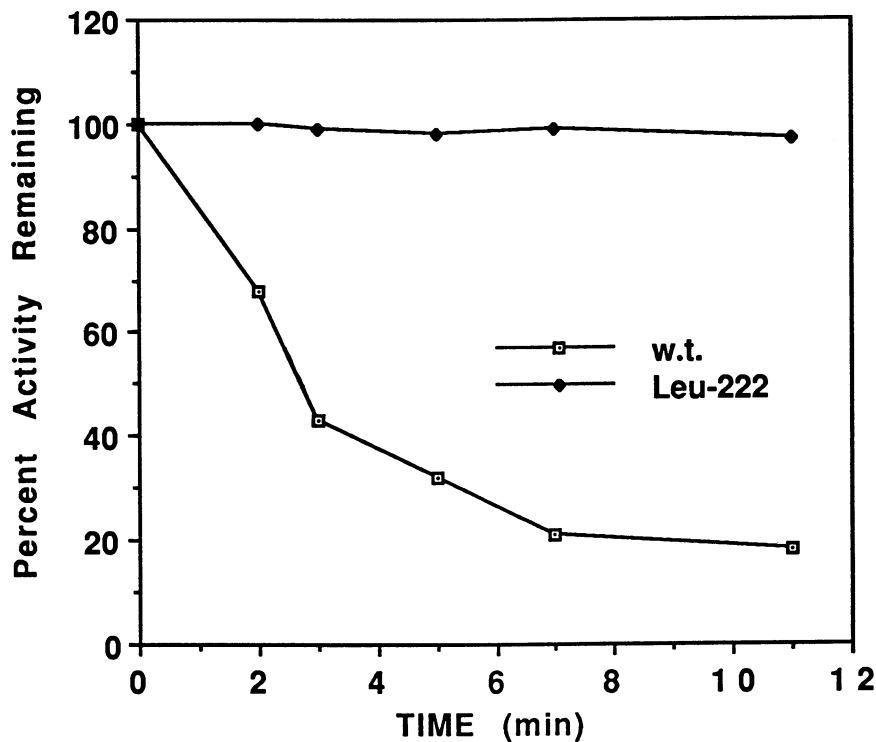


Figure 4. Oxidative stability of subtilisins. Purified wild type or variant subtilisin was mixed with 0.3% H_2O_2 in 0.1M Tris buffer (pH 8.6). At the times indicated aliquots were removed and tested for remaining enzymatic activity (2).

Subtilisin expression in *B. subtilis* is controlled at several levels. Production begins during the transition stage between vegetative growth and sporulation. Though the exact mechanisms which activate expression are not clear, there are a number of known genes which will enhance levels of protease (10). Regulation appears to be primarily at the transcriptional level with both positive (enhancer) and negative (repressor) control elements (11). Figure 5 shows a typical production curve for subtilisin. Enzyme begins appearing after the cessation of logarithmic growth and increases as the culture progresses through the sporulation process. The relationship between sporulation and enzyme production remains unclear. Strains of *B. subtilis* which are lacking the subtilisin gene appear to sporulate normally (12). However, many of the mutations which affect spore production also affect the ability of the cells to produce the enzyme. Again, the control is at the level of messenger RNA production (13).

Table II is a partial list of known *Bacillus* genes which have an effect upon subtilisin production. Henner and coworkers (14) placed beta-galactosidase under the control of the subtilisin promoter. By using promoter deletions, they were able to identify regions of the promoter which are regulated by *sacU*, *sacQ* (present designation *degU* and *degQ* respectively, see Table II) and *hpr*.

Table II. Genetic Loci of *Bacillus subtilis* Which Regulate Subtilisin Production

GENE	PROPOSED ROLE
<i>spoOA</i>	General sporulation control
<i>spoOH</i>	Sporulation specific sigma factor
<i>degU</i>	Transcriptional enhancer
<i>degQ</i>	Transcriptional enhancer
<i>prtR</i>	Transcriptional enhancer
<i>senN</i>	Transcriptional enhancer
<i>abrB</i>	Repressor
<i>hpr</i>	Repressor
<i>sin</i>	Repressor

The subtilisin gene and its upstream regulatory regions are shown in schematic diagram in Figure 6. The mature gene is preceded by both a pre and pro sequence (29 and 106 amino acids respectively) which are removed during the secretion of the enzyme. Maturation of prosubtilisin to mature subtilisin appears to be due to a self-processing event (15).

In order to develop a host strain for production of a variety of subtilisin enzymes, it was first necessary to delete the endogenous alkaline and neutral proteases (12,16). The strain was then constructed to contain the optimal combination of subtilisin regulatory genes which were compatible with the proposed fermentation and recovery process. Once this strain had been produced, the recombinant enzyme

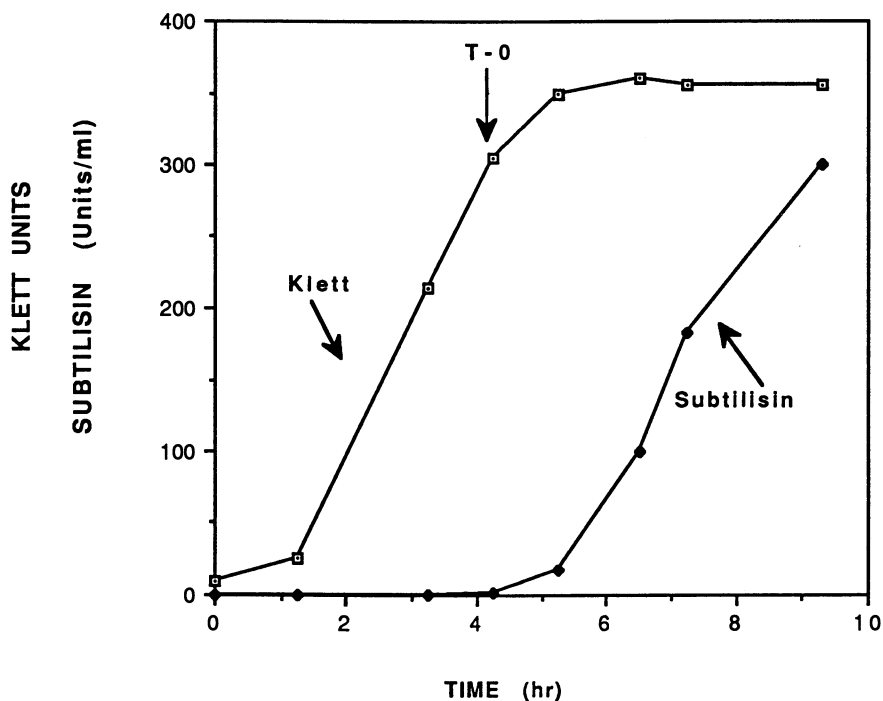


Figure 5. Production of subtilisin. A culture of *B.subtilis* was grown in supplemented nutrient broth (13) and subtilisin production was followed. T-0 indicates the time of first departure from logarithmic growth. Subtilisin levels are indicated as arbitrary units/ml.

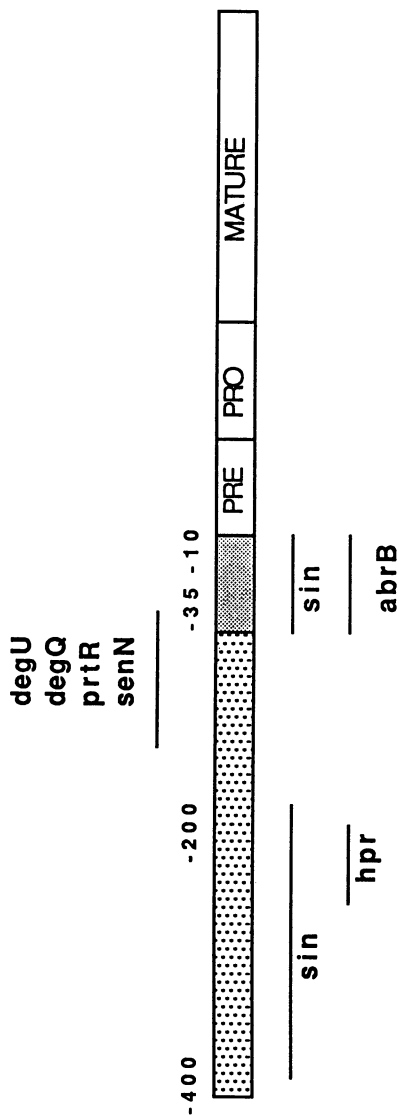


Figure 6. Regulation of the subtilisin promoter. Shaded areas refer to the promoter and upstream regions of the subtilisin gene. Numbers above the figure indicate base pairs upstream. Activators are shown above the figure, repressors below. Lines indicate possible regions of the DNA involved in binding the regulatory proteins.

under the control of the *B.subtilis* subtilisin promoter was cloned into the host organism. The strain was then moved into fermentation development for production optimization studies. After the initial fermentation process had been optimized, the production process was independent of the enzyme being produced. Therefore, production of alternative subtilisin variants (and other proteins as well) was no longer dependent upon individual strain improvement or fermentation development. When new enzymes were introduced, the time required to take them to commercial scale had been shortened considerably.

Summary

Recombinant DNA technology has proven to be an effective means of producing enzymes for large scale commercial manufacture. The ability to genetically engineer preferred characteristics into a naturally occurring enzyme can help avoid extensive natural isolate searches for new enzymes. More importantly, it is now possible to tailor make enzymes for specific customers' needs. Since a protein engineering project includes production of a library of "variant" enzymes, the ability to identify new important activities will be limited solely by the ingenuity of applications screening methods.

Production of laboratory and pilot scale quantities of a newly identified enzyme (either naturally occurring or engineered) can be facilitated by cloning and expressing the gene in an alternate host system. This host may be different from the host for final product of the commercial product (ie. *E.coli* vs *Bacillus spp.*).

By developing a series of generic host production organisms, fermentation development time can be minimized when these host systems are used for production of multiple products. The investment of time and resources in developing the initial fermentation and recovery system can be recovered in subsequent programs in the form of more rapid commercial development timelines.

Genetic engineering techniques have had a significant impact upon the research, development and commercialization phases of projects in the industrial enzyme business. The technology has proven successful and will play an even greater role in the future in expanding the present markets.

Literature Cited

1. Bryan, P.N.; Rollence, M.L.; Pantoliano, M.W.; Wood, J.; Finzel, B.C.; Gilliland, G.L.; Howard, A.J.; Poulos, T.L. *Proteins* **1986**, *1*, 326.
2. Estell, D.; Graycar, T.; Wells, J. *J. Biol. Chem.* **1985**, *260*, 6518.
3. Arbige, M.V.; Pitcher, W.H. *Trends in Biotechnology*. **1989**, *7*, 330.
4. Wells, J.A.; Ferrari, E.; Henner, D.J.; Estell, D.A.; Chen, E.Y. *Nucleic Acids Res.* **1983**, *11*, 7911.
5. Markland, F.S.; Smith, E.L. *J. Biol. Chem.* **1967**, *242*, 5198.
6. Wright, C.S.; Alden, R.A.; Kraut, J. *Nature* **1969** *221*, 235.
7. Markland, F.S.; Smith, E.L. In *The Enzymes*; Boyer, P.D., Ed.; Academic: New York, **1971**; Vol. III, p561.

8. Wells, J.A.; Vasser, M.K.; Powers, D.B. *Gene* **1985**, *34*, 315.
9. Wells, J.A.; Estell, D.A. *Trends Biol. Sci.* **1988**, *13*, 291.
10. Henner, D.J.; Yang, M.; Band, L.; Shimotsu, H.; Ruppen, M.; Ferrari, E. *Proc. 5th Intl. Symp. on Genetics of Industrial Microorganisms* **1986**, p81.
11. Valle, F.; Ferrari, E. In *Regulation of Prokaryotic Development* Smith, I; Slepecky, R.; Setlow, P., Eds. American Society for Microbiology: Washington, D.C., **1989**; p 131.
12. Stahl, M.; Ferrari, E. *J. Bacteriol.* **1984**, *158*, 411.
13. Ferrari, E.; Henner, D.J.; Perego, M.; Hoch, J.A. *J. Bacteriol.* **1988**, *170*, 289.
14. Henner, D.J.; Ferrari, E.; Perego, M.; Hoch, J.A. *J. Bacteriol.* **1988**, *170*, 296.
15. Power, S.D.; Adams, R.M.; Wells, J.A. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 3096.
15. Yang, M.Y.; Ferrari, E.; Henner, D.J. *J. Bacteriol.* **1984**, *160*, 15.

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Chapter 8

Enzymes from Solid Substrates

Recovering Extracellular Degradative Enzymes from *Lentinula edodes* Cultures Grown on Commercial Wood Medium

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Recovering enzymes from white-rot fungi grown on wood could offer the opportunity to produce a powerful set of enzymes for research and development, which are uniquely capable of carrying out a range of extensive or highly-specific modifications to lignocellulose. Recently, we demonstrated that cultures of the edible mushroom *Lentinula edodes* grown on a commercial wood medium secrete a range of important degradative enzymes (ligninases, cellulases, hemicellulases, oligosaccharidases, proteinases, etc.) in quantities sufficient to isolate and characterize. Here we summarize the purification strategy found most effective to recover them and suggest how the enzymes and knowledge gained could be used.

Producing and characterizing microbial degradative enzymes has important practical significance. It can provide new enzymes for use in commercial applications (1) or supply information that permits the more effective use or improvement of microbial cultures. Current applications with isolated enzymes typically use a single or a limited number of enzymes (2). In contrast, applications requiring the use of whole cultures can depend on several enzymes, whose identities and roles are often poorly understood. Potential applications in lignocellulose conversion are especially likely to require multiple enzymes with specificities reflecting the complexity of lignocellulose (3,4) and the conversion desired. Such applications could be more easily developed if researchers had better access to a larger range of useful enzymes.

Here we summarize our work towards developing a method that could efficiently generate a unique set of enzymes in quantities sufficient for research and development by recovering them from wood-grown cultures of *Lentinula* (= *Lentinus*) *edodes* (Berk.)Sing. We discuss the rationale behind the method, the strategy found most effective for recovery, the unique challenges encountered when purifying enzymes from wood extracts, alternative approaches for use with problem extracts or alternative fungal systems, some characteristics of three enzymes purified so far, and give examples for the use of the enzymes and knowledge gained.

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Approach and Rationale

Our recovery method was based on the idea that a superior enzyme set may be obtained if the enzymes were those secreted by a single microorganism growing on the same substrate targeted for enzyme degradation or modification. Our rationale was that this should help ensure that the appropriate enzymes are induced and that they are sufficiently compatible to function effectively in the degradation of a complex polymer like lignocellulose (*i.e.*, the substrate specificities, reaction end-products, and associated binding constants of the individual enzymes must form a functional synergistic system and the enzymes must not be unduly sensitive to endogenous proteinases or oxidases or create undue difficulties (*e.g.*, inhibitory end-products) for the other necessary enzymes).

Having chosen lignocellulose as substrate, we were concerned about the likelihood of producing an appropriate range of enzymes by any method other than solid substrate fermentation. In nature, intact lignocellulose is degraded primarily by solid substrate fermentation, which exposes cultures to a specialized set of conditions difficult to obtain otherwise (5,6). The elevated aeration, elevated concentrations of natural products, slow feeding of nutrients, localized nutrient stress, and localized accumulation of reaction end-products occurring during solid substrate fermentation could markedly affect the enzymes expressed.

A wood medium and a white-rot basidiomycete was selected to produce enzymes. This is because wood is among the most difficult of lignocellulosic materials to degrade and because these microorganisms have evolved to become preeminent wood degraders. Such fungi are capable of producing the entire range of enzymes necessary to completely depolymerize lignocellulose (7). Their enzymes have presumably been selected to cope with elevated lignin content, troublesome polyphenols, and severe diffusion hindrance imposed by lignocellulose matrixes. Finally, growth under the low nitrogen-source content and competitive microorganisms typically present in wood should have selected for efficient, markedly active enzymes that are resistant to proteinase inactivation.

Model System

There are two major reasons why *L. edodes* cultures grown on a commercial wood medium was chosen as the model white-rot system: (I) we typically could detect much higher enzyme levels (8) and had much less difficulty in isolating them from *L. edodes* cultures (9) than from other fungi of current research importance (*e.g.*, *Phanerochaete chrysosporium* and *Phlebia tremellosa*) and (II) wood bioconversion by *L. edodes* is an important process. The cultivation of *L. edodes* on underutilized hardwoods (*e.g.*, scrub oak logs or particles) is currently the single largest commercial bioconversion process using wood (10,11). The fungus produces shiitake, which is the dominant commercial mushroom of the Eastern hemisphere, and a new rapidly-expanding crop in the Western hemisphere. The direct production of such protein crops from lignocellulose and agricultural wastes could become a widely important cash crop and source of food in the future (12).

Recovery Strategy

A simple effective strategy was developed to isolate enzymes from wood-grown cultures of *L. edodes* consisting of the following steps: (a) selecting appropriate cultures, (b) extracting enzymes, (c) removing large molecular weight contaminants, (d) removing interfering polyphenols, (e) removing small molecular weight contaminants, (f) initial fractionation by anion exchange chromatography, and (g)

subsequent fractionation steps as required for individual enzymes. Below we summarize these steps and how the enzyme preparations were stored.

Selecting Cultures. Factors such as the fungal strain, growth medium, moisture content, temperature, aeration level, and culture age can markedly effect the enzyme levels present (Figure 1). Thus, the only reliable method that we found to ensure that the enzymes targeted for extraction were present was to select cultures based on prior experience. Because the strain, medium, and growth conditions are often fixed for a given culture type, we will only outline here two factors that should be considered in selecting cultures. These include lignocellulose particle size and culture age.

The mean lignocellulose particle size in the growth medium effects the efficiency and timing for extraction. The high relative surface area of small particles provides for increased enzyme extraction and earlier extraction (increased nutrient accessibility results in enhanced growth). However, if the particles are too small they can limit aeration and suppress fungal growth.

Studies with *L. edodes* showed that culture age should be carefully selected. Several enzymes showed staged patterns that correlated with changes in age or nutrient status (Figure 1). Whereas, in spite of markedly higher fungal content, other enzymes showed similar levels throughout much of the life cycle, presumably resulting from nutrient regulation (*e.g.*, suppression by enzyme reaction end-products). If desiring a nearly complete set of enzymes in a single extract, we have found it best to extract younger cultures (~ 15 to 30 days old) or cultures not yet in marked nutrient stress (*e.g.*, N-starvation; < 90 days old) be used (8). However, if adequate levels of the targeted enzyme(s) are present, older cultures should not be overlooked. Large quantities of spent cultures suitable for extraction could perhaps be obtained from commercial mushroom farms on a routine basis at little or no cost.

Extracting Enzymes. It is important to understand the limitations of extracting enzymes from lignocellulosic substrates. Given the typical hollow, pore-filled structure of wood and other lignocellulosic materials (4,7), we can assume that only the enzymes present on the exposed hydrated surfaces of such solid substrates will be extracted. And although accessible, the enzymes will not necessarily all be extracted at the same efficiencies. Notoriously tight-binding enzymes, such as cellobiohydrolases (13; visualized in Figure 1 with cellulose azure or solka-floc cellulose as substrate), are likely to be extracted in low yield, thus underestimating their concentrations in the original cultures. Below we discuss the extraction procedure used for *L. edodes* and some observations made while assaying the enzymes present in crude extracts.

Extraction Procedure. We found that distilled water or dilute buffers were usually the preferred extractant. While adding salts, organic solvents, and mild wetting agents sometimes extracted more activity (*e.g.*, 10 to 50%), the increased extraction of polyphenols frequently resulted in decreased enzyme recovery during subsequent purification. Detergents or other agents that break fungal cell membranes should probably be avoided because the release of intracellular proteins could greatly complicate the recovery of extracellular enzymes. The extraction was best done with a solvent volume just large enough to permit effective extraction (typically 1:2 to 1:4 culture to solvent (w/v); 14). Undue extract dilution should be avoided. Besides taking longer to process larger sample volumes, substantial increases in enzyme loss to labware surfaces can occur with dilute extracts.

After adding the solvent, we found that incubating for 1 to 2 hr with intermittent shaking or gentle mixing was adequate for extraction (15). This was followed by decanting, pressing excess liquid from the solid substrate, and removing any remaining wood particles by filtering and/or centrifugation. We had little problem

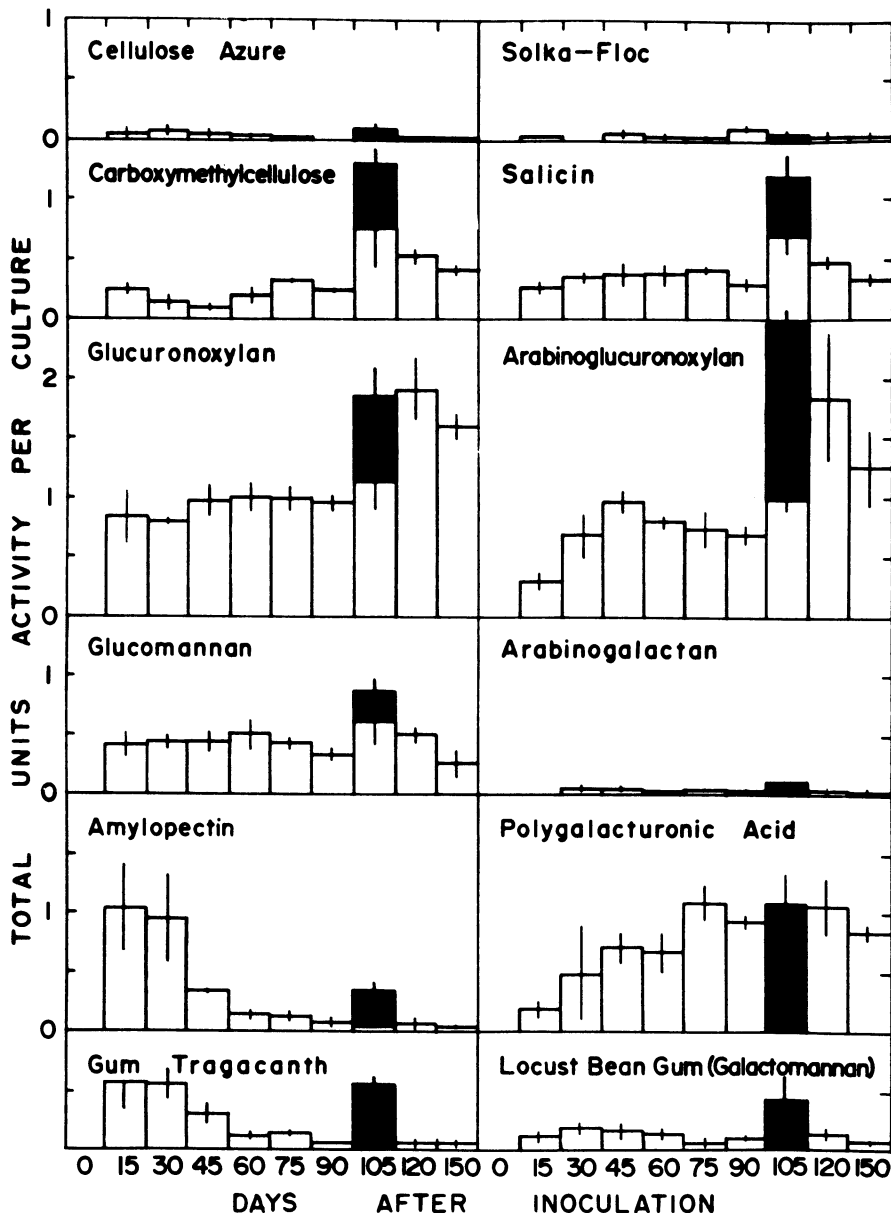


Figure 1. Enzyme activities in crude extracts made from cultures of different ages detected with substrates selected for cellulases, hemicellulases, amylases, and pectinases. Cultures were grown at 22°C. Black bars indicate activity for 105-day-old cultures given a 12-day-long cold shock at 5°C starting on day 90. (Reproduced with permission from ref. 8. Copyright 1985 American Society for Microbiology.)

with polyphenols precipitating the enzymes present in the initial crude extracts, perhaps due to the presence of an unknown fungal wetting agent.

Assays with Crude Extracts. Assays of the activities present in crude culture extracts were useful to indicate the enzymes available for recovery. Extracts from *L. edodes* typically exhibited a wide range of enzyme activities present in quantities apparently sufficient for isolation and characterization (Table I).

However, we have observed that values obtained with crude extracts were only qualitative. Often, they did not accurately estimate the quantities of the individual enzymes present. Inhibitors were typically present that caused the underestimation of certain enzymes (*e.g.*, ligninases; Table II) and that could potentially mask less dominant enzymes. Also, certain polysaccharidases (*e.g.*, hemicellulases) were often overestimated due to the action of non-specific or synergistic enzymes (*e.g.*, other hemicellulases or cellulases) (9,14). This artifact resulted in low apparent recovery of a given activity and only moderate increases in specific activity upon purification of the major corresponding enzyme present, in spite of the fact that SDS polyacrylamide gels indicated good recovery and substantial removal of contaminants (14).

Removing Large Molecular Weight Contaminants. The crude fungal extracts here can potentially contain polysaccharides, such as fungal β -(1,3)-D-glucan slimes (16) and soluble wood hemicelluloses, which can interfere with enzyme concentration and purification. Fortunately such materials were only a minor problem with *L. edodes*. When encountered, the most convenient method found to reduce polysaccharide content was ultrafiltration through membranes sized significantly larger than the largest enzyme anticipated. We used 300,000 or 500,000 mwt cut-off membranes because most fungal extracellular degradative enzymes should be significantly smaller than 200,000 mwt. When prodigious quantities of fungal slimes are present, some researchers congeal them by freeze-thawing or by adding polar organic solvents (*e.g.*, 20% v/v ethanol) after which the slime is skimmed off. Extracts treated in this way could potentially benefit from subsequent ultrafiltration.

Removing Interfering Polyphenols. The most troublesome problem encountered was high polyphenol content. If not removed, this dark-colored wood extractive decreased resolution during chromatographic separations, quickly render expensive chromatographic media (*e.g.*, HPLC columns) nearly useless, and often precipitated enzymes during subsequent purification steps (14,15). We found it best to remove the bulk of this material prior to the first concentration step.

Traditional methods to remove polyphenols include polyphenol adsorption with polyvinylpyrrolidone or protein precipitation with either ammonium sulfate or organic solvents. After testing many alternatives, the most effective method developed was to precipitate and centrifuge the polyphenols away from the enzymes using a minimal concentration (*e.g.*, 0.1 to 0.3 %) of a water-soluble polycation bioprocessing aid such as polyethylenimine (14,15) or Biocryl BPA-1000 (see Forrester, I.T. *et al.* chapter in this symposium book). The concentration of precipitating agent used was determined by titration, using color removal (A_{400nm}) as an indicator. An alternative removal method was to absorb the sample to a small quantity of a diethylaminoethyl (DEAE) or quaternary ammonium (QM) anion exchange resin in dilute buffer and batchwise displace the enzymes with a salt solution (*e.g.*, 500 to 1000 mM NaCl), after which the spent resin was discarded (9).

Removing Small Molecular Weight Contaminants. Ultrafiltration in hollow fiber membrane cartridges was the best method found to remove small molecular weight materials (15). It also served to concentrate the enzymes and change the buffer (via diafiltration) to that required for the next purification step. A 10,000 mwt

Table I. Enzyme Activities Extracted from *Lentinula edodes* Cultures Grown on a Commercial Wood Medium and Assayed in a Crude Extract

<i>Substrate used</i>	<i>Activity type</i>	<i>U/kg culture</i> ^a
Cellulose azure	"exo/endo-cellulase"	65
Acid-swollen cellulose	"exo/endo-cellulase"	1,230
Carboxymethylcellulose	"endo-cellulase"	1,980
PNP- β -D-cellobioside	"exo-cellulase"	710
Salicin	"cellobiase"	1,980
PNP- β -D-glucopyranoside	" β -D-glucosidase"	1,160
Glucuronoxylan	"xylanase"	4,680
Arabinoglucuronoxylan	"xylanase"	4,790
Glucomannan	"mannanase"	3,120
PNP- α -L-arabinofuranoside	" α -L-arabinosidase"	539
PNP- β -D-mannopyranoside	" β -D-mannosidase"	102
PNP- α -D-galactopyranoside	" α -D-galactosidase"	582
Laminarin	"laminarinase"	5,538
Pachyman (β -(1,3)-D-glucan)	"pachymanase"	3,260
Pustulan (β -(1,6)-D-glucan)	"pustulanase"	1,030
Chitin	"chitinase"	142
PNP-N-acetyl- β -D-glucosaminide	" β -D-glucosaminidase"	298
α -Tolidine + O ₂ (air)	"laccase"	880
Veratryl alcohol + H ₂ O ₂	"ligninase"	40
PNP-phosphate	"acid phosphatase"	99
Hide powder azure	"acid proteinase"	1,846

^a One U equals one international unit, except for substrates releasing azure dye, where it equals that activity liberating 0.1 OD at 595 nm (1 cm cuvette) per min. (Adapted with permission from ref. 9. Copyright 1990 Society of Fermentation Technology, Japan.)

Table II. Purification Table for the Manganese Peroxidase (Ligninase) Isolated from *Lentinula edodes* Cultures Grown on a Commercial Wood Medium^a

Purification step	Total volume (ml)	Protein concentration (mg/ml)	Units per ml	Total units	Specific activity (U/mg)	Yield (%)	Fold purified
Crude culture extract	14,500	0.04	1.4	20,160 ^b	35	100	1.0
PEI clarification	13,750	0.04	1.9	25,990 ^b	51	129	1.5
Concentration and diafiltration ^c	255	0.89	95	24,220	107	120	3.1
DEAE chromatography	54	1.01	294	15,990	292	79	8.4
Hydrophobic interaction HPLC	53	0.37	244	12,990	660	65	19.0

^a Assayed with *o*-tolidine as substrate in the presence of H₂O₂ and Mn²⁺.

^b An inhibitor is present in the crude culture filtrate that gives 22% inhibition of peroxidase activity.

^c Ultrafiltration was carried out with a 30,000 mol wt cut-off membrane.

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cut-off membrane was used to obtain complete enzyme sets. Whereas, a membrane of a larger cut-off size was used to obtain a single or few a target enzymes (*e.g.*, 30,000 for a 44,600 mwt ligninase or 75,000 for a 86,000 mwt acid phosphatase). The larger cut-off sizes gave quicker filtration and removed some of the smaller protein contaminates, without any significant loss of the target enzyme(s).

Initial Fractionation. The most effective method found for the initial fractionation of the complex enzyme mixtures present in wood-culture extracts was anion exchange chromatography eluting with salt gradients (9,14,15). This permitted further sample concentration by functioning as a solid phase extractant during loading. It also showed higher sample capacity and better separations than other methods.

Although effective, residual polyphenols in crude samples resulted in less separation than possible with this method. Such binding often resulted in peaks containing several different activities (9). And increased sample loading often broadened and reduced the number of peaks (9,14). Due to these interferences, two different scales of anion exchange chromatography were used. Analytical separations were used to gather information about the enzymes present and preparative separations were used to purify enzyme quantities sufficient for characterization.

Analytical HPLC columns loaded with small samples gave the best separations and useful information (9). Screening the resulting column fractions with a series of enzyme substrates indicated the minimum number of different enzymes present, the salt concentrations at which enzymes tended to elute, where multiple enzymes were likely to be co-eluting (thus requiring further separation), and the substrates most useful to distinguish between the different enzymes present (Figures 2, 3 and 4).

Preparative separations with larger sample volumes were carried out in conventional chromatography columns that had been hand-packed with DEAE or QM anion exchange resins (14,15). This gave less separation than did the analytical method (compare Figure 5 with 4). However, the preparative separation was adequate for our purpose and it permitted removal of any polyphenols that remained bound to the column. Polyphenol removal was carried out after a series of runs by discarding the small portion of dark brown-colored resin from the top of the resin bed. Few subsequent troubles were encountered with the low polyphenol content present after this chromatography step. Although our fractionation schemes have typically used one anion exchange chromatography step, fractions containing many proteins could perhaps benefit from a subsequent fractionation step on a preparative anion exchange HPLC column prior to using another method.

Subsequent Fractionation Methods. Subsequent purification of a given enzyme to yield a single band on SDS gels typically required only one or two additional separation methods. Those found most useful included hydrophobic interaction (*e.g.*, with ligninase; Figure 6) and preparative isoelectric focusing (*e.g.*, with xylanase; 14). Also showing promise was gel permeation chromatography using a resin having maximal separation ability between 20,000 and 90,000 mwt. Such sizing methods should perhaps be used as the final fractionation step due to their low sample capacities and the frequent similarities in enzyme size.

Storage. Purified proteins were readied for storage by changing to a dilute buffer that would not interfere with subsequent characterization using diafiltration in pressurized stirred ultrafiltration cells or collodion bags. The solutions were then frozen at -20°C. We did not find it necessary to add agents such as 20% glycerol to protect the enzymes against ice crystal formation. Enzymes stored in this fashion were markedly stable. No significant activity loss (< 3%) resulted from freeze-thawing the enzymes several times or from allowing them to remain frozen for several months (14).

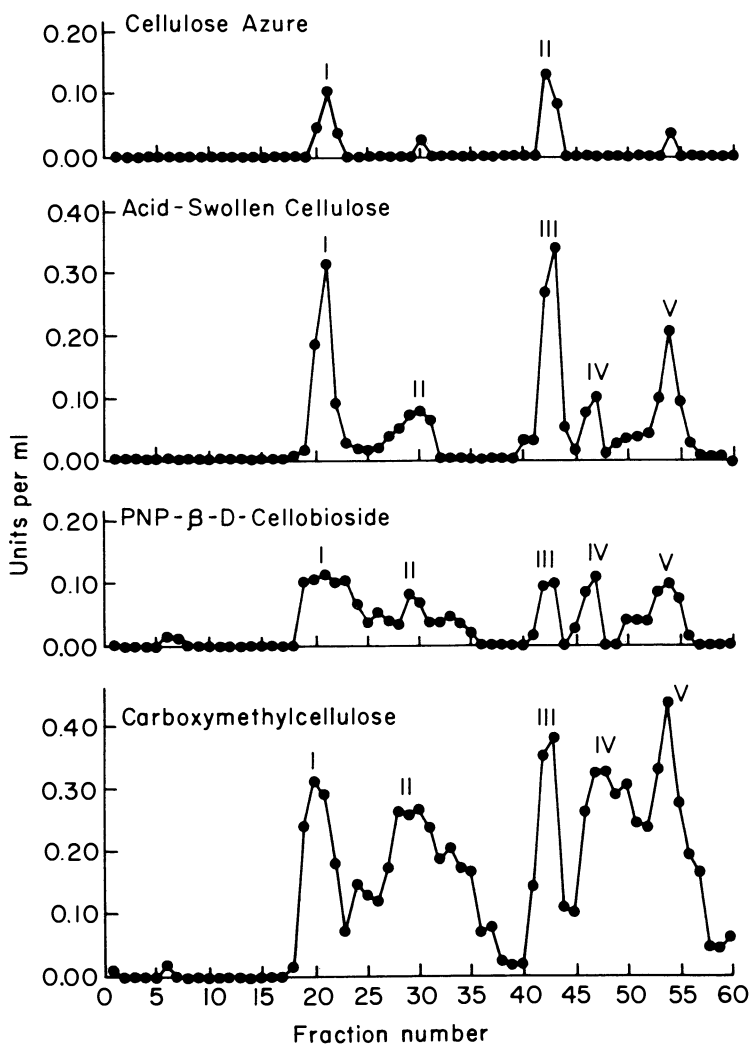


Figure 2. Enzyme activities in analytical QM anion exchange column fractions detected with substrates selected for cellulases. Major peaks are numbered. (Reproduced with permission from ref. 9. Copyright 1990 Society of Fermentation Technology, Japan.)

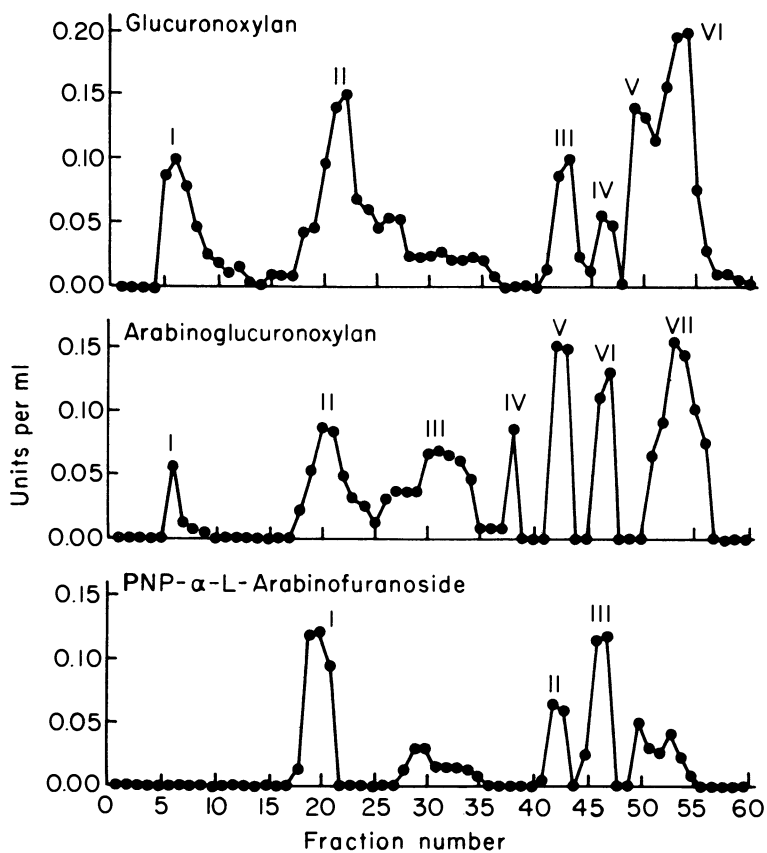


Figure 3. Enzyme activities in analytical QM anion exchange column fractions detected with substrates selected for hemicellulases. Major peaks are numbered. (Reproduced with permission from ref. 9. Copyright 1990 Society of Fermentation Technology, Japan.)

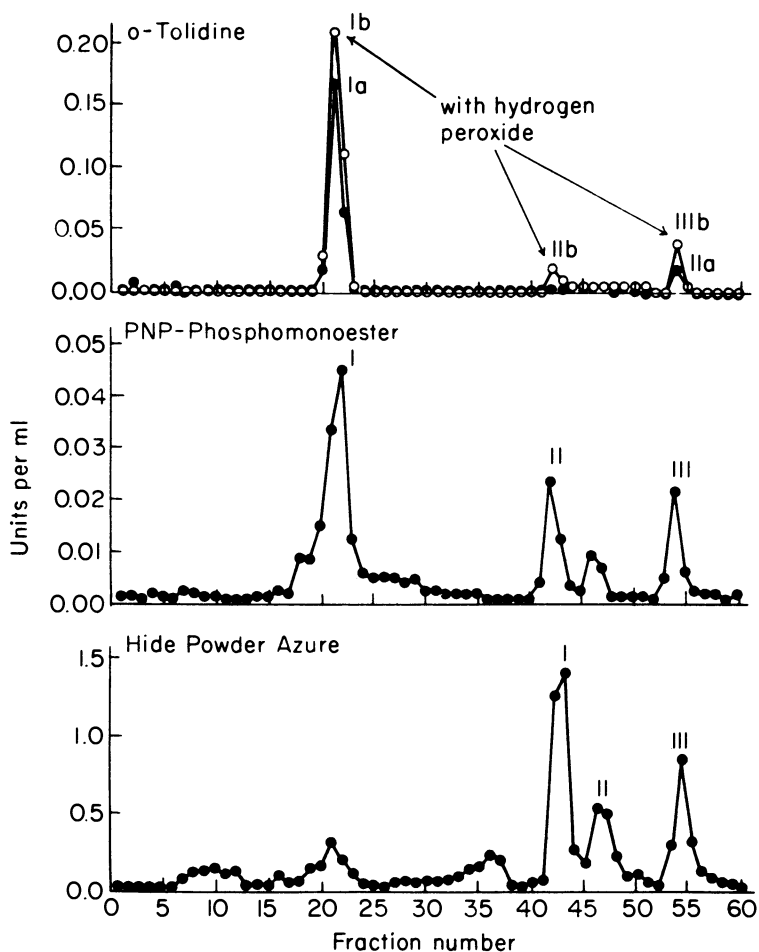


Figure 4. Enzyme activities in analytical QM anion exchange column fractions detected with substrates selected for laccases, peroxidases (including ligninases), acid phosphatases, and acid proteinases. Major peaks are numbered. (Reproduced with permission from ref. 9. Copyright 1990 Society of Fermentation Technology, Japan.)

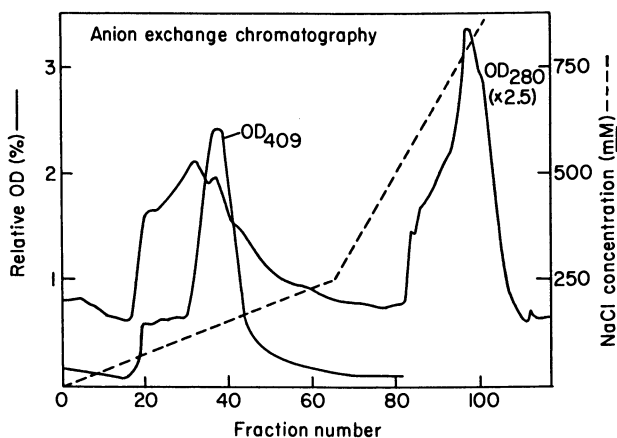


Figure 5. Optical absorbance at 280nm and 409nm (denoting heme-iron containing ligninases) for fractions from the preparative DEAE anion exchange column and salt gradient used to fractionate the enzymes in a crude culture extract. (Reproduced with permission from ref. 15. Copyright 1990 Springer-Verlag.)

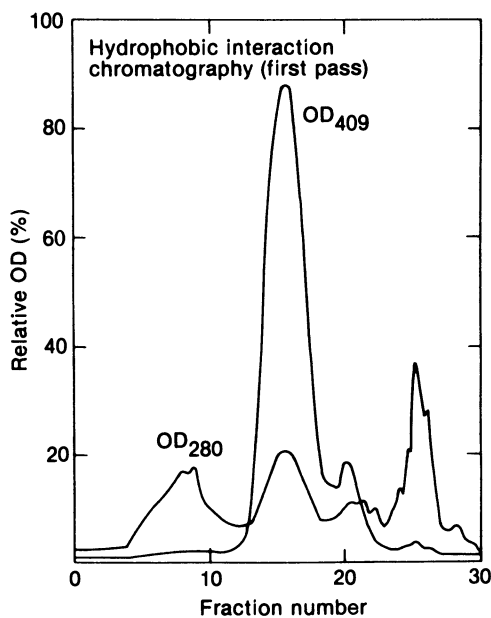


Figure 6. Optical absorbance at 280nm and 409nm (denoting heme-iron containing ligninases) for fractions from the TSK phenyl hydrophobic interaction column used to further purify the ligninase peak taken from the preparative anion exchange column shown in Figure 4. (Reproduced with permission from ref. 15. Copyright 1990 Springer-Verlag.)

Recovered Enzyme Characteristics

The value and potential usefulness of a new enzyme depends on its properties and the extent to which it has been characterized. The initial characterization of an enzyme often involves the determination of its pH optimum, stability, gross physical properties, and substrates. The enzymes of *L. edodes*, typically show pH optima between 3.5 and 5.0, maximal activity at 50 to 60°C, little activity loss until over 70°C, and high relative specific activities (9,14). Below we will highlight some of the other characteristics determined for the major ligninase, β -(1,4)-D-xylanase, and α -(1,3)-L-arabinosidase purified from wood-grown cultures of *L. edodes*.

The only major ligninase detected was a single manganese peroxidase with a molecular weight of 44,600 and isoelectric point of 3.2 (15). It was a heme-iron protein containing one protoporphyrin IX group per enzyme. It oxidized Mn^{2+} to Mn^{3+} using hydrogen peroxide as oxidant. The enzyme was stimulated by pyrophosphate and certain C₂- to C₄-organic acids that chelate and stabilize Mn^{3+} . In the presence of such stabilizers, the Mn^{3+} -chelate generated was capable of oxidizing lignin and lignin model compounds (15,17). The N-terminal sequence of the enzyme showed significant homologies with the manganese (61.7%) and lignin peroxidases (53.2% to 46.8%) reported from *P. chrysosporium* (Figure 7). We suspect that the enzyme functions in lignin degradation by oxidizing manganese to form Mn^{3+} chelates that readily diffuse into wood and oxidatively depolymerize lignin (17).

The major xylanase detected was non-debranching β -(1,4)-D-endoxylanase with a molecular weight of 41,000 and isoelectric point of 3.6 (14). The enzyme was markedly specific for xylans. It showed high specific activity (310 U/mg protein at 40°C) with oat spelt arabinoxylan and aspen wood glucuronoxylan, much reduced activity with β -(1,3)- and β -(1,6)-D-glucans, and no activity with β -(1,4)-D-glucans including acid-swollen cellulose or carboxymethylcellulose. The enzyme showed no oligosaccharidase activity with *p*-nitrophenol sugar analogs. Lacking debranching activity, the enzyme requires accessory enzymes (18,19) to degrade native xylans.

The only major accessory enzyme purified thus far for the xylanase was an α -(1,3)-L-arabinosidase with a molecular weight of 66,000 and isoelectric point of 4.1 (publication in preparation). The enzyme was not capable of acting on intact xylans. However, it acted on the end-products of arabinoxylan (but not glucuronoxylan) degradation by the xylanase, doubling the total extent of degradation.

Future Research

The characterization and use of fungal degradative enzymes produced on solid substrates is a promising area that deserves more research. The first three enzymes isolated from *L. edodes* should be characterized further. Additional enzymes important in lignocellulose degradation should also be isolated and characterized, such as additional hemicellulase accessory enzymes (*e.g.*, α -(1,2)-D-glucuronidase, deacetylase, demethylase, and β -D-xylosidase; 18,19) and cellulases (9). Research is outlined below that could be carried out using the enzymes and information gained.

Enzymes. The most important use for an enzyme set produced by the methods described here would perhaps be in determining the roles of individual enzymes in depolymerizing or modifying lignocellulose or lignocellulose-derived fibers. Besides helping to understand lignocellulose degradation, such information could be used to develop a range of important new commercial applications. Examples with high potential impact include efficient fuel and chemical feedstock production (20), improving paper pulp (*e.g.*, biobleaching kraft pulp or de-inking and restoring

Enzyme	Sequence & Sequence homology	Homology (%)
<i>Lentinula edodes</i>		
MnP ("MnP1")	AVGSDGTVPD[S]VQYDFIPLAQDLTAXLFE-NQXGEXAHERLAXXFHD	
<i>Phanerochaete chrysosporium</i>		
MnP ("MnP1") (Pribnow et al. 1989)	AVCPDGGTRVSHAAACCAFIPLAQDLQETJFQ-NECGEDAHEVIRLTFHD	61.7
MnP ("MnP1") (Pease et al. 1989)	AVCPDGGTRVTNAACCAFIPLAQDLQETLFO-GDCGEDAHEVIRLTFHD	61.7
LP ("CLG5") (deBoer et al. 1987)	ATCSNGK[V]FAASCCTFWNVLSDIQENLFGGCGGAFAHESIRLVFHD	53.2
LP ("H8") (Smith et al. 1988)	ATCSNGKTVGDAASCCAFDVLDDIQQLFHGGCGGAFAHESIRLVFHD	51.1
LP ("O282") (Schalch et al. 1989)	ATCANGIATVGDASCCAFDVLDDIQENLFGGCGGAFAHESIRLVFHD	48.9
LP ("CLG4") (deBoer et al. 1987)	ACP DGVHTASNAACCAWF[V]VLDDIQQLLFGGCGGAFAHEA[RM]VFHD	46.8

Figure 7. N-terminal sequence for the first 47 amino acids of the manganese peroxidase (MnP) isolated from *L. edodes* cultures grown on a commercial wood medium and homology with the deduced amino acid sequences for the MnP's or lignin peroxidases (LP's) reported from *P. chrysosporium*. Boxed areas show regions of sequence identity. Homology is the total length of identical or conservative substitutions. (Reproduced with permission from ref. 15. Copyright 1990 Springer-Verlag.)

strength to recycled pulp), treatment of pulping wastes (21), and increasing animal feed digestibilities (22).

Information. The most immediate use of the information gained here would be in improving shiitake cultivation. An understanding of the enzymes produced by *L. edodes* can be used to improve shiitake cultivation in variety of ways. Knowledge of the preferred substrates for its dominant enzymes can be used to help select the individual components for its cultivation media. This information can also pinpoint the degradative weaknesses of the fungus so that effective strategies can be designed to select or improve commercial strains (23). For example, once rate-limiting enzymes are known, antibodies could be made against them and ELISA methods developed to rapidly screen for strains with enhanced activity. Or N-terminal sequence information could be used to create oligonucleotide probes for use in molecular genetic programs designed to increase enzyme expression (15).

The techniques described here could be adapted to producing enzymes from other fungi or for gaining information necessary to improve other solid-substrate fermentations. This latter area is important because, even though the degradative enzymes are likely to effect process economics, we have little direct knowledge of those used by microorganisms growing on solid substrates. Examples of potential fungal applications based on lignocellulose conversion include the biopulping of wood (24-26), degradation of environmental toxins (e.g., polychlorinated biphenyls and pentachlorophenol; 27,28), aerobic reduction of waste cellulose and other wood-derived materials prior to landfilling, and production of fungal conidia as biopesticides (e.g., *Beauveria bassiana* to control of Gypsy moth or grasshoppers; personal communication, C. Bradley, Mycotek, Inc., Butte, MT).

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Literature Cited

1. Munro, I. G. *Biologist* **1985**, *32*, 289-93.
2. Beuchat, L. R. *Food and Beverage Mycology*, 2nd Ed.; van Nostrand Reinhold: New York, 1987.
3. Jeffries, T. W. In *Wood and Cellulosics: Industrial Utilization, Biotechnology, Structure and Properties*; Kennedy, J. F.; Phillips, G. O.; Williams, P. A., Eds.; Ellis Horwood Ltd: Chichester, West Sussex, England, 1987; pp 213-30.
4. Coté, W. A. In *The Structure, Biosynthesis and Degradation of Wood. Recent Advances in Phytochemistry, Vol. 11*; Loewus, F. A.; Runeckles, V. C., Eds.; Plenum Press: New York, 1977; pp 1-44.
5. Mudgett, R. E. In *Manual of Industrial Microbiology and Biotechnology*; Demain, A. L.; Solomon, N. A., Eds.; American Society for Microbiology: Washington, D.C., 1986; pp 66-83.
6. Hesseltine, C. W. *Internat. Biodeterior.* **1987**, *23*, 79-89.
7. Kirk, T. K. In *The Filamentous Fungi, Vol. 4, Fungal Technology*; Smith, J. E.; Berry, D. R.; Kristiansen, B., Eds.; Edward Arnold: London, 1983; pp 266-95.
8. Leatham, G. F. *Appl. Environ. Microbiol.* **1985**, *50*, 859-67.
9. Mishra, C.; Leatham, G. F. *J. Ferm. Bioengin.* **1990**, *69*, 8-15.

10. Leatham, G. F. *Forest Prod. J.* **1982**, *32*, 29-35.
11. Leatham, G. F.; Leonard, T. J. In *Shiitake Mushrooms: a National Symposium and Trade Show*, Alternative Crop Center, University of Minnesota, St. Paul, 1989; pp 19-28.
12. Campbell-Platt, G.; Cook, P. E. *J. Appl. Bacteriol. Symp. Suppl.* **1989**, 117S-31S.
13. Reese, E. T. *Process Biochem.* **1982**, *17*, 2-6.
14. Mishra, C.; Forrester, I. T.; Kelley, B. D.; Burgess, R. R.; Leatham, G. F. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 226-32.
15. Forrester, I. T.; Grabski, A. C.; Mishra, C.; Kelley, B. D.; Strickland, W. N.; Leatham, G. F.; Burgess, R. R. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 359-365.
16. Wessels, J. G. H.; Sietsma, J. H. In *Encyclopedia of Plant Pathology, Vol. 13B, Plant Carbohydrate II, 2nd Ed.*; Springer Verlag: Berlin, 1981; pp 352-94.
17. Forrester, I. T.; Grabski, A. C.; Burgess, R. R.; Leatham, G. F. *Biochem. Biophys. Res. Comm.* **1988**, *157*, 992-99.
18. Biely, P. *Trends Biotechnol.* **1985**, *3*, 286-90.
19. Dekker, R. F. H. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: New York, 1985; pp 505-31.
20. Eriksson, K.-E. *Tappi J.* **1985**, *68*, 46-55.
21. Eriksson, K.-E.; Kirk, T. K. In *Comprehensive Biotechnology, Vol. 3*; Robinson, C. W., Ed.; Pergamon Press: Toronto, 1985; pp 271-94.
22. Wong, T. M.; Tan, L. U. L.; Saddler, J. N. *Microbiol. Rev.* **1988**, *52*, 305-17.
23. Lu, S.-I.; Leonard, T. J.; Dick, S.; Leatham, G. F. *Micologia Neotropical Aplicada* **1989**, *1*, 5-9.
24. Myers, G. C.; Leatham, G. F.; Wegner, T. H.; Blanchette, R.A. *Tappi J.* **1988**, *71*, 105-8.
25. Leatham, G. F.; Myers, G. C.; Wegner, T. H.; Blanchette, R.A. *Tappi J.* **1990**, *73(4)*, 249-55.
26. Leatham, G. F.; Myers, G. C.; Wegner, T. H. *Tappi J.* **1990**, *73(5)*, 197-200.
27. Eaton, D. C. *Enz. Microb. Technol.* **1985**, *7*, 194-96.
28. Mileski, G. J.; Bumpas, J. A.; Jurek, M. A.; Aust, S. D. *Appl. Environ. Microbiol.* **1988**, *54*, 2885-89.

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Chapter 9

Production of *Trichoderma reesei* Cellulase System with High Hydrolytic Potential by Solid-State Fermentation

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For efficient and complete hydrolysis of cellulose, cellulase systems must contain endoglucanase, exoglucanase, and β -glucosidase in the right proportions. The cellulase systems produced under typical laboratory culture conditions are usually deficient in β -glucosidase. We show here that with solid-state fermentation, as fungal cultures are adapted to grow in nature, *Trichoderma reesei* QMY-1 produces high levels of cellulase enzymes, often having higher activities and more optimal β -glucosidase levels than with other methods. Consequently, this cellulase system has high hydrolytic potential. The presence of cotton hydrolyzing activity in this cellulase system could also be related to its high hydrolytic potential.

For complete hydrolysis of cellulose to glucose, cellulase systems must contain the following enzymes: (i) endoglucanase (1,4- β -glucan glucohydrolase, EC 3.2.1.4) (1), (ii) exoglucanase (1,4- β -glucan cellobiohydrolase, EC 3.2.1.91) (2,3), and (iii) β -glucosidase, EC 3.2.1.21 (β -D-glucoside glucohydrolase or cellobiase) (4,5). Because of the synergy of these enzymes, cellulose is efficiently hydrolyzed to glucose (6,7). Because the hydrolysis of cellulose is due to the synergistic action of endo- and exoglucanases, we refer to their activity in this paper as filter-paper cellulase (FP cellulase) (8).

The cellulase systems of hypercellulase mutants of *T. reesei* produced under typical laboratory culture conditions are generally deficient in β -glucosidase. During the hydrolysis of cellulose, cellobiose accumulates, which inhibits the cellulases (9). From the hydrolysis results reported by various researchers (10-12), we can deduce that a cellulase system having a ratio of FP cellulase activity to β -glucosidase activity (as assayed on salicin) close to 1:1 is necessary to obtain the highest rate of hydrolysis and the highest glucose content in the hydrolysate.

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A literature survey indicated that very little work has been done to produce an optimal cellulase system as described above. Here, we used solid-state fermentation (SSF) to achieve this objective. SSF processes, such as the "koji" process, have been used extensively for amylase production on wheat bran in Japan; its application was extended to cellulase production on wheat bran and lignocellulosic materials by Toyama (13). Since then, wheat bran has become an important substrate for producing various products by SSF (14-20). In this study, we tested various lignocellulosic substrates for the production of cellulase and β -glucosidase from *T. reesei* QMY-1 by SSF.

Materials and Methods

Pretreatment of Substrate. Several different lignocelluloses were pretreated with NaOH. This pretreatment partially solubilizes the hemicelluloses and lignin and swells the cellulose so that the organism can utilize it for its growth and for production of a cellulase system in SSF. The treated lignocelluloses were not washed. The NaOH treatment is done with a minimum amount of water so that, after the addition of nutrient solution and inoculum, the moisture content is less than 80% wt/wt and there is no free water in the medium. More water was added to make suspensions of different lignocellulosic substrates of the desired concentration (1% or 5%) for liquid-state (submerged) fermentation (LSF).

Microorganism. We used *T. reesei* QMY-1 derived from *T. reesei* QM 9414 by Chahal (21) for this work.

Nutrients and Culture Conditions. Nutrients described by Mandels and Weber for cellulase production were supplied in concentrated form (22), but proteose peptone was replaced with yeast extract (Difco Laboratories, Detroit, Mich.). The quantity of nutrients required for each substrate was determined at the rate of their carbohydrate content. The required amount of concentrated nutrient salt solution (5 ml for wheat straw and 5.7 ml for chemithermomechanical pulp, or CTMP) was added to 5 g of substrate. The concentrated salt solution contained the following, dissolved in 200 ml of water: KH_2PO_4 , 28 g; $(\text{NH}_4)_2\text{SO}_4$, 19.6 g; urea, 4.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.2 g; CaCl_2 , 4.2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 70 mg; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 21.84 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 19.6 mg; CoCl_2 , 28 mg; and yeast extract, 7 g. A full concentration of the nutrients means the complete required quantity of the nutrients mentioned above, whereas a one-half concentration is one-half of that quantity. All the flasks were autoclaved at 121°C for 20 min, after the nutrient salt solution was mixed with the substrate.

Inoculum. Inoculum of *T. reesei* QMY-1 was produced on the modified medium as described above but containing 1.5% glucose, with the nutrient salt solution diluted accordingly. For inoculation of each flask containing 5 g of substrate, 5 ml of 2-day-old culture was used. For SSF experiment, the inoculum was spread on the surface of the substrate.

Culture conditions. All the SSF cultures were incubated at 30°C in a humidified incubator (about 80% relative humidity), whereas the LSF cultures were incubated at the same temperature on a shaker at 200 rpm.

Extraction of the cellulase system. The culture of SSF from each flask (originally 5 g of substrate) was mixed well with more water to bring the final weight of the mixture (mycelium plus unutilized lignin, cellulose, and hemicelluloses) to 100 g. Tween 80 was added at a rate of 0.1%. The mixture was shaken for 0.5 h and centrifuged. The supernatant was used for enzyme determination. We estimated that about 7% to 10% cellulases remained adsorbed on the residues (mycelium and unutilized cellulose, hemicelluloses, and lignin) when the residues were suspended in water and Tween 80 as before and the supernatant was tested for cellulase titer.

Analyses. Cellulase enzyme activities were determined as described earlier (21). Enzyme activities were expressed as international units (IU), i.e., μ mole glucose produced per minute. Cotton hydrolyzing activity was expressed as mg of glucose released in 24 hours per ml of enzyme.

Sugars were estimated by using a Beckman 344 gradient high-pressure liquid chromatograph with an Altex 156 refractive index detector and a Spherogel 7.5% carbohydrate column with a flow rate of 0.5 ml/min in the mobile phase of water at 80°C. The sugar samples were appropriately diluted before injection.

Results and Discussion

Role of SSF in Cellulase Production: Effect of Substrate

Wheat straw. Wheat straw ground to 20 mesh was treated with 2% NaOH solution (wt/vol) in 1:2 (solid:liquid) ratio at 121°C for 0.5 h (i.e., 4 g NaOH/100 g wheat straw). *Trichoderma reesei* QMY-1 was grown on pretreated wheat straw in SSF as well as in LSF under otherwise identical culture conditions. The SSF was carried out with full nutrient concentrations in one set and with one-half nutrient concentrations in the other set to evaluate the possible deleterious effects of elevated osmotic pressure. *T. reesei* QMY-1 produced FP cellulase of 8.6 IU/ml (430 IU/g cellulose or 172 IU/g substrate) in 22 days. This showed that the organism was able to tolerate the high salt concentrations required in the SSF. In contrast, when the nutrients were supplied in one-half concentration, FP cellulase activity dropped to 6.7 IU/ml (335 IU/g cellulose or 134 IU/g substrate). However, the maximum enzyme activity was obtained one week earlier (14 days) than that obtained with full salt concentrations (Table I).

When wheat straw was fermented in LSF, the FP cellulase level reached 6 IU/ml (300 IU/g cellulose or 120 IU/g substrate) (Table I) by day 11, decreasing thereafter. This showed that SSF was better than LSF for cellulase production when using wheat straw.

Table I. Cellulase Production on Wheat Straw with *Trichoderma reesei* QMY-1

Fermentation time (days) ^a	FP cellulase (IU/ml)	FP cellulase (IU/g cellulose) ^b	FP cellulase (IU/g substrate) ^b
1. Solid-state fermentation ^c			
(a) Full nutrient concentrations			
22	8.6	430	172
(b) One-half nutrient concentrations			
14	6.7	335	134
2. Liquid-state fermentation (5% conc.) ^d			
11	6.0	300	120

a. Time when maximum enzyme activities were obtained.

b. Enzyme activity (IU/g cellulose or substrate)

$$= \frac{\text{Total enzyme activity (IU)}}{\text{Weight of cellulose or substrate used (g)}}$$

c. SSF = 5 g alkali-treated wheat straw containing 2 g cellulose with only 20 g H₂O. Moisture content of 80%. Enzymes extracted by shaking the 5 g solids in 100 ml of H₂O to make it comparable to that of LSF for uniformity in presentation of enzyme equivalent to that of LSF.

d. LSF = 5 g alkali-treated wheat straw containing 2 g cellulose in 100 ml medium.

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Aspen Wood Treated with NaOH. Aspen wood ground to 20 mesh was treated with 3% NaOH solution (wt/vol) in 1:2 (solid:liquid) ratio at 121°C for 1 h (i.e., 6 g NaOH/100 g wood) (23). Because the lignin content in wood is high (about 25%) compared to that of wheat straw (about 14%), the treated wood was washed to remove the partially solubilized lignin. Partially solubilized hemicelluloses were also washed out. In this case, a maximum FP cellulase of 6.4 IU/ml (194 IU/g cellulose or 128 IU/g substrate) was obtained in 20 days in SSF. A FP cellulase of only 2.5 IU/ml (76 IU/g cellulose or 50 IU/g substrate) was obtained in 15 days in LSF (Table II). The low enzyme activities and yields achieved on wood as compared to that on wheat straw might be caused by the removal of hemicelluloses and lignin and possibly because of its structural differences with wheat straw. Our earlier findings indicated that the presence of hemicelluloses in the medium helps to increase the cellulase production (21). However, as before, SSF proved to be a better system, giving higher enzyme activity per unit (ml) volume and also higher yields per unit (g) cellulose/substrate than LSF.

Aspen Wood Treated with Chemical-Thermomechanical Process. Aspen wood was pulverized into "chemithermomechanical pulp" (CTMP) by a chemical-thermomechanical process (24). During this process, very little hemicellulose and lignin are removed. The CTMP was used without any

Table II. Cellulase Production on Aspen Wood with *Trichoderma reesei* QMY-1

Fermentation time (days)	FP cellulase (IU/ml)	FP cellulase (IU/g cellulose)	FP cellulase (IU/g substrate)
1. Solid-state fermentation			
20	6.4	194	128
2. Liquid-state fermentation (5% wt/wt)			
15	2.5	76	50

- Time when maximum enzyme activities were obtained.
 - 5 g pretreated wood (66% cellulose) in each flask.
 - Cellulase enzymes were extracted as explained for Table I.
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further treatment in one experiment, in another, it was treated with 2% NaOH.

A maximum FP cellulase of 6.3 IU/ml (191 IU/g cellulose or 126 IU/g substrate) was obtained on NaOH-treated CTMP after 20 days in SSF. On untreated CTMP, the FP cellulase remained about 5 IU/ml from 20 to 26 days of fermentation and then increased to 7.2 at 30 days of fermentation (Table III). This indicated that CTMP was a good substrate for cellulase production in SSF even without the mild NaOH treatment.

Table III. Cellulase Production on Aspen Pulp (CTMP) with *Trichoderma reesei* QMY-1

Fermentation time (days)	FP cellulase (IU/ml)	FP cellulase (IU/g cellulose)	FP cellulase (IU/g substrate)
1. CTMP treated with 2% NaOH			
20	6.3	191	126
2. CTMP, untreated			
20	5.0	151	100
26	5.6	170	112
30	7.2	218	144

- Time when maximum enzyme activities were obtained.
- 5 g (dry wt) of CTMP (66% cellulose) in each flask.
- Cellulase enzymes were extracted as explained in Table 1.
- CTMP was treated with 2% NaOH solution (wt/vol) at 121°C for 1 h with 1:2 (solid:liquid) ratio (i.e. 4 g NaOH/100 g CTMP). No washing was done.

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The FP cellulase per unit (ml) volume and enzyme yield per unit (g) cellulose or substrate obtained on wheat straw, wood, and CTMP in SSF were higher than those obtained in LSF on wheat straw and wood (Tables I, II, and III). And wheat straw proved to be a better substrate than wood for cellulase production in SSF. This could be attributed to the polysaccharides (cellulose and hemicelluloses) of wheat straw being more readily available for the organism's growth and cellulase synthesis than those of wood. The hemicelluloses and cellulose were presumably not as available in wood, because of its high lignin content and high cellulose crystallinity, as in wheat straw.

Role of SSF on the Composition of Cellulase System

Our first detailed analysis of the cellulase system produced on wheat straw in SSF indicated that the activities of various enzymes were as follows (IU/ml): FP cellulase, 8.6; β -glucosidase, 10.6; and xylanase, 190-480. The FP cellulase : β -glucosidase ratio was 1:1.23 (21). These results encouraged us to compare the composition of the cellulase systems produced in SSF and LSF on different substrates.

Wheat straw. FP cellulase per unit volume in LSF was very low at 1% wheat straw. It doubled when the wheat straw concentration was raised to 5%. But the FP cellulase per unit weight of wheat straw produced at 5% concentration was reduced to almost one-half that obtained on 1%. Similar observations were made with the β -glucosidase and xylanase activities (Table IV). The low productivity of all the enzymes could be the result of an O₂ transfer problem in the thick fermentation medium in the LSF.

On the other hand, the FP cellulase per unit weight of wheat straw in SSF was as high as under the best conditions of LSF of 1%. In addition, the β -glucosidase and xylanase activities increased to 2.5 and 1.6 times per unit weight of wheat straw in SSF, respectively (Table IV). Another advantage of SSF is that very high enzyme activities per unit volume (ml) can be obtained by using a small amount of water to extract the enzyme system.

The most important advantage of SSF was that the ratio of FP cellulase : β -glucosidase obtained was 1:0.8, which is very close to the recommended ratio of 1:1 (10-12). On the other hand, a very low FP cellulase : β -glucosidase ratio (1:0.26 and 1:0.32) was obtained in LSF whether the wheat straw concentration was 1% or 5%.

Wheat bran. Profuse growth of *T. reesei* QMY-1 was observed within first 5 or 3 days of SSF on wheat bran without any additional nutrients or with nutrients from the Mandels and Weber medium (22), respectively. In spite of good growth on wheat bran without additional nutrients, very low activities of all the enzymes were obtained (Table IV). On wheat bran with nutrients, the activities of FP cellulase, β -glucosidase, and xylanase increased to 8.3, 5.1, and 13875 times, respectively, but the activities of all the enzymes still remained very low compared to those obtained on wheat straw in SSF or LSF (Tables I and IV).

The FP cellulase : β -glucosidase ratio obtained on wheat bran in SSF was close to the recommended ratio of 1:1, but the FP cellulase per unit volume was quite low. Similarly, on either wheat bran or wheat straw in LSF, the ratio of FP cellulase : β -glucosidase was low.

Table IV. Composition of Cellulase Systems of *Trichoderma reesei* QMY-1 produced on Wheat Straw and Wheat Bran Under Liquid and Solid-State Fermentation

Time of fermentation (days) ^a	FP cellulase		β-glucosidase		FP Cellulase: β-glucosidase ratio		Xylanases	
	IU/ml	IU/g substrate ^b	IU/ml	IU/g substrate ^b	IU/ml	IU/g substrate ^b	IU/ml	IU/g substrate ^b
Liquid state fermentation (LSF)^c								
1% (i)	1.9	190	0.5	50	1:0.26	138	13,800	
5% (ii)	4.7	94	1.5	30	1:0.32	288	5,760	
Solid-state fermentation (SSF)^d								
28	9.0	180	7.2	125	1:0.8	1125	22,500	
Wheat bran without additional nutrients in SSF								
5	0.15	3.1	0.15	3.0	1:1	0.006	0.12	
Wheat bran with additional nutrients in SSF								
12	1.29	25.8	0.78	15.6	1:0.6	83.28	1665	
Wheat bran (2%) without additional nutrients in LSF								
6	0.61	31	0.25	12.5	1:0.4	43.2	2160	
Wheat bran (2%) with additional nutrients in LSF								
6	0.72	36	0.27	13.5	1:0.33	56.8	2840	

a. Time of fermentation when maximum enzyme activities were recorded.

b. Enzyme activity (IU/g substrate) = Total enzyme activity (IU) + Original dry weight of substrate (g)

c. LSF (i) = 1 g alkali-treated wheat straw (containing 0.4 g cellulose) in 100 ml medium.

LSF (ii) = 5 g alkali-treated wheat straw (containing 2 g cellulose) in 100 ml medium.

d. SSF = 5 g alkali-treated wheat straw (containing 2 g cellulose) with 20 g H₂O.

Moisture content was 80% wt/wt. Enzymes extracted by shaking the solids in 100 ml of H₂O to make it comparable to that of LSF (ii) for uniformity in presentation of enzyme equivalent to that of LSF (ii). [Part of data from Chahal, D.S., *Trans. Mycol. Soc. R.O.C.*, 1990 (Tables 1 and 2) (in press)].

Although wheat bran proved to be a good substrate for organism growth because its nutrients are readily available (starch, protein, etc.), it supported low productivity for the cellulase system enzymes in SSF and LSF. Toyama showed that when starch and protein were removed from wheat bran (13), cellulase production increased considerably in SSF on the residue composed of mostly lignocellulosic materials. Similarly, critical examination of the data presented by Shamala and Sreekantiah showed that the cellulase activity of *Aspergillus ustus* was low on wheat bran as compared to that on rice straw and when wheat bran was mixed with rice straw the cellulase production dropped correspondingly (see Table VI) (17). The analysis of the data of these researchers supports our hypothesis that starch or other compounds present in wheat bran might be suppressing the synthesis of cellulase system enzymes (13,17).

Pro-cell (steam-exploded aspen wood). Pro-cell, obtained from Stake Technology, was used as obtained in one experiment and treated with 2% NaOH (wt/vol) with 1:2 solid to liquid ratio in another set (i.e., 4 g NaOH/100 g Pro-cell) (25). Enzyme activities of 3.0 IU/ml of FP cellulase and 1.3 IU/ml of β -glucosidase were obtained on Pro-cell without NaOH treatment (Table V). However, FP cellulase and β -glucosidase increased to 1.7 and 3.1 times, respectively, when Pro-cell was treated with NaOH. The FP cellulase : β -glucosidase ratio also increased to almost 2 times on NaOH treated Pro-cell than that obtained on untreated Pro-cell. High xylanase activities of 328 IU/ml and 404 IU/ml were obtained on untreated and treated Pro-cell, respectively. High cotton hydrolyzing activity of 52.6 units/ml was also noticed in the case of NaOH treated Pro-cell. The cotton hydrolyzing activity is the same as C₁ activity necessary for splitting the microfibrils from the crystalline cellulose (26,27).

Table V. Composition of Cellulase System Produced on Pro-cell with *Trichoderma reesei* QMY-1 in SSF

FP cellulase (IU/ml)	β -glucosidase (IU/ml)	FP cellulase: β -glucosidase ratio	Xylanases (IU/ml)	Cotton hydrolyzing activity (units/ml)
1. Pro-cell without NaOH treatment 3.0	1.3	1:0.43	328	24.4
2. Pro-cell treated with 2% NaOH 5.3	4.1	1:0.77	404	52.6

- Pro-cell = steam exploded wood supplied by Dr. J.D. Taylor, Stake Technology Ltd. Oakville, Ontario, Canada.

- SSF for 10 days when maximum enzyme activities were obtained.

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Table VI. Composition of Cellulase Systems Produced in Solid-State Fermentation

Substrates	Organism	Enzyme activity IU/g dry wt substrate			
		FP cellu- lase	β -gluco- sidase	Xyla- nases	Refer- ence
1. Wheat straw	<i>T. reesei</i> QMY-1	180	125	22,500	___*
2. Wheat bran without nutrients	<i>T. reesei</i> QMY-1	3.1	3	0.12	___*
3. Wheat bran with nutrients	<i>T. reesei</i> QMY-1	25.8	15.6	1,665	___*
4. Wheat straw	<i>T. reesei</i> QMY-1	172	212	5,300	(21)
5. Pro-cell (wood)	<i>T. reesei</i> QMY-1	106	82	8,080	(23)
6. Wheat bran	<i>Aspergillus ustus</i>	3.7	60	615	(17)
7. Rice straw	<i>Aspergillus ustus</i>	5.8	15.8	740	(17)
8. Wheat bran and rice straw (1:1)	<i>Aspergillus ustus</i>	4.6	40.7	398	(17)
9. Rice straw (recycled)	Various microorganisms	14	48	1,431	(17)
10. Wheat bran (recycled)	Various microorganisms	8	81	788	(18)
11. Rice straw (recycled 5 times)	<i>Sporotrichum pulverulentum</i>	9	15.8	385	(18)
12. Wheat straw and wheat bran (80:20)	<i>Trichoderma harzianum</i>	18	NA	NA	(19)
13. Straw & bran (1:1)	<i>Aspergillus niger</i>	NA	NA	2,500	(20)

- * ___ Chahal, D.S., *Trans. Mycol. Soc. R.O.C. 1990* (Table 3) (in press).
 - Results from Toyama's work could not be compared here because of the different cellulase units he used (13).
 - NA= Not available.

Comparison of Composition of Cellulase Systems of Various Fungi Produced in SSF

The comparison of cellulase system enzyme activities of various fungi produced in SSF indicated that *T. reesei* QMY-1 seems to be the best of the species tested for the production of a cellulase system containing high activities of all the enzymes, i.e., FP cellulase, β -glucosidase, and xylanases (Table VI). Although xylanases are not normally considered as the part of a cellulase system for cellulose hydrolysis, these enzymes are required when cellulose and hemicelluloses are found together in lignocelluloses. Therefore, the cellulase system of *T. reesei* QMY-1 produced under SSF should be suitable for the hydrolysis of lignocelluloses into monomeric sugars.

Hydrolytic Potential of Cellulase Systems Produced with Different Substrates

Hydrolysis of delignified wheat straw up to 90% was obtained in 96 hours with the cellulase system produced in SSF with wheat straw. It took only 72 hours to obtain over 90% hydrolysis of wheat straw with cellulase system produced with SSF on Pro-cell (Table VII). It is interesting to note that the quantity of cellobiose in the hydrolysate obtained with the cellulase system produced on Pro-cell was higher than that of the cellulase system produced on wheat straw. This is consistent with the observation that cellulase system produced on Pro-cell had a lower ratio of FP cellulase : β -glucosidase (1:0.77) as compared to that produced on wheat straw (1:1.2). However, this cellulase system had a faster hydrolysis rate; it took only 72 hours to obtain over 90% hydrolysis. This might be related to cotton hydrolyzing activity of this cellulase system (Table V).

When wheat straw hydrolysis with the cellulase system of *T. reesei* QMY-1 produced in SSF was compared to that of corn stover hydrolysis with the cellulase system of *T. reesei* Rut-C30 produced in LSF, the latter cellulase system was able to hydrolyze corn stover only up to 60% although it had a very high FP cellulase : β -glucosidase ratio (Table VII) (28,29). Similarly the cellulase system of the same mutant, obtained from fed-batch fermentation (LSF) of washed steam-exploded wood, was able to hydrolyze washed steam-exploded wood only up to 36% (30), even though the FP cellulase : β -glucosidase ratio was higher (1:1.48) than that of recommended ratio (1:1). However, the hydrolysis was increased to 86% (95 g sugar/L) when a very high load of FP cellulase (60 IU/g substrate) was used. In these cases, the high FP cellulase : β -glucosidase ratio was obtained by manipulating the culture conditions in LSF.

Conclusions

We concluded from our study that *T. reesei* QMY-1 produced a cellulase system with higher cellulase activities and more optimal β -glucosidase levels by using SSF. The cellulase systems produced in SSF also had higher hydrolytic potential than those produced in LSF, even when they had high FP cellulase : β -glucosidase ratios. High cotton hydrolyzing activity could be related to the high rates and high yields of hydrolysis. Since the discovery of exoglucanase (1,4- β -glucan cellobiohydrolase), determination of cotton

hydrolyzing activity in cellulase systems has been ignored. We have recently begun studies on the determination of cotton activity with cellulase systems produced under different culture conditions to determine its effect on cellulose hydrolysis.

Table VII. Comparison of Hydrolysis of Delignified Wheat Straw with Cellulase Systems of *Trichoderma reesei* QMY-1 Produced Under Solid-State Fermentation^a

FP cellulase : β -glucosidase ratio	Glucose (g/l)	Cello- biose (g/l)	Xylose (g/l)	Arabi- nose (g/l)	Total sugars	Hydro- lysis ^b (%)
Cellulase system from wheat straw (SSF) ^c						
1:1.2	68.18	3.19	26.71	1.67	99.75	89.77
Cellulase system from Pro-cell (SSF) ^d						
1:0.77	61.3	8.3	31.8	----	101.4	91.26
Cellulase system from cellulose (LSF) ^e						
1:1.8	----	---	----	----	-----	60.00 ^f
Cellulase system from washed steam-exploded wood (LSF, Fed-batch) ^g						
1:1.48	----	---	---	----	40.00	36.00 ^h

a. Delignified wheat straw at 10% conc. was hydrolyzed with 20 IU FP cellulase/g substrate.

b. Hydrolysis % = $\frac{\text{Total wt of sugar produced (g/L)} \times 0.9}{\text{wt of substrate (g/L)}} \times 100$

c. Data from Chahal (21).

d. Data from Chahal (25).

e. Data from Tangnu et al. (28,29).

f. Hammer milled and acid-treated corn stover at 5% conc.

g. Data from Martin et al. (30, Fig. 7).

h. Hydrolysis with 20 IU FP cellulase/g washed steam-exploded wood (10% conc) in 48 hr.

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Literature Cited

1. King, K.W.; Vessal, M.I. *Adv. Chem. Ser.* **1969**, *95*, 7-25.
2. Wood, T.M. *IV Intl. Ferm. Symp.*, Terui, G., Ed; Society of fermentation Technology, Japan, Osaka, Japan, March 19-25; **1972**, pp. 711-18.

3. Wood, T.M. *Biochem. J.* **1972**, *128*, 1183-92.
4. Shewale, J.G. *Intl. J. Biochemistry* **1982**, *14*, 435-44.
5. Woodward, J.; Wiseman, A. *Enzyme and Microb. Technol.* **1983**, *4*, 73-79.
6. Wood, T.M.; McCrae, S.I. *Biochem. J.* **1978**, *171*, 61-72.
7. Wood, T.M.; McCrae, S.I. *Adv. Chem. Sr.* **1979**, *181*, 181-209.
8. Mandels, M.; Andreotti, R.; Roche, C. *Biotechnol. Bioeng. Symp.* **1976**, *6*, 21-33.
9. Mandels, M.; Dorval, S.; Medeiros, J. In *Proc. Ann. Symp. Fuels from Biomass*; Shuster, W.W., Ed.; Rensselaer Polytechnic Institute: Troy, New York, **1978** vol. 2, pp. 627-38.
10. Chahal, D.S.; McGuire, S.; Pikor, H.; Noble, G. *Biomass.* **1982**, *2*, 127-38.
11. Mandels, M.; Medeiros, J.E.; Andreotti, R.E.; Bisset, F.H. *Biotechnol. Bioeng.* **1981**, *23*, 2009-26.
12. Sternberg, D.; Vijaykumar, P.; Reese, E.J. *Can. J. Microbiol.* **1977**, *23*, 139-47.
13. Toyama, N. *Biotechnol. Bioeng. Symp.* **1976**, *6*, 207-19.
14. Kumar, P.K.R.; Lonsane, B.K. *Process Biochem.* **1987**, *22*, 139-43.
15. Thakur, M.S.; Karanth, N.G.; Nand, K. *Appl. Microbiol. Biotechnol.* **1990**, *32*, 409-13.
16. Silman, R.W. *Biotechnol. Bioeng.* **1980**, *22*, 411-20.
17. Shamala, T.R.; Sreekantiah, K.R. *Enzyme Microb. Technol.* **1986**, *8*, 178-82.
18. Shamala, T.R.; Sreekantiah, K.R. *Enzyme Microb. Technol.* **1987**, *9*, 97-101.
19. Deschamps, F.; Giuliano, C.; Asther, M.; Huet, M.C.; Roussos, S. *Biotechnol. Bioeng.* **1985**, *27*, 1385-88.
20. Deschamps, F.; Huet, M.C. *Appl. Microbiol. Biotechnol.* **1985**, *22*, 177-80.
21. Chahal, D.S. *Appl. Environ. Microbiol.* **1985**, *48*, 205-10.
22. Mandels, M.; Weber, J. *Adv. Chem. Sr.* **1969**, *95*, 391-414.
23. Chahal, D.S. In *Biotechnology and Renewable Energy*, **1986**. Moo-Young, M.; Hasnain, S.; Lampty, J. Eds. pp. 57-69.
24. Chahal, D.S.; Ishaque, M.; Chahal, P.S.; Lemay, J.; Lo, S.-N.; Valade, J.L. *Biotechnol. Bioeng. Symp.* **1985**, *15*, 387-97.
25. Chahal, D.S. *VII Intl. Symp. on Alcohol Fuels*; **1986**, Editions Technip, Paris, pp. 48-53.
26. Reese, E.T.; Siu, S.G.H.; Levinson, H.S. *J. Bacteriol.* **1950**, *59*, 485-97.
27. Reese, E.T. In *Recent Advances in Phytochemistry* **1977**, *11*, 311-67.
28. Tangnu, S.K.; Blanch, H.W.; Wilke, C.R. Lawrence Berkeley Laboratory, University of California, Preprint 11074 for W.S. Dept. of Energy, contract W-7405-ENG-48, **1980**.
29. Tangnu, S.K.; Blanch, H.W.; Wilke, C.R. *Biotechnol. Bioeng.* **1981**, *23*, 1837-49.
30. Martin, R.S.; Blanch, H.W.; Wilke, C.R.; Sciamanna, A.F. *Biotechnol. Bioeng.* **1986**, *28*, 564-569.

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Chapter 10

Role of Statistically Designed Experiments in the Development of Efficient Downstream Processes

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The following are required for the development of efficient downstream processes. 1) Integration of the fermentation with the downstream processing. 2) Understanding of how each step in the process affects the other steps. 3) Making each step as robust to incoming sample variations as possible. 4) Consistently producing maximum quality product from each fractionation step. This requires the process has clear goals and objectives, the starting material and the final product are well defined in a measurable way and the affect of each processing step on the product is well understood. The use of statistically designed experiments to make the process robust will be discussed. Several types of fractionation techniques for recovery and the future direction of downstream processing will be outlined.

This paper will focus on the use of statistically designed experiments to develop effective purification processes in the most time and cost efficient fashion. Downstream processing and the recovery of proteins by several different techniques have been discussed in other articles (1-3) and will not be discussed here.

Typical biotechnology purification processes have steps such as fermentation, separation of cells from the growth medium, purification of the product of interest and storage of the product. In each step of the process there may be several factors (eg. salt concentrations, flow rates, pressure, time and temperature) that are key to the proper operation of that step in the process. If there are 7 steps, each with 3 important factors that can be run at a high or low level there are $2^{(7 \times 3)} = 2.1 \times 10^6$ different possible combinations to evaluate in determining the most efficient process. This number doubles if one wants to repeat the results. Obviously, this number of experiments is cost prohibitive and impractical to conduct. Through the use of statistically designed experiments there are ways to obtain the required information from a significantly fewer number of experiments. The use of statistically designed experiments has been outlined in several books (4-6) and specifically for application

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in biotechnology by Haaland (6). Classes on the subject have been offered by several organizations (7-9).

In statistically designed experiments, parameters are changed intentionally to determine how the responses change. In this way, the factors (variables) that affect 1) the location of the mean of the responses, 2) the variation around the mean of the responses, 3) the mean and the variation, or 4) have no effect on the responses, can be identified. The designed experiments, through the use of orthogonal, fractional factorials, also allow for the determination of interactions (synergistic effects) between factors. The factors that are resistant to changes in the other factors (robust) can also be determined by these types of experimental designs. With these data, a process which meets the goal of being on target with the least amount of variation can be developed.

The use of full factorial designs (all possible combinations) allows for the complete definition of the system. However, this approach is usually resource demanding and cost prohibitive (ie. too many experiments need to be completed). The use of fractional factorial designs allows for the identification of the important factors (the needed information) for only a fraction of the resources. Fractional factorials are a defined fraction of the full factorial design. For example, in a one half fractional factorial design, one half of the number of runs of the full factorial design would be completed. Through the use of balanced (orthogonal) designs, the appropriate fraction of the full factorial can be completed so that the desired information can be obtained from the experiment. An orthogonal design is a set of experimental conditions in which the levels of each of the factors is balanced over the other factors (10). Table I illustrates an orthogonal array in which each factor has a high (1) and low (0) setting at which the experiment can be run.

Table I: An example of an orthogonal design

run	Factor A X_1	Factor B X_2	Factor C X_3	Responses
1	0	0	0	y1
2	0	0	1	y2
3	0	1	0	y3
4	0	1	1	y4
5	1	0	0	y5
6	1	0	1	y6
7	1	1	0	y7
8	1	1	1	y8

With the above design each run is an experiment performed at the settings noted in columns X_1 , X_2 , and X_3 , with each run having a response (y1 - y8). For example, if one were looking for enzymatic activity, the responses y1 to y8 would be in units of activity per mg of protein. Notice that for factor A at the low levels (0), the levels for B and C are the same as the levels when A is at its high level (1), i.e. the design

is balanced (orthogonal). When the responses for each run where A was at its low setting are averaged and compared to those averaged when A was at its high setting the effects of the other changing variables (B and C) cancel out. In other words, by subtracting the mean of the responses for when A was at its low setting from the means of the responses when A was at its high setting, the effect of A on the process can be determined without interference from the effect of B and C. This difference is commonly scaled (by dividing by 2) and referred to as the "half effect". The half effect for each single factor (A, B, C), each two factor interaction (AB, AC, BC), and the three factor interaction (ABC) can be determined by this method (11).

The design also allows for determination of how factor A is affected by the changes in the other factors. If there is a small variation around the mean value when A is at the low setting then the low setting of factor A is considered resistant to changes in the other factors (robust). If the value is also close to the target value then the signal to noise ratio (S/N) will be high (12). Data obtained from statistically designed experiments such as these lead to the rapid development of robust and efficient processes.

Statistically designed experiments have been important in determining optimal plating efficiencies of primary breast tumor cells for *in vitro* assays (13). Glacken, et.al., used statistically designed experiments in modeling mammalian cell culture systems to optimize and help overcome some of the barriers to scale-up of the system (14). Statistically designed experiments have also been used by Dunn (15) to explore ligand protein binding. In the manufacturing of enzymes for biomass conversion, these techniques would be directly applicable to maximizing yield and specific activity along with minimizing costs and time for the manufacturing through optimization of the process. A few examples of other areas of interest in biomass conversion that could benefit from the use of designed experiments would be in determining the mechanism of action of a specific enzyme, determining the synergistic affects between enzymes, cofactors and/or reaction conditions, and determining an efficient immobilization or crosslinking procedures. Because our business deals with biopharmaceuticals, the examples in this paper will come from that field. However, the techniques can be used wherever there is a need to efficiently gather information-rich data.

In this paper, we report the use of experimental design in the development of a large scale manufacturing process for ImuVert, a biologic response modifier. ImuVert is currently in human clinical trials for the treatment of human tumors. ImuVert is derived from *Serratia marcescens* and is a combination of ribosomes and lipid vesicles derived from the cell membranes of the bacterium. The activity of ImuVert has been demonstrated in both *in vitro* (16-17) and *in vivo* (human clinical) studies (Jaekle, K. A. *J. Clin. Oncol.*, in press). Our goal is to have an efficient manufacturing process that produces a high yield of a highly active product with the least amount of lot to lot variation. The objective is then to determine how each of the important factors in the process affect the yield and composition of the product. We have used full and fractional factorial designs to determine the important individual factors and two factor interactions for each unit operation. As a result, we have been able to set operating limits for the efficient functioning of the operation. The process steps to be discussed are fermentation medium development and cell lysis

development. We also discuss the future of experimental design in process development.

Materials and Methods

ImuVert Manufacturing. The cells were harvested from the fermentation medium and washed by centrifugation, lysed with a French pressure cell and the cell debris was removed by centrifugation. The cell lysis supernatant was then layered on a sucrose gradient and the ImuVert pelleted by centrifugation. The pellet was then resuspended in buffer A (see below) to a nucleic acid concentration of 2 mg/mL.

Fermentation procedure. Twelve hours prior to the inoculation of the fermenter, 1 L of medium was inoculated with a 2 ml glycerol stock of *Serratia marcescens*. Bacterial cultures were grown at 36°C for 12 hours.

Thirteen liters of medium was sterilized for 20 min. at 121°C in a 20 L Chemap fermenter. The medium was cooled to 36°C and maintained at that temperature throughout the fermentation. The pH was maintained at 6.9 with 5M NaOH. The dissolved oxygen concentration was maintained at the prescribed level by agitation RPM adjustments (min. 400, max 700 RPM) and the addition of air/O₂. The glucose concentration was maintained between 10 to 20 g/L.

A 3.6% v/v inoculum of the 12 hour preculture was added to the fermenter and the fermentation was monitored hourly for optical density at a wavelength of 660 nm, glucose concentration and wet weight of cells per liter. The culture was allowed to grow for 5 hours and then stopped by rapidly cooling the culture to 0°C by running the culture through a stainless steel cooling coil packed in an alcohol/ice bath.

Media. Vitamins and trace metals (Factor A, experiment 1) were added at 0.5 mL/L from the following stock, choline chloride 10 g/L (Sigma), pantothenic acid 0.5 g/L (Sigma), riboflavin 0.18 g/l (Sigma), biotin 0.1 g/L, folic acid 0.5 g/L (Sigma) and niacin 2.75 g/L (Spectrum) along with 5 mL/L of the following stock, ZnSO₄·7H₂O 0.22 g/l (Sigma), CaCl₂ 0.55 g/l (Sigma), MnCl₂·4H₂O 0.5 g/l (Sigma), FeSO₄ 0.5 g/L (Sigma), (NH₄)₆Mo₇O₂₄·4H₂O 0.1 g/L (Sigma), CuSO₄·5H₂O 0.16 g/L (Sigma) and CoCl₂ 0.16 g/L (Sigma) or yeast extract 10 g/L (Marcor) was used.

Amino acids (Factor B, experiment 1) were supplied as casein peptone 35 g/L (Marcor) or casamino acids 25.8 g/L (Marcor).

Dissolved O₂ (Factor C, experiment 1) was maintained at either 10 - 20 % or 20 - 30 %.

Buffers and trace elements (Factor D, experiment 1) were either NaH₂PO₄ (Spectrum, USP) at 1.53 g/L, Na₂HPO₄ (Spectrum, USP) at 19.5 g/L, KH₂PO₄ (Spectrum, USP) 0.74 g/L, NaNH₄HPO₄ (Spectrum, USP) 1.11 g/L, and MgSO₄·7H₂O (Spectrum, USP) at 0.15 g/L or Kosers citrate (Difco) at 12.3 g/L

Glucose concentration was maintained by addition of 50% w/v glucose solution (Spectrum, USP).

Buffer A. 0.02M MgSO₄ (Spectrum, USP), 0.05M NH₄Cl (Spectrum, USP) and 0.02M Tris-hydroxymethyl aminomethane (Spectrum,USP), pH 7.6 at 4°C.

Assay procedures. Vesicle to ribosome ratio measurements. The membrane vesicles (about 200 nm) were separated from the ribosomes (about 25 nm) by gel filtration chromatography. Toyo Soda TSK-75 Toyopearl (Supelco) was packed to a bed height of 20 cm in a 1.5 x 50 cm column (Bio-Rad) at a flow rate of 3-4 mL/min with 3-4 column volumes of Buffer A. The flow rate was adjusted to 2 mL/min and one column volume of buffer A was run through it. A 0.5 mL sample of 2mg/mL RNA final product was loaded on the column and run at 2 mL/min. The effluent was monitored at a wavelength of 280 nm and recorded on a strip chart recorder. The vesicle to ribosome ratio was calculated by dividing the peak height of the vesicle peak by the peak height of the ribosome peak. The acceptable range for ImuVert is 0.75 ± 0.15 .

Determination of nucleic acid yield. Cleared lysate or ImuVert was diluted to a concentration which had an absorbance between 0.4 and 0.5 at 260 nm and the absorbances at 280 and 260 nm were measured. The nucleic acid concentration in solution was then calculated by the method of Warburg and Christian (18). The yield of nucleic acid was calculated by determining the percent of nucleic acid in the lysate that was isolated in the product. The acceptable range for ImuVert manufacturing is 10.5 ± 1.5 .

Results

Example number 1. Medium for fermentation. The goal of this experiment was to develop a defined fermentation medium that was usable for the manufacture of ImuVert. The objectives were to evaluate the effect of replacing citrate with a phosphate buffer, yeast extract with vitamins and minerals, casamino acids with casein peptone and high or low O₂ levels on the final yield and composition of ImuVert.

Experimental Design 1. Due to the large scale of each run, an experimental design with a minimum number of runs was employed to obtain the desired information. The one half fractional factorial in Table II was set up to determine the effect of each factor.

Table II: Medium Component Experimental Design

	FACTOR A	FACTOR B	FACTOR C	FACTOR D
run	vitamin/yeast	casein/casamino	O ₂	buffer
1	vitamin	casein peptone	hi ^a	phosphate
2	yeast extract	casein peptone	hi	citrate
3	vitamin	casamino	hi	citrate
4	yeast extract	casamino	hi	phosphate
5	vitamin	casein peptone	lo	citrate
6	yeast extract	casein peptone	lo	phosphate
7	vitamin	casamino	lo	phosphate
8	yeast ext.	casamino	lo	citrate

^ahi = 20 to 30% dissolved oxygen, lo = 10 to 20% dissolved oxygen.

Figure 1 illustrates the half effect results for the vesicle to ribosome ratio and the yield of nucleic acid in the product. The half effect is a measure of the change in the response as a function of the variable under study. For example, a half effect of one for yield would represent a change of two percent in the yield (eg. 10 to 12%). Half effects of less than 0.7% for yield and 0.06 for vesicle to ribosome ratio are considered not significant. The largest effects on the yield were the change in the amino acids, the change in the buffers and the two factor interaction (synergistic effects other than the two effect added together), AB. The percent dissolved oxygen or the changing from yeast extract to vitamins and minerals did not significantly affect the yield, as single factors. For the vesicle to ribosome ratio the largest effects were the change from yeast extract to vitamins and the two factor interactions, AC, CD and BC. Changing the dissolved oxygen percent, the buffers or the amino acid types did not significantly affect the vesicle to ribosome ratio, as single factors. From these data it can be concluded that the combinations of individual factors (interactions) were important concerns in ImuVert production along with the single factors yeast extract or vitamins (for vesicle to ribosome ratio), amino acids, and buffers (for yield).

In this particular experimental design, the two factor interactions were confounded with another two factor interaction and a deconfounding experiment was required to resolve the effects. As an example of a deconfounding experiment, the following full factorial (Table III) was run to illustrate how to resolve the AB interaction from the CD interaction (ie. the affect of combining yeast extract or vitamins and minerals with the different amino acid types from the affect of combining the different dissolved oxygen percents with the different buffer types). In this case, the A and B factors (yeast extract and casein peptone) were held constant and the C and D factors (percent dissolved oxygen and buffers) were varied.

Table III: Medium Component Deconfounding Experimental Design

	FACTOR C	FACTOR D
run	O ₂	buffer
1	hi ^a	phosphate
2	lo	phosphate
3	hi	citrate
4	lo	citrate

^ahi = 20 to 30% dissolved oxygen, lo = 10 to 20% dissolved oxygen.

The results of a deconfounding experiment that distinguishes between the AB and the CD interaction were displayed in Figure 2. These data indicate that the interaction between the percent dissolved oxygen and buffers (CD) was important for the vesicle to ribosome ratio (Figure 2b) and not for the yield (Figure 2a). Since the CD interaction is not important to the yield the interaction between yeast extract and amino acid types (the AB interaction in the first experiment) must have been important for the yield. In this way, the affect of each interaction on the responses can be determined.

Confirmation of the results obtained in the above experiments was obtained by completing two additional runs using yeast extract, casein peptone, potassium citrate and

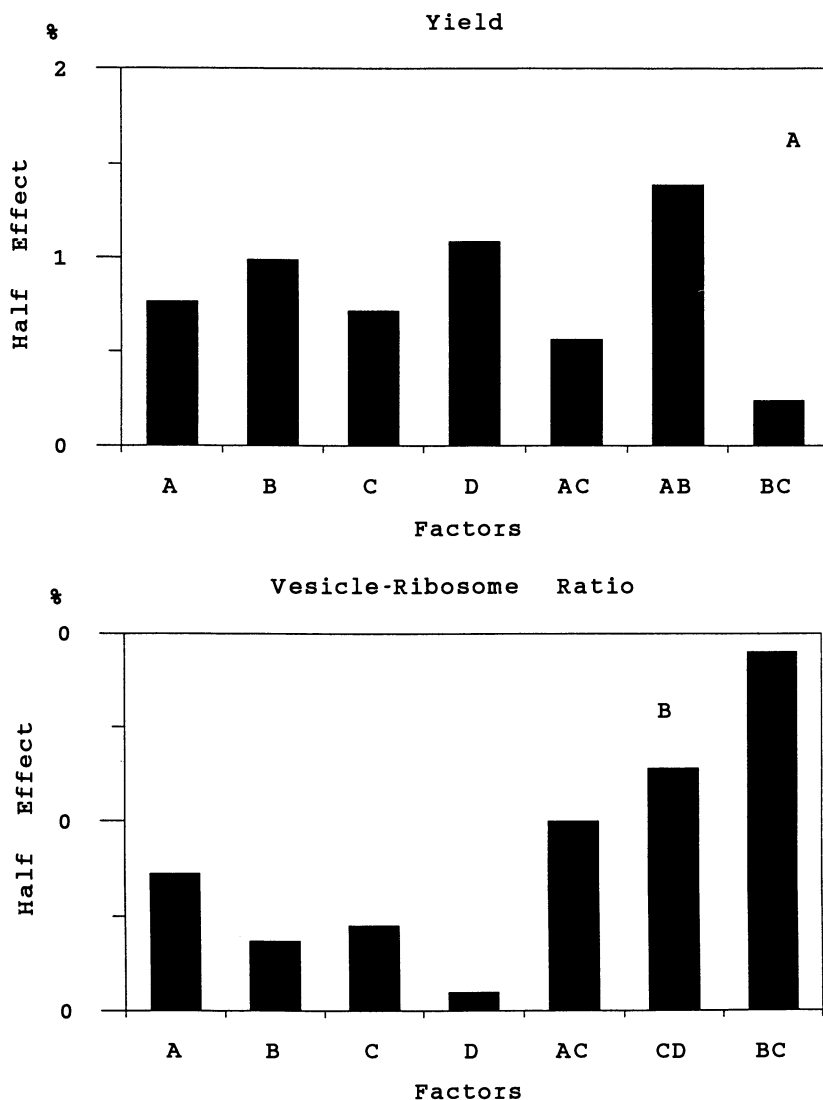


Figure 1. Half Effect Results for Yield (A) or Vesicle to Ribosome Ratio (B). A is the effect of vitamins and yeast extract. B is the effect of casein peptone and casamino acids. C is the effect of dissolved O_2 . D is the effect of citrate and phosphate. AB is the interaction of yeast extract/vitamin and mineral with casein peptone/casamino acids. AC is the interaction of yeast extract/vitamins and minerals with dissolved O_2 . BC is the interaction of casein peptone/casamino acids with dissolved O_2 . CD is the interaction effect of dissolved O_2 with the citrate or phosphate.

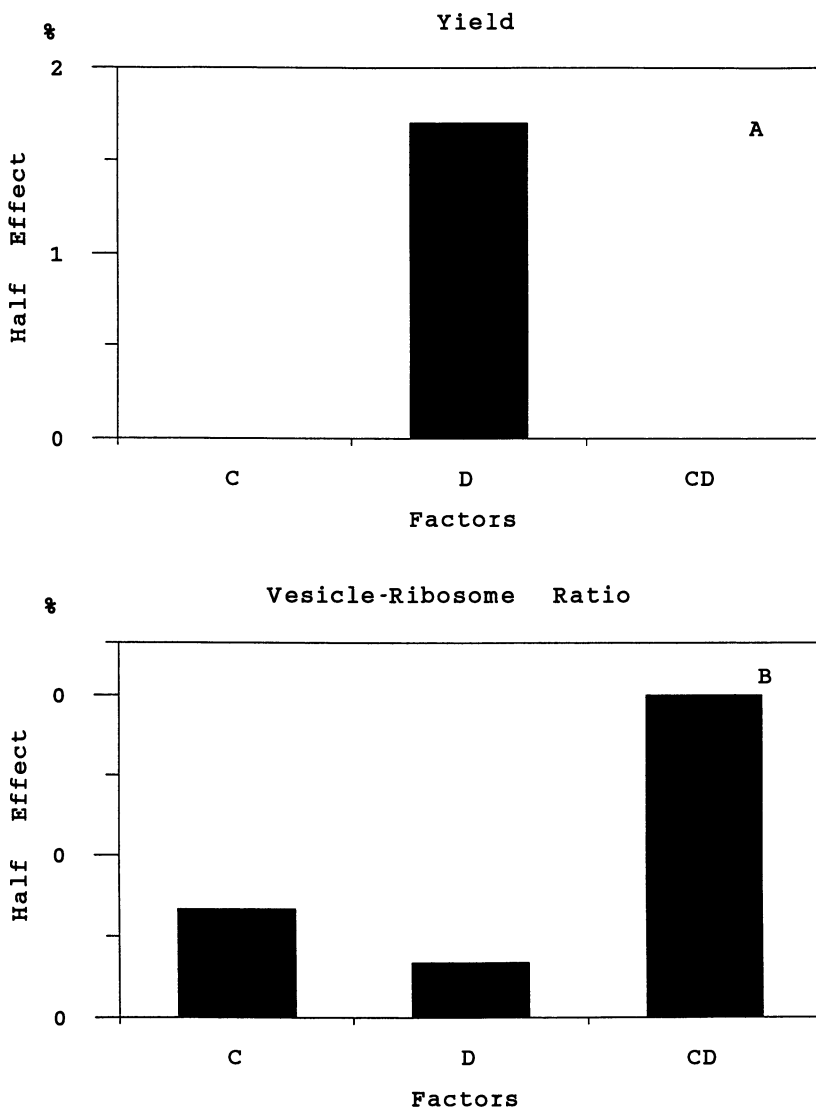


Figure 2. Half Effect Results for Yield (A) or Vesicle to Ribosome Ratio (B) deconfounding experiment. C is the effect of type of buffer on the system. D is the effect of dissolved O_2 on the system. CD is the interaction of dissolved O_2 with the buffers.

20 to 30 % dissolved oxygen. The results were a vesicle to ribosome ratio of 0.65 ± 0.05 and a yield of 9.4 ± 0 . The components identified as being critical by this design have put us on target with minimal variability in the system (see the assay procedures section of the methods for the acceptable limits for the responses).

Example number 2. Cell Lysis with a Manton-Gaulin Press. The goal was to find a scalable lysis procedure for the manufacturing of ImuVert. The objective was to determine how changes in pressure (6000 to 10000 psi) and the number of passes (1 to 10) of the bacterial suspension through the Manton-Gaulin homogenizer affect the final specifications for ImuVert. The solution exiting the press was collected in a flask that was in an alcohol/ice bath and constantly stirred to cool the solution rapidly. **Experimental design 2.** The experiment that was conducted as a full factorial with three levels. This design consisted of 9 experiments plus a control constituting a set of manipulations which could be completed in one day with minimal resource allocation. A three level design tests for nonlinear responses while a two level design assumes that the response is linear between the high and low set points, see Table IV.

Table IV: Lysis Experimental Design

Run	FACTOR A	FACTOR B
	passes	pressure
1.	1	6000
2.	1	8000
3.	1	10000
4.	5	6000
5.	5	8000
6.	5	10000
7.	10	6000
8.	10	8000
9.	10	10000

Figure 3 displays the results obtained from the Manton-Gaulin experiment. The graphs illustrate that the largest effect on both the yield and vesicle to ribosome ratio was the number of times the solution was passed through the homogenizer and the response was nonlinear. The signal to noise ratio was plotted on the same graphs to illustrate which of the responses was closest to the target with the least amount of variation. The larger the signal to noise ratio the closer the data was to the target value with the least variation in the data. In this way, the S/N was useful in deciding which conditions were appropriate to consistently produce a high quality product.

Figure 4 illustrates the interaction between the number of passes and pressure for the responses yield and vesicle to ribosome ratio. If there was no interaction between these two effects then the lines would be parallel. However, at the higher pressures (8000 and 10000 psi) and 10 passes the increase in the yield was greater than at the lower pressure (figure 4a). At 10 passes the 10000 psi pressure the increase in the vesicle to ribosome ratio was greater than at the lower pressures (figure

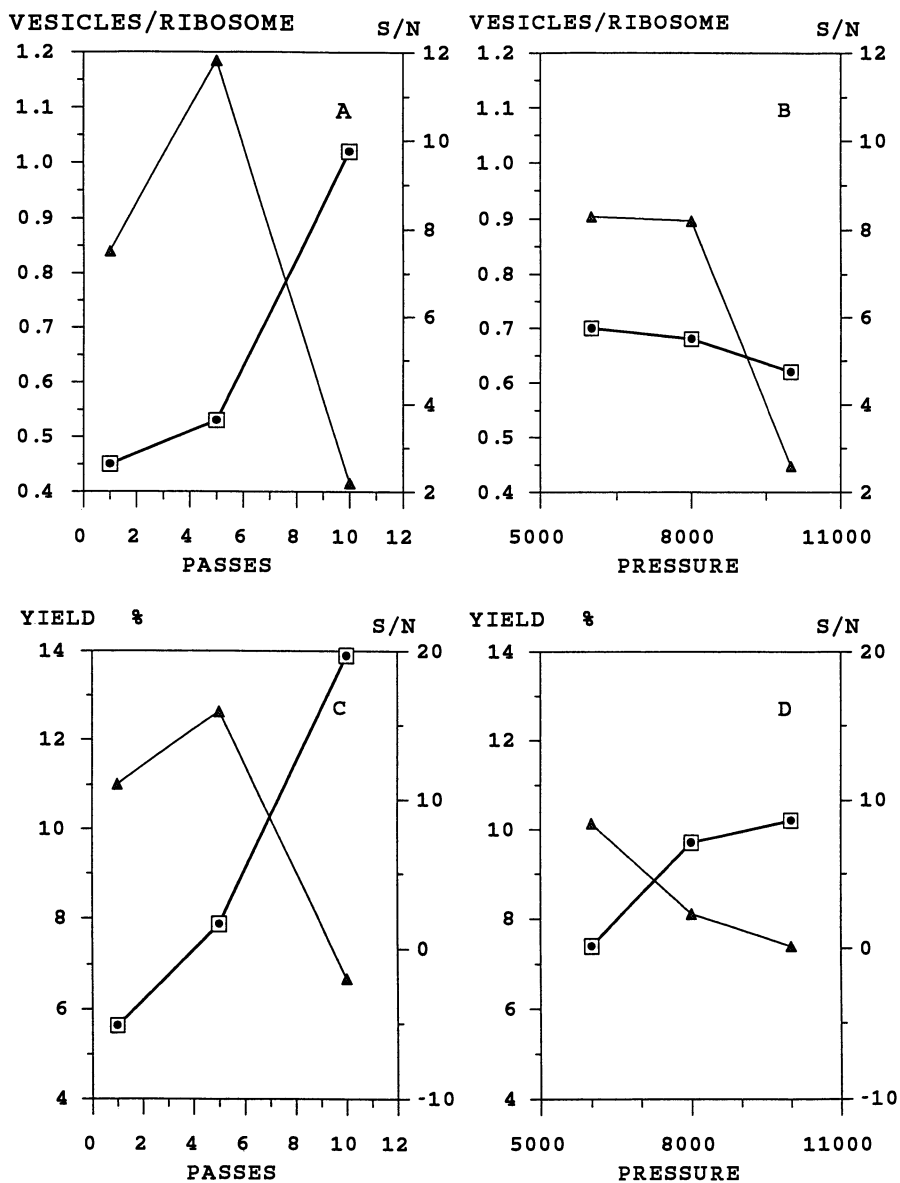


Figure 3. Single Factor Effect Plots and Signal to Noise Ratios. A. \square is the effect of passes on vesicle to ribosome ratio. \blacktriangle is the signal to noise ratios for the data points. B. \square is the effect of pressure on vesicle to ribosome ratio. \blacktriangle is the signal to noise ratios for the data points. C. \square is the effect of passes on yield. \blacktriangle is the signal to noise ratios for the data points. D. \square is the effect of pressure on yield. \blacktriangle is the signal to noise ratios for the data points.

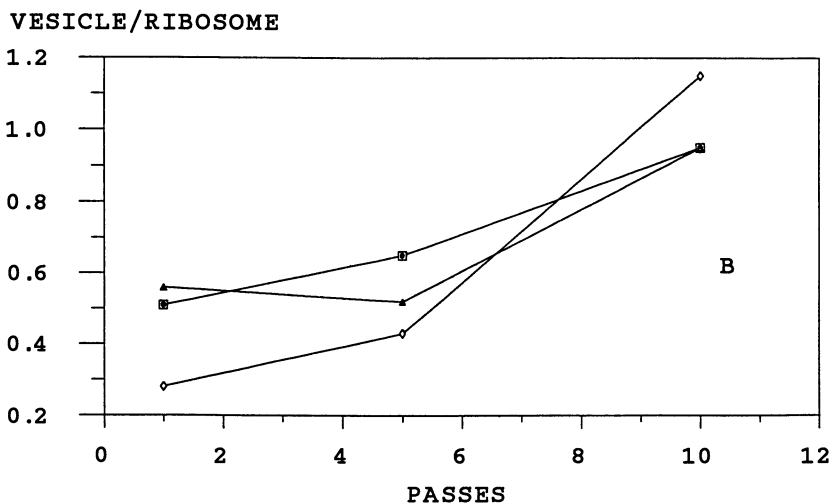
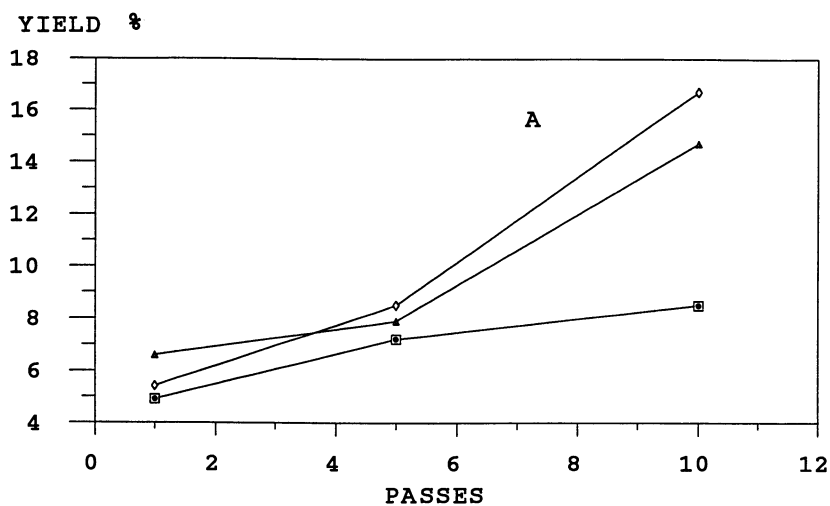


Figure 4. Interaction Of Pressure With Passes. A, Yield. \square is the effect of 6000 psi. \blacktriangle is the effect of 8000 psi. \diamond is the effect of 10000 psi. B, vesicle to ribosome ratio. \square is the effect of 6000 psi. \blacktriangle is the effect of 8000 psi. \diamond is the effect of 10000 psi.

4b). This means the interaction between high pressure and high passes was important in the lysis of the bacteria and needs to be considered in the manufacturing of ImuVert.

Figure 4a also illustrates that the pressure has only a small effect on the yield at 5 passes. This means that response (yield) was robust to the changes in the pressure at this number of passes and if the only concern was yield then the specification for the pressure settings used in manufacturing could be loosened. However, the vesicle to ribosome ratio was not robust to changes in pressure (figure 4b), and the pressure setting that put the preparation on target must be used (6000 psi) if composition was also important. The results of the experiment demonstrate the power of these designs in being able to define the factors which require strict control and which can be relaxed.

The conditions closest to the desired specifications were 5 passes at 6000 psi. A confirmation run was conducted separately to verify the appropriate conditions. Table V illustrates the results of the confirmation run and confirms that the condition chosen produces product which is in our target range (see assay procedures) and produces product similar to the standard method used to manufacture ImuVert.

Table V: Confirmation Runs Comparing Two Methods of Lysis

run	vesicle/ribosome	yield
French press(control)	0.8	9.9%
Mantin-Gaulin	0.9	9.7%

Discussion

In these experiments we have balanced the resource allocations with the depth of data necessary for each of the processes. In addition, we were able to obtain the necessary information to complete the task efficiently. In classical experimentation, one factor was changed until the optimum was found and then the next set of experiments were done at the new optimum, while changing a second variable. This procedure continued until all the variables were "optimized". With classical experimentation, the true "optimum" was rarely found. This was because only a limited number of experiments were done at each level which did not adequately explore the possible solutions, and therefore, the possibility of missing the true optimum was high. In the experiments described in this study, the interactions were extremely important and may have been missed using a traditional approach. The above examples underscore the need for designed experiments with their ability to determine how each factor affects the system, and how each of the other factors interact with that individual factor.

This type of experiment also provides information on the direction to follow if the product is not close enough to its target value after the first round of experiments. In other words, the graphs provide insight into the direction to proceed to reach the target of interest. The data also indicate which factors had the largest effect (the

fastest way to reach the target) and those that were robust (resistant) to changes in the other factors.

As suggested by Haaland (19), in using these statistically designed experiments, we employed problem solving techniques (defining the problem with clear goals and objectives), gathered information-rich data (designed experiments) and used the analysis of the data to design the next set of experiments. In this way, we have used the available resources in an organized and efficient fashion.

The future direction of designed experiments in a biotechnology setting is two-fold;

1. To examine an entire process from beginning to end (raw material suppliers to stability of the final product) as a unit operation. With this frame of reference, the important factors in the entire process and their effect on each other can be determined. The use of initial screening experiments on each of the individual steps would identify the important factors to include in the overall design. This is the only practical way to determine how the process is going to react at the high and low set points for each of the important factors in the process while the other important factors are at either their high or low set points.

2. To use the information gained from these types of experiments to model the process. The model can then be optimized and the tolerances set with the only requirement being computer time and confirmation experiments. The large number of experiments that would be required to perform the tolerance design study can be eliminated by this procedure.

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Literature Cited

1. Scawen, M. D.; Hammond, P. M. *J. Chem. Tech. Biotechnol.* **1989**, *46*, 85-103.
2. Wheelright, S. W. *J. Biotechnol.* **1989**, *11*, 89-103.
3. *Guide to Protein Purification*; Deutscher, M. P., Ed.; Methods in Enzymology; Academic Press: San Diego, CA, 1990; Vol. 182.
4. Barker, T. B. *Quality by Experimental Design*; Marcel Dekker: New York, NY, 1985.
5. Schmidt, S. R.; Launsby, R. G. *Understanding Industrial Designed Experiments*; CQG Printing: Longmont, CO, 1988.
6. Haaland, P. D. *Experimental Design in Biotechnology*; Marcel Dekker: New York, NY, 1989.
7. *Quality Engineering by Design - The Taguchi Approach*; The Center for Quality and Applied Statistics, Rochester Institute of Technology: Rochester, NY, 1989.
8. *Understanding Industrial Designed Experiments*; Launsby and Associates, Colorado Springs, CO, 1989.
9. *Strategy of Experimentation*; DuPont Specialty Services: Wilmington, DE, 1989.

10. Schmidt, S. R.; Launsby, R. G. *Understanding Industrial Designed Experiments*; CQG Printing: Longmont, CO, 1988; p 3-3.
11. Barker, T. B. *Quality by Experimental Design*; Marcel Dekker: New York, NY, 1985; pp 198.
12. Barker, T. B. *Engineering Quality By Design - Interpreting the Taguchi Approach*; Marcel Dekker: New York, NY, 1990; p 20.
13. Besch, G. J.; Tanner, M. A.; Howard, S. P.; Wolberg, W. H.; Gould, M. N. *Cancer Res.* **1986**, *46*, 2306-2313.
14. Glacken, M. W.; Fleischaker, R. J.; Sinskey, A. J. *Biotechnol. Bioeng.* **1986**, *28*, 1376-1389.
15. Dunn, G. *Stat. Med.* **1988**, *7*, 805-815.
16. Warren, R. P.; McCall, C. A.; Urban, R. W. *Mol. Biother.* **1989**, *1*, 145-151.
17. Warren, R. P.; McCall, C. A.; Urban, R. W. *Mol. Biother.* **1989**, *1*, 323-327.
18. Warberg, C. *Biochem. Z.* **1942**, *310*, 348.
19. Haaland, P. D. *Experimental Design in Biotechnology*; Marcel Dekker: New York, NY, 1989; p 15.

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Chapter 11

Enhanced Utility of Polysaccharidases through Chemical Cross-Linking and Immobilization

Application to Fungal β -D-Glucosidase

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We have investigated two related methods for enhancing the usefulness of *Aspergillus niger* β -D-glucosidase: chemical crosslinking for increased activity half-life at elevated temperatures and immobilization on ion-exchange resins for activity recovery from cellulose hydrolysis reactors. In earlier work, we showed that glutaraldehyde crosslinking produced modified β -D-glucosidase demonstrating an 80-fold increase in half-life at 65°C. We have now determined that this modified enzyme possesses broader pH profiles as well. Immobilization of β -D-glucosidase by adsorption on anionic exchange resins is discussed as an economically viable method of enzyme recovery. Attachment of the enzyme to several resins is presented. However, the focus of the work was on the adsorption of the enzyme to Macrosorb KAX DEAE, due to the outstanding characteristics possessed by this immobilized enzyme (IME) system. These characteristics included activity loading, activity retention, and increased thermal half-life. It must be noted that this IME system is not acceptable for process applications due to the prohibitively high cost of the Macrosorb resin; it is however a benchmark for the technical evaluation of other anion exchange resins.

Polysaccharide degrading enzymes have a long history of commercial application in food processing, horticulture, agriculture, and protein research. As with most other industrial enzymes, the economic use of polysaccharidases often depends on obtaining the maximum activity lifetime in the process environment and/or securing a recovery system that permits the sensible reuse of active enzymes from process streams.

Cost sensitivity studies have shown that the successful commercialization of cellulase-based processes, such as the conversion of cellulose to fermentable sugars, is highly dependent on the cost of enzyme production (1). Because fungal β -D-glucosidase (EC 3.2.1.21) is the most labile enzyme in this system under process conditions (2), and key to efficient saccharification of cellulose, this enzyme was targeted for application of stabilization technology, both through chemical modification and immobilization to solid supports.

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Stability Enhancement via Chemical Crosslinking

Reticulation of enzymes, or the addition of external, intramolecular braces by crosslinking the protein with bi- or poly-functional reagents, has been shown to increase the chemical, thermal, and mechanical stability of many enzymes of commercial interest (3-7). A wide variety of protein-crosslinking reagents are now readily available (8). In addition to the glutaraldehyde and carbodiimide/diamine systems widely used to connect amino and carboxyl groups (4), respectively, there are now bifunctional reagents with N-hydroxysuccinimide and imidate moieties (9) for reaction with sulfhydryls, and glyoxals for reaction with arginine (10). The recent introduction of the extremely reactive nitrene- and carbene-generating reagents (11,12) to the list of tools for protein modification means that even the relatively inert peptide nitrogens and aliphatic hydrogens of amino acid side chains are subject to chemical crosslinking.

When considering the cost of the sophisticated bifunctional reagents, glutaraldehyde (at a cost of approximately 12¢/lb) emerges as the most probable choice for large-scale application in cases where it has been found to be effective. The chemistry of glutaraldehyde crosslinking is still somewhat unclear, even though many attempts have been made to study the nature of the stable glutaraldehyde-amine crosslink in proteins (13,14). Richards and Knowles (13) for example, convincingly pointed out that the properties of the glutaraldehyde-induced crosslinks were not consistent with the chemistry of the Schiff base reaction. Recent work by Johnson (15,16) using size exclusion chromatography to analyze glutaraldehyde-amine reaction systems, showed that pyridines and pyridine polymers were produced. These polymers are of considerable dispersity in chain length and present a wide range of distances potentially crosslinked. This characteristic of glutaraldehyde reactions may be used to an advantage in stabilizing proteins that are less sensitive to small changes in tertiary structure or that have sheltered active sites.

In earlier work (17) we showed that a straightforward reaction scheme utilizing glutaraldehyde can successfully modify *Aspergillus niger* β -D-glucosidase to produce a form of the enzyme that demonstrates an 80-fold increase in half-life at 65°C. When a similar attempt was made to stabilize glucoamylase from *A. niger*, no significant enhancement was observed (18).

In the current study we report the investigation of the thermal denaturation behavior of glutaraldehyde modified β -D-glucosidase by differential scanning microcalorimetry (DSC), along with the effect of glutaraldehyde crosslinking on the pH/activity-stability of the enzyme at elevated temperatures.

Enhanced Enzyme Utilization through Immobilization

Overview. The immobilization of biocatalysts by either direct binding or physical retention has been reviewed extensively by Hartmeier (19), Woodward (20), Rosevear (21), and recently by Swaisgood (22). Methods for immobilization by binding can be further divided into: (i) binding to carriers (i.e., either by ionic, adsorptive, or covalent mechanisms) and (ii) binding to each other or to other proteins (i.e., homo- and heteroconjugates). Methods of physical retention include: (i) matrix entrapment in beads, fibers, or sheets and (ii) membrane enclosure in either microcapsules or membrane reactors. The primary reasons for using immobilization technology in industry, apart from special research applications, includes enhanced separation of enzymes from process streams, increased practical concentrations of catalytic activity, potential advantages in the kinetics of enzymes used in multi-enzyme process when held in close spatial proximity, and an enhancement in stability. The use of immobilized enzymes occurs widely in the pharmaceuticals, food, fine chemicals, and medical industries. Experiences reported in the literature have shown that the properties of an

enzyme after immobilization or crosslinking cannot always be inferred from those found for previously studied enzymes.

Polysaccharidases. Polysaccharide degrading or transforming enzymes represent a major component of the U.S. market volume of commercial enzymes (e.g., \$130 million in a total market of \$340 in 1986)(19). Industrial polysaccharidase enzymes have been successfully immobilized on many supports, a few examples of which include glucoamylase on aluminum oxide (23), dextransucrase on DEAE-Sephadex (24), β -lactamase on Sepharose (25), and β -galactosidase on nylon-acrylate copolymer (26). Cellulases represent another class of polysaccharide degrading enzymes subjected to immobilization. Successful attempts to yield active, soluble immobilized cellulase systems include carbodiimide crosslinking to high molecular weight polyvinylalcohol (27) and hexamethylene diisocyanate crosslinking to polyethyleneglycols (28). Cellulases have been entrapped within hydroxyethyl methacrylate (29) and acrylamide gels (30). In other applications cellulases have been immobilized on insoluble carriers. These systems include CNBr-activated Sepharose and Affi-Gel 15 (31), glutaraldehyde-activated nylon (32), and ion exchange resins such as MacroSorb (33), among others. Woodward concludes in a recent review (20); however, that immobilization of cellulases to insoluble supports -- or most types of entrapment -- lead to a severely limited hydrolysis system due to the greatly reduced access of enzyme to substrate. This concern extends to application of immobilized forms of other true polymer or insoluble substrate degrading enzymes as well.

β -D-glucosidase. β -D-glucosidase catalyzes the hydrolysis of cellobiose and some aryl-glucopyranosides, including the two most commonly used analytical substrates: p-nitrophenyl- β -D-glucopyranoside and 4-methylumbelliferyl- β -D-glucopyranoside. β -D-glucosidase has long been recognized as the limiting enzyme in the efficient degradation of cellulosic materials to glucose (34,35). This is due to two factors: (i) the hydrolysis intermediate, cellobiose, is a strong inhibitor of the cellulolytic enzymes and (ii) the commonly utilized cellulolytic enzyme systems are deficient in β -D-glucosidase. Although good evidence exists that cellulase enzymes may be recycled by virtue of their strong affinity for cellulosic substrates (i.e., via cellulose residue recovery from final process hydrolysis stages), the cost-effective recovery of the expensive β -D-glucosidase from process streams may only be accomplished using insoluble, enzyme-laden supports. There have been innumerable studies describing immobilized enzyme (IME) systems over the past two decades, with more than 100 describing immobilization of β -D-glucosidases alone. A review of the most relevant works through 1988 was recently included in a minireview of immobilized cellulases (36). These studies have involved the entire spectrum of immobilization techniques, from co-entrapment of β -D-glucosidase with yeast cells in calcium alginate beads (37), to adsorption through the polysaccharide moiety of the protein to Concanavalin A sepharose (38), and glutaraldehyde attachment to chitosan (39). However, these researchers have either not utilized or have not completely characterized systems that could be applied at process scale. In other words, these studies have dealt entirely with IME systems that do not fit all the required criteria for an economically viable process. These process relevant criteria included cost of support, cost of immobilization, activity loading, activity retention, mechanical stability, and IME recoverability. The previous work appears to fall short on one or more of these criteria; thus, the issue of an economically viable IME system for β -D-glucosidase recovery remains open and in need of resolution.

During the past year, several new studies have been published on β -D-glucosidase IME systems that attempt to fulfill these process-relevant criteria. Immobilization of β -D-glucosidase by attachment to polyurethane foam (40) has shown some promise as an economical IME system due to the support's resistance to enzymatic and microbial degradation, advantageous physical properties, and improved kinetics. However, the potential for fouling by particulate matter exhibited by foams renders these supports

suspect for use in a reactor with real lignocellulosic materials. An attempt to immobilize β -D-glucosidase onto magnetic particles has recently been reported (41). The enzyme was immobilized onto polyethyleneimine/glutaraldehyde-activated magnetite (PAM) and titanium (IV) oxide (TiO_2)-coated magnetite (TAM) to use their magnetic properties in recovery. Both IMEs suffered from low activity yield upon immobilization and had less than optimal physical recovery even though they were magnetic, rendering them impractical for process utilization. An *in situ* immobilized, cell associated β -D-glucosidase from *Aspergillus phoenicis* grown as beads in shake flasks would have a potential use in a cellobiose hydrolysis reactor (42); however, application to real lignocellulosic reactors may be limited due to the mechanical shear sensitivity. One study showing promise for process scale application is the adsorption of β -D-glucosidase from *Trichoderma reesei* onto sandy alumina (43) due to the combination of low support cost, realistic activity loading and retention, and excellent mechanical characteristics. Sandy alumina bound nearly 70 pNPG units/g support while improving the enzyme's kinetics and displaying adequate operational stability. These studies are the most promising investigations reported within the past year, but none of these studies fully characterize the process scale potential of the IME system. Unfortunately, an IME system that fulfills all of the relevant criteria for process utilization will most probably not be reported in a traditional journal, but in the claims of a patent application.

In the work presented we will discuss an anion exchange resin immobilized β -D-glucosidase system. Anion exchange resins possess very favorable physical and chemical characteristics. Some commercially available resins are inexpensive (\$6 to \$8/lb), have excellent mechanical stabilities, high recovery potentials (due to their macroreticulate size and high densities), and adequate loading potentials at their cost/lb. The focus of this study is the use of the resin Macrosorb KAX DEAE for binding β -D-glucosidase. Macrosorb is not a candidate for industrial use due to the high support costs, but the exceptional physicochemical characteristics of this support provide a case study against which other anionic exchange resin IMEs can be technically evaluated in the search for an economically viable IME system for use in a large scale ethanol fermentation.

Materials and Methods

Chemicals and Reagents. All common reagents and buffer components used in this study were of reagent-grade and were obtained from Fisher or VWR Scientific. Glutaraldehyde (EM grade, 25% aqueous solution) and p-nitrophenyl- β -D-glucopyranoside were obtained from Sigma Chemical Co. Macrosorb KAX DEAE resin was obtained from Sterling Organics. Amberlyst A-26, IRA-900, and IRA-93 anion exchange resins were obtained from Sigma. Water used to prepare solutions was deionized using a Nanopure water system.

The commercial cellulase preparation used in this study was Genencor GC 123 (lot # D7246), which demonstrates 90 IFPU/mL at 45°C.

Instrumentation. A Pharmacia BioPilot Column Chromatography system was used to perform large-scale size exclusion chromatography (SEC) with an 11.3 x 90 cm BioProcess column packed with Sephacryl S-200 HR gel. High performance size exclusion (HPSEC) and ion exchange chromatography (HPIEC) were conducted with Pharmacia Superose 6 and 12 (HR 10/30) and Mono-Q (HR 5/5) columns respectively, equipped with Beckman model 520 system controller and Beckman model 110B HPLC pumps.

β -D-glucosidase preparations were concentrated and dialyzed with an Amicon model DC-2 Hollow Fiber Ultraconcentrator equipped with H1P10-20 or H1P100-20 cartridges.

Resin-bound enzyme was packed in Pharmacia C 10/20 jacketed columns for kinetic analysis. Glucose concentrations in hydrolyzates were determined with a YSI Model 27 glucose analyzer from Yellow Springs Instruments.

Enzyme Purification. β -D-glucosidase preparations were used in three grades, based on state of enzyme purity, during the course of this study. All grades of β -D-glucosidase were derived from the *Aspergillus niger* commercial enzyme, Novozym SP188 (Novo Ltd., batch DCN-01).

Diafiltered β -D-glucosidase. This enzyme grade was prepared by the exhaustive ultradialysis of Novozym SP188 with 10 mM phosphate buffer pH 6.5. Amicon H1P10-20 hollow fiber cartridges were used for this purpose.

SEC purified β -D-glucosidase. This enzyme grade was prepared by applying diafiltered SP188 to the Sephacryl S-200 column. The column was operated at a flow rate of 15 mL/min in 10 mM phosphate buffer pH 6.5 with 100 mM NaCl. β -D-glucosidase activity was found in an early eluting peak which proved to be approximately 92% β -D-glucosidase by SDS-polyacrylamide gel electrophoresis (PAGE)(data not shown).

High purity β -D-glucosidase. The high purity enzyme grade for kinetic and DSC studies was prepared by a sequential HPSEC and HPIEC procedure, previously reported by Baker et al. (17), using the Sephacryl S-200 and Mono-Q columns, respectively. This approach yields a β -D-glucosidase preparation of approximately 99% purity by SDS-PAGE.

Enzyme Activity Measurements. β -D-glucosidase activity was determined according to the method of Wood (44) as aryl- β -D-glucosidase by the hydrolysis of p-nitrophenyl- β -D-glucopyranoside (pNPG). In general, the concentration of p-nitrophenolate anion was determined from the extinction at 410 nm under alkaline conditions induced by the addition of 2 M Na_2CO_3 following a 30-min incubation period of enzyme and pNPG. Cellulase assays followed the method described by Ghose (45).

pH-Dependence of Thermal Inactivation of Native and Crosslinked Enzyme. A series of aliquots of enzyme (48.5 nanograms/mL in a volume of 0.6 mL in 2.0 mM buffer, 0.1 M NaCl, at each of the test pH values) were transferred to 13 x 100-mm screw-capped culture tubes and preincubated in water baths set at 55°, 60°, 65° or 70°C. At appropriate time intervals, assay of remaining activity in each aliquot of a given series was initiated by removing the tube from the bath at preincubation (test) temperature, adding 0.4 mL of a solution of pNPG (5.0 mg/mL in 25 mM acetate, pH 5.0, 0.1 M NaCl), mixing, and incubating for 1 hour in a bath at the assay temperature of 45°C. Assays were terminated by addition of 2.0 mL of 1.0 M Na_2CO_3 , and the release of p-nitrophenol was quantitated by measuring the absorbance of the resulting solution at 420 nm.

Buffers used for preincubation of the enzyme aliquots at the various pH values were glycolate/NaOH (pH 3.5 and 4.0), acetate/NaOH (pH 5.0), MES/NaOH (pH 6.0), MOPS (pH 7.0), Tris (pH 8.0) and boric acid/NaOH (pH 9.0). The preincubation buffers were adjusted at room temperature (20°C) to the values indicated above; the effect of temperature on the pH was then measured over the range from 20°C to 75°C, and the activity half-life determined at each temperature was reported at the actual pH of the preincubation.

Differential scanning calorimetry (DSC) of native and crosslinked β -D-glucosidase. The sample of glutaraldehyde-stabilized β -D-glucosidase was produced essentially as detailed earlier (17), i.e., by treatment of the enzyme with glutaraldehyde at pH 9.0 in borate for

30 min, followed by treatment with KBH_4 to reduce the presumed Schiff-base adducts formed by reaction of the aldehyde with enzyme amino groups. After diafiltration to remove excess reagents and exchange of the treated protein into 10 mM acetate, pH 5.0, 0.1 M NaCl, the sample was incubated for 5 hours at 65°C. This heat treatment was sufficient to denature essentially all of the non-stabilized enzyme remaining in the sample (17). Denatured protein was then removed by concentration, precipitation and centrifugation. Both samples, native and stabilized enzyme, were then exchanged into 40 mM acetate, pH 5.0, 0.1 M NaCl for DSC.

DSC thermograms were obtained with an MC-2 scanning microcalorimeter (Microcal, Inc., Northampton, MA) using the DA-2 data acquisition and analysis software package (Microcal, Inc.) on an IBM PC-XT microcomputer. The scan rate was 55°C per hour in both cases; protein concentrations were 0.65 mg/mL for the native sample and 0.29 mg/mL for the glutaraldehyde-treated enzyme. In both cases, the sample was rescanned after cooling and the second thermogram obtained, which showed no trace of the protein-unfolding endotherms seen in the original scan, was subtracted from the original thermogram to remove any intrinsic instrumental effects as well as those effects due to any lack of reproducibility in sample-loading technique between sample and reference cells.

Activity and Protein Binding Studies. All four anion exchange resins tested were exhaustively water washed and de-fined. Amberlite IRA-900, Macroorb KAX DEAE, and Amberlyst A-26 resins were titrated to pH 5.0 with 50 mM sodium acetate buffer pH 5.0, and then exchanged into 10 mM sodium acetate pH 5.0 through several washes. Titration with 0.01 N HCl was required for the Amberlite IRA-93 because it was purchased in a free-base form. Once the resin was titrated to pH 2 the same sodium acetate exchange procedure given above was appropriate. Once all resins were in 10 mM sodium acetate, 0.5-g samples were placed into tubes containing varying amounts of H1P10-20 ultrafiltered Novozym SP188 in 10 mM sodium acetate pH 5.0. These samples were incubated for 18 h at 22°C (this period of time was determined to be twice the minimum incubation time required for complete adsorption), the amounts of protein and activity adsorbed were then determined by difference from the initial solution concentrations. To determine enzyme inactivation during immobilization, the protein was eluted with 10 mM sodium acetate buffer pH 5.0 with 1.0 M NaCl. Protein concentrations were obtained with the BCA protein assay kit from Pierce. Activity was measured by the pNPG assay described above.

Since protein adsorption to an anion exchange resin is reversible and does not constitute a classical immobilization, the ability of the resins to retain activity under various conditions must be determined. Macroorb KAX DEAE bound β -D-glucosidase was tested with solutions of primary interest for their final application. Several batch washes of a 1% w/v slurry were required to ensure complete equilibrium elution for a given concentration, as determined from the absence of pNPG units in subsequent washes. Several salt solutions of typical fermentation media components were tested. These included 3 mM to 50 mM solutions of MgSO_4 , KH_2PO_4 , NaCl, and sodium acetate. Also, incubations with cellulase solutions were tested to determine if the proteins present in a cellulose hydrolysis would displace the β -D-glucosidase. Both of these displacement studies were carried out at 22°C and 40°C.

Stability and Performance of Bound Enzyme. The stability of the IME was determined by two methods. One measurement of bound activity was obtained using traditional cellulose hydrolysis experiments (described below). In the other method, direct kinetic parameter measurements were obtained using a recirculating differential (RDR) reactor system following the method of Ford et al. (46).

The operational stability of the IME was determined by the performance of the IME in a real cellulose hydrolysis reactor. 50-mL tubes were charged with 2.5 g α -cellulose (50 g/L) in 10 mM sodium acetate pH 4.8. Tetracycline and cycloheximide

were added to a final concentration of 40 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$, respectively, to prevent microbial contamination. Genencor GC 123 cellulase was used at a concentration of 20 IFPU/g cellulose with various quantities of IME (300 pNPG U/g wet resin beads at 45°C) to produce ratios of 0, 0.25, 0.50, 1.0, 2.0, and 4.0 pNPG U/IFPU at 45°C. These solutions were incubated with mixing by gentle inversion at 45°C for 120 h. Both glucose and cellobiose were measured over time by direct glucose analysis (YSI model 27 glucose analyzer, Yellow Springs Instruments) and HPLC, respectively. The β -D-glucosidase activity released from the resin was assayed by pNPG activity in the free solution.

The temperature and pH stability of the Macrosorb KAX DEAE bound enzyme was determined in a recirculating differential reactor system that consisted of a water jacketed Pharmacia C 10/20 chromatography column packed with 0.2 g of IME. Substrate solution was delivered from a water-jacketed beaker with a MasterFlex pump that provided flow rates up to 50 ml/min. The cellobiose concentration did not exceed 3 g/L (9 mM) to minimize substrate inhibition. The flow rate necessary to minimize the external mass transfer limitation was determined by the achievement of a constant initial hydrolysis rate with respect to superficial linear velocity of the substrate solution (46). This flow was then utilized for all subsequent studies. The substrate solution pH remained at 4.75 during the temperature stability study, while the temperature of the water jackets ranged from 25° to 75°C. The activation energy was determined from data obtained during the temperature optima study. Rate constants for assays at 20° to 60°C were used in Arrhenius analysis. Temperature half-life studies were conducted at 60°C, pH 5. Assays at 55°C were run a 1.5 h intervals. The elapsed time that the resin was exposed to 60°C was used to determine the activity half-life. Operating then at 55°C, the pH of the substrate solutions was varied from pH 4.65 to 5.70. The lower limit was set at pH 4.65 due to losses of β -D-glucosidase from the support as the enzyme approached its isoelectric pH (i.e., pH 4.50). The YSI glucose analyzer was used for substrate conversion assay.

Kinetics of Bound and Free Enzyme. The kinetics of the IME were obtained with the recirculating differential reactor system as described above. The appropriate flow rate, the temperature optimum, and pH optimum as described above were used to most accurately establish the kinetic parameters for this IME enzyme. Substrate solutions from 3 to 150 mM cellobiose in 10 mM sodium acetate were appropriate for this portion of the study. Results were analyzed with the ENZFIT software package (Elsevier Publishers) that permits precise Lineweaver-Burk regressions.

Results and Discussion

pH-Dependence of Thermal Stability of Native and Intramolecularly Crosslinked β -D-Glucosidase. Figure 1 (A through D) compares the survivability of the activities of native and glutaraldehyde-crosslinked β -D-glucosidase when exposed to a combination of pH and temperature stress, prior to measurement of surviving activity by assay at a standard pH and temperature. The plots with linear ordinate scales (Figure 1A and 1C) dramatically show the magnitude of the changes in stability when either the preincubation pH or preincubation temperature, or both, are changed. The plots with logarithmic ordinates facilitate comparison of the overall shapes of curves representing sets of half-lives differing greatly in magnitude. The logarithmic treatment is particularly advantageous in the cases of the 65° and 70°C curves for the native enzyme and the 70°C curve for the crosslinked enzyme, whose curves are reduced to near-invisibility by the scaling requirements of the respective linear plots.

Crosslinking with glutaraldehyde has been shown earlier (17) to result in an 80-fold increase in half-life of the β -D-glucosidase under thermal stress at (65°C) pH 5.0.

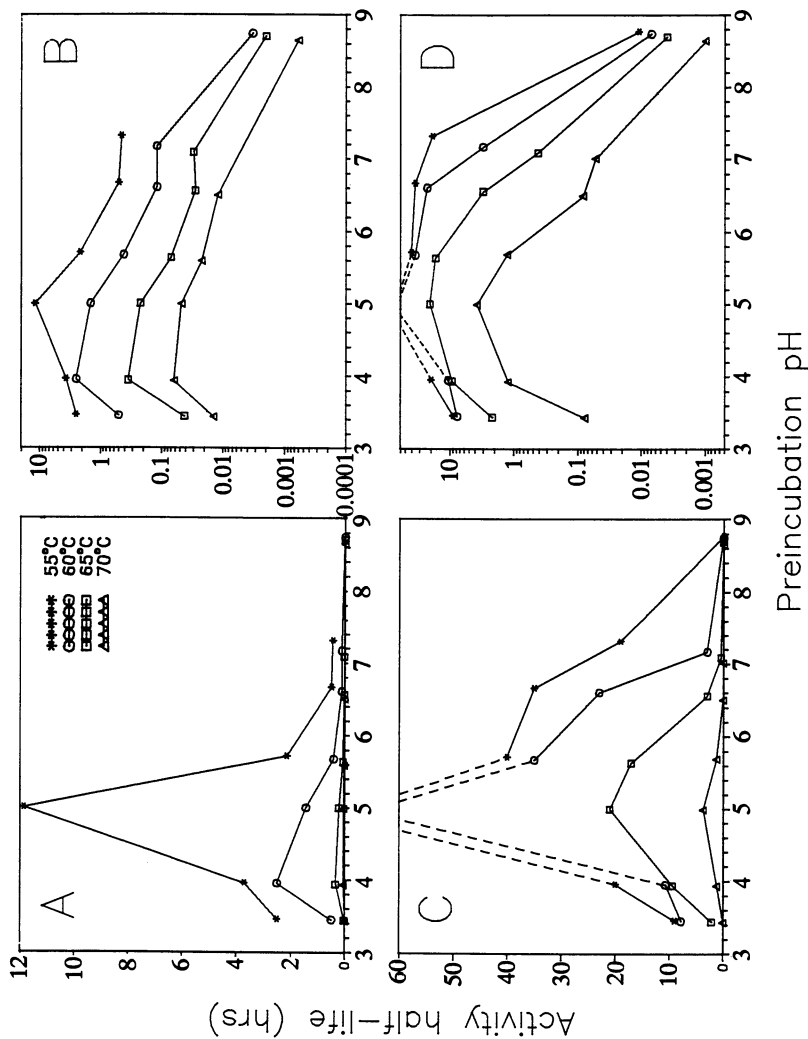


Figure 1. pH-dependence of the half-life for loss of activity in native and glutaraldehyde-modified β -D-glucosidase upon preincubation at the indicated pH values and at a range of temperatures from 55 to 70 °C, with subsequent assay at pH 5.0, 45 °C. A,B: Linear-ordinate and logarithmic-ordinate plots, respectively, for the glutaraldehyde-modified enzyme.

The DSC thermal-unfolding curves shown in Figure 2 indicate that this activity/stabilization is accompanied by (and possibly directly connected with) a significant stabilization of the overall three-dimensional structure of the protein molecule, in that the structure-breaking endotherm for the crosslinked enzyme occurs at a temperature some 6°C higher than the corresponding endotherm for the native enzyme. Given these findings, it would not seem entirely unreasonable to suppose that a crosslink or crosslinks giving the structure greater resistance to disruption under thermal stress might also stabilize the enzyme against denaturation under pH stress, and therefore might not only greatly increase the stability of the enzyme at the pH of optimum stability for the native protein, but might also result in a broader plateau of pH-stability. The activity/decay data presented in Figure 1 were collected to explore this possibility.

The results plotted in Figure 1 (B) and (D) seem to indicate that while crosslinking may produce some broadening of the pH-stability optimum at the lower temperatures used in this study, especially at 55°C and pH values below 8.0, the broadening effect is greatly reduced at temperatures as high as 70°C. In fact at the pH extreme of 8.6 to 8.8, the half-life of the crosslinked enzyme is little more than double the half-life of the native enzyme, regardless of the stress temperature used in the range from 55° to 70°C. It would appear that for pH values for which the native form of this particular enzyme is itself rather stable, this particular chemical modification is capable of spectacular enhancements of the thermal stability, but is ineffective for enhancing the stability of the enzyme under pH conditions at which the native enzyme is markedly unstable.

Immobilization of β -D-glucosidase on Ion Exchange resin.

Activity and Protein Binding Studies. Figure 3 shows the protein and activity loading isotherms for Novozym SP188 on the three anionic exchange resins. Obviously the Macrosorb KAX DEAE possess the highest loading capacities, retaining 2.5- and 6-fold more protein and activity than IRA-93. The anionic displacement of enzyme from these resins showed (data not shown) that all three resins are very sensitive to SO_4^{2-} at concentrations of 3 mM and above, with 50% to 60% activity removal at 10 mM MgSO_4 . This is not surprising, because SO_4^{2-} is divalent, nor is it a serious problem due to the fact that only a 1 mM SO_4^{2-} concentration is required in ethanol-producing fermentations. All the resins were much less sensitive to the other metal anions tested with no losses from Macrosorb at 20 mM concentrations, and 5% to 10% activity losses for the others in 20 mM solutions. There was little (5% to 8%) displacement of the β -D-glucosidase by the cellulase proteins even at unrealistically high protein concentrations (1500 IFPU/L free cellulase, i.e., no substrate for binding). These aspects, combined with Macrosorb's superior physicochemical properties, excellent recovery potential (36), and low internal diffusion resistance make it an excellent case study for the evaluation of other IME systems. In other words, this IME system will become a tool against which other attempts to immobilize β -D-glucosidase on anionic exchange resins can be measured. To successfully use this IME system in this manner, a complete data base is required. Therefore, the complete evaluation of Macrosorb KAX DEAE bound β -D-glucosidase is our first step toward selection of an economically viable IME system for use in a large scale ethanol production.

Stability and Performance of Macrosorb-Bound Enzyme. The hydrolysis of α -cellulose into glucose by Genencor GC 123 cellulase alone and cellulase supplemented with two ratios each of free and immobilized β -D-glucosidase is presented in Figure 4. The increase in conversion upon supplementation is notable (approximately 20% higher for the 1:1 ratio), and the similarity in performance by free and immobilized enzyme is striking. The absence of cellobiose in the media as determined by HPLC indicated that the hydrolysis was limited by the cellulase enzyme activity. The ratio at which the supplementation effect begins to diminish is approximately 0.5 pNPG U/IFPU at 45°C

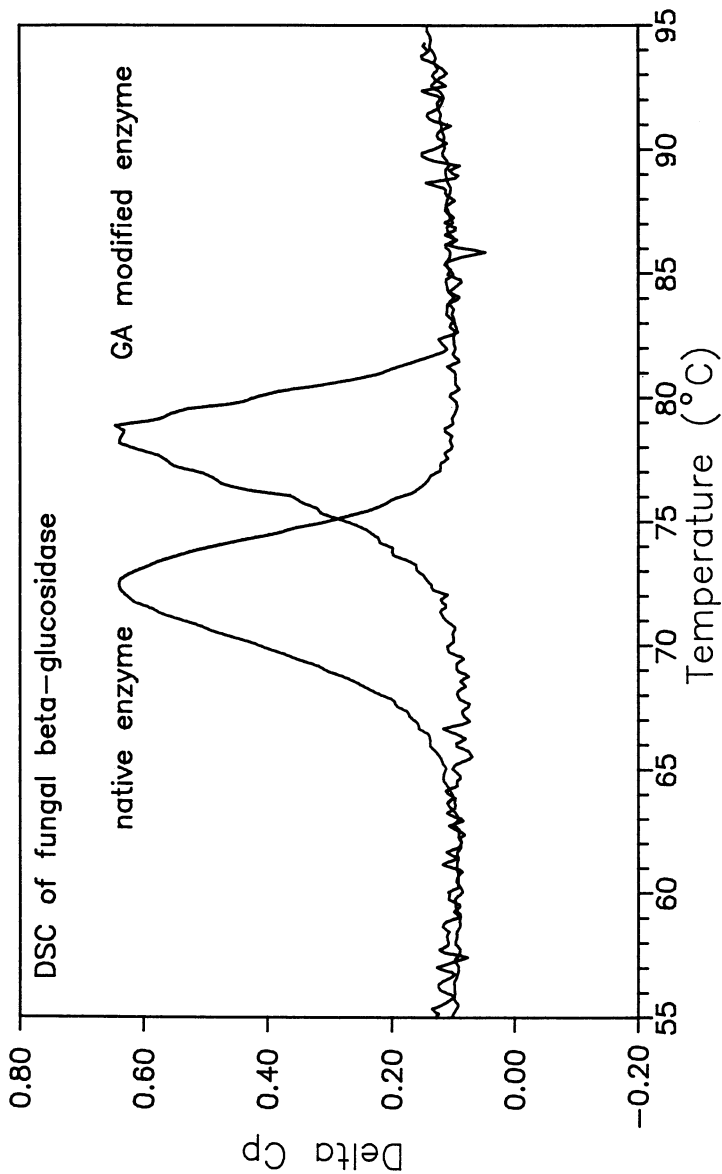


Figure 2. Comparison of DSC thermograms for native and glutaraldehyde (GA)-crosslinked β -D-glucosidase at pH 5.0 in 10mM NaCl. In order to facilitate comparison of the peaks, the differential heat capacity values plotted for the native enzyme are the actual values multiplied by a factor of 0.571.

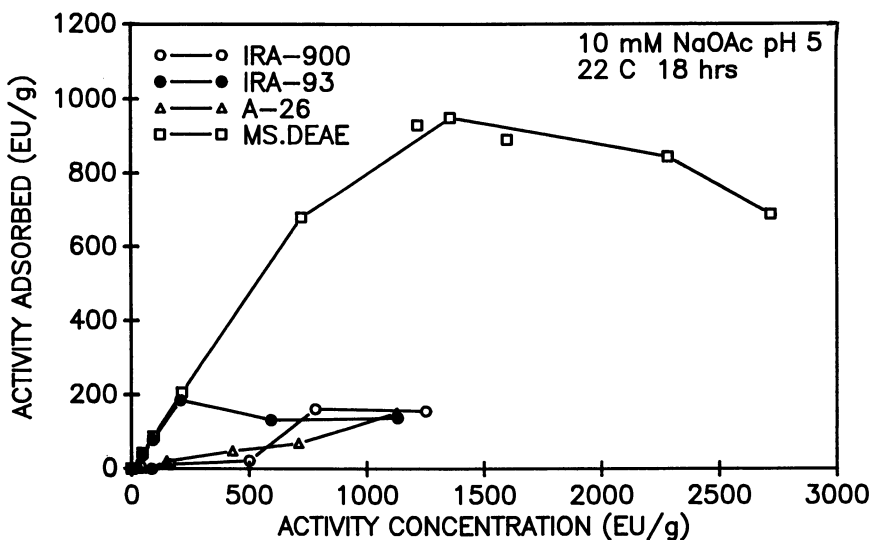
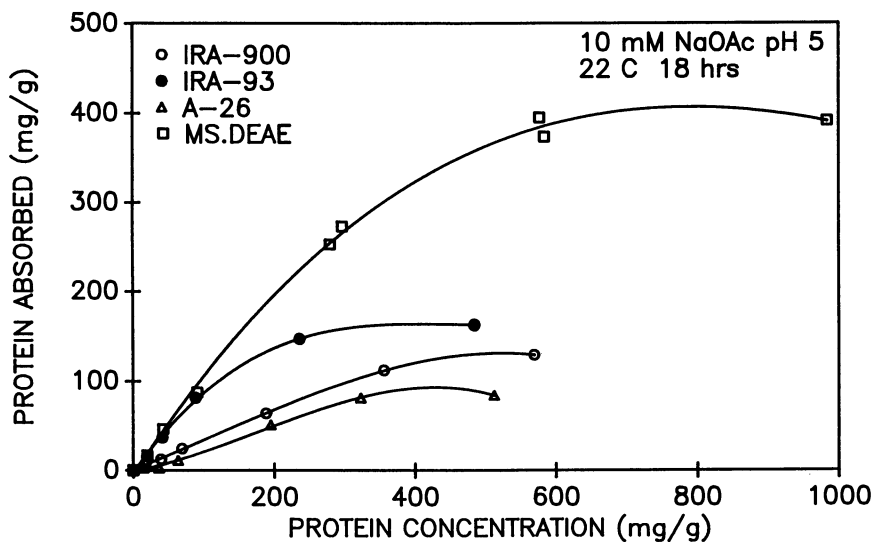


Figure 3. Protein (upper) and activity (lower) loading of four anion exchange resins with Novozym SP188 β -D-glucosidase in 10 mM sodium acetate pH 5.0. Samples were incubated for 18 h at 22°C.

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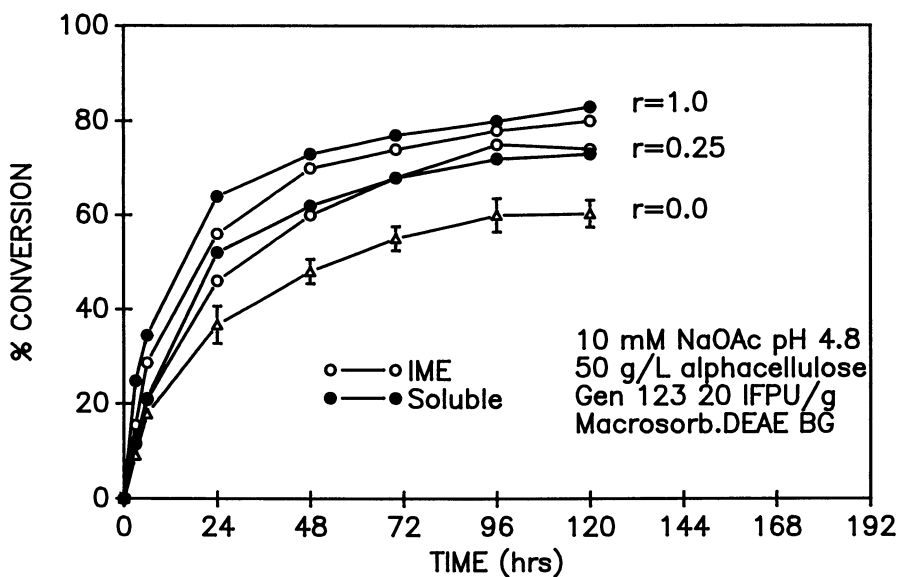


Figure 4. Conversion of 50 g/L α -cellulose to glucose by Genencor GC 123 cellulase supplemented to various ratios ($r = \text{pNPG U at } 45^\circ\text{C}/\text{IFPU at } 45^\circ\text{C}$) of free and immobilized β -D-glucosidase. Nonsupplemented cellulase is included as a control. Reactions were carried out in mixed tube with 10 mM sodium acetate pH 4.8 at 45°C .

(data not shown). It is important to note that no pNPG units were eluted from the IME during the hydrolysis, and there is no loss of activity due to thermal denaturation during the 120-hour incubation at 45°C in the controls. Apparently no attrition of the IME occurred (admittedly due in part to the gentle mixing). The IME was recovered (95%-100% by weight) by simple decantation of the media. These are exceptional operational characteristics for an IME in a mixed reactor.

The temperature and pH stabilities of the free and immobilized enzyme appear in Table I, along with the kinetic parameters. A flow rate of ≥ 30 mL/min was found to be adequate to minimize the external mass transfer effects (data not shown). The temperature optimum of the IME was higher than that of the free enzyme, due to the increase in thermal stability of the enzyme as determined by the increase in the activity half-life. This increase in thermal stability was most likely due to the rich hydrogen bonding environment influencing the immobilized enzyme. We may also conclude that the pH optimum was shifted down to a more acidic value, compared to the free pH optimum (i.e., pH 4.50), due to the electrostatic partitioning of H_3O^+ by the positively charged microenvironment inside the IME. The actual value cannot be determined, however, because of the limitation imposed by the elution of activity at pH 4.55. The decrease in activation energy upon immobilization indicates that the immobilized activity was not as sensitive to temperature changes as the free enzyme.

Table I. Comparison of the Physicochemical Properties of Free (Novozym SP188) and Macrosorb KAX Immobilized β -D-glucosidase

Property	Free β -D-glucosidase ^a	Macrosorb KAX Immobilized
Temperature Optimum	60-70°C	65-75°C
pH optimum	4.5	between ^b 4.55 and 4.70
Stability (half-life at pH 5.0 and 60°C)	1.4 h ^c	9.0 h
Activation energy (kJ/mol/K)	52.5	30.0
Apparent K_m (mM cellobiose)	5.6	10.1
V_{max}	33.7 ^d	117 ^e
$V_{max}/\text{apparent } K_m$	6.0	11.7

^aFrom ref. 47. ^bGiven as range from pH 4.55 because enzyme elutes from resin at pH values less than this. ^cFrom ref. 17. ^d($\mu\text{mol}/\text{min}/\text{mg}$ protein). ^e($\mu\text{mol}/\text{min}/\text{mg}$ bound protein).

Kinetics of Free and Macrosorb-Bound enzyme. The results of the Lineweaver-Burk analysis of the initial rates for free and immobilized enzyme appear in Table I. The increase in the substrate affinity parameter K_m due to some internal mass transfer limitations in the IME as no substrate-matrix interactions were present. The increase

in V_{\max} may be due to the normalized activity for protein content. MacroSorb KAX DEAE binds β -D-glucosidase more specifically than other proteins, therefore the activity to protein ratio is higher in the resin than in stock solution. The first order rate constant for Michaelis-Menten kinetics, V_{\max}/K_m describes the kinetics of cellobiose hydrolysis at low ($[S] < K_m$) substrate concentrations. This low substrate concentration region is of primary interest for IME use in cellulose hydrolysis because of the sensitivity of cellulases to cellobiose inhibition. Supplementation of β -D-glucosidase is necessary for the maintenance of low cellobiose levels for most enzymatic cellulose hydrolysis reactions (35). Therefore, the effect of immobilization on this ratio is a more appropriate measure of the process relevant kinetic performance of the IME than either parameter alone. The increase in the ratio upon immobilization would indicate an expected increase in performance of the IME in a real cellulose hydrolysis. However, the cellulose hydrolysis ratios (see Figure 4) are based on activities, and not on total protein, therefore the similarity in performance of free and immobilized enzyme was not unexpected.

Conclusions

We have reported the successful development of two related methods for enhancing enzyme utilization. Fungal β -D-glucosidase was used as the test enzyme for these studies. Enhanced thermal and pH stability was shown for chemically crosslinked, soluble β -D-glucosidase. Use of this enzyme was also enhanced through immobilization for recycle. Immobilization of the test enzyme was also shown to significantly increase the activity half-life of the enzyme at elevated temperature (i.e., 60°C). Each methodology has its own shortcomings, however, including extra initial costs and losses of activity. Ultimately, the benefits of each technology must be weighed against the costs, both in terms of economics and enzyme activity recovery. As mentioned previously, an *a priori* estimate of the benefits of either of these technologies based on a new enzyme system is difficult, but the potential for these technologies are significant.

Acknowledgements. This work was funded by the Biochemical Conversion Program at the DOE Biofuels and Municipal Waste Technology Division.

Literature Cited

1. Wright, J. D.; Power, A. J.; Douglas, L. J. *Biotechnol. Bioeng. Symp.* 1986, 17, 285-302.
2. Durand, H.; Soucaille, P.; Tiraby, G. *Enzyme Microb. Technol.* 1984, 6, 175-180.
3. Mozhaev, V. V.; Martinek, K. *Enzyme Microb. Technol.* 1984, 6, 50-59.
4. Schmid, R. D. *Adv. Biochem. Eng.* 1979, 12, 41-118.
5. Torchilin, V. P.; Maksimenko, A. V.; Smirnov, V. N.; Berezin, I. V.; Klivanov, A. M.; Martinek, K. *Biochim. Biophys. Acta.* 1978, 552, 277.
6. Torchilin, V. P.; Martinek, K. *Enzyme Microb. Technol.* 1979, 1, 74-82.
7. Johnson, R. E.; Adams, P.; Rupley, J. A. *Biochem.* 1978, 17, 1479-1484.
8. Conn, M. *Meth. Enzymol.* 1983, 103, 49-59.
9. Moreland, R. B.; Dockter, M. E. *Biochem. Biophys. Res. Commun.* 1981, 99, 339-346.
10. Politz, S. M.; Noller, H. F.; McWhirter, P. D. *Biochem.* 1981, 20, 372-378.
11. Ji, T. H.; Kiehm, D. J.; Middaugh, C. R. *J. Biol. Chem.* 1980, 255, 2990-2993.
12. Chowdhry, V.; Vaughan, R.; Westheimer, F. H. *Proc. Natl. Acad. Sci.* 1976, 73, 1406-1408.

13. Richards, F. M.; Knowles, J. R. *J. Mol. Biol.* **1968**, *37*, 231-233.
14. Korn, A. H.; Fearheller, S. H.; Filachione, E. M. *J. Mol. Biol.* **1972**, *65*, 525-529.
15. Johnson, T. J. A. In *Science of Biological Specimen Preparation*; Mueller, M.; Becker, R. P.; Boyde, A.; Wolosewick, J. J., Eds.; SEM Inc.: AMF O'Hare, Chicago, IL, 1986; p. 51.
16. Johnson, T. J. A. *European J. Cell. Biol.* **1987**, *45*, 160-169.
17. Baker, J. O.; Oh, K. K.; Grohmann, K.; Himmel, M. E. *Biotech. Lett.* **1988**, *10*, 325-330.
18. Tatsumoto, K.; Oh, K. K.; Baker, J. O.; Himmel, M. E. *Appl. Biochem. Biotechnol.* **1989**, *20/21*, 293-308.
19. Hartmeier, W. *Immobilized Biocatalysts*; Springer-Verlag: Berlin, 1986.
20. Woodward, J. *Immobilized Cells and Enzymes, A Practical Approach*; IRL-Press: Oxford, UK, 1985.
21. Rosevear, A.; Kennedy, J. F.; Cabral, J. M. S. *Immobilised Enzymes and Cells*; Adam Hilger: Bristol, UK, 1987.
22. Swaisgood, H. E.; Horton, H. R. In *Biocatalysts in Agricultural Biotechnology*; Whitaker, J. R.; Sonnet, P. E., Eds.; American Chemical Society: Washington, DC, 1989; pp 242-261.
23. Krakowiak, W.; Jach, M.; Korona, J.; and Sugier, H. *Starch/Starke* **1984**, *36*, 396-398.
24. Ogino, S. *Agric. Biol. Chem.* **1970**, *34*, 1268-1271.
25. Pastorino, A. M.; Dalzoppo, D.; Fontana, A. *J. Appl. Biochem.* **1985**, *7*, 93-97.
26. Beddows, C. G.; Guthrie, J. T.; Abdel-Hay, F. I. *Biotechnol. Bioeng.* **1981**, *23*, 2885-2889.
27. Rao, M.; Mishra, C. *Biotech. Lett.* **1984**, *6*, 319-322.
28. Kumakura, M.; Kaetsu, I. *Biotech. Lett.* **1985a**, *7*, 773-778.
29. Kumakura, M.; Kaetsu, I. *Studia Biophysica* **1985b**, *107*, 103-107.
30. Roy, P. K.; Roy, U.; Duke, D. K. *J. Chem. Tech. Biotechnol.* **1984**, *34B*, 165-170.
31. Chim-anage, P.; Kashiwagi, Y.; Magae, Y.; Ohta, T.; Sasaki, T. *Biotechnol. Bioeng.* **1986**, *28*, 1876-1978.
32. Jain, P.; Wilkins, E. S. *Biotechnol. Bioeng.* **1987**, *30*, 1057-1062.
33. Woodward, J.; Bales, J. C. In *Bioproducts and Bioprocesses*; Fiechter, A.; Okada, H.; Tanner, R., Eds.; Springer: Berlin, 1989; pp 87-101.
34. Wood, T. M.; McCrae, S. I. In *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis*; American Chemical Society: Washington, DC, 1979; pp 181-209.
35. Sternberg, D.; Vijayakumar, P.; Reese, E. T. *Can. J. Microbiol.* **1977**, *23*, 139-147.
36. Woodward, J. *J. Biotechnol.* **1989**, *11*, 299-312.
37. Hahn-Hagerdal, B. *Biotechnol. Bioeng.* **1984**, *26*, 771-774.
38. Woodward, J.; Marquess, H. J.; Picker, C. S. *Prep. Biochem.* **1986**, *16*, 337-352.
39. Desai, J. D.; Ray, R.; Desai, A. *J. Ferment. Technol.* **1986**, *64*, 255-258.
40. Chakrabarti, A. C.; Storey, K. B. *Appl. Biochem. Biotechnol.* **1989**, *22*, 263-278.
41. Dekker, R. F. H. *Appl. Biochem. Biotechnol.* **1989**, *23*, 25-39.
42. Reczey, K.; Persson, I.; Tjerneld, F.; Hahn-Hagerdal, B. *Biotechnol. Techniques* **1989**, *3*, 205-210.
43. Fadda, M. B.; Dessi, M. R.; Rinaldi, A.; Satta, G. *Biotechnol. Bioeng.* **1989**, *33*, 777-779.
44. Wood, T. M. *Biochem. J.* **1971**, *121*, 353-362.
45. Ghose, T. K. *Pure and Appl. Chem.* **1987**, *59*, 257-268.
46. Ford, J.R.; Lambert, A.H.; Cohen, W.; Chambers, R.P. *Biotechnol. Bioeng. Symp.* **1972**, *3*, 267-284.
47. Dekker, R. F. H. *Biotech. Bioeng.* **1986**, *28*, 1438-1442.

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Chapter 12

Bioprocessing Aids in the Recovery of Proteins from Biomass

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The extraction of enzymes from biological tissues has been examined using a three step process involving an initial clarification, employing bioprocessing aids such as the Biocryls to remove non-protein contaminants, followed by ultrafiltration for concentration and then a final chromatographic purification. DNA, RNA, cell-wall debris and pigmented compounds in particular are eliminated by this process. The method has been used for bacterial, plant, fungal and animal tissues.

The inherent complexity and chemical diversity of biomass predicts the recovery and purification of proteins from such a source will be an extremely challenging task. We have been studying this problem with a long-term research goal of identifying and establishing experimental conditions which may be applicable for protein purification from all biomass systems.

Our approach has been to divide protein purification into three distinct steps. These being Step I, the collection and clarification of extracts from the original biomass; Step II, the concentration of the clarified stream; and Step III, the chromatographic purification of the specified protein. It has been our experience that Step I, in the removal of either non-protein contaminants or superfluous proteins, is critical to the design and effectiveness of the latter two Steps. As to be expected, different types of biomass contain widely varied non-protein contaminants. However, these components are commonly; particulate debris, pigmented organics, and biochemicals such as oligosaccharides, lipids, pyrogens and nucleic acids (1-3). The downstream effects of these types of contaminants assumes greater importance when the biomass processes are transformed into production-level systems. Various chemical agents, including the Biocryls, polyethylenimine and chitosan, have been under study in our laboratory as so-called bioprocessing aids in Step I, to remove the contaminating species found in biomass.

In this paper we report on the application of the three step approach to protein purification in two different biomass systems (i) a bacterial homogenate and (ii) a plant homogenate. We also discuss the advantages which accrue, in each case, from the incorporation of bioprocessing aids into Step I, an experimental strategy for evaluating the adaptation of bioprocessing aids into a protein purification protocol, and the general utility of these techniques to protein recovery from biomass.

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Bioprocessing Aids

The researcher wishing to study and characterize the mix of enzymes associated with the conversion of a specific biomass is often confronted with a familiar problem. The key enzymes of interest are present only in very minute amounts and are extremely intractable to separation from the overwhelming mass of chemically-complex biomass. In our laboratory, we confronted this problem during the recovery and characterization of the extracellular enzymes produced by a white-rot fungus, *Lentinula edodes*, grown on a commercial wood medium. More complete details of the issues involved in that program are presented in this monograph (4) and elsewhere (1, 5-7). Suffice to say, the key to the success of the *L. edodes* program was the utilization of chemical agents, such as the Biocryl BPA-1000 or polyethylenimine (PEI), to remove tannins, polyphenols and debris from the wood extracts. We now have extended this line of research to examine a broad range of biomass systems, using various chemical agents for enhancement of bioprocessing and enzyme recovery.

Selective precipitation of a protein from a biomass stream, although a desirable method for purification of an enzyme (8), is often an impracticable option due to the high concentrations of non-protein contaminants. In considering that problem, we have adopted a different approach, that is to introduce a procedure --ideally very early in the biomass processing system -- which may remove selectively the non-protein components. The procedure we sought to satisfy those criteria was the addition of a chemical agent to the crude biomass in Step I, resulting in flocculation and selective precipitation of the undesirable components, but at the same time leaving the target protein (enzyme) in the resultant supernatant phase. This general class of chemical agent is defined as a bioprocessing aid. Some of the chemical and physical properties of compounds which have been investigated as potential bioprocessing aids are summarised in Figure 1. Other materials which could be classified as bioprocessing aids include; polyvinylpyrrolidone, the Whatman CDR product, as well as Chitoplex and Evalsan (9,10).

The application of PEI as a bioprocessing aid in protein purification has been covered in a comprehensive review by Jendrisak (11). Although in some situations PEI does initiate the flocculation of non-protein contaminants in Step I, without simultaneous precipitation of the target protein, in most cases the PEI causes coincident precipitation of the protein with nucleic acid, from the crude biomass. Further careful processing is then necessary to selectively dissociate the protein from the complex nucleic acid aggregation. This approach does not satisfy the conditions we sought for Step I. Chitosan, which is derived from chitin by deacylation, has been of limited use in bioprocessing (12), it is available commercially in various size ranges. The form we have found most useful has an average molecular weight of ~750,000. Chitosan like PEI however, is an example of a water-soluble, cationic polymer of high molecular weight and charge density which initiates the flocculation of nucleoprotein complexes. The need for chitosan to be solubilized in acidic conditions may also restrict its widespread application for biomass processing.

Chemical agents which more closely approach the criteria we sought for use in Step I, are the Biocryl bioprocessing aids (BPAs). These materials, are crosslinked acrylic or styrenic polymer particles with diameters of 0.1 or 0.2 microns. Surface functional groups are strongly basic quaternary ammonium (BPA-1000), weakly basic tertiary amine (BPA-1050) or strongly acidic sulfonic acid (BPA-2100). These charged particles adsorb oppositely charged species such as cell debris, nucleic acids, contaminant proteins, color bodies and enzymes in a wide variety of fermentation broths, cell or tissue extracts and homogenates. A high molecular weight, water soluble formulation is also available. This material, BPA-5020, has a weakly basic tertiary amine functionality. In some situations it may act synergistically with another polycationic bioprocessing aid such as BPA-1000 (3).

Unlike water soluble polymers such as PEI or chitosan, the particulate BPAs are shear stable and pumpable. The particles, along with the adsorbed materials, can be effectively removed from the suspending medium by either membrane filtration or centrifugation. The BPAs have been designed to have specific functional groups on the surface and in the case of the strong anionic and cationic species, maintain their charge over a broad pH range. When used in conjunction with membrane filtration (13) they reduce the development of a concentration polarization layer on the membrane surface resulting in enhanced flux rates. When an appropriately sized membrane is chosen, the particulate BPAs are maintained in the retentate producing a product stream free of the filter aid.

Given the broad pH range over which the BPAs can be used, it is also possible to specifically target a product protein for removal with the BPA, leaving the contaminants behind. This is accomplished by adjusting the pH of the medium so that the target protein alone (or with few contaminants) is bound. The product bound BPA can then be removed by centrifugation or in ultrafiltration it can be eluted and concentrated in a single step.

Materials and Methods

Growth and Lysis of *Escherichia coli*. The *E. coli* strain used in this work was RAJ201 harboring the plasmid pRAJ255 (14). This plasmid contains the gene for β -glucuronidase, *uidA*, under the control of the *lac* promoter and operator (14). Cultures of *E. coli* were grown in a 10 L fermenter on 2YT, induced with IPTG at 8 hours (O.D.₅₅₀ = 5), harvested at 23.5 hours (O.D.₅₅₀ = 8), and the cell paste stored frozen at -70° C until they were to be lysed. Upon thawing of the cell paste the material was suspended in phosphate lysis buffer A [20 mM potassium phosphate (pH 7.1), 10 mM 2-mercaptoethanol] at a concentration of 1.0 g wet weight cells in a total volume of 5 ml. The bacteria were lysed by a double passage through a Gaulin homogenizer at 10,000 psi, with cooling of the cellular material in a dry ice:ethanol bath after each passage. Lysis was estimated to be >95% by phase contrast light microscopy. The resulting *E. coli* homogenate was stored, until required, at -70° C.

Alfalfa Lysis. Whole alfalfa (*Medicago sativa*) plant tissue was broken and cell rupture was by shear in a rotary device. Juice expression was by a batch type press, as described elsewhere (17). The resulting alfalfa extract was stored, until required, at -20° C.

Fractionation and Analysis of Extracts

Extract Preparation. The *E. coli* extract was diluted four-fold with buffer A and the alfalfa extract was diluted 2.5 fold with 50 mM sodium phosphate buffer pH 7.1. The pH of the diluted alfalfa juice was adjusted with 1 M NaOH to a final pH of 7.1 before proceeding with the next bioprocessing step.

Step I: Standardized BPA Assessment Protocol. A 1.0–1.2 ml aliquot of extract was added to each of a series of plastic microfuge tubes (No. 72.690 Sarstedt, W. Germany). The series was titrated with BPA-1000 or BPA-1050 [TWS-3088B] (TosoHaas, Philadelphia, PA). Addition of 5–100 μ l of BPA is usually sufficient but greater quantities may be required depending on the nature of the extract and degree of contamination. The BPA treated extracts were mixed thoroughly, allowed to react for 5 minutes [0° C for *E. coli* extract, 22° C for alfalfa extract], and centrifuged (700–1000 g for 2 min). Pellets were then discarded and the supernatant was assayed for turbidity, enzyme activity [β -glucuronidase (GUS) or peroxidase activity], protein, RNA, and DNA. This standardized BPA assessment protocol is shown schematically in Figure 2.


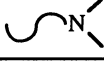

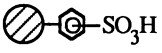
	Physical Form	Functionality	Structure	Size
BIOCRYL BPA-1000	Stable Suspension 10% Solids	Quaternary Amine		~0.1 micron
BIOCRYL BPA-5020	Water Soluble Polymer 2% Solids	Tertiary Amine		High MW >200,000
BIOCRYL BPA-1050	Stable Suspension 10% Solids	Tertiary Amine		~0.1 micron
BIOCRYL BPA-2100	Stable Suspension 10% Solids	Sulfonic Acid		~0.2 micron
Polyethylenimine	Stable Solution 10% Solids	Secondary Amine	$\text{H}_2\text{N}-\left[\begin{array}{c} \\ \text{C}-\text{C}-\text{N} \\ \quad \\ \text{H} \quad \text{H} \end{array} \right]_n-\begin{array}{c} \\ \text{C}-\text{C}-\text{H} \\ \quad \\ \text{H} \quad \text{N}_2\text{H} \end{array}$	High MW 30-40,000
Chitosan	Stable Solution 1% Solids	Primary Amine	$\text{H}_2\text{N}-\left[\begin{array}{c} \text{G} \\ \\ \text{G}-\text{G} \\ \quad \\ \text{NH}_2 \quad \text{NH}_2 \end{array} \right]_n-\text{G}$	High MW 750,000

Figure 1. Characteristics of bioprocessing aids.

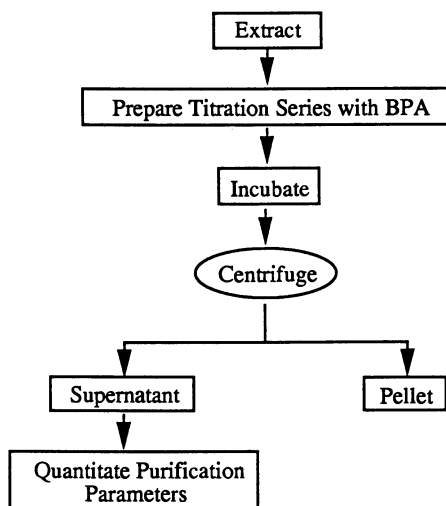


Figure 2. Schematic of the standardized BPA assessment protocol.

Step II: Diafiltration. Extracts to be fractionated by HPLC were diafiltered into buffer A using Centricon 10 microconcentrators (Amicon Div., Grace & Co., Danvers, MA) and then passed through 0.22 μm filters.

Step III: HPLC. Samples (500 μl) of the concentrated, filtered extracts (1.7–3.6 mg protein) were applied (1 ml/min.) to a 7.5 mm x 75 mm TSK DEAE-5PW column coupled to a gradient HPLC system consisting of a #210A sample injection valve, a #126 dual pump, a #165 dual-channel variable wavelength detector, and an IBM-PC-based data system/controller running System Gold software (Beckman Instruments, Inc., Waldwick, NJ). Elution buffers were buffer A and buffer A plus 1.0 M KCl (buffer B). Protein elution and column re-equilibration was achieved with a gradient of buffer A/B [% (v/v)A/min]: 100/0; 100/5; 20/45; 0/46; 0/48; 100/50; 100/65; using a flow rate of 1 ml/min and collecting 1 ml fractions. Protein elution was monitored at 280 nm.

Assays. The turbidity of *E. coli* extracts and alfalfa extracts was quantitated spectrophotometrically at 600 nm and 680nm respectively. GUS activity was assayed as outlined by Novel and Novel (15), except that activity was monitored at 400 nm ($\epsilon^\circ = 9.6 \times 10^3$). Peroxidase activity was determined at 37°C with *o*-tolidine using a final concentration of 0.67 mM *o*-tolidine-HCl (Sigma Chemical Co., St. Louis, MO) and 1 mM H_2O_2 in 20 mM sodium phosphate buffer pH 7.1. The change in absorbance was measured at 600 nm, $\epsilon^\circ = 6.34 \times 10^3$ (18). Protein was determined by the biuret reaction; RNA by the orcinol method; and DNA by the diphenylamine method (16).

Electrophoresis. Electrophoresis was performed on a Pharmacia Phast System (Piscataway, NJ). Protein purity was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). A Phast System 10-15% gradient polyacrylamide gel was run and stained with Coomassie brilliant blue G250 according to standard Phast System methods. Protein mobility was compared to an SDS-7 standard (Sigma Chemical Co., St. Louis, MO), containing seven proteins in the molecular weight range of 14,000 to 66,000.

Results

BPA Treatment of *E. coli* Extracts. The purpose of these experiments was to develop a general approach to Step I of our protein purification scheme, as applied to bacterial homogenates. This goal was considered particularly relevant given the importance of bacterial systems as a biomass as well as a valuable resource for enzyme recovery. In our particular case, we sought to develop a simple and efficient clarification procedure for bacterial homogenates (using *E. coli* as the test biomass) that would remove, ideally, cell debris and nucleic acids while leaving the enzyme of interest in the soluble fraction of the clarified extract. For our program GUS was used as the target enzyme because of the ease with which it is assayed, because of its stability, and because of the common use of GUS as a genetic marker in plant transformation systems. Previously, PEI had been used to precipitate proteins such as RNA polymerase along with the nucleic acids (11). In that approach to bioprocessing the desired protein was differentially released from the nucleoprotein pellet by step-wise batch extractions with buffers of increasing salt concentration. It would appear that such an extraction adds a time consuming and potentially expensive step to a large scale protein purification. Therefore, it was considered desirable to develop a method that eliminated the salt extraction step yet still resulted in a clarified extract suitable for use directly in subsequent purification steps.

Our approach to the experimental problem was based on the concept of varying the

pH and/or ionic strength to prevent binding of the target enzyme to the BPAs while simultaneously inducing aggregation and precipitation of the cell wall debris and nucleic acids. The first step in this process was to titrate the *E. coli* extract with the BPA at neutral pH, in the absence of exogenous salt, to determine the extent of separation of GUS from nucleic acids and cellular debris which could be achieved. It was determined that at a BPA-1000 concentration of 4000 ppm, nucleic acids were almost completely removed and turbidity was reduced by approximately 50% (Figure 3). Unfortunately in these experiments, the GUS activity was found to coprecipitate with the nucleic acids as did 75% of the total cellular protein. Increasing the amount of added BPA-1000 continued to reduce the turbidity and the amount of protein. The clearing effect of increasing amounts of BPA-1000 on the *E. coli* extract is shown in Figure 4. Also of note was the steady increase in the size of the pellet with increasing additions of BPA-1000. Similar results were obtained with BPA-1050 except that a final concentration 2000 ppm higher than for BPA-1000 was required.

Effect of pH, salt and mixing sequence. It was ascertained that changes in pH were unsuccessful in achieving differential precipitation of GUS and nucleic acids. At pH 5.5, GUS activity and nucleic acids were less well separated than at pH 7.1. Utilization of more alkaline conditions was of restricted value since at pH 9.0 GUS was unstable. Only at pH 8.0, and only with BPA-1050, was some separation possible between GUS and nucleic acids precipitation. In view of the limited benefits of this experimental approach, we decided to investigate the effect of exogenous salt on the flocculation by the Biocryls of GUS in the *E. coli* extracts. At pH 7.1, using a constant BPA-1000 concentration of 4000 ppm, but varying exogenous KCl between 300 mM and 500 mM, it was observed that nucleic acids were almost completely removed from the extract while greater than 95% of the GUS activity remained in the supernatant (Figure 5). Almost identical results were obtained with BPA-1050 except that the optimum BPA concentration was higher, requiring 6000 ppm BPA-1050. Subsequently a sample of the untreated *E. coli* extract and the extract processed with BPA-1000 and exogenous 300 mM KCl were analyzed separately by HPLC. The GUS activity in both samples was found to elute from the DEAE column at about 300 mM KCl and furthermore the SDS-PAGE analysis showed that samples contained approximately 50% of protein as GUS (Figure 6). The SDS-PAGE gel also showed that in the unfractionated extracts, prior to HPLC, the BPA-1000 treatment (in the presence of 300 mM KCl) does not decrease the amount of GUS compared to the crude *E. coli* extract untreated with BPA.

It was also noted during the course of the exogenous KCl addition studies that the sequence in which the salt, dilution buffer, extract, and BPA were added had a profound effect on the turbidity of the resulting extracts. Initially, experiments were performed by adding the BPA to the diluted extract. We found subsequently that a five fold decrease in turbidity could be achieved by mixing diluting buffer, salt, and BPA first, and then adding extract to this mixture (Figure 7). These results were surprising and suggest that dilution and ionic environment may influence the ability of positively charged BPA to react with particulate material such as the cell wall and lipid components present in bacterial cell debris. Further studies will be required to fully investigate this interesting observation. By comparison the altered mixing protocol decreased protein content slightly but had no effect on GUS activity or nucleic acid content (Figure 7). Using the new protocol we studied the effect of centrifugal force on achieving clarification of the untreated and BPA-1000 (or BPA-1050) treated *E. coli* extracts containing 300 mM KCl. As centrifugal force increased, the turbidity of the resultant supernatant phase for both the untreated and BPA treated samples decreased (Figure 8A). However, the turbidity in the BPA treated samples was 5–10 fold lower than in the untreated sample at all centrifugal forces tested. In fact, the turbidity in the treated samples at the lowest centrifugal force (~80 g) was less than that in the untreated

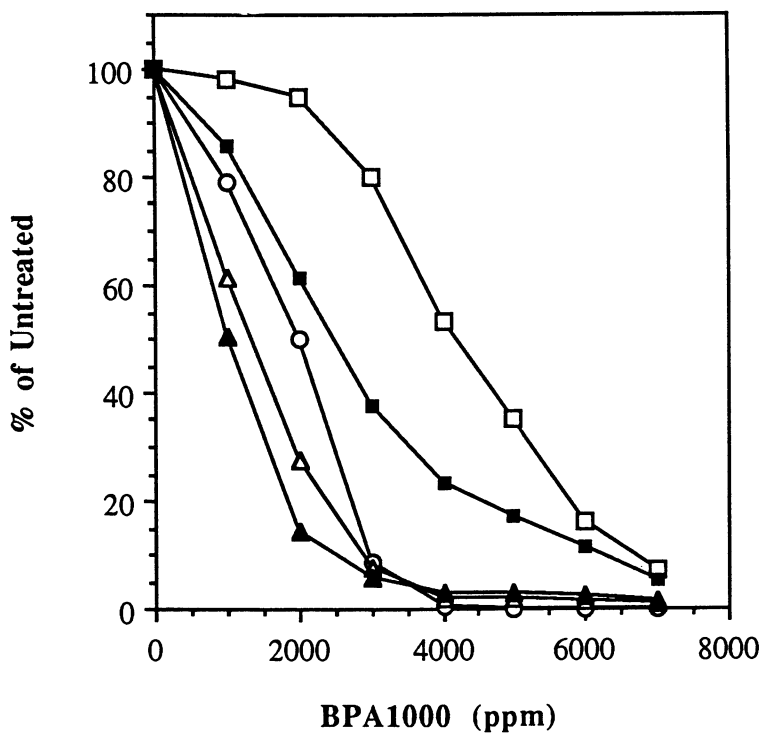


Figure 3. Titration of *E. coli* extract with BPA-1000. Results are expressed as a percentage of those for the untreated extract and are plotted against the added BPA-1000 expressed in parts per million (ppm). Legend: □ = turbidity; ○ = GUS activity; ■ = protein; △ = RNA; ▲ = DNA.

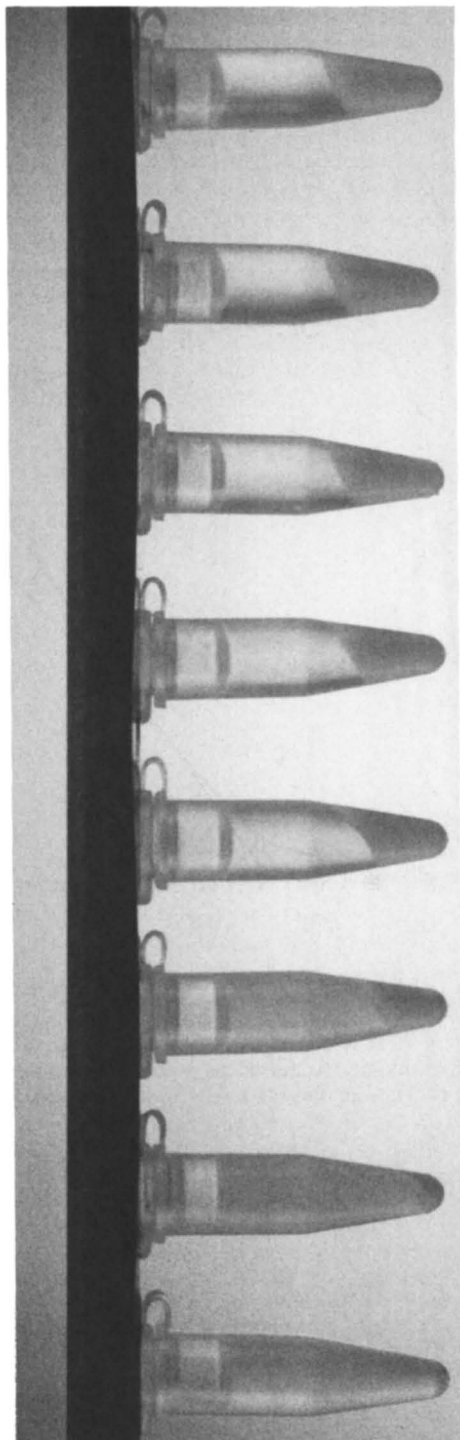


Figure 4. Photograph of *E. coli* extract titrated with BPA-1000 as in Figure 3. The BPA-1000 concentrations from left to right are 0, 1000, 2000, 3000, 4000, 5000, 6000, and 7000 ppm.

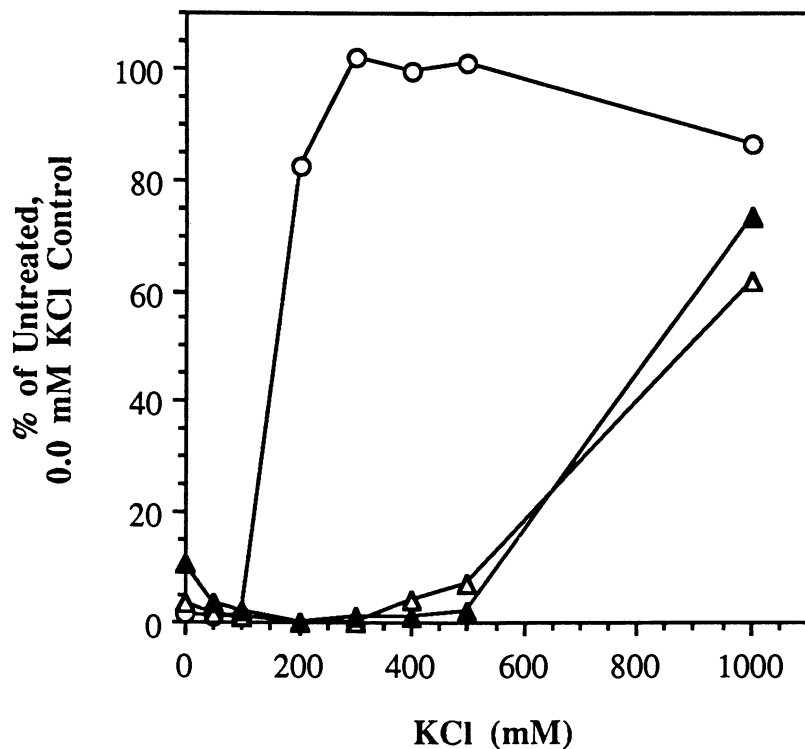


Figure 5. Clarification of *E. coli* extract in the presence of increasing concentrations of KCl. Results are expressed as a percentage of those for the untreated sample (0 mM KCl). Legend: ○ = GUS activity; △ = RNA; ▲ = DNA.

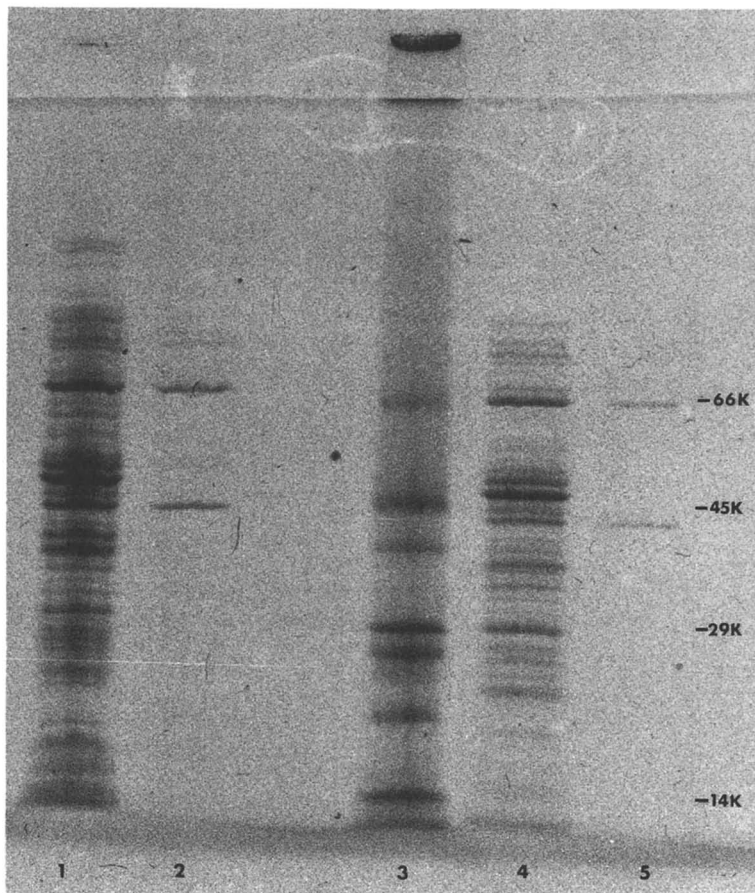


Figure 6. 10-15% SDS polyacrylamide gel of: lane 1 — untreated *E. coli* extract (0 mM KCl); lane 2 — the peak GUS fraction from the HPLC separation of untreated extract; lane 3 — Sigma SDS-7 molecular weight standards (approximate molecular weights of 4 standards are indicated on the right hand edge of the figure); lane 4 — BPA-1000 treated *E. coli* extract (4000 ppm BPA-1000 and 300 mM KCl); lane 5 — the peak GUS fraction from the HPLC separation of BPA-1000 treated extract. GUS migrates slightly slower than the 66,000 molecular weight standard.

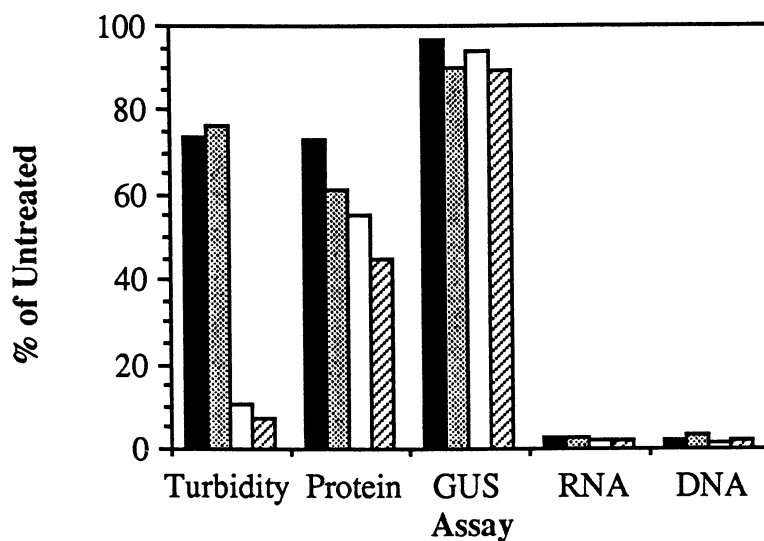


Figure 7. The effect of the mixing order in the BPA treatment protocol on the clarification parameters. Results are expressed as a percentage of those for the untreated sample. All samples contained 300 mM KCl. BPA-1000 was used at a concentration of 4000 ppm and BPA-1050 was used at a concentration of 6000 ppm. Legend: ■ = BPA-1000 treated extract, BPA added last; ▨ = BPA-1050 treated extract, BPA added last; □ = BPA-1000 treated extract, extract added last; ▩ = BPA-1050 treated extract, extract added last.

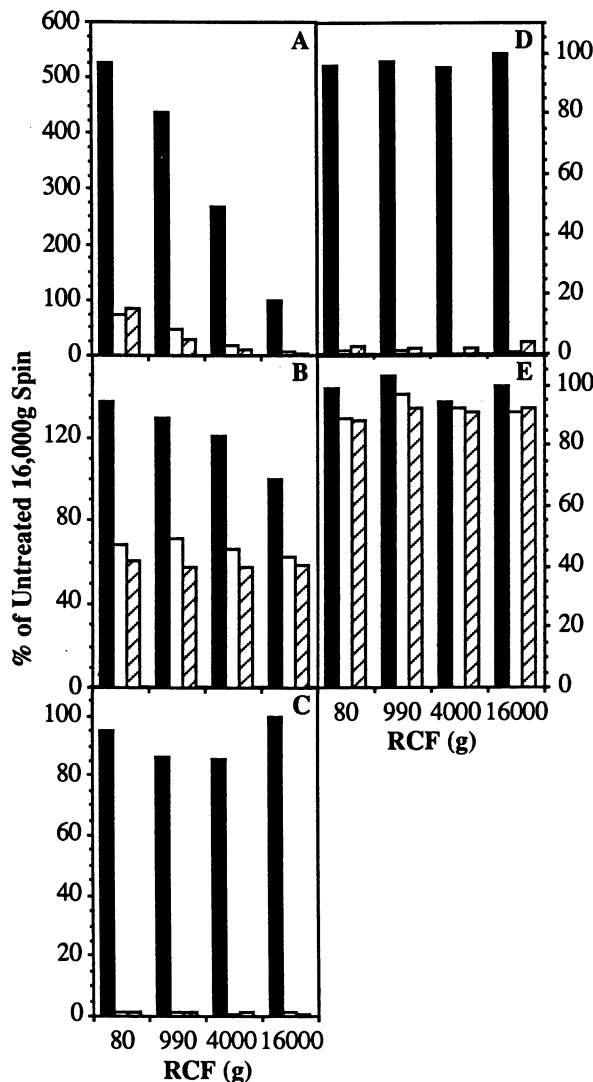


Figure 8. The effect of centrifugal force in the BPA treatment protocol on the clarification parameters. Results are expressed as a percentage of those for the untreated sample spun at 16,000 g. All treatments involved 2 min centrifugation. The KCl, BPA-1000, and BPA-1050 concentrations are the same as in Figure 7. A: turbidity versus relative centrifugal force (RCF); B: protein versus RCF; C: RNA versus RCF; D: DNA versus RCF; E: GUS activity versus RCF. Legend: ■ = untreated extract; □ = BPA-1000 treated extract; ▨ = BPA-1050 treated extract.

sample at the highest force (16,000 g). Protein content in the untreated samples decreased in total by approximately one third as the centrifugal force increased, but the protein content in the treated samples was largely unchanged by centrifugal force (Figure 8B). As indicated in Figures 8C, D and E, the RNA and DNA content, and the GUS activity, in contrast to turbidity, are largely unaffected by the centrifugal force.

BPA-1000 Treatment of Alfalfa

Alfalfa juice as expressed directly from whole *M. sativa* tissue is an extremely dark green viscous solution, high in protein content as well as undesirable components such as pigments, secondary products and the phenolic group of flavonoids and phenylpropanoids. Previous large-scale processes for the recovery of protein from alfalfa juice have involved heat precipitation (30–80°C) and coagulation or acid precipitation of the proteins (20). However, heat or acid treatment is not feasible in most cases where biologically active protein (enzymes in particular) are to be purified from the plant. Therefore, we examined the feasibility of using bioprocessing aids to selectively precipitate the plant tissue contaminants (consistent with the Step I processing strategy) with the added goal of leaving the desired protein(s) in solution. It was also hoped this goal could be achieved under conditions suitable for the maintenance of enzymatic activity. For our investigations, peroxidase was selected as the target enzyme since alfalfa tissue contains several peroxidase isozymes (19) and a simple procedure has been established to assay that enzyme (5,18).

The results of BPA-1000 treatment of the alfalfa extract are summarized in Figure 9. Clarification of the extract could be assessed very effectively using spectrophotometry, due to the distinctive absorbance peak of the plant pigments at 680 nm. With increasing additions of BPA-1000, the absorbance at 680 nm was reduced to less than 5% of its untreated level indicating extremely efficient removal of pigment and debris (Figure 10). The concentration of nucleic acids remaining in solution after BPA-1000 treatment was also significantly reduced. At ~3000 ppm the amount of RNA remaining in solution was less than 55% of that in the untreated sample and DNA had been reduced to only 3% of its initial concentration. The differential in the effectiveness of RNA vs DNA precipitation could possibly be due to the formation of large DNA concatamers which increase the interaction and subsequent flocculation of DNA by the BPA. Although BPA-1000 effectively removed pigment, debris, DNA and to a lesser extent RNA, almost 90% of the total protein and greater than 100% of the peroxidase activity remained in solution at ~3000 ppm BPA. It should be noted that our laboratory has frequently observed greater than 100% enzymic activity of a biological extract after treatment with BPA in Step I (2,5). It is possible that these higher levels of enzymatic activity could reflect the removal of specific enzyme inhibitors by the Biocryl.

Conclusions

We have found it possible to remove greater than 95% of the nucleic acids and 80–90% of the turbidity in *E. coli* extracts using BPA-1000 or BPA-1050. At the same time, greater than 95% of the target enzyme, GUS, remains in the clarified extract. This differential removal of debris, cell wall fragments and nucleic acids was accomplished by treating the extracts with BPAs in the presence of varying concentrations of exogenous salt. The obvious advantage of this procedure is a simplification over the procedure commonly used for PEI. Such a simplification could save considerable time and expense particularly when a system for the recovery of enzymes from bacterial biomass is transformed into a large scale, commercial operation. In addition, this procedure could prove generally useful for other bacterial proteins since greater than 90% of the nucleic acids are removed from the *E. coli* extracts over at least the concentration range of 0–500 mM KCl. Since proteins generally do not have the charge density of nucleic acids, it should be feasible to establish a salt concentration that

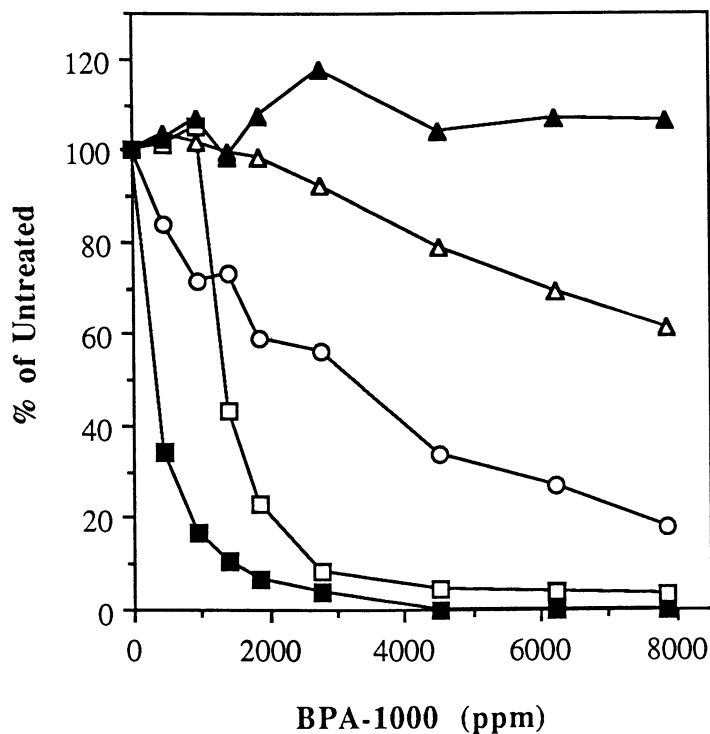


Figure 9. Titration of alfalfa extract with BPA-1000. Results are expressed as in Figure 3. Legend: \square = turbidity; \circ = RNA; \blacksquare = DNA; \triangle = protein; \blacktriangle = peroxidase activity.

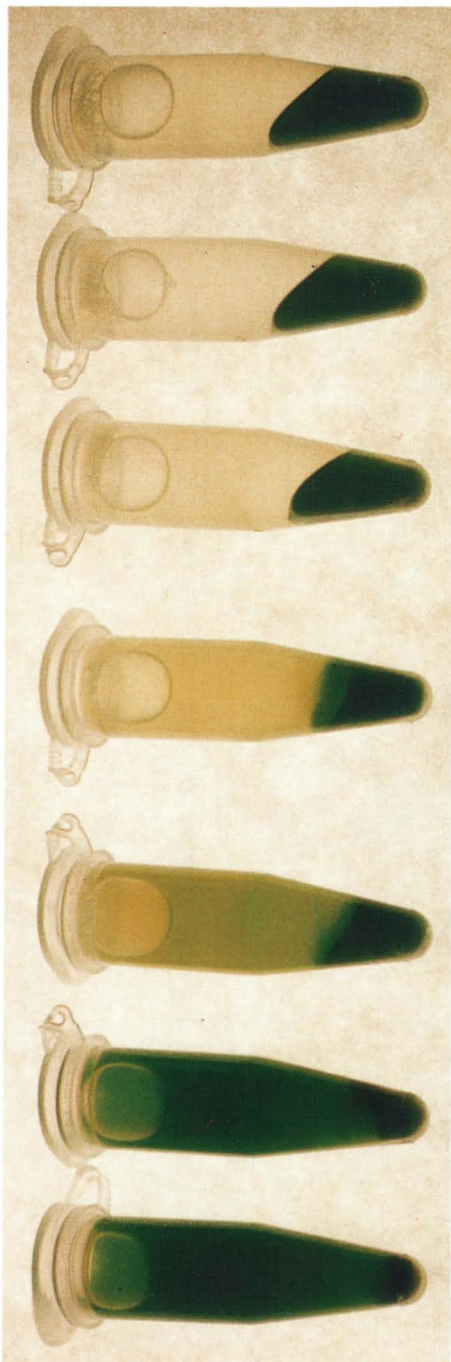


Figure 10. Photograph of alfalfa extract titrated with BPA-1000 as in Figure 9. The BPA-1000 concentrations from left to right are 0, 475, 950, 1425, 1900, 2850, and 3800 ppm.

prevents binding of the protein of interest to the BPA but facilitates binding, and flocculation, of the nucleic acids. Should salt alone not prove effective, a combination of salt plus changes in pH — either to the pI of the protein or to the pK of the BPA — may prove useful.

Another advantage to the incorporation of bioprocessing aids into the Step I procedure is that the clarified extract can be used directly for subsequent purification steps even without the use of a Step II system to dewater or concentrate the process stream. These factors are especially relevant when HPLC systems are used in Step III for the chromatographic procedures. Nucleic acids, pigmented organics and especially cellular debris can very quickly foul an HPLC column. This is an even more important consideration for large scale protein purification schemes where the volumes of material and costs of the operation are greatly increased (3).

A final advantage to the use of bioprocessing aids in bacterial biomass processing is the low centrifugal forces needed to clarify the extract. Typically, untreated *E. coli* extracts are clarified by a 30,000 g centrifugation. After BPA treatment, the *E. coli* extracts are clarified better by an 80 g centrifugation than by a 14,000 g centrifugation of untreated extract. Centrifugal forces of 14,000 g or 30,000 g are easy to generate for laboratory-scale purifications but for industrial-scale purification strategies, where the processing stream is vast, such centrifugation systems would normally be impractical. The results obtained in this study indicate that bioprocessing aids in the Step I clarification of bacterial extracts could improve low speed centrifugation and therefore may offer adaptation for bacterial fractionation on an industrial scale.

Based on the above advantages, we believe that incorporation of bioprocessing aids into Step I of a purification scheme could be used as a general approach to clarifying bacterial homogenates and the recovery of enzymes from bacterial biomass.

Recovery of biologically active proteins from plant biomass is problematic due to the large quantity of cellular debris, nucleic acids, photosynthetic pigments and phenolic compounds (21). Removal of these contaminants in a Step I process is generally necessary in order to facilitate the subsequent bioprocessing of Step II and Step III, such as ultrafiltration and chromatography. The second series of experiments covered in this paper was designed to address those issues, employing a bioprocessing aid in Step I of a strategy for the recovery of enzymes from plant biomass. The experimental results which were obtained for peroxidase in alfalfa extracts indicate that bioprocessing aids are of considerable benefit in the clarification of crude plant extracts. This area of research is the subject of ongoing work by our laboratory.

Although this report focuses on the processing of bacterial homogenates and plant extracts, all or part of the three step protein purification strategy, has been successfully applied by our laboratory to a variety of biomass sources. These sources include bacterial culture fluids (2), animal tissue extracts, animal secretions, animal tissue culture fluids (3), fungal culture fluids (1,5), fungal extracts (22), yeast extracts and yeast culture fluid (3). The full potential for the use of bioprocessing aids in the recovery of proteins from biomass has yet to be realized and further research in this area is continuing.

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Literature Cited

1. Forrester, I.T.; Grabski, A.C.; Kelley, B.D.; Strickland, W.N.; Leatham, G.F.; Burgess, R.R. In *Biotechnology in Pulp and Paper Manufacture*; Kirk, T.K.; Chang, H-M., Eds.; Butterworths: London, UK, 1990, pp 407-414.
2. Forrester, I.T.; Grabski, A.C.; Strickland, W.N. In *Fermentation Technologies : Industrial Applications*; Yu, P-L., Ed.; Elsevier Applied Science: London, UK, 1990, pp 403-406.
3. Fletcher, K.; Deley, S.; Fleischaker, R.J.; Forrester, I.T.; Grabski, A.C.; Strickland, W.N. In *Separations for Biotechnology*; Pyle, D.L., Ed.; Elsevier Applied Science: London, UK, 1990, Vol. 2, pp 142-151.
4. Leatham, G.F.; Forrester, I.T.; Mishra, C. In this volume.
5. Forrester, I.T.; Grabski, A.C.; Mishra, C.; Kelley, B.D.; Strickland, W.N.; Leatham, G.F.; Burgess, R.R. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 359-365.
6. Forrester, I.T.; Grabski, A.C.; Burgess, R.R.; Leatham, G.F. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 992-999.
7. Mishra, C.; Forrester, I.T.; Kelley, B.D.; Burgess, R.R.; Leatham, G.F. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 226-232.
8. Lightfoot, E.N.; Gibbs, S.J.; Cockrem, M.C.M.; Athalye, A.M. In *Protein Purification Micro to Macro*; Burgess, R.R., Ed.; UCLA Symposia on Molecular and Cellular Biology New Series; Alan R. Liss: New York, NY, 1987, Vol. 68; pp 75-97.
9. Muzzarelli, R.A.A.; Weckx, M.; Filippini, O. *Carbohydr. Polym.* **1989**, *11*, 293-306.
10. Muzzarelli, R.A.A.; Weckx, M.; Fiippini, O.; Lough, C. *Carbohydr. Polym.* **1989**, *11*, 307-320.
11. Jendrisak, J. In *Protein Purification Micro to Macro*; Burgess, R.R., Ed.; UCLA Symposia on Molecular and Cellular Biology New Series; Alan R. Liss: New York, NY, 1987, Vol. 68; pp 75-97.
12. Persson, I.; Lindman, B. In *Flocculation in Biotechnology and Separation Systems*; Attica, Y.A., Ed.; Elsevier: Amsterdam, Netherlands, 1987, pp 457-466.
13. Cabral, J.M.S.; Robinson, E.M.; Cooney, C.L. U.S. Patent 4 830 753, 1989.
14. Jefferson, R.A.; Burgess, S.M.; Hirsh, D. *Proc. Natl. Acad. Sci. USA.* **1986**, *83*, 8447-8451.
15. Novel, M.; Novel, G. *J. Bacteriol.* **1976**, *127*, 406-417.
16. Hanson, R.S.; Phillips, J.A. In *Manual of Methods for General Bacteriology*; Gerhardt, P.; Murray, R.G.E.; Costilow, R.N.; Nester, E.W.; Wood, W.A.; Krieg, N.R.; Phillips, G.B. Eds.; American Society for Microbiology: Washington, DC, 1981, pp 328-364.
17. Koegel, R.G.; Fomin, V.I.; Bruhn, H.D. *Trans. A.S.A.E.* **1972**, *16*, 712-716.
18. Leatham, G.F.; Stahmann, M.A. *J. Gen. Microbiol.* **1981**, *125*, 147-157.
19. Quiros, C.F. *Crop Sci.* **1980**, *20*, 262-264.
20. Straub, R.J.; Basken, K.E.; Koegel, R.G.; Bruhn, H.D. *Trans. A.S.A.E.* **1977**, *20*, 649-652.
21. Loomis, W.D. In *Methods in Enzymology*; Fleischer, S.; Packer, L. Eds.; Academic Press: New York, NY, 1974, Vol. XXXI, pp 528-544.
22. Folgert, J.; Forrester, I.T.; Bush, R.K. *J. Allergy Clin. Immunol.* **1990**, *85*, 153.

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Chapter 13

Chromatography in Enzyme Isolation and Production

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Recent advances in the development of chromatographic media and equipment have made chromatography a viable choice for protein isolation and purification. Gels more stable to chemical (i.e., concentrated sodium hydroxide) and mechanical pressure allow more efficient separations with greater reproducibility, and increased column cycles using clean-in-place (CIP) procedures. This paper describes the application of different chromatographic methods (i.e., affinity, ion exchange, and gel filtration) to large-scale protein purification, along with the merits of various column designs presently available. The benefits of process development and production automation will also be reviewed.

Many methods have been developed to separate substances of interest either from natural sources or from synthetic or fermentative processes. One method that has been successful in more applications than any other is chromatography. Chromatography has been used to separate small substances such as salts, minerals, and organic compounds (e.g. drugs) to more complex biological molecules such as proteins and nucleic acids. Chromatography is presently carried out both in aqueous and organic solvents, using a wide variety of supports from paper to dextrans, and silica to agarose.

Chromatography has gained a special place in the separation of proteins and other labile complex biological substances due especially to its typically gentle nature. These procedures can provide well-defined separations of similar polypeptides while yielding a high percentage of the original biological activity of the materials separated. Furthermore, chromatographic separation procedures can be utilized for both analytical purposes where milligram quantities are isolated, to production scale where tens to thousands of grams per year are separated. Due to the labile nature of most proteins, separations requiring biologically active products are almost always carried out in aqueous solvents. The best success has been using a stationary, hydrophilic, non-interactive base support matrix physically or chemically modified for specific applications with biological agents to be separated in the mobile phase. This allows predictable and reproducible chromatography with the separation mode selected for that matrix by its manufacturer (e.g., ion exchange, affinity, etc.).

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Chromatography of material such as proteins has occasionally been carried out in batch mode. Here the proteins are adsorbed onto a chromatographic medium by mixing the sample and the media in a common vessel prior to specific elution of the various adsorbed proteins. The mixture's components are subsequently eluted from the media by various types of elution schemes (e.g., salt and/or pH steps or gradients, specific affinity elution, etc.).

Chromatography also has its own special equipment requirements that vary dependent on the type and scale of the procedure, and the efficiency level desired from the separation. This paper deals predominantly with chromatographic separations as they pertain to large-scale industrial processes and their special needs and considerations.

Chromatographic Techniques and Their Applications

Successful application of chromatographic techniques relies on resolution, or the resolving power of the particular technique used. Resolution is defined by the relation of selectivity and efficiency of the chromatographic gel media (*1*). Selectivity is a function of the mode of separation of the gel (i.e., gel filtration, ion exchange, etc.) and efficiency is a function of the support matrix (i.e., particle shape, size distribution, mechanical stability, density of interactive chemical groups, etc.). Each of the various modes of chromatographic separation have unique advantages that dictate where and when in a purification process these techniques should be used.

Initial Product Capture. Initial chromatographic steps involve product capture or concentration. The feed stream, coming from fermentation, cell culture or tissue extraction, is generally clarified using filtration or a bioprocessing agent. After clarification, the most versatile and commonly applied chromatographic procedure is ion exchange (IEX)(2) on a solid matrix. IEX's popularity is due to its ability to handle large volumes of feed in a short time using a relatively small amount of a gel medium. Selection of proper loading conditions can insure that the percentage of the desired product bound is as high as possible while minimizing unwanted by-products. Elution with salts using either stepwise or linear gradients is then used to fractionate the bound products. Effectively designed IEX procedures can give tremendous product enrichment during the first chromatographic step.

Another practical, but currently less utilized, technique for use at initial stages is hydrophobic interaction chromatography (HIC). Also a concentrating technique, HIC requires high ionic strength to promote protein adsorption making it a good technique to follow a salt precipitation step, or an initial IEX concentrating step, where the salt concentration in the eluted product fraction is high. Proteins are then eluted (in HIC) by reduction in eluent ionic strength.

Both IEX and HIC can be used in batch mode where the chromatographic media and feed are mixed, the mixture put in a vessel, and a simple one step elution is used to displace the product (and co-eluting contaminants). The advantages of batch adsorption are minimal equipment requirements, and its simplicity to perform. However, it is very cumbersome at large scales and requires much operator manipulation of the media and the product. Loading the feed on IEX or HIC solid phase media packed in a column provides an efficient process where loading and elution of the product occurs in a controlled environment where the gel is not handled during processing. Moreover, because less gel is required, more protein can be loaded on gel packed in a column than in free solution (3). Even though the equipment costs (columns, pumps, etc.) are higher than batch mode, this cost is quickly recovered from savings in buffer and gel media. Savings in operator time is also realized because these steps are easily automated.

After the initial isolation or capture step, the product is in a more workable

volume (and buffer) and is then ready for further fractionation. Depending on the requirements for the next step, the product buffer can be changed using a desalting or diafiltration step prior to application on the next chromatographic column.

Product Resolution. The selection of the separation step or steps, and the sequence of those steps, that follow initial isolation require as much knowledge of the protein as possible. This will enhance the ability to apply techniques whose selectivity allow the most efficient, most economic, and best achievable resolution of the product.

Combining chromatographic techniques in a logical sequence affords the most efficient process (see Fig. 1). Chromatographic media properties such as capacity, recovery, resolving power and selectivity should all be considered in establishing the sequence. There are two design rules commonly adhered to by process development chemists. First is to utilize higher capacity techniques such as IEX, HIC or affinity earlier in the scheme when higher volumes are commonly found. The second is to use different chromatographic techniques for each step to take advantage of the different selectivity types available.

The most commonly used resolving techniques in a process are IEX and affinity (4). Both techniques lend themselves easily to scale-up and provide the greatest resolving power. HIC is gaining popularity, but is limited in both application and predictability. IEX is by far the most versatile of the resolving techniques as it can be applied to the greatest number of proteins under the greatest variety of conditions (2). Manipulation of buffer conditions such as pH, ionic strength and elution schemes (i.e., stepwise or linear gradient) affords tremendous potential in the ability to optimize IEX separations. An optimized IEX separation is relatively inexpensive to operate since simple salts are commonly used for product elution. In addition, since base-stable IEX gels are commercially available, cleaning and depyrogenation are effected with inexpensive agents such as sodium hydroxide or ethanol. From a regulatory standpoint, it is also very easy to document the removal of both the eluents and cleaning agents from IEX column effluents.

Affinity chromatography, although not as versatile as IEX, offers the highest resolution available where the technique is applicable. Using a column filled with an immobilized ligand that is specific only to the product, a single pass of the product fraction over that column can often yield product pure enough to require only a final polishing step. Affinity chromatography is particularly attractive because of the simplicity of its operation. As this technique is most often carried out as an adsorption/desorption technique, it is no more complicated to operate than gel filtration. However, unlike gel filtration, very large feed volumes can be processed on a relatively small column in a very short time yielding small volumes of a relatively concentrated product. This allows unsophisticated, relatively inexpensive equipment and control systems to be used. The major drawbacks of affinity chromatography are the high cost of affinity gels and the labile nature of some affinity agents (e.g., proteins and antibodies). Also, the immobilized ligand is often sensitive requiring the user to clean and depyrogenate the gel with agents much more expensive than common cleaning agents such as sodium hydroxide. There is also the requirement to effect removal of eluent and trace amounts of column ligand from the product and the additional time and costs needed to validate those procedures. Nevertheless, the resolving power of affinity makes its use very compelling since the economy of operation and product purity achieved provides savings which can far outweigh the higher initial and operating costs of the procedure.

Gel filtration is the last of the major chromatography techniques commonly applied in the resolving portion of a process. Of all the techniques discussed thus far in this chapter, gel filtration offers the lowest resolution. The separation is based solely on Stoke's radius of the protein molecule and is the most sensitive to flow rate and sample volume. To achieve significant resolution among sample components, the sample volume should be no greater than five percent of the column bed volume.

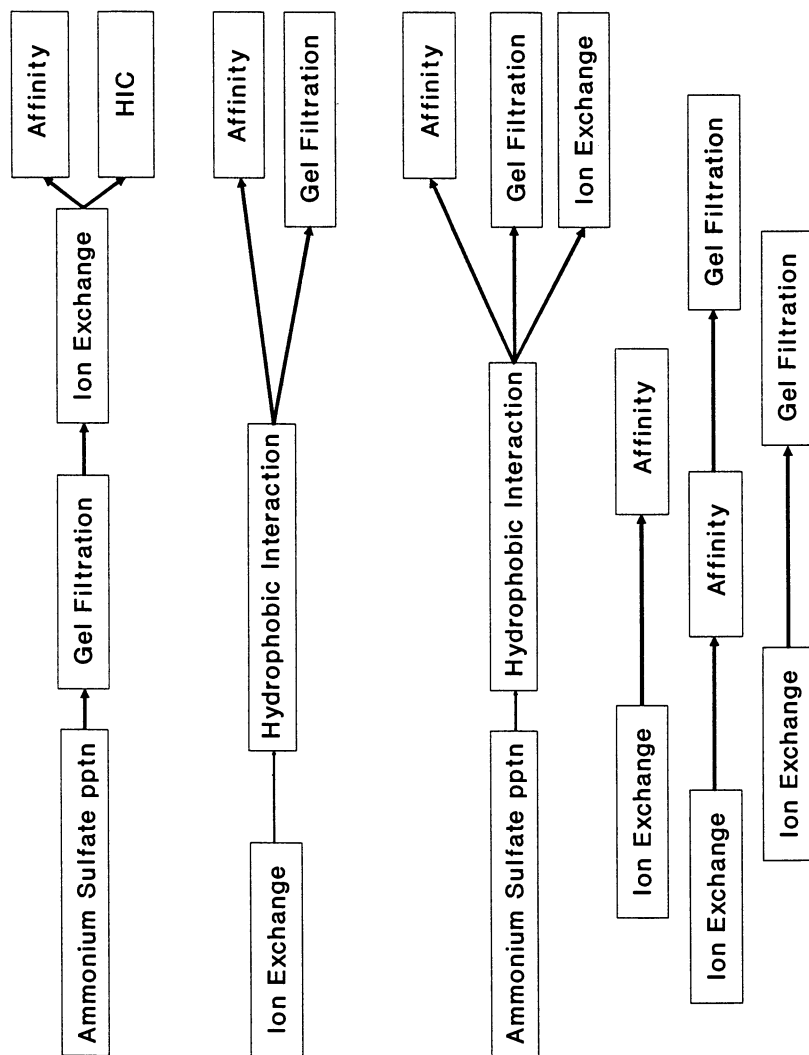


Figure 1. Examples of logical sequences of chromatographic steps.

Obviously, with large sample volumes, the column and gel requirements result in a costly process. It is for these reasons that gel filtration is selected as the last or among the later resolving steps in a process, where both the sample volumes smaller, and the mixture to be fractionated is far less complex.

Less product manipulation between chromatographic procedures (i.e., buffer exchange, etc.) reduces total process time and increases overall product yield. This is accomplished by considering the compatibility of the targeted techniques. For instance, the need for buffer exchange, concentration or lyophilization of the product can be reduced by placing diluting techniques after concentrating ones (i.e., gel filtration after IEX or affinity). Physical handling of the product can be minimized further increasing the yield of the process. Other common examples include following IEX with HIC, or salt precipitation with HIC where the product fraction from the previous column can be directly applied to the second column with minimal preparation.

Polishing. This last process step prepares the product for final formulation or for actual sale. It is designed to remove any aggregated protein, remove residual chromatographic eluent(s), and place the product into a specific solvent. These requirements are admirably served by gel filtration. At this point, the sample volume is small and the product fraction to be applied is fairly clean. The gel and column equipment requirements are now within reason and, the clean samples result in much longer gel life.

IEX can also be used for polishing. Although usually too costly to be applied earlier in a process, high performance ion exchange chromatography (HPIEC) has been used to polish small quantities of high value proteins. HPIEC offers high efficiency which increases resolution resulting in high purity products. IEX has also been used for final concentration followed by a step elution of the product with a volatile buffer (e.g., ammonium bicarbonate) allowing lyophilization for storage or sale.

Chromatographic Supports

Chromatographic resolution is also dependent on column efficiency (*I*). Column efficiency is directly dependent on the nature of the support matrix and how well that support is packed in its column. Available chromatographic supports are based on dextran, agarose, polystyrene, acrylic, cellulose, silica gel and a variety of other polymers. Although cellulosic supports are manufactured in both microcrystalline and beaded forms, most supports are beaded. Newer supports may use hybrid bead construction where the base support is coated with a second material (e.g., dextran or silica coated with agarose).

The most effective supports used in the separation of proteins all have certain common characteristics. They should be hydrophilic as separations are almost always carried out in aqueous buffers. Supports must be inert in that nonspecific binding is minimized. It is also desirable that the support does not contribute to the separation in ways different from the active groups attached to it. This helps to insure predictability and reproducibility of the separations among different manufactured lots of chromatographic media.

The support should be stable to common cleaning and sanitizing agents such as sodium hydroxide. Cleaning allows the maximum number of separation cycles on any one column while preventing product contamination across product lots. Caustic cleaning also sanitizes the gel and helps remove pyrogens. Stability to mild organic solvents (e.g., alcohols, acetone or acetonitrile) is also desirable. These solvents can be used for removal of lipids and other hydrophobic contaminants from the gel, enhancing the solubility of certain feed stream components or simply for their bacteriostatic effect. Lastly, a good process support should have good mechanical properties. Many of the better cross-linked supports can handle linear flow rates in excess of 250 cm/hr without

compression of the packed column bed and are also not subject to shrinking and swelling with changes in ionic strength. These characteristics allow all the chromatographic regeneration and cleaning procedures to be performed in place in the column thus eliminating the need to unpack the column every time the support needs to be cleaned or regenerated.

Chromatographic Equipment

Careful selection of equipment used to carry out chromatography is as important as the selection of the technique itself. Flexibility, ease of manipulation and ability to automate are important. Different techniques have different requirements for column configuration, all require the maximum throughput for process efficiency and economy, and all benefit from automation of the individual process steps.

In the case of IEX, HIC or affinity chromatography, the desired column configuration is one of short bed height and large cross sectional area (1). This will allow high throughputs with low pressure drops reducing the cost of both the column and the pumping and support systems. However, more importantly, high throughputs will reduce the process time reducing labor and utility costs. The added advantage of short bed heights is the ability to use softer support matrices with less fear of restriction of eluent flow resulting from overcompression of the gel bed.

In contrast, gel filtration requires taller thinner columns (1), the height determined by the resolution required. Newer less compressible gels can be packed as high as 50 cm without any significant reduction in manufacturer's specified flow rate ranges (with gradual reductions corresponding to increased bed heights) (5). Older and softer more compressible gels require multiple columns to be placed in series to attain the same overall bed height (6). This configuration combines both the benefit of high throughput achieved with low bed heights and the resolution obtained with longer columns.

The three basic column designs are presently commercially available (Fig. 2a-c). The first two are straight through tube designs and differ in the end piece construction. The first of these two uses fixed end pieces and holds a specified amount of gel. The amount of gel used in the packing slurry must be sufficient to fill the column as no end piece adjustment is possible. The second of the straight through designs utilizes an adjustable end piece. The obvious advantage is that the top column end piece (adaptor) can be adjusted to the top of the gel bed after packing compensating for any inaccuracies in calculating the amount of gel present in the packing slurry. However, columns of this design are more difficult to clean and sanitize as there are more dead spaces around the adaptor than in the fixed-column design. The third design is the radial flow column where flow moves from the outside of the cylinder to the inside. This provides high throughput and the ability to use soft gels. Unlike the straight through designs, it is difficult to pack this column efficiently. Hence, its best applications are affinity and simple IEX or HIC where the separation is a simple on and off procedure.

The Basics of Scaling-Up Chromatographic Separations

In this brief treatise the discussion of the scale-up of chromatographic processes can not be fully covered. I will attempt to summarize a few of the general rules that most process development chemists attempt to follow to make the transition from the bench to the pilot and production scale easier and more efficient.

One primary goal is to conserve selectivity throughout scale-up. This means attempting to use the same separation chemistry in development that will eventually be used in production. This avoids the need to re-optimize in each stage of the scale-up process. It is desirable to use the same support matrix to avoid unforeseen matrix effects causing unnecessary modification of developed procedures.

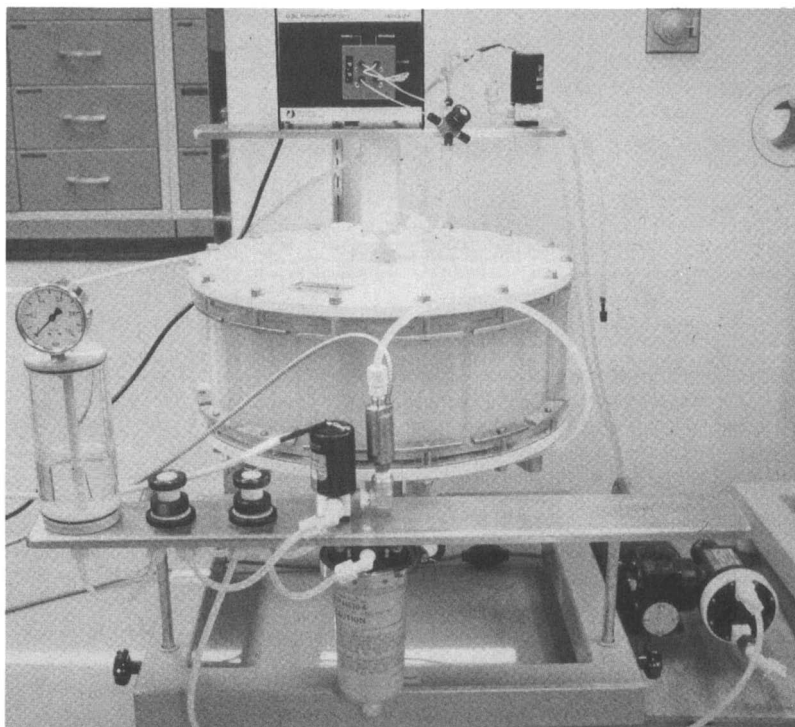


Figure 2a. Example of a typical fixed end-piece column.

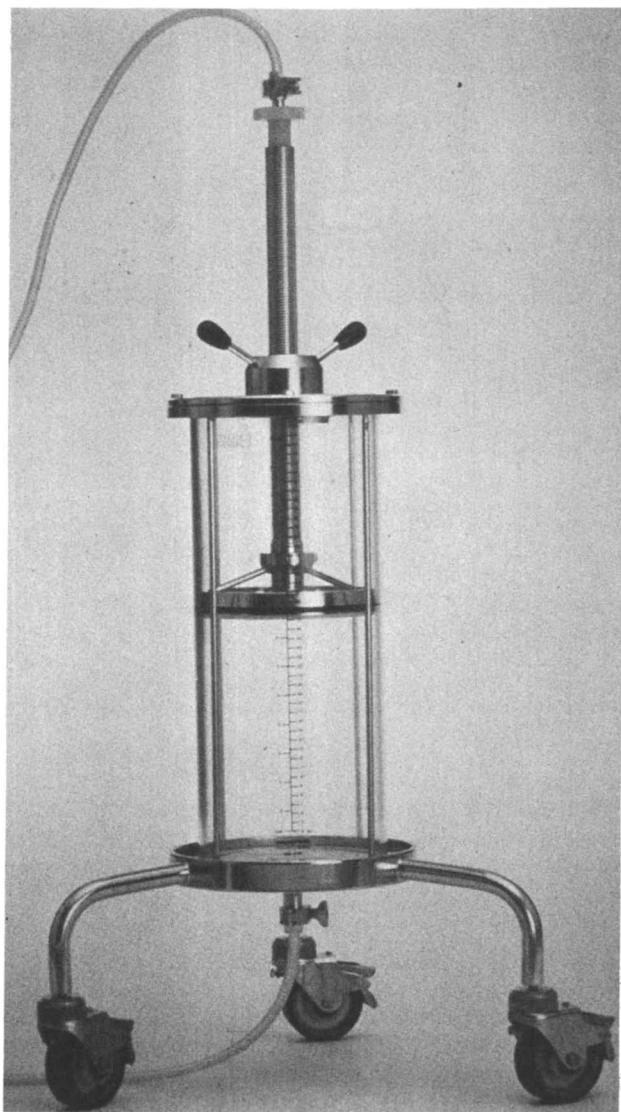


Figure 2b. Example of a typical adjustable end-piece column.

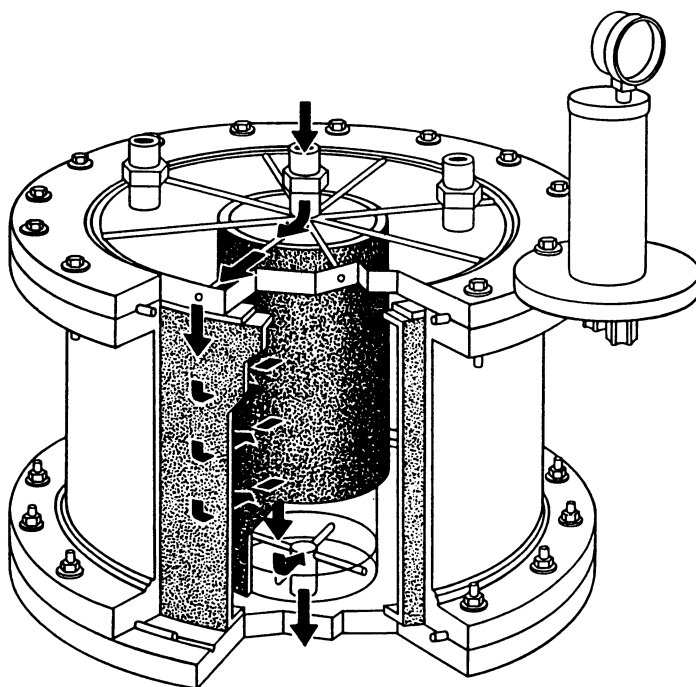


Figure 2c. Cutaway diagram of a typical radial flow column. (Reproduced with permission from ref. 8. Copyright 1989 Astor Publishing Corporation.)

Retention of linear flow rate is crucial to the scale up of any chromatographic technique. It is important to maintain the same ratio of the sample volume and the elution volume to the column bed volume, and to keep the sample concentration constant. Adherence to these principles avoids variation in available dynamic capacity due to changes in the point of "sample breakthrough" (7). It also helps maintain the desired and expected peak shapes making design of process operation easier. Maintaining column dimensions that remain proportional throughout the scale-up process insures predictable results. Due to wall effects on both fluid flow through the column and support of the gel matrix, columns with a minimum diameter of 10 cm must be used in the initial steps of process development (wall effects are negligible at this diameter).

Application of the diagnostic tools of HETP (height in equivalent theoretical plates) and peak symmetry throughout the scale up process will insure that the maximum performance of the selected gels is attained (1). Information from these diagnostic techniques can also serve as a standard for evaluating newly packed columns. HETP and peak symmetry measurements, when compared to a set standard, can also indicate an aging column and provide a basis for the establishment of a standard operating procedure in the production process.

Literature Cited

1. *Process Chromatography, A Practical Guide*; Sofer, G. K.; Nystrom, L.-E., Eds.; Academic Press: San Diego, CA, 1989; App. A.
2. Scopes, R. *Protein Purification Principles and Practice*; Springer-Verlag: New York, NY, 1982.
3. Spears, R. M. Ph.D. Thesis, University of Maryland, College Park, MD, 1982.
4. Bonnerjea, J.; Oh, S.; Hoare, M.; Dunnill, P. *Bio/Technology* **1986**, *4*, 954-958.
5. Haff, L. A.; Easterday, R. L. *J. Liquid Chromatogr.* **1978**, *1*.
6. Cooney, J. N. *Bio/Technology* **1984**, *2*, 41-43, 46-51, 54-55.
7. Christer-Jansson, J. *Adv. Biochem. Eng.* **1982**, *25*, 53.
8. Saxena et al. *BioPharm* **1989**, *2*(3), 48.

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Chapter 14

Lignin Peroxidase

Catalysis, Oxycomplex, and Heme-Linked Ionization

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Lignin peroxidase, secreted by the white-rot fungus *Phanerochaete chrysosporium* in response to nutrient deprivation, catalyzes the H₂O₂-dependent oxidation of non-phenolic aromatic substrates. The present report summarizes the kinetic and structural characteristics of lignin peroxidase isozymes. Our results indicate that the active site of lignin peroxidase is more electron deficient than other peroxidases. As a result, the redox potential of the heme active site is higher, the heme active site is more reactive and the oxycomplex is more stable than that of other peroxidases. Also discussed is the heme-linked ionization of lignin peroxidase.

Lignin biodegradation has been studied extensively in the basidiomycete *Phanerochaete chrysosporium*. The degradation of lignin by this organism is catalyzed by extracellular peroxidases in a H₂O₂-dependent process (1). The two classes of peroxidases found in the extracellular fluid of ligninolytic cultures of *P. chrysosporium* include lignin peroxidase (ligninase) (2) and manganese-dependent peroxidase (3, 4). To better understand the complex process of lignin biodegradation, basic research has been conducted to characterize these enzymes with respect to their structure and biochemical properties. The information gained from these studies has added to our knowledge on how the heme reactivity of hemoproteins is controlled.

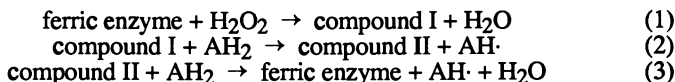
Under nitrogen limiting conditions, *P. chrysosporium* secretes at least six lignin peroxidase isozymes and four Mn-dependent peroxidase isozymes (1). These isozymes are glycoproteins, each containing one molecule of protoporphyrin IX as the prosthetic group (1). Their molecular weights range from 38-45 kD and pI values range from 3.3-4.9 (1, 5, 6). Lignin peroxidase isozymes are designated as H1 (pI = 4.7), H2 (pI = 4.4), H6 (pI = 3.7), H7 (pI = 3.6), H8 (pI = 3.5) and H10 (pI = 3.3) (1), and Mn-dependent peroxidase isozymes as H3 (pI = 4.9), H4 (pI = 4.5), H5 (pI = 4.1-4.3) and H9 (pI not known) (1). With the exception of isozyme H1, all lignin peroxidase isozymes are phosphorylated on their carbohydrate moiety in the form of mannose 6-phosphate. In contrast, none of the Mn-dependent peroxidase isozymes are phosphorylated (5). Amino acid sequences obtained from cDNA cloning show that with the exception of sequence near the active

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site, which include the proximal and distal histidine residues, lignin and Mn-dependent peroxidases have very low homologies to plant and other fungal peroxidases (7-11).

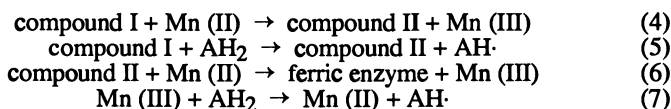
Catalytic Cycle of Lignin Peroxidase

Formation and Reactivity of Compounds I and II. The catalytic cycle of lignin peroxidase is similar to other peroxidases (12):



Compound I is a two-electron oxidized enzyme intermediate containing a oxyferryl iron and a porphyrin cation radical while compound II is an one-electron oxidized intermediate (13). With lignin peroxidase, as with other peroxidases, the substrate oxidation products are free radicals which undergo nonenzymatic disproportionation reactions to give rise to the final products.

Mn-dependent peroxidase differs from lignin peroxidase in that it utilizes Mn (II) as the main substrate (14). The oxidized manganese ion, Mn (III), carries out the oxidation of organic molecules. Compound I of Mn-dependent peroxidase is able to oxidize Mn (II) to Mn (III) as well as some phenolic compounds; the compound II can only oxidize Mn (II) (14):



In spite of the difference in substrate specificity, lignin and Mn-dependent peroxidases share some very unique features in that the pH optimum for both enzymes is very low (1, 15) and they are able to oxidize substrates of high redox potential that are not readily oxidized by other peroxidases (1). The latter property implies that the lignin and Mn-dependent peroxidase intermediates compound I and compound II are of higher redox potential than those of other peroxidases. This is supported by the electrochemical data (6). The redox potentials of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple of lignin and Mn-dependent peroxidases are about -140 mV and -90 mV at pH 7.0, respectively. These values are significantly higher than those for other peroxidases, such as -270 mV for horseradish peroxidase (HRP) and -195 mV for cytochrome c peroxidase (CCP); the redox potentials for lignin and Mn-dependent peroxidase catalytic intermediates are thus predicted to be higher than other peroxidases.

Effect of pH on Lignin Peroxidase Catalysis. The oxidation of organic substrates by lignin peroxidase (V_{max}) has a pH optimum equal to or possibly below 2. Detailed studies have been performed on the pH dependency of many of the individual reactions involved in catalysis. The effect of pH on the reaction rates between the isolated ferric enzyme, compounds I or II and their respective substrates has been studied. Rapid kinetic data indicate that compound I formation from ferric enzyme and H_2O_2 is not pH dependent from pH 2.5-7.5 (15, 16). Similar results are obtained with Mn-dependent peroxidase (14). This is in contrast to other peroxidases where the pK_a values for the reaction of ferric enzyme with H_2O_2 are usually in the range of 3 to 6 (12).

Although compound I formation is not influenced by pH, reactions of compounds I and II are significantly affected by pH. These reactions are acid-catalyzed (16, 17). The rate constant for the oxidation of veratryl alcohol or ferrocyanide by lignin peroxidase compound I is 10^3 times greater at pH 3.5 than at pH 6.0. The enhancement in rate is of the same magnitude for compound II reacting with veratryl alcohol. Therefore, the observed pH dependency for V_{\max} in catalysis is due to the pH-dependent reactions between the compounds I and II and the reducing substrates.

Oxycomplex of Lignin Peroxidase

Formation and Structure of Oxycomplex. Compound III is formed when peroxidase is incubated with excess H_2O_2 (12). The structure of peroxidase compound III has been well established as the oxygenated form of ferropoxidase ($Fe^{2+}O_2$), the same as oxymyoglobin. Therefore, it is more accurately referred to as the oxycomplex.

The ferrous form of lignin peroxidase can be generated through a one-electron reduction of the ferric lignin peroxidase under anaerobic conditions using dithionite or the photoreductant deazoflavin (18, 19). The oxycomplex is generated when the ferropoxidase is mixed with O_2 gas, air, or the O_2 -containing buffer (19). The pure oxycomplex can also be obtained by mixing the ferric enzyme with excess H_2O_2 . The excess H_2O_2 is then removed by treatment with catalase, followed by passage through a Sephadex G25 column (21). There is also evidence to suggest that the oxycomplex can be formed from the reaction between ferric peroxidase and superoxide (21).

The spectrum of lignin peroxidase oxycomplex is characterized by a Soret peak at 416 nm and two visible peaks at 543 and 578 nm (Figure 1) (19, 21). The spectrum for the oxycomplex is virtually the same regardless of how the oxycomplex is formed, whether from the oxygenation of the ferropoxidase or the reaction of ferric enzyme with excess H_2O_2 . However, a small discrepancy is noticeable in the spectrum of the oxycomplex formed from the reaction of ferric peroxidase with excess H_2O_2 without removal of the excess H_2O_2 (21). Such a spectrum exhibits a Soret peak at 418 nm as shown in Figure 1. Nakajima and Yamazaki (22) clearly showed that in the presence of H_2O_2 , there is an equilibrium between compound II and compound III:



Since compound II has a Soret peak at 420 nm and the oxycomplex 416 nm, the equilibrated solution of compound II, H_2O_2 , and compound III should exhibit an intermediate absorption peak. The existence of such an equilibrium has been established with HRP (22) and was also observed with lignin peroxidase (Cai, D., and Tien, M., unpublished results; 23). Wariishi and Gold (23) recently proposed the existence of a new enzyme intermediate when compound III is formed in the presence of H_2O_2 . They referred to this intermediate as compound III* and suggested it to be a complex between compound III and H_2O_2 . The existence of such an intermediate was based primarily on the Soret spectral properties where a 419 nm maximum was observed. In contrast, we see no evidence for existence of such a complex. The observed 419 nm absorbance maximum can be best explained by the existence of both compounds II and III in solution.

Stability of Oxycomplex. The oxycomplex of various peroxidases and other hemoproteins shows dramatic difference in stability, or the rate of

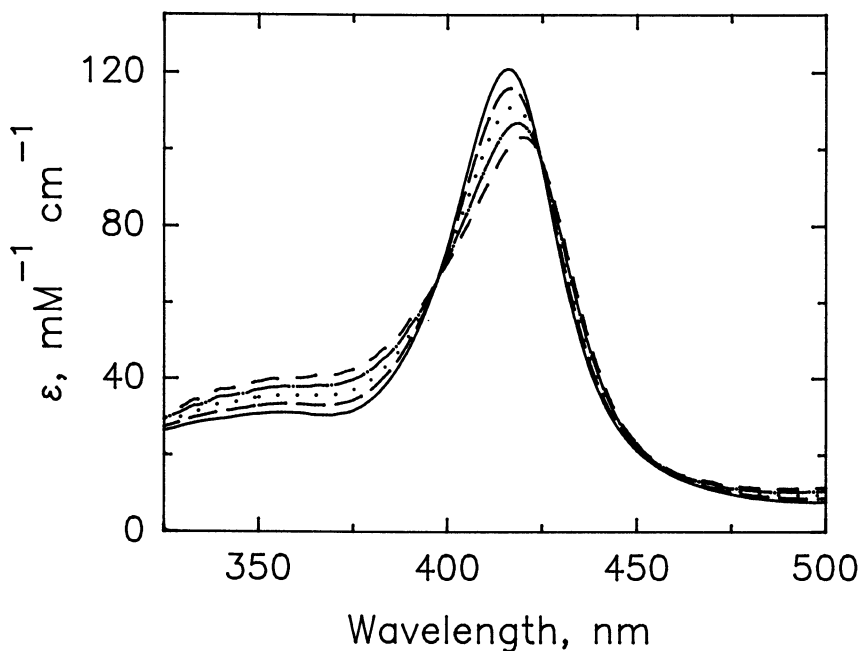


Figure 1. Absorption spectra of lignin peroxidase oxycomplex and compound II. Also shown is the calculated spectra of the mixtures of oxycomplex and compound II. In descending order of the Soret peak extinction coefficient, 100% oxycomplex (416 nm) is shown in solid line; the mixture of 75% oxycomplex and 25% compound II (417 nm) in long dashed line; the mixture of 50% oxycomplex and 50% compound II (417 nm) in dotted line; the mixture of 25% oxycomplex and 75% compound II (419 nm) in dashed-dot line; and 100% compound II (420 nm) in medium dashed line.

autoxidation (19). The oxycomplex of lignin peroxidase isozyme H1 autoxidizes to the ferric enzyme with a $t_{1/2} = 25$ min at 25 °C, pH 7.2. The oxycomplex of isozyme H8 is unusually stable under the same conditions with a $t_{1/2}$ too long to accurately measure. However, the $t_{1/2}$ for HRP oxycomplex autoxidation is only 4 min at 20 °C. Oxymyoglobin is more stable than oxypoxidases with a half-life of several hours.

Factors that can affect the rate of oxycomplex autoxidation have been studied with HRP. When reconstituted with different 2,4-substituent deuteriohemes, the autoxidation of HRP oxycomplex decreases as the electron density on the heme decreases (24). The observation that lignin peroxidase oxycomplex is more stable than that of HRP but less stable than that of oxymyoglobin implies that the heme active site of lignin peroxidase is more electron deficient than HRP and less than myoglobin. This conclusion is supported by the results from electrochemical studies which have suggested that the electron density in lignin peroxidase active site is lower than HRP and higher than myoglobin (6).

The electron density on hemoprotein active site is thought to be influenced by the axial ligand (25). In lignin peroxidase, HRP, CCP and myoglobin the axial ligand is the proximal histidine residue. The N₅ of the proximal histidine is ligated to the fifth coordinate of the heme iron. Because of the direct interaction with the heme iron, the axial histidine has a major impact on the heme electronic environment. Protein crystallographic studies show that the proximal histidine of CCP and myoglobin is H-bonded to a neighboring electron-donating group (26, 27). In myoglobin this group is the carbonyl of Leu89 on the peptide backbone, whereas in CCP it is the carboxylate side chain of Asp235, which is further H-bonded to Trp191. Hence, the effect of the proximal histidine on the heme environment can be further related to the strength of the H-bonding network. Although there are no crystallographic data available for HRP, spectroscopic studies have suggested that the H-bond in HRP is somewhat stronger than that of CCP and much stronger than that of myoglobin (28). Spectroscopic studies also indicate that the strength of the H-bond in lignin peroxidase is greater than in myoglobin but weaker than in HRP (29, 30). This is summarized in Table I.

Table I. Comparison of the Effects of the Proximal Histidine H-bonding Strength on Some Hemoprotein Properties

	HRP ^a	LP	Mb
H-bonding of Proximal His	Strong	Weaker	Weak
Heme Electron Density	High	Lower	Low
Redox Potential	Low	Higher	Highest
Stability of Higher Oxidation States	Stable	Less stable	Least stable
Stability of Oxycomplex	Unstable	More stable	Most Stable

^aAbbreviations used: HRP, horseradish peroxidase; LP, lignin peroxidase; Mb, myoglobin.

Reactivity of the Oxycomplex. The oxycomplex of lignin peroxidase does not react with veratryl alcohol, one of the lignin peroxidase substrates. Furthermore, the stability of the oxycomplex is not affected by veratryl

alcohol (20). However, in the presence of H_2O_2 , veratryl alcohol is oxidized in solutions containing compound III (19). The apparent activity of the oxycomplex solution is more than 80% of that of the native enzyme. When H_2O_2 is present in 1:1 stoichiometry to oxycomplex, the oxycomplex will be readily oxidized back to the ferric enzyme, resulting in the oxidation of veratryl alcohol (20).

Although compound III can be formed from the reaction of compound II with H_2O_2 , it is not usually formed during catalysis where H_2O_2 and the other substrate are present in excess since the reaction of compound II with the reducing substrates is much faster than with H_2O_2 . When only H_2O_2 is present and the reducing substrate is absent, the oxycomplex is formed. In the absence of a reducing substrate, a high concentration of H_2O_2 also leads to enzyme inactivation. For lignin peroxidase, the inactivation is biphasic and dependent on H_2O_2 concentration, indicating that the inactivation is a result of bimolecular reactions between lignin peroxidase and H_2O_2 (20). The inactivation will not occur if the excess H_2O_2 is removed (20); instead the oxycomplex will autoxidize to the ferric enzyme with more than 95% recovery (19).

Heme-Linked Ionization in Lignin Peroxidase

Studies on the effect of pH on peroxidase catalysis, or the heme-linked ionization, have provided much information on peroxidase catalysis and the active site structure. Heme-linked ionization has been observed in kinetic, electrochemical, absorption spectroscopic, proton balance, and Raman spectroscopic studies. Kinetic studies show that compound I formation is base-catalyzed (12). The pK_a values are in the range of 3 to 6. The reactions of compounds I and II with substrates are also pH-dependent with pK_a values in a similar range (12). Ligand binding (e.g. CO, O_2 or halide ions) to ferrous and ferric peroxidases is also pH-dependent. A wide range of pK_a values has been reported (12). The redox potentials of $\text{Fe}^{3+}/\text{Fe}^{2+}$ couples for peroxidases measured so far are all affected by pH. The pK_a values are between 6 and 8, indicative of an imidazole group of a histidine residue (6, 31-33).

A detailed study on heme-linked ionization was performed by Yamazaki *et al.* (34). They quantitated the proton balance between five different oxidation states of HRP using pH titration techniques, and found that the heme-linked ionization was associated with all oxidation states with a similar pK_a , equal to 6-8. Another effective way to demonstrate the heme-linked ionization is to look for the pH-dependent absorption spectral changes of a certain oxidation or ligation state. Although the absorption change due to a pH change is very small, they have been documented with all oxidation states and many ligation forms of HRP and some oxidation states of CCP (35). Using the Raman spectroscopic technique, the frequency shift of the Fe-His (the proximal His) stretching line is pH-dependent with HRP, CCP, Japanese peroxidase and turnip peroxidase (36). All pK_a values agree closely with that for a histidine residue. These compelling data strongly suggest that heme-linked ionization is associated with the ionization of the distal histidine.

There is some experimental evidence showing the effects of heme-linked ionization on lignin peroxidase. The redox potential of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple of lignin and Mn-dependent peroxidases is pH-dependent, as is the absorption spectrum of the ferrous form of the lignin and Mn-dependent peroxidases (6). The pK_a values determined from both experiments were 6.6. Detailed studies were performed studying O_2 binding to the ferrous lignin peroxidase (19). The pK_a for the ionization was measured at different

temperatures and the ΔH for ionization calculated. The pK_a is equal to 6.6 at 20 °C and the ΔH_{ion} is 7.2 kcal/mol. Both values are in excellent agreement with those for the imidazole of a histidine residue. Therefore, it is clear that the imidazole group of the distal histidine is the ionizable group responsible for all of the pH effects in the ferrous lignin and Mn-dependent peroxidases.

However, the distal histidine apparently has no effect on lignin and Mn-dependent peroxidase compound I formation. Although all active site amino acid residues that are proposed to participate in compound I formation of peroxidase (37) are conserved in lignin and Mn-dependent peroxidases, the lack of pH dependence may be a result of some inherent structural and conformational differences between lignin and Mn-dependent peroxidases and other peroxidases.

Acknowledgments

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Literature Cited

1. Tien, M. *CRC Crit. Rev. Microbiol.* **1987**, *15*, 141-168.
2. Tien, M.; Kirk, T. K. *Science* **1983**, *221*, 661-663.
3. Glenn, J. K.; Gold, M. J. *Arch. Biochem. Biophys.* **1985**, *242*, 329-341.
4. Paszczynski, A.; Huynh, V.-B.; Crawford, R. *FEMS Microbiol. Lett.* **1985**, *29*, 37-41.
5. Kuan, I.-c.; Tien, M. *J. Biol. Chem.* **1989**, *264*, 20350-20355.
6. Millis, C. D.; Cai, D.; Stankovich, M. T.; Tien, M. *Biochemistry* **1989**, *28*, 8484-8489.
7. Tien, M.; Tu, C.-P. *D. Nature* **1987**, *326*, 520-523.
8. de Boer, H. A.; Zhang, Y. Z.; Collins, C.; Reddy, C. A. *Gene* **1987**, *60*, 93-102.
9. Andrawis, A.; Pease, E.; Kuan, I.-c.; Holzbaaur, E.; Tien, M. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 673-680.
10. Pribnow, D.; Mayfield, M. B.; Nipper, V. J.; Brown, J. A.; Gold, M. H. *J. Biol. Chem.* **1989**, *264*, 5036-5040.
11. Pease, E. A.; Andrawis, A.; Tien, M. *J. Biol. Chem.* **1989**, *264*, 13531-13535.
12. Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1976**, *19*, 187-251.
13. Dunford, H. B. *Adv. Inorg. Biochem.* **1982**, *4*, 41-68.
14. Wariishi, H.; Dunford, H. B.; MacDonald, I. D.; Gold, M. H. *J. Biol. Chem.* **1989**, *264*, 3335-3340.
15. Andrawis, A.; Johnson, K. A.; Tien, M. *J. Biol. Chem.* **1988**, *263*, 1195-1198.
16. Harvey, P. J.; Palmer, J. M.; Schoemaker, H. E.; Dekker, H. L.; Wever, R. *Biochim. Biophys. Acta* **1989**, *994*, 59-63.
17. Marquez, L.; Wariishi, H.; Dunford, H. B.; Gold, M. H. *J. Biol. Chem.* **1988**, *263*, 10549-10552.
18. Gold, M. H.; Kuwahara, M.; Chiu, A. A.; Glenn, J. K. *Arch. Biochem. Biophys.* **1984**, *234*, 353-362.
19. Cai, D.; Tien, M. *Biochemistry* **1990**, *29*, 2085-2091.

20. Cai, D.; Tien, M. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 464-469.
21. Shimizu, N.; Kobayashi, K.; Hayashi, K. *Biochim. Biophys. Acta* **1989**, *995*, 133-137.
22. Nakajima, R.; Yamazaki, I. *J. Biol. Chem.* **1987**, *262*, 2576-2581.
23. Wariishi, H.; Gold, M. H. *J. Biol. Chem.* **1990**, *265*, 2070-2077.
24. Makino, R.; Yamada, H.; Yamazaki, I. *Arch. Biochem. Biophys.* **1976**, *173*, 66-70.
25. Falk, J. E. *Porphyrin & Metalloporphyrins*; Elsevier: New York, NY, 1964.
26. Takano, T. *J. Mol. Biol.* **1977**, *110*, 537-568.
27. Finzel, B. C.; Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1984**, *259*, 13027-13036.
28. de Ropp, J. S.; Thandabal, V.; La Mar, G. N. *J. Am. Chem. Soc.* **1985**, *170*, 8268-8270.
29. Mino, Y.; Wariishi, H.; Blackburn, N. J.; Loehr, T. M.; Gold, M. H. *J. Biol. Chem.* **1988**, *263*, 7029-7036.
30. Kuila, D.; Tien, M.; Fee, J. A.; Ondrias, M. R. *Biochemistry* **1985**, *24*, 3394-3397.
31. Harbury, H. A. *J. Biol. Chem.* **1957**, *2*, 1009-1024.
32. Conroy, C. W.; Tyma, P.; Daum, P. H.; Erman, J. E. *Biochim. Biophys. Acta* **1978**, *537*, 62-69.
33. Makino, R.; Chiang, R.; Hager, L. P. *Biochemistry* **1976**, *15*, 4748-4754.
34. Yamada, H.; Yamazaki, I. *Arch. Biochem. Biophys.* **1974**, *165*, 728-738.
35. Yamazaki, I.; Hayashi, Y. *Arch. Biochem. Biophys.* **1978**, *190*, 446-453.
36. Kitagawa, T. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G. Ed.; John Wiley and Sons: New York, NY, 1988, Vol. 3; pp 97-133.
37. Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199-8205.

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Chapter 15

Structure and Regulation of Manganese Peroxidase Gene from *Phanerochaete chrysosporium*

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Manganese peroxidase (MnP) is one of two lignin-degrading enzymes secreted by the white-rot basidiomycete *Phanerochaete chrysosporium*. MnP is an H_2O_2 -dependent heme peroxidase. The enzyme oxidizes Mn^{2+} to Mn^{3+} , which in turn oxidizes the terminal phenolic substrates. Several cDNAs encoding MnP isozymes have been isolated and characterized. A cDNA probe was used to isolate and characterize a gene encoding MnP. Comparison of the cDNA and genomic sequences reveal 6 introns, all containing less than 72 base pairs. The appearance of MnP activity in the extracellular medium depends on the presence of Mn. In addition, MnP mRNA is only detected in cells grown in the presence of Mn. If Mn is added to 4-day-old nitrogen-limited Mn-deficient cultures, extracellular MnP activity appears within 6 hours and MnP mRNA appears within 1 hour. These results indicate that Mn is involved in the transcriptional regulation of the MnP gene.

Lignin, a heterogeneous, phenylpropanoid polymer, is the most abundant renewable aromatic material. It comprises 15-30% of woody plant cell walls, forming an encrusting matrix surrounding the cellulose, which retards the microbial depolymerization of that most prevalent natural polymer (1,2). Thus the degradation of lignin plays a key role in the earth's carbon cycle (1-3). White-rot basidiomycetous fungi are the only known organisms which are capable of degrading lignin completely to CO_2 and H_2O in pure culture. The white-rot basidiomycete *Phanerochaete chrysosporium* degrades lignin during secondary metabolic (idiophasic) growth, the onset of which is triggered by limiting cultures for nutrient nitrogen (4-6). Under ligninolytic conditions *P. chrysosporium* secretes two extracellular heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP) which, along with an H_2O_2 generating system, are apparently the major components of its lignin-degradative system (4-7). A

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considerable amount of evidence from model substructure studies suggests that LiP and MnP are responsible for the initial degradation of lignin by this organism (4,6-8). The structure and mechanism of lignin peroxidase have been studied extensively (4,6,7,9,10).

Structural and Catalytic Properties of Manganese Peroxidase

The second ligninolytic enzyme, MnP, has been identified (11), purified and characterized (13-16). MnP is an H_2O_2 -dependent heme glycoprotein ($M_r \sim 46,000$) with an iron protoporphyrin IX prosthetic group. MnP catalyzes the Mn^{II} -dependent oxidation of a variety of phenols and phenolic lignin model compounds (6,8,13-15,17). Electronic absorption (6,13,14), EPR and resonance Raman (18) evidence indicates that the heme iron in the native protein is in the high-spin, pentacoordinate, ferric state, suggesting that the heme environment is similar to that of horseradish peroxidase (HRP) (19). In recent work (18), EPR spectra of ^{14}NO - and ^{15}NO - adducts of ferrous MnP were compared with those of HRP. Coordination of a histidine as the fifth ligand to the heme iron (proximal His) was confirmed from the pattern of superhyperfine splittings of the NO signals centered at $g \approx 2.005$.

It has been demonstrated that Mn^{II} is the preferred substrate for MnP (13-17). The enzyme oxidizes Mn^{II} to Mn^{III} and the Mn^{III} produced, complexed with a suitable carboxylic acid ligand (12-16), diffuses from the enzyme and in turn oxidizes the organic substrates (6,8,13-17). Thus the Mn ion participates in the reaction as a diffusible redox couple (Fig. 1) rather than as an enzyme-binding activator. In support of this concept, we have demonstrated that chemically prepared Mn^{III} complexed with a carboxylic acid ligand such as malonate or lactate mimics the reactivity of the enzyme (6,8,14,15).

Our spectral and kinetic characterization of the oxidized intermediates of MnP compound I (MnPI), and MnP compound II (MnPII) indicate (Fig. 1) that the oxidation states and catalytic cycle of MnP are similar to LiP and HRP (6,15,16,19). MnPI contains two oxidizing equivalents over the native ferric enzyme. The first equivalent resides in the $Fe^{IV}=\text{O}$ structure (19); the second equivalent resides in a porphyrin π -cation radical [$P^{\cdot+}$]. Reduction of compound I by one equivalent yields MnPII (Fig. 1); spectral evidence indicates that MnPII contains an $Fe^{IV}=\text{O}$ structure (15,16). Our kinetic evidence also suggests that carboxylic acid chelators such as lactate or malonate facilitate the dissociation of the Mn^{III} from the enzyme (16).

Structure of the Manganese Peroxidase Gene

A *P. chrysosporium* cDNA library was prepared in the expression vector λ GT11 using RNA isolated from ligninolytic mycelia (20). The first MnP cDNA encoding MnP isozyme I (MnP-1) ($pI = 4.9$) was isolated using polyclonal antibody prepared against purified MnP-1 and sequenced using the dideoxy chain termination method. The deduced mature protein contains 357 amino acids (aa) with a calculated M_r of 37,439. This is $\sim 81\%$ of the experimentally determined molecular weight with the discrepancy due to glycosylation (6,17,20). The deduced mature protein is preceded by a 21-aa signal peptide. Southern blot analysis (20) indicates that MnP-1 is a member of a family of *P. chrysosporium* MnP genes, and subsequently a second MnP cDNA encoding a related gene was sequenced (21; Gold, M. H.; Mayfield, M. B.; Nipper, V. J.; Pribnow, D., unpublished results).

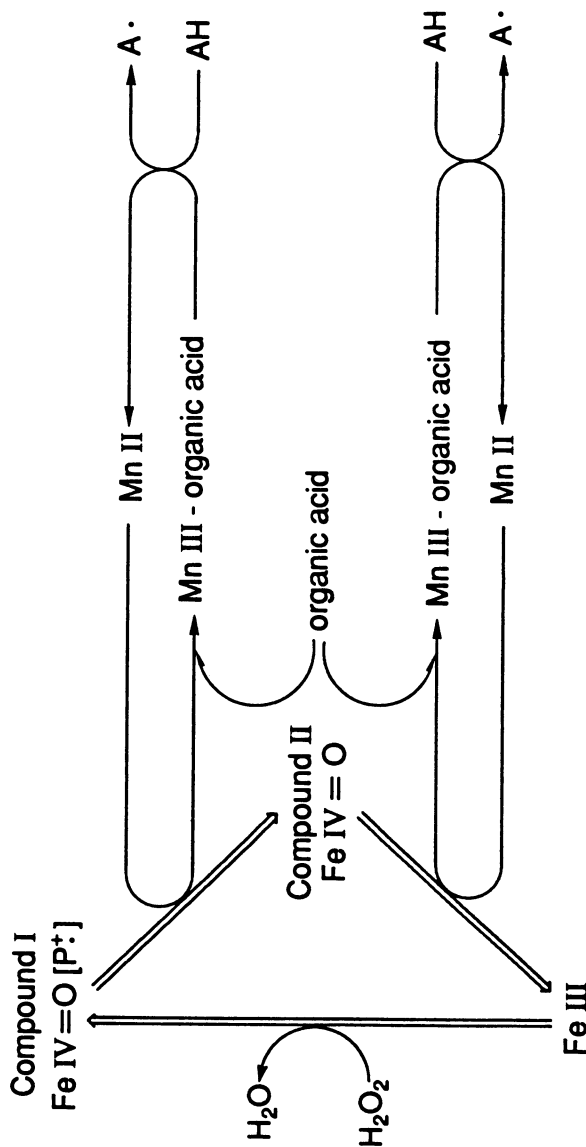


Figure 1. Catalytic cycle of manganese peroxidase. The Mn^{III} produced is chelated by organic acids and the complex diffuses from the enzyme surface. This Mn^{III} -organic acid complex in turn oxidizes phenolic substrates in lignin and other substrates. AH = phenolic lignin substructure.

Most plant peroxidases contain 2 His and an Arg which are essential for activity (22,23). Our recent EPR and resonance Raman evidence (18; see above) demonstrates the presence of a proximal His ligated to the heme iron of MnP-1. Comparative sequence analysis (Fig. 2) indicates that the sequences flanking the proximal His and the distal His and Arg are conserved in the MnP-1 protein. In addition, very little variation is observed in the locations of the proximal His (residue 176) and the distal His and Arg (residues 46 and 42, respectively), suggesting that all of these peroxidases evolved from a common precursor.

The MnP-1 cDNA clone was used to probe a *P. chrysosporium* genomic DNA library in the vector λ EMBL-3, and a MnP-1 genomic clone was isolated and characterized (Godfrey, B. J.; Mayfield, M. B., Brown, J. A; Gold, M. H., Gene, in press). The sequence includes 2539 base pairs (bp). Comparison of the cDNA and genomic sequences reveal 6 introns (Fig. 3) varying in size from 57 to 72 bp. The intron splice junction sequences of the MnP-1 gene all adhere to the GT---AG rule. Despite the strong homology in the coding regions between the MnP-1 gene (20) and several LiP genes (24-26), there is little similarity in the locations of introns. Codons for the distal His (His 46) and distal Arg (Arg 42)--amino acid residues thought to be involved in the heterolytic cleavage of H_2O_2 during MnP compound I formation (16,20-22,24,25)--are together in exon 3 in the MnP-1 gene, but are split by intron 2 in the LiP genes (Fig. 3). The codon for the proximal His (His 173) is located in exon 5 of the MnP-1 gene and in exon 6 of the LiP gene.

MnP lacks the putative 7-aa propeptide ending in the dibasic aa pair Lys-Arg, found between the signal peptide and the N-terminus of the mature LiP proteins (27); the sequences encoding the MnP-1 signal peptide and N-terminus of the mature protein are on the same exon. In the LiP gene, the sequence encoding the signal peptide is separated from that encoding the propeptide by an intron (Fig. 3) (27).

The 5' upstream region of the MnP-1 gene contains a TATAAA element 81 bp upstream of the translation initiation codon. In addition, three inverted CCAAT elements (ATTGG) (28) are found at positions -181, -195, and -304 with respect to the initiation codon. We are analyzing the 5' upstream sequence of the MnP-1 gene for other possible regulatory sequence elements.

Regulation of the Expression of Manganese Peroxidase by Manganese

The ligninolytic system of *P. chrysosporium* is expressed during secondary metabolic (idiophasic) growth, the onset of which is triggered by limiting cultures for nutrient nitrogen (4,5). LiP and MnP activity appear in the extracellular medium only during the secondary metabolic phase of growth (4,13,20,24). Northern blot analysis has demonstrated that the expression of LiP (24) and MnP (20,21) isozymes is controlled at the level of gene transcription by nutrient nitrogen.

As discussed above, Mn is the primary substrate for MnP (13-15). Our results demonstrate that the expression of MnP is regulated by Mn and by nitrogen. As shown in Figure 4, accumulation of MnP activity in the extracellular medium of nitrogen-limited cultures depends on the presence of Mn, confirming previous results from our lab and others (29-32). In contrast, comparisons of the mycelial biomass production from cultures grown in the presence and absence of Mn indicate that Mn has no significant effect on primary growth (32). Addition of Mn to the cell-free extracellular medium of cultures grown in the absence of Mn does not

Distal histidine																			
MnP	37	A	H	E	V	I	R	L	T	F	H	D	A	I	A	I	S	R	53
LiP	38	A	H	E	S	I	R	L	V	F	H	D	S	I	A	I	S	P	54
HRP	33	A	A	S	I	L	R	L	H	F	H	D	C	F	V	N	G	C	49
CCP	43	G	P	V	L	V	R	L	A	W	H	T	S	G	T	W	D	K	59

Proximal histidine																
MnP	163	P	F	E	V	V	S	L	L	A	S	H	S	V	A	176
LiP	166	E	L	E	L	V	W	M	L	S	A	H	S	V	A	179
HRP	160	S	S	D	L	V	A	L	S	G	G	H	T	F	G	173
CCP	164	D	R	E	V	V	A	L	M	G	A	H	A	L	G	177

Figure 2. Comparison of *P. chrysosporium* MnP-1 and other peroxidases at regions near the proximal and distal histidines. The peroxidase sequences used were manganese peroxidase (MnP) (20), cytochrome c peroxidase (CCP) (38), horseradish peroxidase (HRP) (39), and LiP (24). Identical amino acids are enclosed in solid boxes, and similar amino acids are enclosed in dashed boxes. (Reproduced with permission from Ref. 20. Copyright 1989, American Society for Biochemistry and Molecular Biology, Inc.)

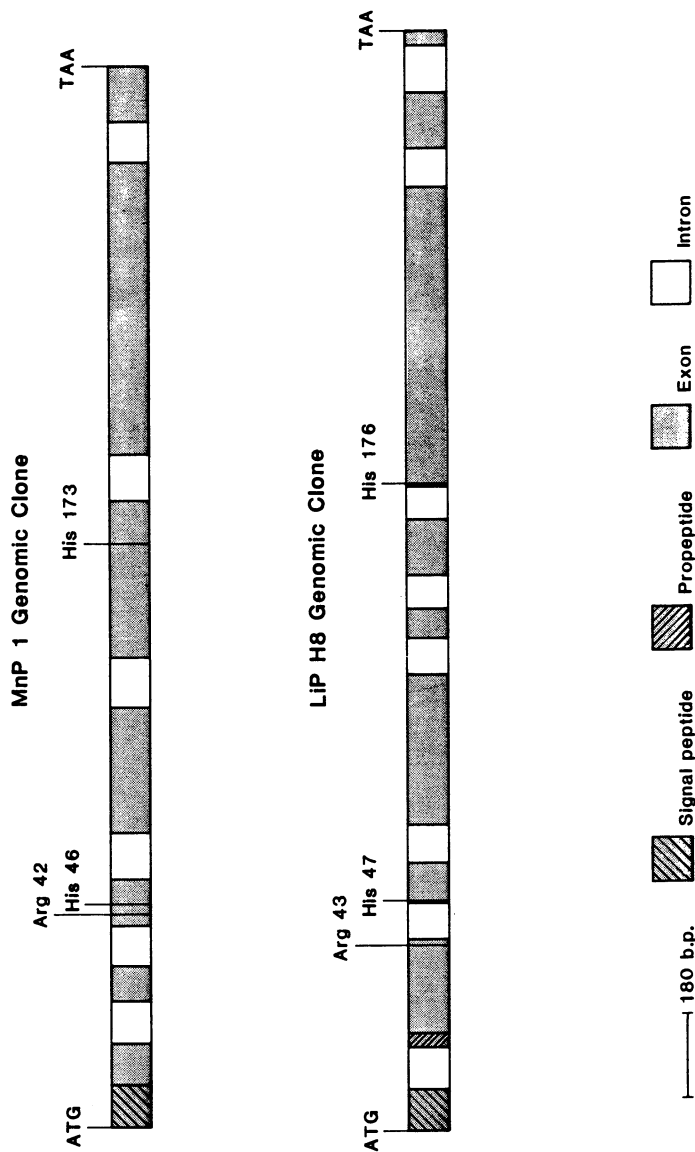


Figure 3. Comparison of the structures of the MnP-1 and LiP-H8 genes (26,27). The structures of the genes from the translation initiation codons to the termination codons are shown. Exons are indicated by shading. The putative signal peptides and propeptide are indicated by crosshatching. The positions of the distal His (His 46 and 47), distal Arg (Arg 42 and 43), and proximal His (His 173 and 176) for MnP-1 and LiP-H8, respectively, are also indicated. (Reproduced with permission from Godfrey, B. J.; Mayfield, M. B.; Brown, J. A.; Gold, M. H. *Gene* 1990, in press. Copyright 1990, Elsevier Science Publishers B. V.)

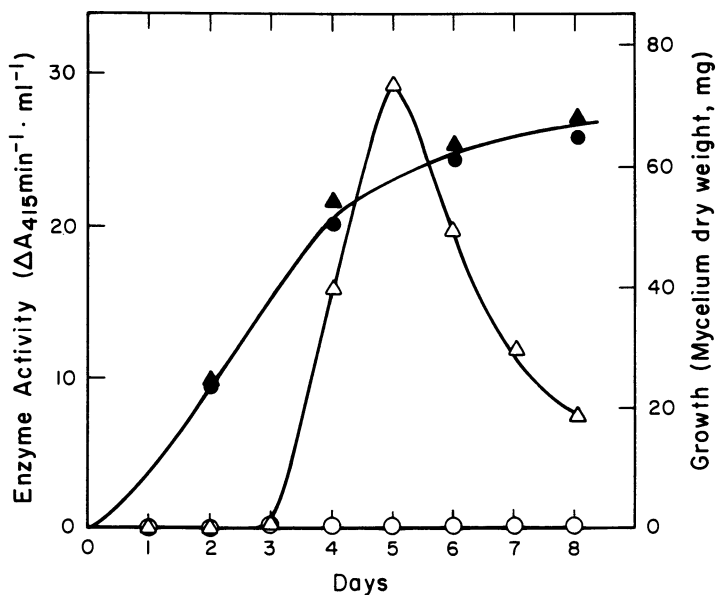


Figure 4. Effect of Mn supplementation on the appearance of extracellular MnP activity. Nitrogen-limited cultures were grown from a conidial inoculation. Extracellular MnP activity from cultures grown in the presence (open triangles) or absence (open circles) of $180 \mu\text{M}$ MnSO_4 was assayed as described (13,32). Dry weights of mycelia grown in the presence (solid triangles) and absence (solid circles) of Mn were also determined. (Reproduced with permission from Ref. 32. Copyright 1990. American Society for Microbiology.)

restore MnP activity, indicating that Mn is not acting as an activator of the enzyme. This was tested directly using Western blotting. MnP antiserum detected both extracellular and intracellular MnP only in cultures grown in the presence of Mn. This indicates that no active or inactive extracellular MnP protein is present in the absence of Mn, and that Mn is not regulating secretion of the enzyme.

When Mn is added to cultures previously grown for 4 days in the absence of Mn, extracellular MnP accumulation ensues within 6 hours and reaches a maximum within 18 hours (Fig. 5). This rapid response to Mn suggests that the Mn effect is probably not indirect—for example, by controlling the rate of nitrogen depletion, and consequently the onset of secondary metabolism and production of MnP. The lack of effect of Mn on mycelial growth also indicates that Mn is not regulating the utilization of nitrogen. The rapid response to the addition of Mn to 4-day-old cultures suggests that the metal affects the synthesis of the MnP protein directly. The inhibitory effects of actinomycin D and cycloheximide (Fig. 5) suggest that Mn is specifically involved in the transcriptional control of the MnP-1 gene. The translational inhibitor cycloheximide would inhibit MnP synthesis regardless of whether Mn affects transcription or translation. However, the inhibitory effect of the RNA synthesis inhibitor actinomycin D on MnP induction (Fig. 5) strongly suggests that Mn is involved in the transcriptional control of MnP synthesis rather than affecting mRNA stability or translation.

Northern blot analysis confirms that Mn exerts its effect at the level of transcription. MnP mRNA is detectable in 4-day-old cultures and reaches a maximum in 5-day-old nitrogen-limited cultures grown in the presence of 180 μ M Mn (Fig. 6a). This is in agreement with the results obtained for MnP activity. In contrast, no MnP mRNA is detectable in cultures grown in the absence of Mn. In addition, when Mn is added to a concentration of 180 μ M to 4-day-old cultures grown in the absence of Mn, Northern blot analysis shows that MnP mRNA appears within 1 hour (Fig. 6b). These results strongly indicate that Mn, the substrate of the enzyme, is regulating the transcription of the MnP gene. This rapid response to Mn supports our argument that Mn is acting directly to control gene transcription rather than indirectly by regulating the utilization of nitrogen.

Clarification of the details of Mn regulation of MnP gene transcription will require further study. However, a model whereby Mn^{2+} binds to and activates a specific transcription factor is an attractive possibility because of the high degree of specificity involved (32). Metal-ion-specific transcription factors occur in the activation of the yeast metallothionein gene by Cu (33), in the activation of the Mer operon by Hg (34), and in the activation of the Fe regulation system in *Escherichia coli* (35). However, other less specific mechanisms for Mn induction are also possible. For example, Mn might regulate a secondary messenger such as cAMP. Mn-specific adenyl cyclases such as have been found in yeast (36) could be regulating cAMP levels. However, the latter mechanisms seem less likely because they would be expected to result in global responses affecting a variety of metabolic functions. We plan to use the MnP gene and the DNA transformation system which we have recently developed for *P. chrysosporium* (37) to elucidate the regulatory sequences and protein factors involved in the Mn control of MnP gene transcription.

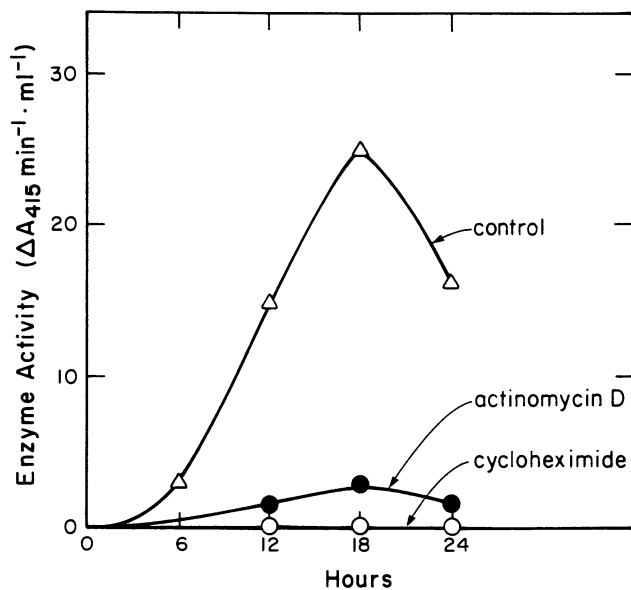


Figure 5. Effect of actinomycin D and cycloheximide on the induction of MnP activity. Mn-deficient cultures were grown for 4 days after which MnSO₄ (180 μM) was added alone (triangles), simultaneously with actinomycin D (50 μg/mL) (solid circles), or with cycloheximide (50 μg/mL) (open circles). Extracellular enzyme activity was assayed at the indicated intervals after the additions, by the ABTS method (13,32). (Reproduced with permission from Ref. 32. Copyright 1990, American Society for Microbiology.)

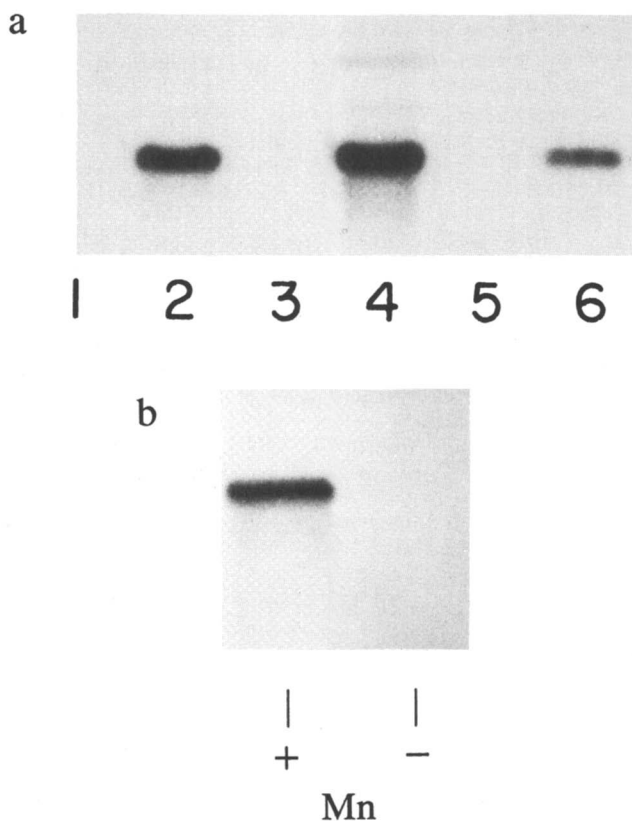


Figure 6. a. Northern blot analysis of *P. chrysosporium* RNA probed with ^{32}P -labeled MnP-1 cDNA. RNA from Mn-deficient (Mn^-) and complete cultures (Mn^+) was isolated, separated by electrophoresis, and probed as described (32). Samples were as follows: day 4, Mn^- (lane 1); day 4, Mn^+ (lane 2); day 5, Mn^- (lane 3); day 5, Mn^+ (lane 4); day 6, Mn^- (lane 5); day 6, Mn^+ (lane 6). (Reproduced with permission from Ref. 32. Copyright 1990, American Society for Microbiology.)

b. Mn induction of MnP gene transcription. Mn-deficient cultures were grown for 4 days, after which MnSO_4 was added to the experimental cultures to a final concentration of $180\ \mu\text{M}$. One hour after the addition of Mn, total RNA was extracted, electrophoresed, transferred to filters and probed with ^{32}P -labeled cDNA (32). These results indicate that Mn induces the transcription of the MnP-1 gene.

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Literature Cited

1. Crawford, R. L. Lignin Biodegradation and Transformation; Wiley: New York, 1981.
2. Sarkanen, K. V.; Ludwig, C. H. Lignins. Occurrence, Formation, Structure and Reactions. Wiley-Interscience: New York, 1971.
3. Freudenberg, K. In Constitution and Biosynthesis of Lignin; Neish, A. C.; Freudenberg, K., Eds.; Springer-Verlag: New York, 1968, pp 47-122.
4. Kirk, T. K.; Farrell, R. L. Annu. Rev. Microbiol. **1987**, 41, 465-505.
5. Buswell, J. A.; Odier, E. CRC Crit. Rev. Biotechnol. **1987**, 6, 1-60.
6. Gold, M. H.; Wariishi, H.; Valli, K. In Biocatalysis in Agricultural Biotechnology; Whitaker, J. R.; Sonnet, P. E., Eds.; American Chemical Society: Washington, DC, 1989, ACS Symposium Series No. 389, pp 127-140.
7. Tien, M. CRC Crit. Rev. Microbiol. **1987**, 15, 141-168.
8. Wariishi, H.; Valli, K.; Gold, M. H. Biochemistry; **1989**, 28, 6017-6023.
9. Marquez, L.; Wariishi, H.; Dunford, H. B.; Gold, M. H. J. Biol. Chem. **1988**, 263, 10549-10552.
10. Wariishi, H.; Gold, M. H. J. Biol. Chem. **1990**, 265, 2070-2077.
11. Kuwahara, M.; Glenn, J. K.; Morgan, M. A.; Gold, M. H. FEBS Lett. **1984**, 169, 247-250.
12. Wariishi, H.; Valli, K.; Renganathan, V.; Gold, M. H. J. Biol. Chem. **1989**, 264, 14185-14191.
13. Glenn, J. K.; Gold, M. H. Arch. Biochem. Biophys. **1985**, 242, 329-341.
14. Glenn, J. K.; Akileswaran, L.; Gold, M. H. Arch. Biochem. Biophys. **1986**, 251, 688-696.
15. Wariishi, H.; Akileswaran, L.; Gold, M. H. Biochemistry **1988**, 27, 5365-5370.
16. Wariishi, H.; Dunford, H. B.; MacDonald, I. D.; Gold, M. H. J. Biol. Chem. **1989**, 264, 3335-3340.
17. Paszczynski, A.; Huynh, V.-B.; Crawford, R. L. Arch. Biochem. Biophys. **1986**, 244, 750-65.
18. Mino, Y.; Wariishi, H.; Blackburn, N. J.; Loehr, T. M.; Gold, M. H. J. Biol. Chem. **1988**, 263, 7029-7036.
19. Dunford, H. B.; Stillman, J. S. Coord. Chem. Rev. **1976**, 19, 187-251.
20. Pribnow, D. G.; Mayfield, M. B.; Nipper, V. J.; Gold, M. H. J. Biol. Chem. **1989**, 264, 5036-5040.
21. Pease, E. A.; Andrawis, A.; Tien, M. J. Biol. Chem. **1989**, 264, 13531-13535.
22. Poulos, T. L.; Kraut, J. J. Biol. Chem. **1988**, 263, 8199-8205.
23. Mazza, G.; Welinder, K. G. Eur. J. Biochem. **1980**, 108, 481-489.
24. Tien, M.; Tu, C.-P. D. Nature **1987**, 326, 520-523.
25. de Boer, H. A.; Zhang, Y. Z.; Collins, C.; Reddy, C. A. Gene (Amst.) **1987**, 60, 93-102.

26. Smith, T. L.; Schälch, H.; Gaskell, J.; Covert, S.; Cullen, D. Nucleic Acids Res. **1988**, 16, 1219.
27. Schälch, H.; Gaskell, J.; Smith, T. L.; Cullen, D. Mol. Cell. Biol. **1989**, 9, 2743-2747.
28. Dynan, W. S.; Tjian, R. Nature **1985**, 316, 774-778.
29. Glenn, J. K., Ph.D. Dissertation, 1986, Oregon Graduate Institute, pp. 132-142.
30. Bonnarme, P.; Jeffries, T. W. Appl. Environ. Microbiol. **1990**, 56, 210-217.
31. Gold, M. H.; Brown, J. A.; Glenn, J. K. J. Cell. Biochem. **1990**, 14CS, 206.
32. Brown, J. A.; Glenn, J. K.; Gold, M. H. J. Bacteriol. **1990**, 172, 3125-3130.
33. Furst, P.; Hu, S.; Hackett, R.; Hamer, D. Cell **1988**, 55, 705-717.
34. O'Halloran, T. V. in Metal Ions in Biological Systems; Sigel, H.; Sigel, A., Eds.; Marcel Dekker, New York, 1989, pp. 105-146.
35. de Lorenzo, V.; Wee, S.; Herrero, M.; Neilands, J. B. J. Bacteriol. **1987**, 169, 2624-2630.
36. Londesborough, J. C.; Nurminen, T. Acta Chem. Scand. **1972**, 26, 3396-3398.
37. Alic, M.; Kornegay, J.; Pribnow, D. G.; Gold, M. H. Appl. Environ. Microbiol. **1989**, 55, 406-411.
38. Kaput, J.; Goltz, S.; Blobel, G. J. Biol. Chem. **1982**, 257, 15054-15058.
39. Welinder, K. G. FEBS Lett. **1976**, 72, 19-23.

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Chapter 16

Regulation of Ligninase Production in White-Rot Fungi

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Carbon, nitrogen and manganese are critical nutritional variables in the production of ligninases including lignin peroxidase (LiP) and manganese peroxidase (MnP) by *Phanerochaete chrysosporium* and other white rot fungi. Excess carbon and nitrogen repress lignin biodegradation. Mn(II) is a specific effector that induces MnP and represses LiP. LiP and MnP also have different sensitivities to carbon and nitrogen supply. Mn(II) regulation is superimposed on carbon and nitrogen regulation and is only apparent when cultures are derepressed for these macronutrients. Supplementing nitrogen during cultivation represses MnP activity but can stimulate LiP production. These findings suggest that the regulatory mechanisms for LiP and MnP isoenzymes differ in several ways.

Lignin peroxidases (LiPs) and manganese peroxidases (MnPs) have been implicated in the biological degradation of lignin by white-rot fungi (1, 2, 3, 4). Their regulation is of interest because such knowledge would help us to better understand the process of lignin biodegradation in nature and because these enzymes might be useful in biological pulping (5) or biological bleaching (6). Nutritional regulation has been studied most extensively.

In *Phanerochaete chrysosporium* and other white-rot fungi, lignin biodegradation and ligninase production occur in response to nitrogen or carbon limitation (7, 8, 9, 10). They are interactive variables with different effects. Carbon limitation causes the rapid onset of lignin mineralization, but it is short-lived as the cells undergo autocatabolism accompanied by a rapid loss of cell dry weight (8). Nitrogen limitation results in a delayed onset of lignin mineralization, occurring only after nitrogen turnover in the cell. (11). Nitrogen limitation is convenient to use in studying lignin

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biodegradation, but in optimizing the production of ligninolytic enzymes, limiting nitrogen also limits the ability of the organism to produce extracellular proteins. Ligninases and other secreted enzymes are degraded by extracellular proteinases, and total enzyme production is limited by the amount of available nitrogen. With balanced carbon and nitrogen supplies one might attain maximal protein production while avoiding carbon catabolite repression.

Recently we have discovered that Mn(II) regulates LiP and MnP in white-rot fungi (12). LiP is formed almost exclusively when Mn(II) is low (1.6 to 0.3 ppm); MnP is formed almost exclusively when Mn(II) is high (40 to 199 ppm). At intermediate levels – and especially the 11 ppm (0.2 mM) level often used for production of lignin-degrading enzymes – both sets of isoenzymes are formed. All isoenzymes of the LiP and MnP families are regulated together, suggesting that common mechanisms are involved.

Manganese affects LiP and MnP isoenzymes in a much more specific manner than do carbon and nitrogen. With Mn(II) regulation, nitrogen and carbon uptake rates are not dramatically altered over the range of Mn(II) levels effective for LiP and MnP regulation; cell dry weight, and oxygen uptake rates are similar at high and low Mn(II) levels (12). The effects of Mn(II) on isoenzyme production are clear-cut; isoenzyme profiles are distinct. These factors make Mn(II) a very useful tool for studying the different effects of LiP and MnP in lignin biodegradation.

Several different white-rot fungi are affected by Mn(II) in a manner similar to that observed with *P. chrysosporium*, indicating that Mn(II) regulation of *P. chrysosporium* is not idiosyncratic (12). In fact, organisms such as *Lentinula edodes*, which was not previously shown to produce LiP, from it in good titer when Mn(II) is present at low concentrations.

Mn(II) also affects the rate of lignin mineralization. The rate that *P. chrysosporium* produces $^{14}\text{CO}_2$ from uniformly ring-labeled synthetic lignin is six times greater at low Mn(II) levels than at high Mn(II) (11). *Phlebia brevispora* also mineralizes synthetic lignin at a significantly higher rate at low Mn(II), and in this organism, laccase, like MnP is more abundant at higher Mn(II) levels (13), suggesting that LiP rather than MnP or laccase is principally involved in lignin biodegradation.

By regulating the level of Mn(II), it should be possible to maximize production of either enzyme. In the present study, we examined the interactive effects of carbon, nitrogen and manganese in regulation of LiP and MnP.

Experimental

Microorganism. All experiments reported here were performed with *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725). Cultures were obtained from the Center for Forest Mycology at the Forest Products Laboratory, Madison, WI, and were maintained as previously described (12).

Cultural conditions. Unless otherwise stated, the initial medium contained per liter: 10 g glucose, 0.2 g diammonium tartrate, 4.6 g sodium D-tartrate dihydrate (adjusted to pH 4.5), 2.0 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg thiamine HCl, 0.5 g Tween 20, and 70 ml of a trace elements solution without MnSO_4 added. The trace elements solution contained per liter: 1.5 g nitroloacetic acid, 3.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g NaCl, 0.1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g CoSO_4 , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.01 g H_3BO_3 , 0.01 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.

Shake flask studies were carried out at 39°C in 125-ml Erlenmeyer flasks containing 40 ml of medium (180 rpm, 2.5 cm dia cycle). The carbon:nitrogen ratio was varied by increasing or decreasing the diammonium tartrate supply, and Mn(II) added as specified. A spore inoculum was used (0.5 to 1.0 10^5 spores/ml final concentration),

and veratryl alcohol (2.5 mM) was added at the time of inoculation. After 48 h of growth, Mn(II) was added to the desired concentration and cultures were flushed daily with pure oxygen for 1 min (flow = 6.5 l/min, 760 mm Hg, 21°C). All other conditions were as previously published (12).

Multiple Variant Analysis. Three variables were changed simultaneously: the initial carbon:nitrogen ratio, the initial carbon concentration, and the initial Mn(II) concentration. To find an optimum to produce LiP activity, a low range of Mn(II) concentrations were used (0 to 0.8 ppm), whereas for the production of MnP activity, a high range of Mn(II) concentrations (0 to 80 ppm) were used. Cultures attained maximal production on different days, so activity was expressed as a percentage of the maximal activity attained under the best conditions.

Enzyme Assays. All enzyme assays were previously described (12).

Results

Carbon and Nitrogen Interactions. We found that MnP activity was repressed more at high nitrogen than was LiP. If the carbon:nitrogen ratio were kept nearly constant, good LiP and MnP production could be maintained over a range of concentrations. In the experiment shown in Fig. 1, initial carbon and nitrogen concentrations were varied while holding Mn(II) at 11.2 ppm. If carbon were increased without increasing nitrogen, and (especially) if nitrogen were increased without increasing carbon, enzyme production decreased. Production of both enzymes increased with increasing carbon and nitrogen up to an apparent optimum range of 3.2 to 4.0 g/l carbon and 0.11 to 0.14 g/l nitrogen. The conditions routinely used in our experiments corresponded to carbon and nitrogen supplies of 4.0 and 0.030, respectively. An optimum carbon:nitrogen ratio of about 30 to 40 is indicated by the data shown, but this will be affected by other nutritional factors.

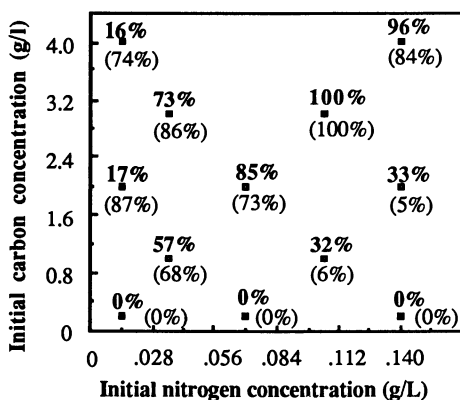


Figure 1. Effect of the initial carbon:nitrogen ratio on the production of peroxidases *P. chrysosporium*. Values in parentheses indicate MnP activity (100% = 900 nmol/min·ml); bold values indicate LiP activity (100% = 187 nmol/min·ml). Each value corresponds to the activity at its maximum. The concentration of Mn(II) employed was 11.15 ppm. All other conditions were as previously published (12).

Carbon and Nitrogen Starvation. Carbon and nitrogen starvation affect LiP and MnP production in different manners. As is shown in Fig. 2, carbon starvation leads

to a rapid appearance of LiP activity, but the effect on MnP activity is much less pronounced. Nitrogen-limited cultures produce both LiP and MnP. Carbon-limited cultures produce mostly LiP. For this reason, the full regulatory effects of Mn(II) can be demonstrated best under nitrogen limitation. Except for the experiments shown in Fig. 4 below, nitrogen was limiting in all experiments to test the effects of Mn(II). In separate experiments, we attempted to examine the effect of Mn(II) under carbon limitation, but no significant MnP or LiP production was obtained (data not shown).

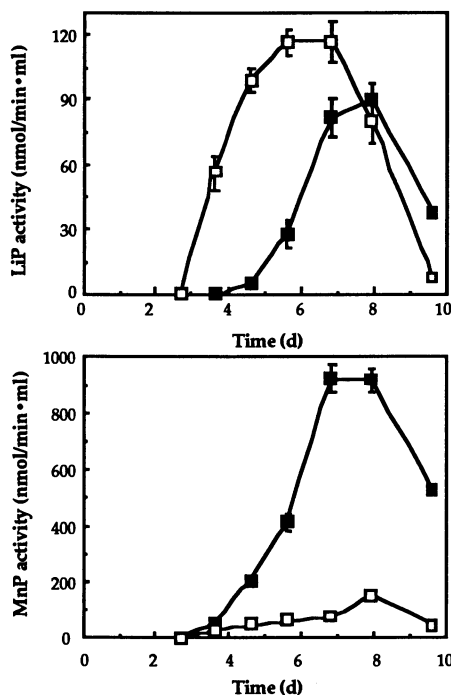


Figure 2. Effects of carbon and nitrogen limitation on the production of LiP and MnP activities. Carbon limited cultures (□); Nitrogen limited cultures (■). Carbon limited cultures contained per liter 0.66 g diammonium sulfate and 2 g glucose. Nitrogen limited cultures were the same as the controls. They contained 0.2 g diammonium tartrate and 10 g glucose. All cultures received 11.2 ppm Mn(II).

Repression by Supplemental Carbon and Nitrogen. Both LiP and MnP appear to be under nitrogen-regulation, but the effects on LiP are more stringent than the effects on MnP. Ammonium tartrate is known to repress lignin degrading enzymes and lignin mineralization when it is added during cultivation. As is shown in Fig. 3, when diammonium tartrate was added on day 4 to cultures actively producing lignin-degrading enzymes, a transient repression of LiP activity was observed followed by increased production. In the case of MnP, however, no significant stimulation was observed.

Interaction of Mn(II) with Carbon and Nitrogen. We attempted to determine an optimal carbon concentration and carbon:nitrogen ratio for LiP and MnP over low and high Mn(II) ranges, respectively. Earlier studies had shown that the optimum Mn(II) was less than 1 ppm for LiP and greater than 25 ppm for MnP (12). We wanted

to know if varying Mn(II) could overcome the repressive effects of glucose and nitrogen.

The regulatory effect of Mn(II) appeared to be separate from the effects of carbon and nitrogen. As is shown in Fig. 4, both LiP and MnP activities were completely repressed when the carbon concentration was high (6 g/l carbon = 15 g/l glucose) and the carbon:nitrogen ratio was low. Both LiP and MnP activities increased with increasing carbon:nitrogen ratio. The effect of Mn(II) was seen at intermediate carbon and nitrogen levels. With LiP, enzyme activity increased as Mn(II) decreased; with MnP, the opposite effect was observed. The optimum Mn(II) concentration for LiP or MnP production appeared to vary with the carbon:nitrogen ratio.

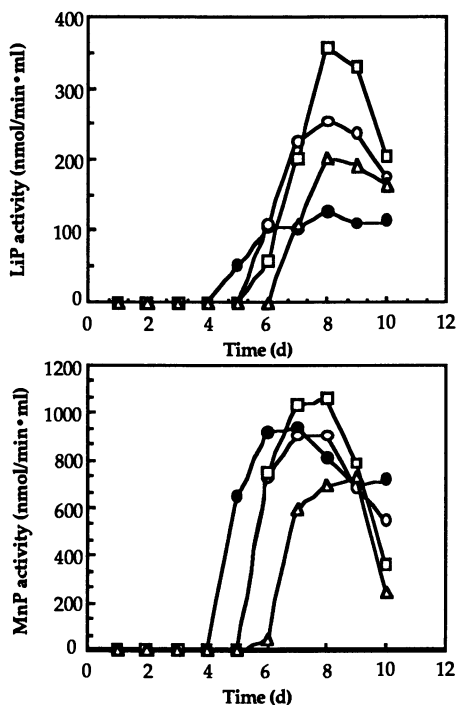


Figure 3. Effects of diammonium tartrate supplements on LiP and MnP production by *P. chrysosporium*. Additional nitrogen was added to triplicate cultures on day 4. Control (●); 2.2 mM (○); 4.4 mM (□); 8.8 mM (△). Glucose and Mn(II) concentrations were 10 g/l and 11.2 ppm, respectively.

No LiP activity was observed at high carbon and low carbon:nitrogen ratio (high nitrogen). Repression of LiP was markedly relieved at each carbon level as the carbon:nitrogen ratio increased (i.e., as nitrogen became limiting), and repression was also relieved when the initial carbon level was reduced while keeping carbon:nitrogen constant. The interactive effects of Mn(II) were ambiguous. At moderate carbon:nitrogen ratios, an optimum was obtained at very low Mn(II) levels (point B on Fig. 4a), but at higher carbon:nitrogen ratios (i.e. under nitrogen limitation) and at higher carbon levels (where nitrogen-limitation would be more effective), moderately higher Mn(II) levels were necessary (point A on Fig. 4a).

A similar pattern of carbon and nitrogen catabolite repression of MnP production was observed. Carbon repression of MnP could be overcome somewhat at moderate carbon and nitrogen levels by increasing Mn(II) (point A, Fig. 4b), but at low carbon and high nitrogen levels, decreasing Mn(II) from 64 to 16 ppm resulted in an almost two-fold increase in MnP activity. Decreasing the carbon supply at a constant carbon:nitrogen ratio did not increase MnP activity as much as the same shift increased LiP (Fig. 4a). Three optima were observed for MnP. The two best (points B and C on Fig. 4b) were both at high carbon:nitrogen ratios, relatively high Mn(II) and low carbon. A third was at a moderate carbon:nitrogen ratio and carbon concentration, but at the highest Mn(II) level employed (point A on Fig. 4b).

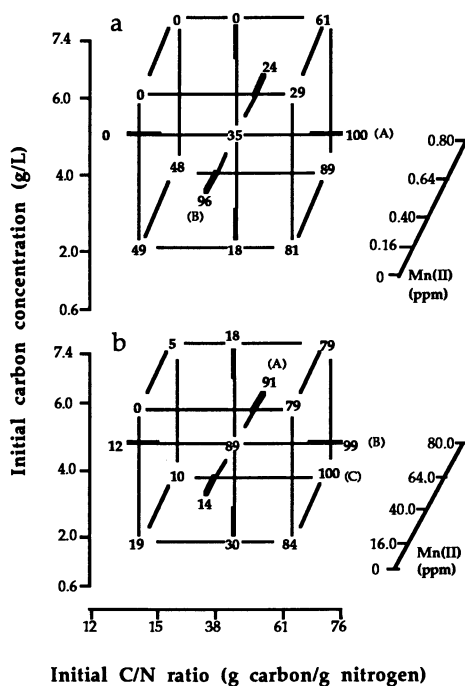


Figure 4. Effects of Mn(II), carbon and nitrogen on the production of peroxidases by *P. chrysosporium*. a) LiP activities over a low range of Mn(II); b) MnP activities over a high range of Mn(II). Activities are given in percent of maximum. LiP 100% = 454 nmol/min•ml; MnP 100% = 874 nmol/min•ml).

Discussion

The experiments depicted in Figs. 1 and 4 did not determine true optima. In the study of carbon and nitrogen interactions, the optima appeared to lie in the range of 2.4 to 4.0 g/l for carbon and 0.084 to 0.14 g/l nitrogen. One point fell in this range, and it was the maximum for this series of experiments but it is not necessarily an optimum. Likewise, in the partial factorial design depicted in Fig. 4, all of the maxima occurred at star points rather than within the matrix, so it is apparent that the optimum or optima lie somewhere outside the limits of the experimental design. Despite these limitations, several useful inferences can be drawn from these data.

First, LiPs appear to be regulated by carbon catabolite repression while MnPs are not derepressed by the carbon limitation used here. Since we know also that DHP mineralization shows a sudden onset in response to carbon starvation in this organism (8), one might conclude that this enzyme system is in some way related to respiratory pathways or the acquisition of metabolic energy. However, no direct evidence to date has shown that *P. chrysosporium* will grow solely at the expense of lignin oxidation. Indeed, cometabolism of carbohydrates or some other carbon source appears to be a necessary requisite. Additional regulatory studies on this enzyme and its relationship to energy-yielding metabolism should be conducted using cellulose or holocellulose as a carbon source. Even though such conditions would result in lower overall enzyme titers, the conditions would be more representative of what occurs in nature.

Second, both LiP and MnP are formed in response to nitrogen starvation, but the onset of enzyme production occurs more slowly than with carbon limitation. Unlike carbon with is virtually always present in excess in wood, albeit in a slowly-utilizable form, nitrogen is often a limiting nutrient (at least in laboratory culture), so both LiP and MnP would tend to be derepressed during growth under such conditions. The slightly greater repressive effects of nitrogen on MnP might not be significant, but they suggest that during the phase of initial invasion, when the fungus is growing at the expense of readily-available sugar and when residual cellular nitrogen should be available, neither LiP nor MnP are formed. It is not clear that nitrogen would be a limiting nutrient during extensive degradation in nature where wood is likely to receive nitrogen from soils and through bacterial nitrogen fixation, but it is also unlikely that nitrogen would be present in great excess under such conditions.

Third, the effects of Mn(II) are only conspicuous when nitrogen is limiting. At low carbon levels, LiP is derepressed, but the very low cell yields make even very small Mn(II) concentrations effective in repressing LiP activity. The genetic regulatory elements are not yet known for these enzymes, but recent studies have shown that Mn regulates expression of MnP by *P. chrysosporium* (14).

Literature Cited

- 1 Tien, M.; Kirk, T. K. *Science* **1983**, *221*:661-663.
- 2 Glenn, J. K.; Morgan, M. B.; Mayfield, M.; Kuwahara, M.; Gold, M. H. *Biochem. Biophys. Res. Comm.* **1983**, *114*, 1077-1083.
- 3 Hunh, V.-B.; Crawford, R. L. *FEMS Microbiol. Lett.* **1985**, *28*, 119-123.
- 4 Glenn, J. K.; Gold, M. H. *Arch. Biochem. Biophys.* **1985**, *242*, 329-341.
- 5 Reid, I.D. *Enzyme Microb. Technol.* **1989**, *11*, 786-803.
- 6 Eriksson, K. E.; Kirk, T. K. In *The Principles of Biotechnology: Engineering Considerations*; Cooney, C., L.; Humphrey, A. E., Eds.; Pergamon:New York, NY, 1985; pp 271-294.
- 7 Keyser, P. J.; Kirk, T. K.; Zeikus, J. G. *J. Bacteriol.* **1978**, *135*, 790-797.
- 8 Jeffries, T. W.; Choi, S.; Kirk, T. K. *Appl. Environ. Microbiol.* **1981**, *42*, 290-296.
- 9 Leatham, G. F.; Kirk, T. K. *FEMS Microbiol. Lett.* **1983**, *16*, 65-67.
- 10 Kirk, T. K.; Croan, S.; Tien, M.; Murtagh, K. E.; Farrel, R. L. *Enzyme Microb. Technol.* **1985**, *8*, 27-32.
- 11 Fenn, P.; Kirk, T.K. *Arch. Microbiol.* **1981**, *130*, 59-65.
- 12 Bonnarne, P.; Jeffries, T. W. *Appl. Environ. Microbiol.* **1990**, *56*, 210-217.
- 13 Perez, J.; Jeffries, T. W. *Appl. Environ. Microbiol.* **1990**, *56* (6), 1806-1812.
- 14 Brown, J. A.; Glenn, J. K.; Gold, M. H. *J. Bacteriol.* **1990**, *172*, 3125-3130.

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Chapter 17

Laccases of the Ligninolytic Fungus *Coriolus versicolor*

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Several laccases from *Coriolus versicolor* were isolated and partially characterized. One actively secreted enzyme, laccase III, was shown to depolymerize a residual protolignin fraction. Physicochemical and kinetic properties of laccase III were also investigated. This enzyme has two copper atoms per protein molecule which are believed to be necessary for catalysis, a molecular weight of 66,000, an N-terminal amino acid sequence of Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-, and four N-linked carbohydrate chains. Laccase III can catalyze the removal of protons from free phenolic hydroxyl groups, as well as the cleavage of the β -O-4 ether linkages following the cleavage of C-C bonds between the α - and β -carbons and between alkyl and phenyl groups. This enzyme acts on lignins to loosen the three-dimensional structure of this high-polymer, permitting depolymerization and solubilization.

Biodegradation Ability of *Coriolus Versicolor*

C. versicolor is a white, wood-rotting fungus that can digest cellulose, hemicellulose, and lignin, the main components in wood. Hardwood decayed by this fungus normally shows a 60% overall loss in weight, with only 35% of the original lignin content remaining. This characteristic indicates that the rate of the lignin decomposition is similar to that of the weight loss of the decayed wood (1). White rot fungi, being able to degrade lignin, produce a color reaction for phenolic substances (Bavendamm reaction) resulting from the action of extracellular phenoloxidases. Brown-rot fungi do not degrade lignin and do not demonstrate this reaction. It is apparent that the laccase secreted only by white-rot fungi brings about the color reaction (2). Ander et al. (3) reported that a mutant from *S. pulverulentum*, which was not able to secrete phenoloxidases, lost the original ability to degrade lignin and other wood components. These results suggest that the phenoloxidases are involved in lignin biodegradation.

Ability of Lignin Degradation of Laccase

Konishi et al. (4,5) reported that milled wood lignin (MWL) treated with the laccase from *C. versicolor* suffered oxidative polymerization, depolymerization producing

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carbonyl groups, and also partial solubilization in culture medium. Ishihara et al. (6) detected 2,6-dimethoxybenzoquinone, low and high molecular products formed by enzymatic oxidation, and an insoluble fraction (e.g., in 90% dioxane solution) when the MWL of maple wood was treated with the laccase of *C. versicolor*. They also reported that after treatment with laccase, vanillic acid and MWL yielded o- and p-quinone structures by oxidative de-methylation (7).

Noguchi et al. (8) synthesized precursors of lignin biosynthesis (i.e., coniferyl and sinapyl alcohols) in which the side chain and methoxy groups were doubly labeled by ^{14}C and ^3H , prepared the DHP (synthetic lignin) from them, and treated the DHP with the laccase. These results showed that about 25% of ^3H s of the α - and β -positions were removed in a ratio of one to one, respectively, and that the methoxyl group of a syringyl unit was de-methylated easier than that of a guaiacyl.

From these results the enzymatic functions of laccase on lignin can be summarized. (i) Laccase can depolymerize lignin, resulting in soluble lignin fractions. (ii) Laccase can polymerize and produce insoluble lignins. (iii) The oxidative reactions brought about by laccase catalyze the formation of carbonyl groups at the α -carbon and carboxyl groups and quinone structures resulting from the de-methylation of methoxy groups. It is therefore concluded that laccase participates in the oxidative degradation of lignin.

Fractionation of Laccases and Their Characterization

To elucidate the properties of laccases involved in the lignin biodegradation, we fractionated crude laccase fractions and isolated pure enzyme fractions. *C. versicolor* was incubated in a glucose-peptone medium including a copper constituent with 30-liter jar-fermentor. An extra-cellular crude enzyme was isolated from the culture medium by precipitation with ammonium sulfate (90% saturation) and subsequently fractionated by gel-filtration using Sephadex G-50. A high molecular weight fraction (F-1) having phenoloxidase activity was isolated by gel filtration and subjected to ion-exchange chromatography using DEAE TOYOPEAL 650M (Figure 1). The primary phenoloxidase fractions, labeled TOYO-F1, -F3, and -F4, corresponded to laccases I, II, and III, respectively. TOYO-F1 and -F4 were further purified by carboxymethyl cellulose and hydroxyapatite chromatography. These fractions showed one band by SDS-polyacrylamide gel electrophoresis (PAGE) (Figure 2). Their molecular weights were found to be 66,000 by SDS-PAGE. Laccase III was concentrated with an ultrafilter and crystallized in a cellophane tube with 5mM sodium thiocarbonate (Figure 3).

Comparison Between Three Laccase Fractions (10,11)

To elucidate some enzymatic characteristics of the isolated laccases I, II, and III, substrate specificities for several simple phenols, electrophoresis patterns, ultraviolet spectra, electron spin resonance spectra, copper content, and immunological similarities were investigated. Tyrosine, tannic acid, gallic acid, hydroquinone, catechol, pyrogallol, p-cresol, homocatechol, α -naphthol, β -naphthol, p-phenylenediamine, and p-benzoquinone as substrates. No differences in the specificities of these substrates was found. The UV spectra for the laccases under study are shown in Figure 4. Laccase III displays three adsorption bands (280, 405, and 600nm), laccase II shows one band (280nm), and laccase I shows two bands (280 and 405 nm). These data appear to indicate differences in chemical structure. The results of the copper content analysis (10) and two-dimensional electrophoresis also indicate that these fractions are completely different proteins (10). Therefore, we may expect differences in substrate specificities between the three laccase fractions for more lignin-like substrates, yet no difference for some simple phenolic substrates.

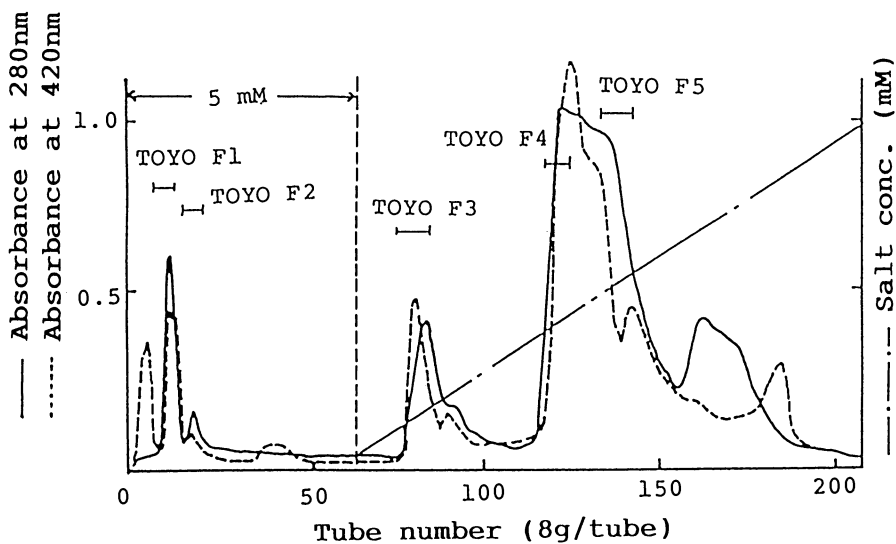


Figure 1. Fractionation of F-1 with DEAE TOYOPEAL 650M.

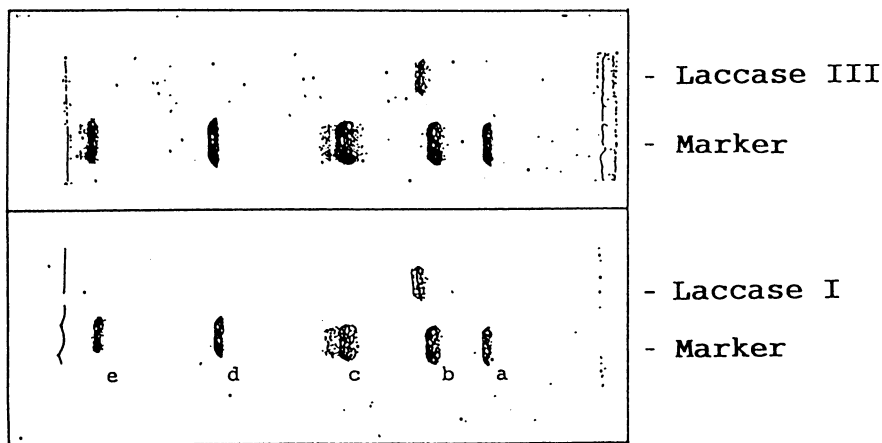


Figure 2. Analysis of laccase I and laccase III by SDS-PAGE (10% polyacrylamide slab gel electrophoresis).

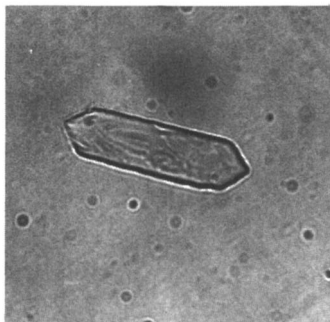


Figure 3. Crystal of laccase III.

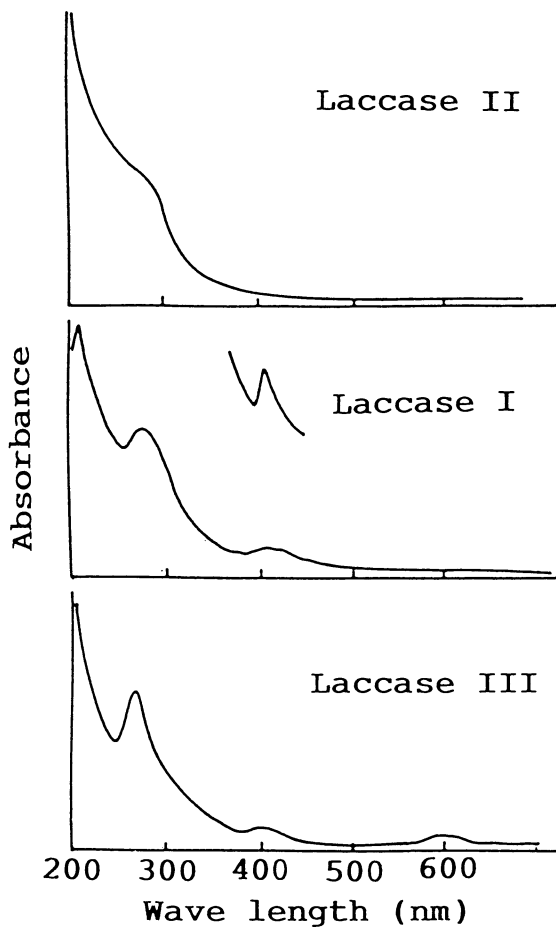


Figure 4. UV-VIS spectra of laccases I, II, and III.

In order to investigate the active sites of these proteins, laccases I and III were subjected to ESR (electron spin resonance) spectroscopic analysis. The ESR spectra shown in Figure 5 indicate clear differences in peaks 2 and 6 which support the concept that the copper atoms in laccases I and III have different conformations in each molecule. Furthermore, immunological similarity between laccases I and III was also investigated. Antibody specific for laccase III was prepared from rabbit serum by conventional methods. When applied to Ouchterlony diffusion plates containing laccase I, no precipitation lines developed (Figure 6). This result showed that there were no conserved epitopes on the surfaces laccases I and III.

The Role of Laccase and Peroxidase on the Lignin Biodegradation

It has been pointed out that phenoloxidases involved in the degradation of lignin by *C. versicolor* may be laccases and/or lignin-peroxidases, including the manganese-dependent peroxidase (for example, ref. 12). To elucidate the role of these enzymes in lignin degradation, the incorporation of isotopic label from DHP (labeled with ^{14}C) into the fungal mycelium (hyphae) was examined. These fractions included a "released" $^{14}\text{CO}_2$ fraction, a DMF-insoluble fraction (filter), a DMF-soluble fraction (filter, DMF-soluble), and a water-soluble fraction (agar) (Figure 7). The culture medium used was limited in nitrogen source (L-N) because the lignin-peroxidase was found to be secreted only under these conditions (Figure 8) (13). It is generally assumed that the biodegradation of solid, high-polymer lignins is initiated by the solubilization and depolymerization, followed by the incorporation of the degraded fragments into the mycelia and finally conversion into CO_2 . The experimental results presented here support the idea that the solid lignin was strongly degraded and that the oxidative polymerization was controlled by the secretion of peroxidases. The amount of the water soluble fraction, reflecting the degree of depolymerization, is almost the same whether or not the peroxidases are secreted to the extracellular region. This fact indicates that laccases can depolymerize and solubilize lignin. Therefore, it is presumed that the main role of peroxidases is the conversion of the soluble lignin fragments into smaller molecules which can be easily incorporated into the mycelium. The metabolism of the incorporated lignin fragments depends on the growth rate of the fungus which is evaluated from the amount of $^{14}\text{CO}_2$ released from both conditions tested [i.e., low-nitrogen (L-N):low-carbon (L-C), and L-N:high-carbon (H-C)]. The best conditions for degradation of the solid lignin resulted from the secretion of laccases and peroxidases into the extracellular region, accompanied by high fungal proliferation (14).

Properties of Laccase III

The purified laccase III was found to migrate on SDS-PAGE as one band which corresponded to a molecular weight of 66,000. It was also found to be a copper-enzyme (Type I) from examination of the UV spectrum shown in Figure 4. Atomic absorption spectroscopy showed that one molecule of laccase III contains two copper atoms. The N-terminal amino acid sequence of laccase III was found by Edman degradation to be Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile---. The amino acid PTHs (phenylthiohydantoin) determined for the N-terminal sequence analysis were cleanly identified by high pressure liquid chromatography, supporting the notion that the isolated laccase III is a pure protein. Microscopic analysis of the crystallized protein showed that its crystal form is a hexagonal plate.

As part of the investigation of the carbohydrate moieties of laccase III, we produced carbohydrate-depleted laccase III by the culture of *C. versicolor* with tunicamycin and by the selective cleavage of the carbohydrate chains with endoglycosidases. Laccases isolated from *C. versicolor* cultures which were treated with tunicamycin were analyzed

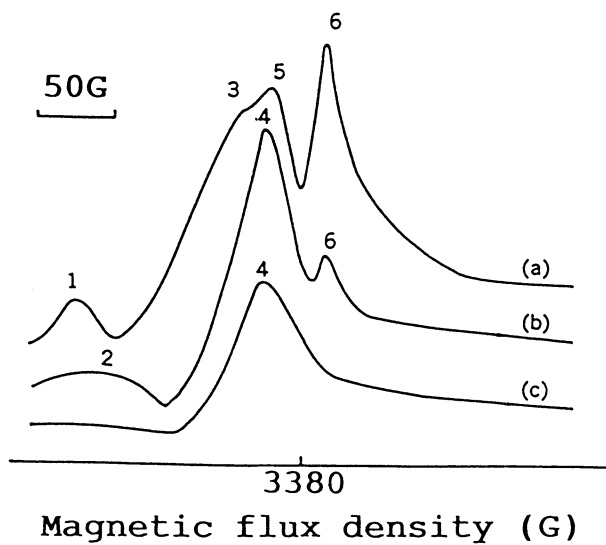
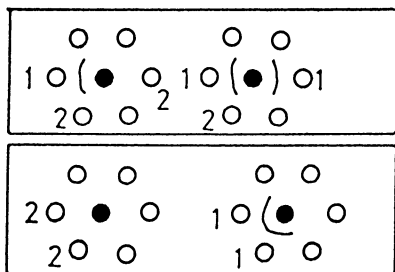
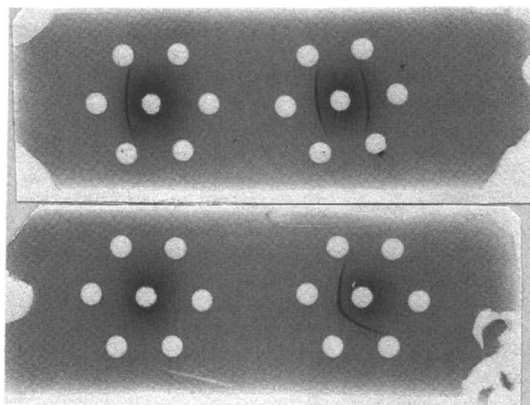


Figure 5. ESR spectra of crude enzyme, laccases I, and III. (a) Crude enzyme, (b) laccase I, (c) laccase III.



- . Antiserum of Laccase III
- 1. Laccase III
- 2. Laccase I

Figure 6. Antigen-antibody reaction between the antisera specific for laccase III and laccases I and III.

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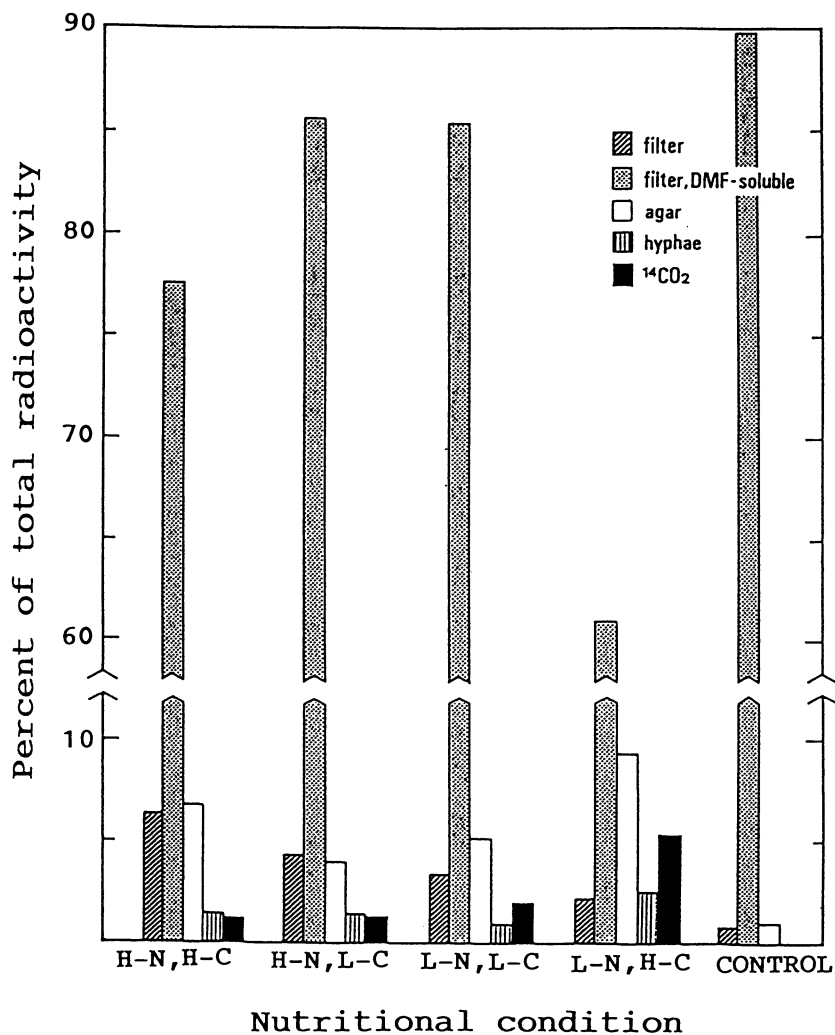


Figure 7. Distribution of ^{14}C of [2- ^{14}C]-DHP after incubation with *C. versicolor*. Average values from four replicate assays are presented.

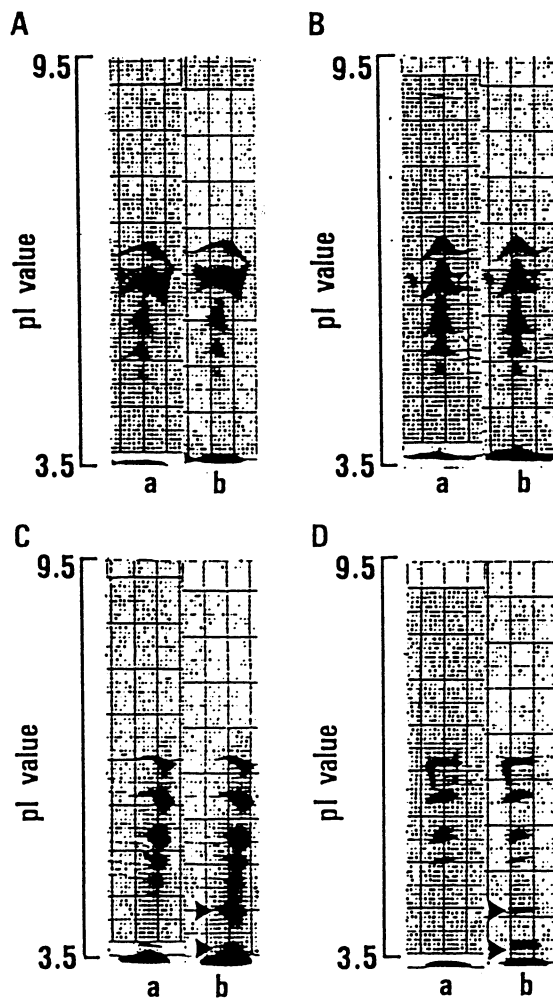


Figure 8. Separation of extracellular enzymes from *C. versicolor* on isoelectric focusing. Nutritional culture conditions were as follows: H-N/H-C (A), H-N/L-C (B), L-N/L-C (C), L-N/H-C (D). The gels were stained with guaiacol (a) and subsequently hydrogen peroxide was added (b).

by SDS-PAGE. The carbohydrate-depleted laccase III was secreted to extracellular region easily and did not accumulate into the mycelium. It is clear that the carbohydrate chains do not participate in the ability of the fungus to secrete the enzyme extracellularly. Laccase III has been shown recently to contain four N-linked carbohydrate chains which are 2-3k daltons in size (15). The carbohydrate-depleted laccase III was shown to be less thermostable, however, and more sensitive to protease attack than the native laccase III.

No differences were found between the native and deglycosylated enzymes for the characteristics of pH range of stability, optimum pH, and adsorption to cellulose and milled wood. It can be concluded that the most important role of the carbohydrate chains in laccase III is the resistance to proteolysis in wood decay (15).

Degradation Mechanism of Lignin Model Compounds by Laccase III (16,17)

To elucidate the reaction mechanism of lignin by laccase III, we first carried out degradation experiments using model compounds which were guaiacylglycerol- β -guaiacyl ether (GOG), guaiacylglycerol- β -syringyl ether (GOS), syringylglycerol- β -guaiacyl ether (SOG) (18), syringylglycerol- β -syringyl ether (SOS) (19), and *o,o'*-dihydrobiphenyl dehydro-dimer of guaiacylglycerol- β -guaiacyl ether (GOG-Dimer). These compounds were degraded by laccase III and the degraded compounds were isolated by column chromatography and TLC (thin layer chromatography). The isolated compounds were then analyzed by gas chromatography-mass spectroscopy, $^1\text{H-NMR}$, and $^{13}\text{C-NMR}$. Degradation pathways were presumed from the identified compounds (see Figure 9).

From the degradation of SOG by laccase, 3-hydroxy-2-(*o*-methoxy phenoxy)-propionic acid (V), 2-(*o*-methoxy phenoxy) ethanol (VI), syringaldehyde (VII), 2,6-dimethoxy benzoquinone-1,4 (IX), guaiacol (VIII), and 4-formyl-6-methoxy benzoquinone-1,2 (XIV) were detected in the first stage of the reaction (after 1.5 hr.). Compounds (VIII), (IX), and (XIV) were found in the late stage (after 10 hr.). It is clear that a main reaction was the cleavage of C-C bonds between the α - and β -carbons in side chains and that a side reaction is the cleavage of alkyl-phenyl bonds. The following reaction being the cleavage of β -O-4 ether linkage.

The degradation products of GOG were (VIII), GOG-Dimer, vanillin (II), and dehydrodivanillin (X). The reaction of GOG by laccase III, therefore, brings about the formation of biphenyl structures, the cleavage of C-C bond between α - and β -carbons, and the cleavage of β -O-4 ether linkages. The mode of these cleavages is similar to that of SOG.

The degradation products of GOS were 1,3-dimethyl pyrogallol (III), 2-(2',6'-dimethoxy phenoxy)-2-propenal (VIII), 2-(2',6'-dimethoxy phenoxy)-3-hydroxypropanal (XII), and GOS-Dimer. These products show that the reaction includes oxidative polymerization and the cleavage of β -O-4 ether linkage following the alkyl-phenyl cleavage. This depolymerization pathway of GOS is also similar to that of SOS (Table I).

Finally, the degradation of the β -O-4 structure type constituting a main linkage in lignin by laccase III includes the cleavage of bonds between α - and β -carbons, and between alkyl and phenyl groups. The selection of both cleavages depends on the β -O-aryl ring structure; specifically, the cleavage between α - and β -positions is prior to the alkyl-phenyl cleavage when the β -O-aryl ring is guaiacyl, and the alkyl-phenyl cleavage is selected when the β -O-aryl ring is syringyl. Both reactions bring about the cleavage of β -O-4 ether linkages and produce new free phenolic hydroxyl groups in lignin.

Compounds (III), (V), (VI), and (VIII) were detected from the degradation of the threo- and erythro-types of SOS. The primary difference between both reactions was the detection of α -(2,6-dimethoxy phenoxy)- β -hydroxy propiosyringone (XIII) in the first stage (5 min) of the degradation of the threo-type compound only. This result showed

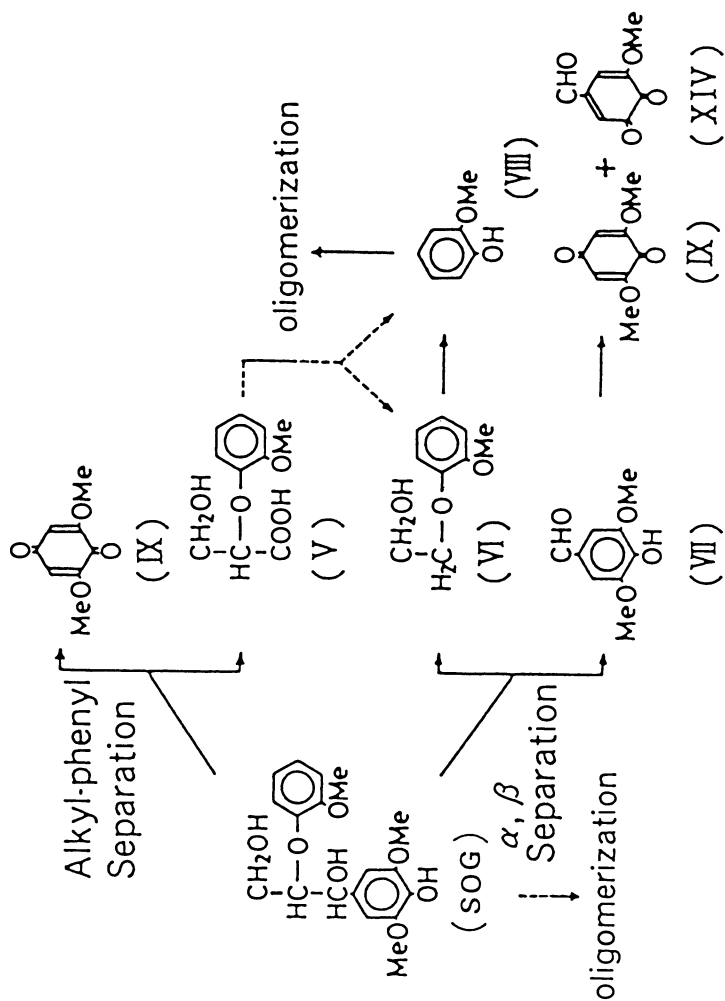


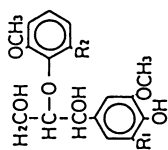
Figure 9. Proposed degradation pathway of (SOG) by laccase III. Broken lines show the assumed paths.

Table I. Degradation Mechanism of Lignin Dimer Models

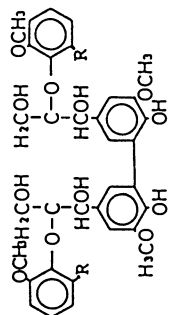
Substrate	Degradation products	Main reaction
GOG	[II], [X], [VIII], [GOG-Dimer]	α , β -side chain cleavage 5-5 bond formation (polymerization)
GOS	[III], [IV], [XII], [GOS-Dimer]	Alkyl-phenyl cleavage 5-5 formation
SOG	[V], [VI], [VII], [VIII], [IX]	α , β -side chain cleavage
SOS	[III], [IV], [XII], [IX]	Alkyl-phenyl cleavage
Erythro	[III], [IV], [XII], [IX]	Alkyl-phenyl cleavage
Threo	[III], [IV], [XII], [IX], [XIII]	α -carbonylic reaction Alkyl-phenyl cleavage
GOG-Dimer	[VIII], [XI]	Alkyl-phenyl cleavage

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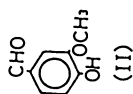
NOTE: Structure Key:



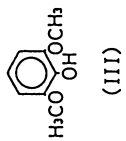
GOG : $R_1 = R_2 = H$
 GOS : $R_1 = H, R_2 = OCH_3$
 SOG : $R_1 = OCH_3, R_2 = H$
 SOS : $R_1 = R_2 = OCH_3$



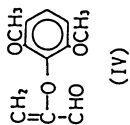
GOG-Dimer: $R = H$
 GOS-Dimer: $R = OCH_3$



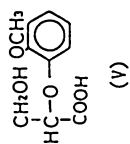
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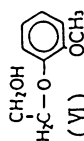
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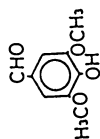
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(V)



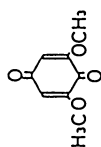
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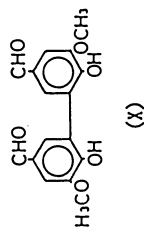
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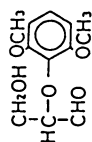
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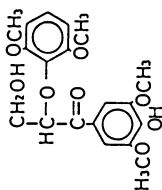
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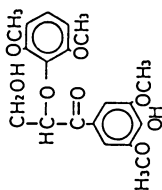
(X)



(XI)



(XII)



(XIII)

that the erythro-type compounds undergo the alkyl-phenyl cleavage directly. The threo-type compounds do not, however, because of steric hindrance factors for the enzymatic reaction. Therefore, threo-type is first converted into a α -carbonyl compound (for example, XIII) losing stereospecificity and then undergoes the alkyl-phenyl cleavage.

The GOG-Dimer formed from the lignin dimer compound (GOG) by laccase III was also degraded by laccase III, resulting in the formation of compounds (I), (VI), and biphenyl dimer of 2-methoxy-1,4-benzoquinone (IX). This indicates that GOG-Dimer also suffers the cleavage of β -O-4 ether linkage via the alkyl-phenyl cleavage.

In conclusion, it is clear that laccase III can cleave the β -O-4 ether linkages in the phenolic subunits of lignin. Considering this cleaving reaction from a viewpoint of lignin degradation, laccase III brings about depolymerization by eliminating one unit from the free phenolic hydroxyl containing end group of the lignin macromolecule. It is characteristic of this depolymerization process to consume one free phenolic hydroxyl group and to produce one. These reactions will be continued. This concept (shown in Figure 10) supports the fact that the lignin in decayed wood always has a constant value of free phenolic hydroxyl groups when subjected to decay and any degree of resulting weight loss (20).

Reactivity of Laccase III for High Polymer Lignin (21)

The kinds of reactions catalyzed by the action of laccase III on high polymer lignins and protolignins are an important issue from the standpoint of the commercial utilization of this enzyme and of the elucidation of wood-rotting mechanism. Milled beech wood was treated with the extracellular enzymes of *C. versicolor*, including laccase III, for 4 days at 35°C and analyzed by the permanganate oxidation method (22). The results can be summarized as follows: (i) 76% of the free phenolic units in lignin were degraded, (ii) 18% of total aromatic units were degraded, (iii) 12% of alkyl-aryl ether linkages were cleaved, (iv) 1% of the degree of condensation was decreased, and (v) 21% of syringyl, 16% of guaiacyl and 18% of p-hydroxyphenyl nuclei were degraded. These results showed that the free phenolic units actively participate in the biodegradation of lignin and that the syringyl units had higher reactivity than the guaiacyl or p-hydroxyphenyl units. These phenomena may be explained by the idea that the protolignin was significantly affected by the laccases and the lignin degradation by the laccase does not include the polymerization reaction.

Beech wood meal (sized to 60 mesh) and wood meal ground by vibration ball milling were treated with laccase III. The klason lignin content and the amount of functional groups of these treated samples were subsequently determined. After exposure to enzyme the klason lignin content of the wood meals decreased by about 1% ($\pm 0.05\%$), but for the ground wood meal samples it decreased by 5%. This shows that the activity of the enzyme on protolignin depends on the accessibility of the lignin substrate. Therefore when wood meals are finely powdered, as the ground wood meal sample, laccase III can catalyze the delignification by 3-5%. However, MWL isolated from the ground wood meals was also treated by laccase III and compared with untreated MWL. The treated sample was found to display a decrease in phenolic hydroxyl groups, an increase in conjugated and unconjugated carbonyl groups, including carboxylic acids, and a higher reactivity for syringyl units. Therefore it was clear that lignin markedly suffers oxidation, but no oxidative polymerization was observed.

To visualize whether or not wood can be delignified by laccase III, ultraviolet photomicrographs (280nm) were taken before and after treatment of 0.5 μm cross-sections of red pine with laccase III (Figure 11). After enzyme treatment areas having an absorbance less than 0.2 in the secondary wall, and an absorbance less than 0.4 in the middle lamella, appeared. Each part of the secondary wall, middle lamella, and cell junction was subjected to ultraviolet microscopy, and absorption curves in the 240-300nm region were determined. The absorption curves of three samples after treatment

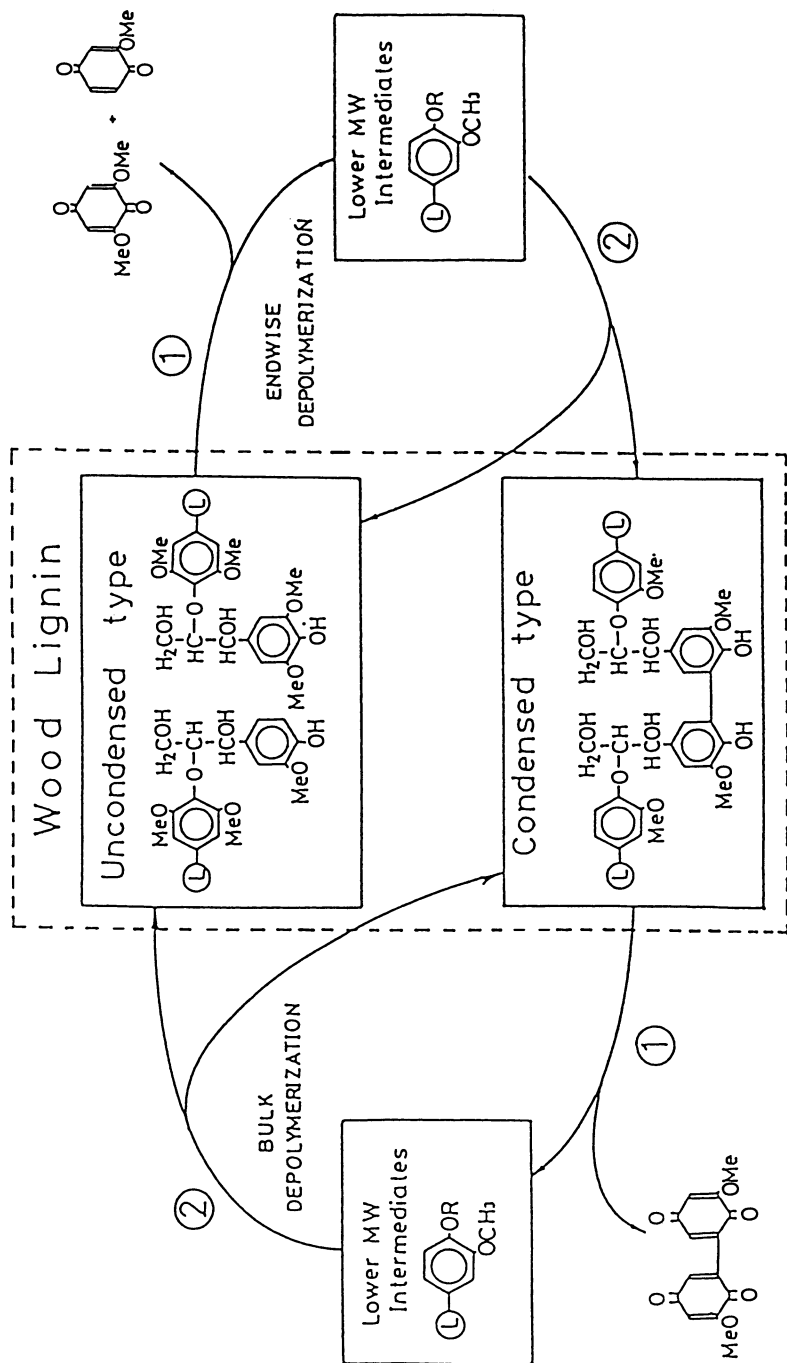


Figure 10. A proposed depolymerization mechanism of lignin by laccase. (1) Side chain cleavage (2) β -O-4 ether cleavage.

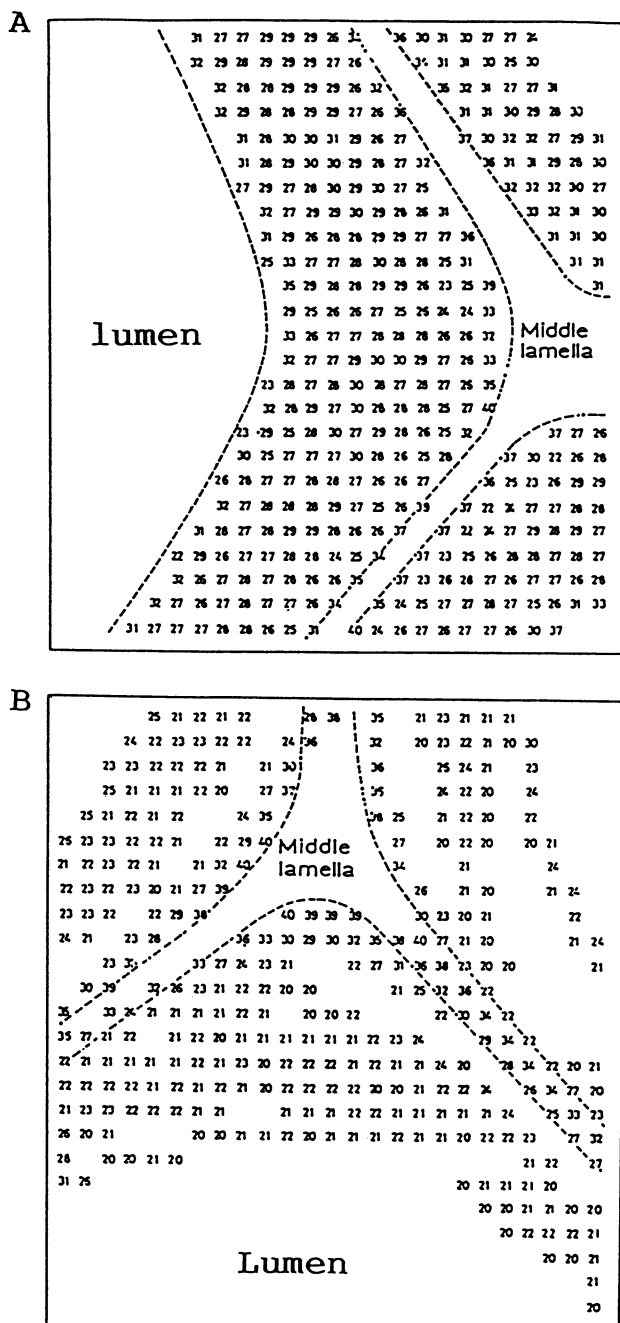


Figure 11. Ultraviolet photomicrographs of 0.5 micron cross-sections of red pine at 280nm. (A) and (B) were taken before and after treatment with laccase III. Absorbance units of 0.20-0.40 were indicated as 20-40.

showed a decrease in value. They demonstrate that protolignin in wood cell walls can also be delignified by laccase III when the sections are thin.

In summary, we conclude that laccase III can degrade a portion of protolignin through depolymerization and solubilization.

Literature Cited

1. Fukuda, K.; Haraguchi, T. *Bull. Exp. For. Tokyo Univ. Agric. Technol.* 1974, 15, 25-29.
2. Higuchi, T. *Adv. Enzymol.* 1971, 34, 207.
3. Ander, P.; Eriksson, K.E. *Arch Microbiol.* 1976, 109, 1-7.
4. Konishi, K.; Inoue, Y.; Higuchi, T. *Mokuzai Gakkaishi.* 1972, 18, 571-576.
5. Konishi, K.; Inoue, Y.; Higuchi, T. *Mokuzai Gakkaishi.* 1974, 20, 26-30.
6. Ishihara, T.; Miyazaki, M. *Mokuzai Gakkaishi.* 1972, 18, 415-419.
7. Ishihara, T.; Miyazaki, M. *Mokuzai Gakkaishi.* 1974, 20, 39-41.
8. Noguchi, A.; Shimada, M.; Higuchi, T. *Holzforschung* 1980, 34, 86-89.
9. Morohoshi, N.; Nakamura, M.; Yoshitake, A.; Katayama, Y.; Haraguchi, T. *Mokuzai Gakkaishi.* 1987, 33, 743-746.
10. Morohoshi, N.; Wariishi, H.; Muraiso, C.; Nagai, T.; Haraguchi, T. *Mokuzai gakkaishi.* 1978, 33, 218-225.
11. Morohoshi, N.; Fujita, K.; Wariishi, H.; Haraguchi, T. *Mokuzai Gakkaishi* 1987, 33, 310-315.
12. Higuchi, T. *Wood Research* 1986, 73, 143-150.
13. Morohoshi, N.; Tamura, R.; Katayama, Y.; Morimoto, M. *Bull. Exp. For. Tokyo, Univ. Agric. Technol.* 1990, 27, 47-56.
14. Iimura, Y. *Proc. 34th Lignin Symposium*, Nagoya, Japan, 1989; pp 125-128.
15. Morohoshi, N. In *Wood Processing and Utilization*; Kennedy, J.F., Ed.; Ellis Horwood Limited: West Sussex, England, 1989; pp 341-346.
16. Morohoshi, N.; Nakamura, M.; Haraguchi, T. *Proc. 30th Lignin Symposium*, Koochi, Japan, December 1985; pp 61-64.
17. Morohoshi, N.; Nakamura, M.; Katayama, Y.; Haraguchi, T. *Proc. 4th ISWPC*, Paris, France, April 1987, Vol. 2; pp 305-311.
18. Wariishi, H.; Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi.* 1987, 33, 892-898.
19. Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi.* 1987, 33, 495-502.

20. Morohoshi, N.; Kosaka, T.; Haraguchi, T.; Fukuda, K. *Bull. Exp. For. Tokyo Univ. Agric. Technol.* 1983, 19, 59-78.
21. Morohoshi, N.; Nakamura, M.; Katayama, Y.; Haraguchi, T.; Fujii, T.; Hiroi, T. *International Biodeterioration* 1989, 25, 7-14.
22. Nakamura, M.; Morohoshi, N.; Haraguchi, T. *Bull. Exp. For. Tokyo Univ. Agric. Technol.* 1989, 26, 79-84.

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Chapter 18

Pilot-Scale Production and Properties of Lignin Peroxidases

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Lignin peroxidase was produced in a 1 m³ bioreactor. The mycelia of *Phanerochaete chrysosporium* was immobilized on a 12 m² carrier lamellae bound on steel wire mesh. The enzyme was harvested with ultrafiltration and the permeate with added nutrients was fed back to the bioreactor through a sterile filter. A total of 1.5 million units of lignin peroxidase was harvested during one month. Lignin peroxidase was purified by ion exchange chromatography. The purified lignin peroxidase was most stable at pH 4.5-5.0. As expected, hydrogen peroxide rapidly inactivated the enzyme. Veratryl alcohol had no observable stabilizing effect on lignin peroxidase.

Lignin together with cellulose and hemicellulose is a structural component of woody plants. It is an aromatic irregular polymer having no repetitive linkages and no definite structure. Lignin effectively protects the woody plants against microbial attack and only a few organisms including rot-fungi and some bacteria are able to degrade it.

The discovery of a lignin degrading enzyme in 1983 (1,2) created excitement and enthusiasm among both the scientific and industrial community. Great expectations were laid on the enzyme's capability for lignin degradation and modification in e.g. pulp and paper industry (3). However, as usual, the story was not at all as simple as expected. The ligninase proved to be a peroxidase (4) and the in vitro reaction with lignin led to polymerization reactions (5). Furthermore, the enzyme was produced only in tiny amounts during secondary (growth limited) metabolism of slowly growing non-agitated cultures of some white-rot fungi, the best producer being *Phanerochaete chrysosporium*. Initially lignin peroxidases were produced in shake flasks in very small quantities, which made it impossible to study their industrial applications. Early lignin peroxidase research has been reviewed by Kirk and Farrell (6), and the mechanism of fungal lignin degradation has recently been reviewed (7).

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A program for large-scale production of lignin peroxidases was initiated at the Swiss Federal Institute of Technology in 1984. It was shown that lignin peroxidase production could be enhanced in the presence of lignin or lignin-like low molecular weight compounds (8). The enzyme was also produced in agitated cultures (9) and immobilized reactors (10). Here we report the pilot-scale production and purification of lignin peroxidases as well as the stability characteristics of the enzyme as an industrial product.

Experimental

Production of Lignin Peroxidase. Medium for the inoculum was rich in yeast extract (25 g/l) and glucose (25 g/l) to promote maximal growth of the mycelia. The inoculum of *Phanerochaete chrysosporium* ATCC 24725 was first cultivated for 3 days at 30°C in five litres of medium divided in five shake flasks. The shake flask batches were transferred to a 100 litre bioreactor and cultivated again for 3 days at 30°C. The batches were stirred and aerated to obtain maximal growth of mycelia.

The medium for enzyme production was completely synthetic and limited in carbon to prevent excessive growth of mycelia. It contained glucose 2 g/l plus ammonia nitrogen, vitamins and trace elements according to Leisola et al.(11). The 100 l inoculum was transferred into 800 l of medium in a 1000 l bioreactor equipped with 12 m² of nylon wool sheets supported by steel wire screen (Figure 1). The medium was continuously stirred with the impeller (100-200 rpm) and saturated with pure oxygen. The temperature was maintained at 37°C.

At 24 hours after inoculation, 350 g veratryl alcohol was added to induce lignin peroxidase production. The veratryl alcohol was diluted with water to a final concentration of 5 % (by weight) and pumped through a sterile filter into the bioreactor. At 123 hours the first lignin peroxidase activity peak appeared. The medium was harvested through a coarse filter to remove possible solids and the enzyme was concentrated on a PCI ultrafilter with 50 000 Da cut-off membranes. The permeate was immediately fed back to the bioreactor through a sterile filter. The whole enzyme harvesting cycle was performed within 4 hours. Veratryl alcohol was not added for the subsequent 5 harvests. After the 6th and 8th harvest more veratryl alcohol was added in an attempt to increase the enzyme production (see Table I). A new portion of glucose was dissolved in the permeate and fed into the bioreactor through sterile filter to start a new enzyme production cycle. The second lignin peroxidase peak appeared at 189 hours. The enzyme harvesting and feeding cycles were repeated until nine batches of lignin peroxidase were collected during 499 hours of cultivation (Table I). No other nutrients than glucose were added during the process.

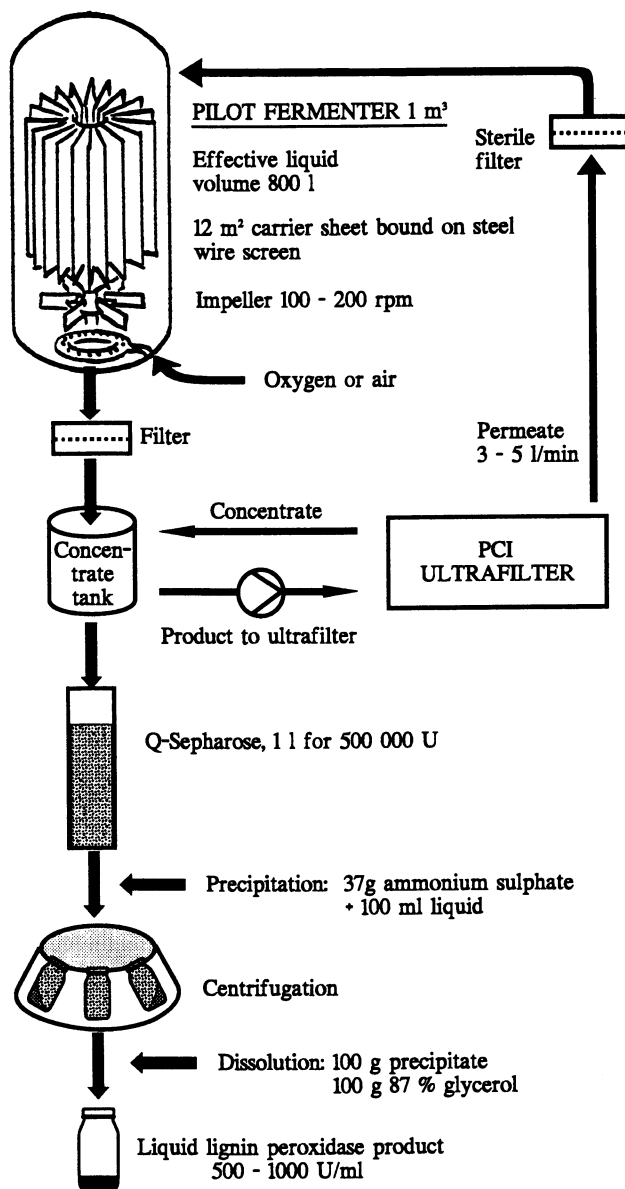


Figure 1. Pilot-scale production of lignin peroxidases

Table I. Pilot Production of Lignin Peroxidase

<i>Time (h)</i>	<i>Lignin Peroxidase (U/l) at Harvest</i>	<i>Concentrate (total U)</i>	<i>Veratryl Alcohol (g)</i>	<i>Glucose (kg)</i>
0	-	-	350	1.6
123	280	202 000		2.4
189	160	138 000		0.6
215	187	78 000		0.8
236	213	189 000		2.4
261	270	216 000		1.1
292	432	334 000	50 ^a	1.0
332	121	93 000		1.4
380	336	183 000	160 ^a	3.2
499	154	117 000		
Total collected units		1 550 000		

^aAdded immediately after the harvest at 292 and 380 h.

Purification

Unfractionated Preparation. Each harvest of the enzyme was concentrated to 40 l and diafiltered with 0.01 M sodium acetate, pH 6.0. Enzyme concentrate was bound to Q-Sepharose and eluted with 0.4 M sodium acetate, pH 6.0. Fractions with activity were pooled, sterile filtered and stored in the cold in 10 mM veratryl alcohol. Before further purification, the solution was dialyzed.

Fractionation by Linear Gradient. It is known that lignin peroxidases can be conveniently separated by their charge properties (12). To find out how the enzymes in our preparation could be separated, analytical ion exchange chromatography with linear gradient elution was used. 16100 U in 188 ml of dialyzed lignin peroxidase in 0.01 M sodium acetate, pH 6.0, was applied to an anion exchange column (Q-Sepharose, Pharmacia, $\varnothing = 1.6$ cm, $h = 9.2$ cm). It eluted as three main peaks with a linear sodium acetate gradient (0.01 to 0.40 M; 1.5 ml/min, 300 min, fractions 1-45; Figure 2). 10 ml fractions were collected and their activity and absorbances at 280 nm and 405 nm were measured. Lignin peroxidase fractions eluted with 0.20 to 0.33 M acetate were pooled and precipitated with ammonium sulphate (37 g to 100 ml). The precipitate was dissolved by adding 87% glycerol (1:1 w/w). Volume of the enzyme solution was 7 ml.

Fractionation by Stepwise Elution. Information obtained from the analytical separation was applied for a preparative purification. Lignin peroxidase concentrate was bound to a Q-Sepharose column ($\varnothing = 5$ cm, $V = 1000$ ml) after ultrafiltration and eluted stepwise with 0.08 M, 0.18 M and 0.28 M sodium acetate, pH 6.0. The fraction which was eluted with 0.28 M buffer ($V = 3.91$, 4400 U/l) was purified further. It was bound to Q-Sepharose and eluted with 0.18 M and 0.3 M sodium acetate. Enzyme in the latter fraction was precipitated and dissolved in glycerol as previously described. The volume was 15 ml.

Enzyme fractions obtained by linear gradient elution and stepwise elution were pooled and used later in this work.

Purity Criteria. Purity of lignin peroxidase was analyzed with analytical anion exchange chromatography using detection at 405 nm. About 4 U of lignin peroxidase was applied to a Pharmacia Mono Q column (HR 5/5, 0.25 x 5.0 cm) and eluted with linear sodium acetate, pH 6.0, gradient (0.01 to 0.55 M, 1 ml/min, 14 min).

Properties of Lignin Peroxidase.

Activity Assays. The standard activity assay mixture of 3 ml contained about 0.1 U/ml lignin peroxidase, 0.4 mM veratryl alcohol (Fluka, purum > 97%) and 0.1 M sodium tartrate, pH 3.0. The reaction was started by adding 15 μ l of 54 mM H₂O₂ to make a final concentration of 0.28 mM in the reaction. The production of veratraldehyde was followed by recording the change of absorbance for 12 seconds at 310 nm in a cuvette which was thermostated to 37°C. The reaction was started 24 seconds before the recording. One unit of lignin peroxidase is defined as the amount of enzyme required to oxidize one μ mol of veratryl alcohol to veratraldehyde in one minute.

For pH and thermoinactivated samples, the conditions were equal to those in the standard assay, except that the concentration of tartrate buffer, pH 3.0, was 0.3 M.

For hydrogen peroxide treated samples, the concentration of H₂O₂ in the mixture was 0.3 to 1.8 mM and concentration of sodium tartrate was 0.3 M. Contact with H₂O₂ causes a period (called the lag time) where no production of veratraldehyde was observed in the assay of activity. Length of the lag time was estimated to be the 24 s + inactive period observed in the graph. After the lag time lignin peroxidase was able to convert veratryl alcohol to veratraldehyde. The highest rate of veratraldehyde production occurred immediately after the onset of the reaction and was defined to be the residual activity.

For veratryl alcohol treated samples, the concentration of veratryl alcohol in the activity assay mixture was 1 - 11 mM, the concentration of tartrate was 0.01, 0.02 or 0.3 M and the enzyme activity at the beginning was 0.2 U/ml. The samples were at 30°C during the reaction.

For determination of the Michaelis constant, the activity of purified lignin peroxidase was measured by using the standard activity assay method except that the concentration of veratryl alcohol was varied between 7 μ M and 2.67 mM.

Stability Studies. For the inactivation treatments the buffers were made by mixing 0.2 M Na₂HPO₄ and 0.1 M tartaric acid to produce the pH values between 3.0 and 7.0.

To study the effect of pH and temperature, 0.25 ml of the lignin peroxidase diluted with water was mixed with 0.75 ml of buffer, pH 3.0 - 7.0 and incubated at temperatures of 30 - 70°C. The protein concentration of the incubation mixture was 50 μ g/ml. After various incubation times (0 - 27 h) the inactivation was stopped by adding 9 ml of cold 0.33 M tartrate buffer, pH 3.0.

To study the effect of hydrogen peroxide, the lignin peroxidase was incubated in buffer, either pH 3.0 or 5.0, at the temperature of either 0°C or 10°C. Protein concentration was 30 $\mu\text{g}/\text{ml}$ and the concentration of H_2O_2 was 0.2-11.6 mM. The incubation time varied between 0 and 295 min. After incubation 0.4 ml of the enzyme sample was pipetted directly into the activity assay mixture.

To study the effect of veratryl alcohol, purified lignin peroxidase or unfractionated enzyme preparation was incubated with buffer, pH 3.0 or 5.0. The concentration of veratryl alcohol in the incubation mixture was 0, 10 or 100 mM. Incubation times were 38 days at 20°C and 40 days at 4°C. The protein concentration of purified enzyme was 80 $\mu\text{g}/\text{ml}$ and of unfractionated preparation 180 $\mu\text{g}/\text{ml}$. The incubation mixtures were sterile filtered to prevent microbial growth.

Mathematical Treatment of the Results. In almost all experiments inactivation followed first order kinetics with a high correlation. The half-lives of lignin peroxidase were calculated from the following equation, where k is a rate constant of inactivation:

$$t_{1/2} = \ln 2/k \quad (1)$$

The Arrhenius equation:

$$k = A \exp (-E_a/RT) \quad (2)$$

was used in this work to express the correlation between temperature and half-life of the enzyme at different pH's. A computer program, RS/1, (13) was used in performing calculations and plotting graphs. The half-life of enzyme activity was determined by plotting the logarithm of residual activity (U/l) versus incubation time. For all the fitted lines in the inactivation experiment concerning temperature and pH the F-value (14,15) was determined by the RS/1 program. The F-value is the single most useful measure of the significance of the fit as a whole. Also the number of points in a graph is taken into consideration in its calculation. F-value increases with increasing significance of the fit. The points in Arrhenius plots, which represent half-lives, were weighted with the related F-value when fitting the lines (Figure 3). On the basis of the equations of these fitted lines in Arrhenius plots, corrected half-lives were calculated, and these corrected values were used when the half-lives of lignin peroxidase were plotted as a function of pH at different temperatures (Figure 4).

Results

Production. The inoculum grew vigorously in the rich yeast extract containing media and produced a thick viscous dispersion in the stirred tank bioreactor. No lignin peroxidase activity could be detected at this stage. When transferred to the 1000 l production bioreactor, the mycelium of *P.chrysosporium* attached completely to the nylon wool sheets within a few hours after inoculation and the medium remained completely clear throughout the cultivation. The enzyme had to be harvested immediately after the maximal activity level was reached due to its

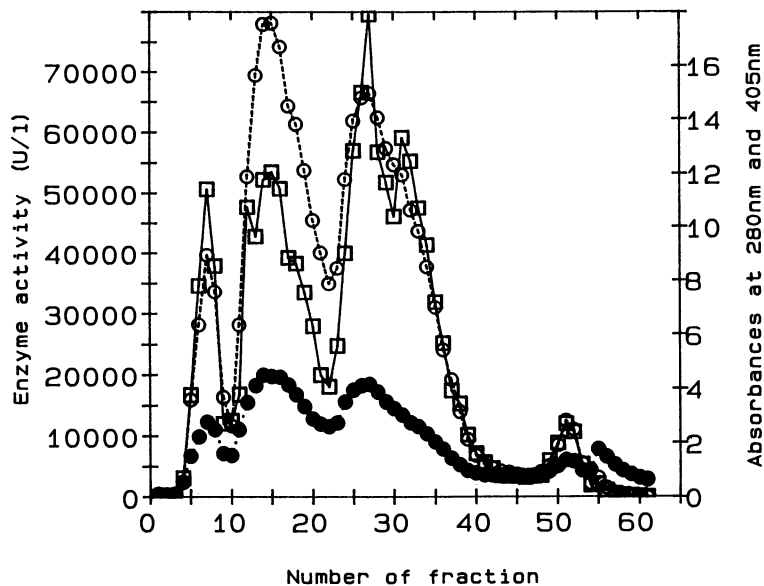


Figure 2. Purification of lignin peroxidases by anion exchange chromatography.
 □ Enzyme activity (U/l); ○ Absorbance at 405 nm; ● Absorbance at 280 nm.

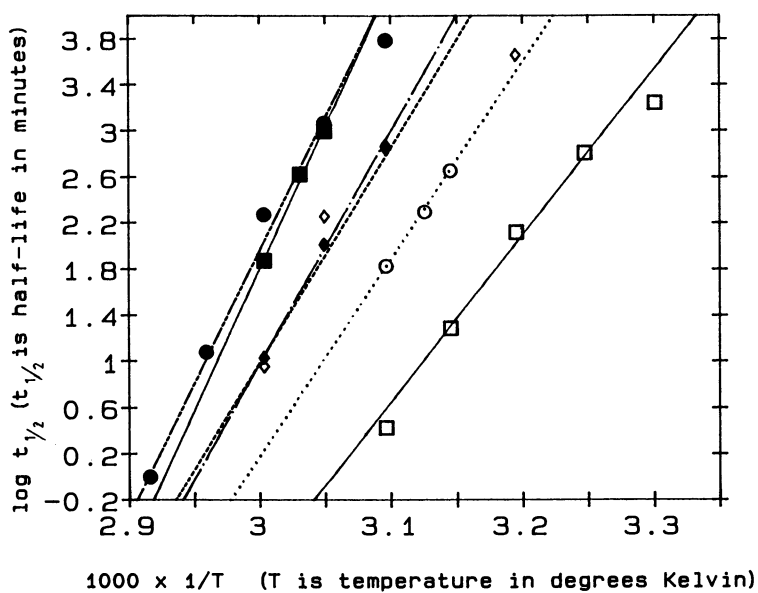


Figure 3. Arrhenius plots. □ pH 3.0; ○ pH 3.5; ◇ pH 4.0; ■ pH 4.5; ● pH 5.0; ◆ pH 6.0.

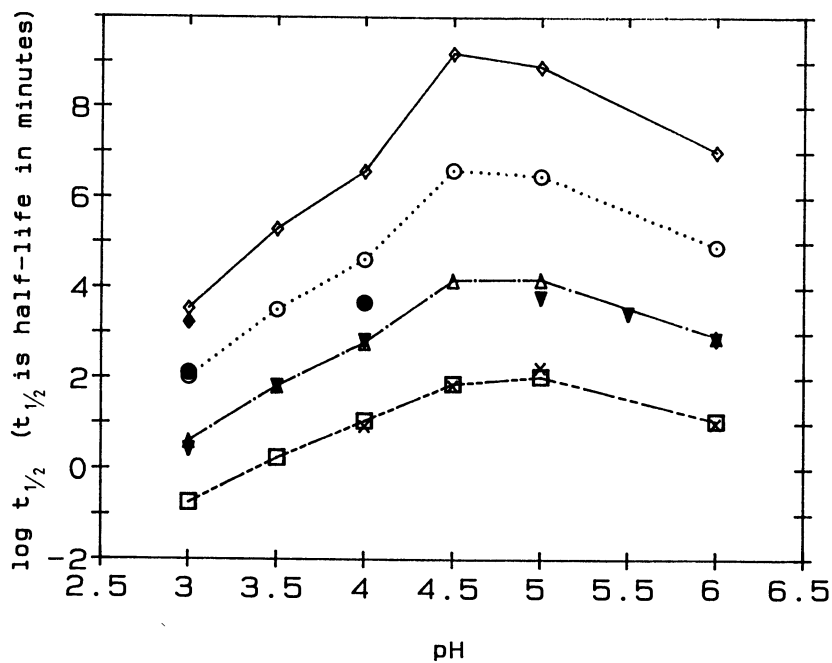


Figure 4. Half-lives of lignin peroxidase as a function of pH at different temperatures. Calculated half-lives: \diamond 30°C; \circ 40°C; \triangle 50°C; \square 60°C. Experimentally obtained half-lives: \blacklozenge 30°C; \bullet 40°C; \blacktriangledown 50°C; \times 60°C.

rapid inactivation. A total of 1.5 million units of lignin peroxidase was collected (Table I). After the first purification step with Q-Sepharose, the yield was 1.12 million units. The cultivation had to be discontinued when spaces between the sheets grew full of mycelia. At this stage both glucose, veratryl alcohol and nutrient addition failed to induce more enzyme production.

Purification. One main peak was observed with detection at 405 nm, when purified lignin peroxidase was analyzed by Mono Q chromatography. The retention time of the main peak was 11.8 min and its area was 98.9% of the total peak area. Possibly the enzyme solution contains, however, two isoenzymes which have very similar properties. The K_m of the purified lignin peroxidase for veratryl alcohol was 139 μ M on the basis of Eadie-Hofstee plot.

Effect of Temperature. The rate of inactivation obeys the Arrhenius equation well within the experimental range (Figure 3) and the plots do not intersect each other, although the slopes differ slightly in a regular manner. If the fitted lines are extrapolated, they intersect at high temperature (about 90°C), and inactivation of lignin peroxidase seems to become independent of pH. The half-lives calculated from the Arrhenius plots are close to the observed values between one and 1250 minutes. When the experimentally obtained half-life is long, the enzyme is slightly inactivated during incubation, and the determination of the half-life is very inaccurate.

Effect of pH. The pH vs. stability curves of Figure 4 are plotted using the half-lives calculated from the Arrhenius plots (Figure 3). Stability is expressed as log $t_{1/2}$ to make possible to visualize the curves at the temperature range (30-60°C). Maximum stability was achieved at pH 4.5 - 5.0. The stability of the enzyme at pH 3.0 which was used for the activity assay was much lower than the stability at pH 4.5.

Effect of Hydrogen Peroxide. Hydrogen peroxide had two different effects on lignin peroxidase. Firstly it caused a lag time in the assay of activity. The length of the lag time before the onset of the reaction varied depending on the hydrogen peroxide concentration, pH and contact time. After the lag time the reaction star-

Table II. Effect of Hydrogen Peroxide on Stability of Lignin Peroxidase

Concentration of H_2O_2 (mM)	Half-life of Lignin Peroxidase Activity (min)			
	pH 3.0		pH 5.0	
	0°C	10°C	0°C	10°C
0	Years ^a	Years ^a	Years ^a	Years ^a
0.2	153		325	
1.0	133	29	286	56
5.0	92	21	193	44
11.6	53		141	

^aEstimation

ted rapidly with the maximal rate. The rate was dependent on the contact time and conditions before the activity assay. The lowest concentration used (0.2 mM) reduced the half-life to a few hundred minutes. Further increase of hydrogen peroxide, up to 11.6 mM, shortened the half-life relatively little (Table II). Lignin peroxidase was inactivated more rapidly in the presence of H₂O₂ at pH 3.0 than at pH 5.0. At the latter pH, the half-lives at 0°C and 10°C were about two times longer. The half-lives at 0°C were nearly four times longer than at 10°C.

Effect of Veratryl Alcohol. At pH 5.0 the purified lignin peroxidase was not inactivated under the conditions tested. At pH 3.0 the enzyme lost its activity when incubated at 20°C for 38 days, and the presence of veratryl alcohol could not stabilize it. 100 mM veratryl alcohol even inactivated the enzyme to some extent. Ionic strength did not significantly affect the activities. The effect of veratryl alcohol was the same when unfractionated enzyme was used. This time the ionic strength in the activity assay mixture affected the activities, probably because one enzyme in the unfractionated preparation is sensitive to high ionic strength (12).

Discussion

In this study we have shown that the production of lignin peroxidases by *P.chrysosporium* can be scaled-up by using the ability of the fungus to grow on solid surfaces. More than 1 500 000 units were produced during one month's production period in a 1 m³ bioreactor involving a total of nine enzyme recovery cycles. The enzyme was conveniently concentrated by ultrafiltration and further purified by ion exchange chromatography.

A detailed scientific study on the properties of the five major isozymic forms of the lignin peroxidases produced in our pilot reactor has recently been published (12). Our purified enzyme in this study is composed of two isozymes having isoelectric points of 3.85 and 3.80 and molecular masses of 42 000. In this study we have characterized the enzyme's stability as an industrial product.

Lignin peroxidases are very stable when stored at pH 4.5 - 5.0 and low temperatures (< 30°C). Their pH stability optimum differs from the activity optimum which is close to 2.5 for most of the isozymes (12). However, the pH-optimum for lignin degradation is the same as the stability optimum for the enzyme, indicating that lignin peroxidase is not the rate limiting factor in lignin degradation. Furthermore some interesting reactions of lignin peroxidase like ester formation from veratryl alcohol methyl ether also proceed optimally at pH 4.5 (16).

Usually the kinetics of a chemical reaction follows the Arrhenius equation so that straight lines can be drawn over a wide temperature range. In this work it has been assumed that dependencies are linear, although the extreme points differ from the lines.

Aitken and Irvine (17) have recently reported on the stability characteristics of lignin peroxidases. They also have shown that the enzyme was most stable at pH 4.5, although higher pH values were not tested. The stability was dependent on protein concentration and veratryl alcohol had a stabilizing effect. The latter result was contrary to our experience.

Lignin peroxidases are rapidly inactivated in the presence of H_2O_2 . The mechanism has been studied in detail by Wariishi and Gold (18). The enzyme is overoxidized in the presence of excess H_2O_2 into an inactive compound III form which can be regenerated e.g. with veratryl alcohol. This was also shown in our experiments. Compound III can further be irreversibly inactivated when exposed to H_2O_2 for longer times (18) which explains why only a small part of the activity was recovered in the presence of veratryl alcohol. Due to oxygen radicals and substrate radicals (especially phenols), lignin peroxidases are also inactivated during their reaction cycle even when excess H_2O_2 is not present (19).

Literature Cited

1. Tien, M.; Kirk, T. K. *Science* 1983, 221, 661-663.
2. Glenn, J. K.; Morgan, M. A.; Mayfield, M. B.; Kuwahara, M.; Gold, M. H. *Biochem. Biophys. Res. Comm.* 1983, 114, 1077-1083.
3. Milgrom, L. *New Scientist* 1985, 16 may, 16-17.
4. Kuila, D.; Tien, M.; Fee, J. A.; Ondrias, M. R. *Biochemistry* 1985, 24, 3394-3397.
5. Haemmerli, S. D.; Leisola, M. S. A.; Fiechter, A. *FEMS Microbiol. Lett.* 1986, 35, 33-36.
6. Kirk, T. K.; Farrell, R. L. *Ann. Rev. Microbiol.* 1987, 41, 465-505.
7. Schoemaker, H. E.; Leisola, M. S. A. *J. Biotechnol.* 1990, 13, 101-109.
8. Leisola, M. S. A.; Meussdoerffer, F.; Waldner, R.; Fiechter, A. *J. Biotechnol.* 1985, 2, 379-382.
9. Leisola, M.; Fiechter, A. *FEMS Microbiol. Lett.* 1985, 29, 33-36.
10. Linko, Y.-Y.; Leisola, M. S. A.; Lindholm, N.; Troller, J.; Linko, P.; Fiechter, A. *J. Biotechnol.* 1986, 4, 283-291.
11. Leisola, M.; Thanei-Wyss, U.; Fiechter, A. *J. Biotechnol.* 1985, 3, 97-107.
12. Glumoff, T.; Harvey, P. J.; Molinari, S.; Goble, M.; Frank, G.; Palmer, J.; Smit, J. D. G.; Leisola, M. S. A. *Eur. J. Biochem.* 1990, 187, 515-520.
13. *RS/1 User's guide*; Bolt Beranek and Newman Inc. Cambridge, MA, 1984; Books 1-3.
14. *RS/1 User's guide*; Bolt Beranek and Newman Inc. Cambridge, MA, 1984; Book 2; pp 168-169.
15. Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters*; John Wiley and sons: New York, 1978; pp 460-461.
16. Schmidt, H.; Haemmerli, S.; Schoemaker, H. E.; Leisola, M. S. A. *Biochemistry* 1989, 28, 1776-1783.
17. Aitken, M. D.; Irvine, R. L. *Biotech. Bioeng.* 1989, 34, 1251-1260.
18. Wariishi, H.; Gold, M. H. *J. Biol. Chem.* 1990, 265, 2070-2077.
19. Harvey, P. J.; Palmer, J. M. *J. Biotechnol.* 1990, 13, 169-180.

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Chapter 19

Chemistry of Lignin Degradation by Lignin Peroxidases

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The chemistry of degradation of lignin by lignin peroxidases from a white-rot fungus, *Phanerochaete chrysosporium*, is reviewed. The enzyme catalyzes side chain cleavage and aromatic ring cleavage of lignin substructure model compounds and lignin preparations. The mechanisms of these reactions are explained on the basis of initial single-electron oxidation of aromatic rings by the enzyme. Degradative reactions of protolignin in wood by the fungus can be attributed to the enzyme.

Lignin biodegradation research has been much developed during the last 15 years, and has evoked much interest in recent years (1,2). Many of these studies have been directed towards establishing the exact chemical mechanisms involved in lignin biodegradation. In these mechanistic studies, simpler lignin substructure model dimers and trimers have been used frequently (3-5), since lignin is a complex and heterogeneous polymer having various intermonomer linkages as well as three different monomeric units. On the other hand, polymeric lignin preparations such as dehydrogenation polymers (DHP), milled wood lignins (MWL) and wood per se are not suitable for the detailed mechanistic studies, but studies of their degradation provide outlines of lignin biodegradation (6). Both the approaches with the substructure models and with the polymers are complementary, and have contributed to the elucidation of the chemistry of lignin biodegradation.

In 1978, Kirk and coworkers established the optimal culture parameters for lignin degradation of the lignin degrading white-rot basidiomycete *Phanerochaete chrysosporium* (7). Later, Tien and Kirk, and Glenn et al. reported the isolation of enzymes from ligninolytic cultures of the fungus (8,9), which are nowadays called ligninase or lignin peroxidase. This enzyme catalyzes the side chain and aromatic ring cleavages of lignin substructure models (8-11), veratryl alcohol (12) and polymeric lignin preparations (8,13,14). The degradative

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reactions of protolignin by *P. chrysosporium* can also be attributed to this enzyme. The mechanisms of these degradative reactions were elucidated based on the experiments with lignin substructure model compounds (15-17). In this review, the chemistry of lignin degradation by lignin peroxidases is discussed.

Degradation of Lignin Substructure Model Compounds by Ligninolytic Cultures of *Phanerochaete chrysosporium*

Since the determination of optimal culture parameters for lignin degradation by *P. chrysosporium* (7), many experiments with lignin substructure model compounds were conducted to find out the reactions to be used for isolation and assay of ligninolytic enzyme activities, and to examine the reaction mechanisms (3-5). In these substructure model studies, β -0-4 lignin substructure models have been used most frequently, since the substructure is the most frequent among lignin substructures (18).

First, Weinstein et al. demonstrated that several of the culture parameters which affect lignin degradation by the fungus have similar effects on the degradation of β -0-4 lignin substructure model dimers (19). Several groups reported the side chain cleavages of β -0-4 dimers, such as 1 and 2, by ligninolytic cultures of the fungus (i.e. optimized for lignin degradation) (19-26). Identified products were as follows (Figure 1A): arylglycerol 10 and α -oxoaryl glycerol 11 from β -0-4 bond cleavage; 4-ethoxy-3-methoxybenzyl alcohol 6, 2-(2-methoxyphenoxy)ethanol 8 and guaiacol 9 from C α -C β cleavage; 1,3-dihydroxy-2-(2-methoxyphenoxy)propane 13 from alkyl-phenyl cleavage; and the α -oxodimer 12 from oxidation at the α (benzyl) position (Figure 1A).

On the other hand, we found that *P. chrysosporium* catalyzes the aromatic ring cleavage of the β -0-4 dimers (27,28). The identified aromatic ring cleavage products are the β , γ -cyclic carbonate of arylglycerol 14, α , β -cyclic carbonate of arylglycerol 15, γ -formate of arylglycerol 16, and methyl oxalate of arylglycerol 17 (Figure 1A).

Most of the degradation products shown in Figure 1A were also detected in the degradation of the β -0-4 dimers by other white-rot fungi, *Coriolus versicolor* (29,30) and *Coriolus hirsutus* (31).

For the side chain cleavage mechanisms, our series of experiments with stable isotopes showed the following results: (i) In β -0-4 bond cleavage to give the arylglycerol 10, deuterium atoms at both C α and C β positions were retained quantitatively (22,32) and the β -oxygen of the arylglycerol 10 and phenolic oxygen of guaiacol 9 were derived quantitatively from the β -0-4 bond oxygen of 1 (Figure 1B) (33). These results clearly show that the arylglycerol 10 was formed via O-C4 cleavage of the β -0-4 bond with retention of hydrogen (deuterium) atoms at C α and C β positions, and that guaiacol 9 was formed via C β -O cleavage of the β -0-4 bond. (ii) Direct hydrolysis of the β -0-4 bond giving rise to the arylglycerol 10 was eliminated, because ^{18}O from H_2^{18}O was not incorporated into the C β position of the arylglycerol 10 (Figure 1B) (24). (iii) 2-(2-Methoxyphenoxy)ethanol 8 was formed via migration of the β -methoxyphenoxy group of 1 from the β -carbon atom to the γ -carbon atom (Figure 1C) (34).

Next to β -0-4 lignin substructure model compounds, β -1 lignin substructure model compounds have been often used for lignin biodegradation studies. This type of compound was found to be degraded

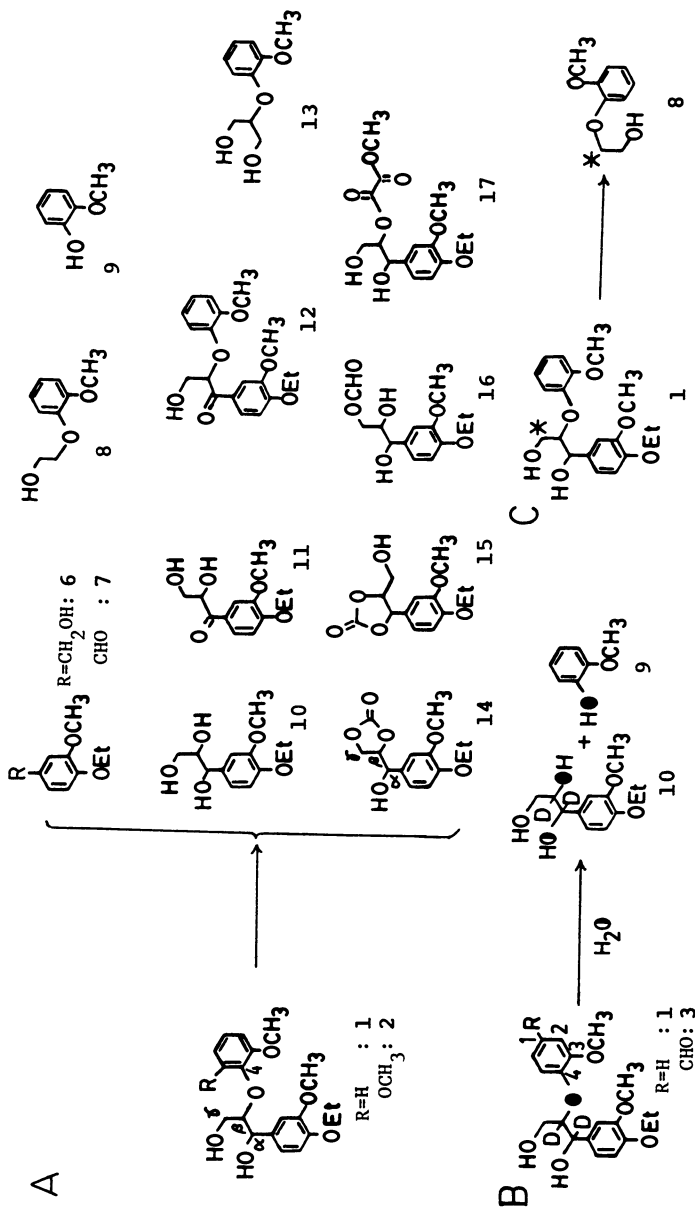


Figure 1. β -O-4 lignin substructure model compounds and products of their degradation by ligninolytic cultures of *Phanerochaete chrysosporium* and by lignin peroxidase. D: ^2H , \bullet and \circ : ^{18}O of β -ether bond and of H_2^{18}O , respectively. B and C show results of stable isotope experiments.

via C α -C β cleavage (for both the non-phenolic and phenolic substrate) and alkyl-phenyl cleavage (for the phenolic substrate) by *P. chrysosporium* (35-38).

Importantly, most of these degradative reactions of lignin substructure models were also suggested to occur in the degradation of protolignin in wood (6).

Degradation of Lignin Substructure Model Compounds by Lignin Peroxidase from *Phanerochaete chrysosporium*

Side Chain and Aromatic Ring Cleavage by Lignin Peroxidase. In 1983, two groups isolated lignin peroxidase (ligninase) from ligninolytic cultures of *P. chrysosporium* (8,9). At first they reported that the enzyme cleaves the C α -C β and β -0-4 bonds of β -0-4 and β -1 lignin substructure models (8,9). Subsequently, we found that the enzyme cleaves the aromatic ring of β -0-4 dimers and a trimer (10,11,39), and Leisola et al. discovered the aromatic ring cleavage of veratryl alcohol by the enzyme (12). The degradation products from the β -0-4 and β -1 dimers and the trimer by the enzyme were almost the same as those by the intact cultures of the fungus (8-11,38-44). From β -0-4 dimers involving 1 and 2, the following products were identified: from C α -C β cleavage, 4-ethoxy-3-methoxybenzaldehyde 7 and phenols derived from the phenoxy group attached at the β -position of the β -0-4 dimer; from β -0-4 bond cleavage, arylglycerol 10 and α -oxoaryl-glycerol 11; from α -oxidation, α -oxo- β -0-4 dimer; and from aromatic ring cleavage, 14, 15, 16, and 17 (Figure 1A).

4-Ethoxy-3-methoxybenzyl alcohol 6, 2-(2-methoxyphenoxy)ethanol 8 and 1,3-dihydroxy-2-(2-methoxyphenoxy)propane 13 were formed from the β -0-4 dimer 1 in intact cultures, but not identified in the *in vitro* system. They are probably formed secondarily by reduction of initial cleavage products formed by the enzyme. In fact, this fungus reduces 4-ethoxy-3-methoxybenzaldehyde 7 and 2-benzyloxyacetaldehyde to the corresponding alcohols, 4-ethoxy-3-methoxybenzyl alcohol 6 and a compound similar to 8, 2-benzyloxyethanol, respectively (23).

Stable isotope experiments similar to those with the intact cultures shown in Figure 1B and 1C were conducted with lignin peroxidase, and the same results as those obtained with the intact cultures were reproduced with the enzyme system (16,42,45,46).

Thus, three important degradative reactions suggested by the studies of chemical analysis of decayed lignin, namely C α -C β cleavage, β -0-4 bond cleavage and aromatic ring cleavage, were demonstrated in degradation of lignin substructure model dimers both by intact ligninolytic cultures of *P. chrysosporium* and by lignin peroxidases. The chemical structures of the products and the stable isotope experiments showed that the degradation of lignin substructure model compounds by intact cells of *P. chrysosporium* is attributed to lignin peroxidases.

Mechanism of Degradation of Lignin Substructure Models by Lignin Peroxidase. Based on the results of the isotope experiments described in the preceding sections and the important findings that lignin peroxidase produces cation radicals from methoxylated benzenes including a lignin substructure model dimer (15,47), mechanisms of C α -C β cleavage and β -0-4 bond cleavage of non-phenolic lignin substructure models by lignin peroxidase were proposed. The proposed

mechanisms for two types of $\text{C}\alpha$ - $\text{C}\beta$ cleavage are shown in Figure 2A. One of them involves initial single-electron oxidation of the B-ring of **1** giving rise to the corresponding cation radical. The electrophilic cation radical formed, in turn, is attacked by a nucleophile, the γ -hydroxyl group, causing migration of the B-ring. Subsequent $\text{C}\alpha$ - $\text{C}\beta$ cleavage gives products **7** and **19** (5,34). The other is initiated by single-electron oxidation of the A-ring. The A-ring derived cation radical intermediate then undergoes cleavage at the $\text{C}\alpha$ - $\text{C}\beta$ bond to give rise to the products **7** and **9** (5,15,16).

Figure 2B shows the proposed mechanisms for β -0-4 bond cleavage. This involves initial single-electron oxidation of the B-ring of **1**. The cation radical intermediate is then attacked by water (pathway I) (15,16) or by the α -hydroxyl group (pathway II) (5). Subsequent decomposition of the intermediates gives rise to the β -0-4 bond cleavage product, arylglycerol **10**.

As for the aromatic ring cleavage mechanisms, we identified another ring cleavage product of β -0-4 dimer **4**, namely the methyl muconate of arylglycerol **18**, which retains all the six carbon atoms derived from the cleaved ring, and, therefore, is suitable for the elucidation of the ring cleavage mechanism (Figure 3) (43). For elucidation of the aromatic ring cleavage mechanism, we investigated several stable isotope experiments: ^{13}C tracer experiments confirmed that **14**-**18** are ring cleavage products (11,43). Experiments with a deuterium labeled β -0-4 dimer showed that demethylation or demethoxylation is not a prerequisite for ring cleavage by the enzyme (11). Oxygen atoms are incorporated into aromatic ring cleavage products from H_2^{18}O and/or $^{18}\text{O}_2$ (Figure 3) (11,17).

Based on the isotope experiments, we proposed mechanisms for aromatic ring cleavage of β -0-4 lignin substructure models as shown in Figure 3. Again, the initial reaction is a single-electron oxidation by lignin peroxidase to give rise to a cation radical intermediate. The intermediate is then attacked by a nucleophile, water, and the resulting radical reacts with dioxygen to give the muconate **18** (5,17). Formation of other ring cleavage products was also explained in the same way (5,17). Later, Miki et al. (48) proposed similar mechanisms for ring cleavage of a β -0-4 dimer by the enzyme. Mechanisms similar to the formation of the muconate shown in Figure 3 were also proposed for the lignin peroxidase catalyzed ring cleavage of veratryl alcohol (49-51).

Mechanisms for other reactions of non-phenolic substrates catalyzed by the enzyme are also understood on the basis of cation radical intermediates (15,52).

Degradation of phenolic lignin substructure models by the enzyme is explained on the basis of initial phenoxy radical formation (38), which has been substantiated by ESR spectroscopy (53). The phenoxy-radical intermediates are degraded to give rise to $\text{C}\alpha$ - $\text{C}\beta$ cleavage products, alkyl-phenyl cleavage products and $\text{C}\alpha$ -oxidation products (38). Also, this type of degradation of phenolic substrates can be mediated by manganese peroxidases (54) and laccases (4,55-58).

Thus, almost all the reactions of lignin substructure model dimers by the enzyme are explained on the basis of cation radical intermediates and their subsequent reactions with nucleophiles such as H_2O and intramolecular hydroxyl groups, and with radicals such as dioxygen (for non-phenolic substrates), or on the basis of phenoxy radical intermediates (for phenolic substrates).

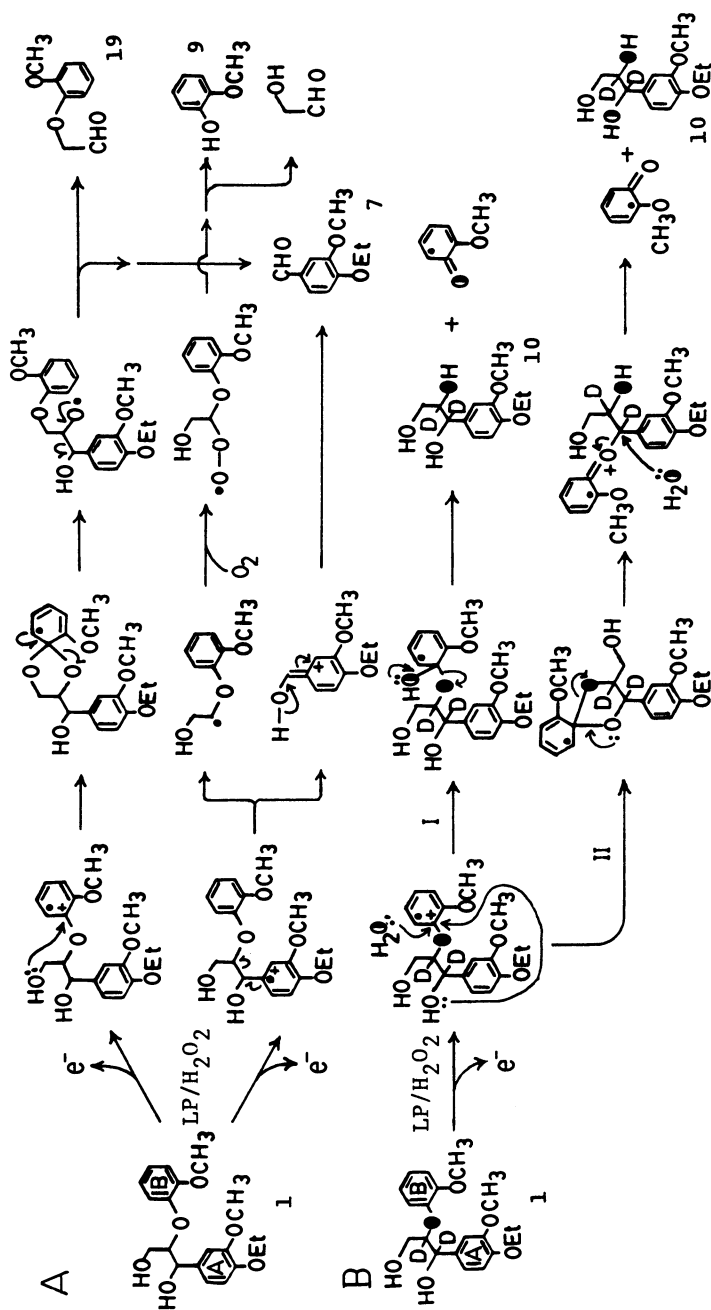


Figure 2. Mechanisms for α -C8 bond cleavage and β -O-4 bond cleavage of β -O-4 dimer 1 by lignin peroxidase/ H_2O_2 system. A: α -C8 cleavage; B: β -O-4 bond cleavage. LP: lignin peroxidase, D: 2H , \bullet and O ; ^{18}O of β -O-4 bond oxygen and of $H_2^{18}O$, respectively.

Lignin peroxidases were isolated from other white-rot fungi such as *Coriolus (Trametes) versicolor* (59,60) and *Phlebia radiata* (61,62). Recently, Kawai et al. showed the degradation of a β -0-4 dimer by a lignin peroxidase preparation from *C. versicolor*, and proposed mechanisms similar to those shown in Figures 2A, 2B and 3 (63).

Degradation of Polymeric Lignin Preparations by Lignin Peroxidases

As described above, the substructure model approach has been used successfully to elucidate the mechanisms of chemical reactions catalyzed by lignin peroxidase. This approach is based on the assumption that reactivities of specific functional groups of the substructure model compounds toward lignin peroxidase are similar to those of the functional groups of protolignin. However, the actual reactivity of protolignin may be influenced by factors other than the reactivity of specific functional groups, such as the polymeric nature of protolignin and intimate coexistence of protolignin with hemicelluloses. These possible factors specific to protolignin are being examined: Experiments with polymeric lignin preparations have been used to investigate (i) the change of molecular weight of the lignin preparation by the enzyme and (ii) the identification of low molecular weight degradation products.

To assess question (ii), we synthesized a synthetic lignin 5 (dehydrogenation copolymer, DHcP, composed of coniferyl alcohol and a $(\beta$ -0-4)- $(\beta$ - β) lignin substructure model trimer) (5,13,14). This DHcP 5 was incubated in the presence of veratryl alcohol with lignin peroxidase, giving rise to aromatic ring cleavage products 14, 15, 16 and 17, β -0-4 bond cleavage products 10 and 11, and $\text{C}\alpha$ - $\text{C}\beta$ cleavage product 7 (Figure 4). All of the degradation products had been identified as products of degradation of β -0-4 dimers (10,11) and a $(\beta$ -0-4)- $(\beta$ -0-4) trimer (39) by the enzyme (c.f. Figure 1A). This result clearly shows that lignin peroxidase degrades polymeric lignin preparation and that, based on the structure of the degradation products, the mechanisms of the reactions producing these products are similar to those for the substructure models shown in Figures 2A, 2B and 3. It is now apparent that the degradative cleavages in protolignin ($\text{C}\alpha$ - $\text{C}\beta$ cleavage, β -0-4 bond cleavage, and aromatic ring cleavage) can be attributed to lignin peroxidases.

Veratryl alcohol, which is biosynthesized by *P. chrysosporium* (64,65) and *C. versicolor* (66), was required for the formation of the degradation products from the DHcP 5. This compound also enhanced the degradation of the β -0-4 dimer 2, although the dimer was degraded by the enzyme without veratryl alcohol (14) as previously reported (10,11). Acceleration by veratryl alcohol of lignin peroxidase oxidation of several compounds was reported (45,67-69). Possible roles of veratryl alcohol as a single electron mediator between the enzyme and substrates, and as a protective reagent against inactivation of the enzyme, were proposed (67,68,70). These may explain the role of veratryl alcohol in the degradation of the DHcP and β -0-4 dimer 2 by the enzyme, but it is still inconclusive, and further investigations are required in the role of veratryl alcohol.

Depolymerization of ^{14}C -methylated spruce lignin and ^{14}C -methylated birch MWL by lignin peroxidase was reported in the paper describing the discovery of the enzyme (8). Glenn et al. also

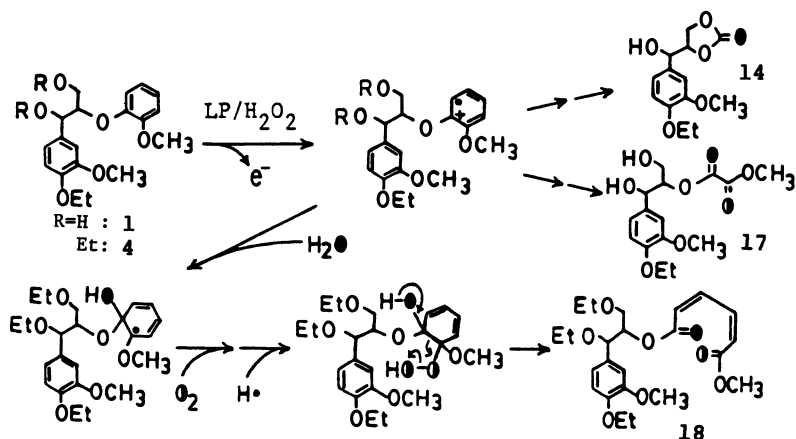


Figure 3. Mechanism for aromatic ring cleavage of β -O-4 dimer by lignin peroxidase/ H_2O_2 system. \bullet and \circ : ^{18}O of $H_2^{18}O$ and $^{18}O_2$, respectively. LP:lignin peroxidase.

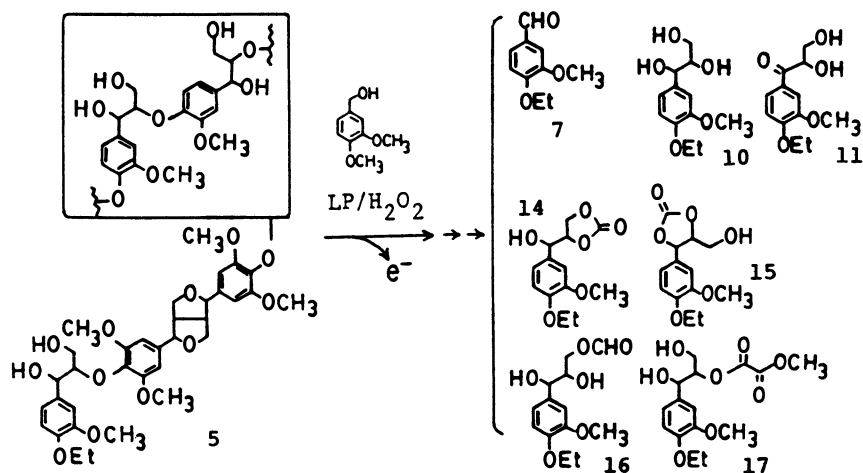


Figure 4. Degradation of DHcP 5 by lignin peroxidase/ H_2O_2 system. In DHcP 5, the portion enclosed with rectangular represents the portion derived from coniferyl alcohol. LP:lignin peroxidase.

mentioned the degradation of a ^{14}C -lignin by the enzyme (9). On the other hand, Haemmerli et al. reported the polymerization of lignin preparations (alkali-isolated straw lignin, dioxane-HCl-isolated straw lignin, and spruce MWL) by lignin peroxidase (69). Similar results were obtained with a synthetic [ring- ^{14}C]lignin (53).

Lignin peroxidase was shown to produce a phenoxy radical from acetosyringone by ESR spectroscopy (53) and to polymerize phenolic compounds such as guaiacol (53) and coniferyl alcohol (71). Accordingly, the further polymerization of the free phenolic, polymeric lignin preparations by the enzyme is probably due to phenolic coupling. On the other hand, the ligninolytic cultures of *P. chrysosporium* degraded non-methylated (free phenolic) lignin preparations (25,72), suggesting an inadequate assay condition and/or existence of a system preventing the polymerization in the intact cultures.

Conclusion

In conclusion, degradative reactions of lignin substructure models in ligninolytic cultures of *P. chrysosporium* are attributed to lignin peroxidase. The enzyme degrades synthetic lignins as well as lignin substructure models, giving rise to $\text{C}\alpha$ - $\text{C}\beta$ cleavage products, β -0-4 bond cleavage products, and aromatic ring cleavage products. Degradative reactions ($\text{C}\alpha$ - $\text{C}\beta$ cleavage, β -0-4 bond cleavage and aromatic ring cleavage) of protolignin by the fungus can be attributed to lignin peroxidase. The mechanisms of degradation by the enzyme are explained by the initial single-electron oxidation of aromatic rings by the enzyme and subsequent decomposition of the cation radicals formed. Also, the results obtained so far show that lignin peroxidase depolymerizes methylated lignin preparations, while the enzyme polymerizes free-phenolic lignin preparations. Further study is required to avoid the polymerization.

Literature Cited

1. *Biosynthesis and Biodegradation of Wood Components*; Higuchi T.; Academic Press: Orlando, FL, 1985.
2. Kirk, T.K.; Farrell, R.L. *Annual Rev. Microbiol.* 1987, 41, 465.
3. Higuchi, T. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: Orlando, FL, 1985, pp 557-578.
4. Higuchi, T. *Wood Research* 1986, No. 73, 58.
5. Umezawa, T. *Wood Research* 1988, No. 75, 21.
6. Chen, C.-L.; Chang, H.-m. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: Orlando, FL, 1985; pp 535-556.
7. Kirk, T.K.; Schultz, E.; Connors, W.J.; Lorenz, L.F.; Zeikus, J. *G. Arch. Microbiol.* 1978, 117, 277.
8. Tien, M.; Kirk, T.K. *Science* 1983, 221, 661.
9. Glenn, J.K.; Morgan, M.A.; Mayfield, M.B.; Kuwahara, M.; Gold, M.H. *Biochem. Biophys. Res. Commun.* 1983, 114, 1077.
10. Umezawa, T.; Shimada, M.; Higuchi, T.; Kusai, K. *FEBS Lett.* 1986, 205, 287.
11. Umezawa, T.; Higuchi, T. *FEBS Lett.* 1986, 205, 293.
12. Leisola, M.S.A.; Schmidt, B.; Thanei-Wyss, U.; Fiechter, A. *FEBS Lett.* 1985, 189, 267.

13. Umezawa, T.; Higuchi, T. *FEBS Lett.* **1989**, *242*, 325.
14. Umezawa, T.; Higuchi, T. *Mokuzai Gakkaishi* **1989**, *35*, 1014.
15. Kirk, T.K.; Tien, M.; Kersten, P.J.; Mozuch, M.D.; Kalyanaraman, B. *Biochem. J.* **1986**, *236*, 279.
16. Miki, K.; Renganathan, V.; Gold, M.H. *Biochemistry* **1986**, *25*, 4790.
17. Umezawa, T.; Higuchi, T. *FEBS Lett.* **1987**, *218*, 255.
18. Sakakibara, A. *Wood Sci. Technol.* **1980**, *14*, 89.
19. Weinstein, D.A.; Krisnangkura, K.; Mayfield, M.B.; Gold, M.H. *Appl. Environ. Microbiol.* **1980**, *39*, 535.
20. Enoki, A.; Goldsby, G.P.; Gold, M.H. *Arch. Microbiol.* **1980**, *125*, 227.
21. Enoki, A.; Goldsby, G.P.; Gold, M.H. *Arch. Microbiol.* **1981**, *129*, 141.
22. Umezawa, T.; Nakatsubo, F.; Higuchi, T. *Agric. Biol. Chem.* **1983**, *47*, 2677.
23. Umezawa, T.; Higuchi, T.; Nakatsubo, F. *Agric. Biol. Chem.* **1983**, *47*, 2945.
24. Umezawa, T.; Higuchi, T. *Agric. Biol. Chem.* **1984**, *48*, 1917.
25. Fenn, P.; Kirk, T.K. *J. Wood Chem. Technol.* **1984**, *4*, 131.
26. Yokota, S.; Umezawa, T.; Higuchi, T. *Mokuzai Gakkaishi*, **1988**, *34*, 65.
27. Umezawa, T.; Higuchi, T. *FEBS Lett.* **1985**, *182*, 257.
28. Umezawa, T.; Kawai, S.; Yokota, S.; Higuchi, T. *Wood Research* **1986**, *No. 73*, 8.
29. Kawai, S.; Umezawa, T.; Higuchi, T. *Agric. Biol. Chem.* **1985**, *49*, 2325.
30. Kawai, S.; Umezawa, T.; Higuchi, T. *Appl. Environ. Microbiol.* **1985**, *50*, 1505.
31. Yoshihara, K.; Umezawa, T.; Higuchi, T.; Nishiyama, M. *Agric. Biol. Chem.* **1988**, *52*, 2345.
32. Umezawa, T.; Higuchi, T. *Wood Research* **1985**, *No. 71*, 25.
33. Umezawa, T.; Higuchi, T. *FEMS Microbiol. Lett.* **1985**, *26*, 123.
34. Umezawa, T.; Higuchi, T. *FEBS Lett.* **1985**, *192*, 147.
35. Nakatsubo, F.; Reid, I.D.; Kirk, T.K. *Biochim. Biophys. Acta* **1982**, *719*, 284.
36. Enoki, A.; Gold, M.H. *Arch. Microbiol.* **1982**, *132*, 123.
37. Kamaya, Y.; Higuchi, T. *Mokuzai Gakkaishi* **1984**, *30*, 237.
38. Yokota, S.; Umezawa, T.; Kawai, S.; Higuchi, T. *Holzforschung* **1990**, in press.
39. Umezawa, T.; Higuchi, T. *Mokuzai Gakkaishi*, **1988**, *34*, 929.
40. Tien, M.; Kirk, T.K. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 2280.
41. Gold, M.H.; Kuwahara, M.; Chiu, A.A.; Glenn, J.K. *Arch. Biochem. Biophys.* **1984**, *234*, 353.
42. Habe, T.; Shimada, M.; Umezawa, T.; Higuchi, T. *Agric. Biol. Chem.* **1985**, *49*, 3505.
43. Umezawa, T.; Higuchi, T. *Agric. Biol. Chem.* **1987**, *51*, 2281.
44. Miki, K.; Renganathan, V.; Mayfield, M.B.; Gold, M.H. *FEBS Lett.* **1987**, *210*, 199.
45. Kuwahara, M.; Glenn, J.K.; Morgan, M.A.; Gold, M.H. *FEBS Lett.* **1984**, *169*, 247.
46. Miki, K.; Renganathan, V.; Gold, M.H. *FEBS Lett.* **1986**, *203*, 235.
47. Kersten, P.J.; Tien, M.; Kalyanaraman, B.; Kirk, T.K. *J. Biol. Chem.* **1985**, *260*, 2609.

48. Miki, K.; Kondo, R.; Renganathan, V.; Mayfield, M.B.; Gold, M.H. *Biochemistry* **1988**, *27*, 4787.
49. Shimada, M.; Hattori, T.; Umezawa, T.; Higuchi, T.; Uzura, K. *FEBS Lett.* **1987**, *221*, 327.
50. Haemmerli, S.D.; Schoemaker, H.E.; Schmidt, H.W.H.; Leisola, M.S.A. *FEBS Lett.* **1987**, *220*, 149.
51. Schmidt, H.W.H.; Haemmerli, S.D.; Schoemaker, H.E.; Leisola, M.S.A. *Biochemistry* **1989**, *28*, 1776.
52. Kawai, S.; Umezawa, T.; Higuchi, T. *FEBS Lett.* **1987**, *210*, 61.
53. Odier, E.; Mozuch, M.D.; Kalyanaraman, B.; Kirk, T.K. *Biochim.* **1988**, *70*, 847.
54. Wariishi, H.; Valli, K.; Gold, M.H. *Biochemistry* **1989**, *28*, 6017.
55. Kirk, T.K.; Harkin, J.M.; Cowling, E.B. *Biochim. Biophys. Acta* **1968**, *165*, 145.
56. Wariishi, H.; Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 892.
57. Kawai, S.; Umezawa, T.; Shimada, M.; Higuchi, T.; Koide, K.; Nishida, T.; Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 792.
58. Kawai, S.; Umezawa, T.; Shimada, M.; Higuchi, T. *FEBS Lett.* **1988**, *236*, 309.
59. Dodson, P.J.; Evans, C.S.; Harvey, P.J.; Palmer, J.M. *FEMS Microbiol. Lett.* **1987**, *42*, 17.
60. Jönsson, L.; Johansson, T.; Sjöström, K.; Nyman, P.O. *Acta. Chem. Scand.* **1987**, *B41*, 766.
61. Kantelinen, A.; Waldner, R.; Niku-Paavola, M.-L.; Leisola, M. S.A. *Appl. Microbiol. Biotechnol.* **1988**, *28*, 193.
62. Niku-Paavola, M.-L.; Karhunen, E.; Salola, P.; Raunio, V. *Biochem. J.* **1988**, *254*, 877.
63. Kawai, S.; Shoji, S.; Nabeta, K.; Okuyama, H.; Higuchi, T. *Mokuzai Gakkaishi* **1990**, *36*, 126.
64. Lundquist, K.; Kirk, T.K. *Phytochem.* **1978**, *17*, 1676.
65. Shimada, M.; Nakatsubo, T.; Kirk, T.K.; Higuchi, T. *Arch. Microbiol.* **1981**, *129*, 321.
66. Kawai, S.; Umezawa, T.; Higuchi, T. *Wood Research* **1986**, *No. 73*, 18.
67. Harvey, P.J.; Schoemaker, H.E.; Palmer, J.M. *FEBS Lett.* **1986**, *195*, 242.
68. Haemmerli, S.D.; Leisola, M.S.A.; Sanglard, D.; Fiechter, A. *J. Biol. Chem.* **1986**, *261*, 6900.
69. Haemmerli, S.D.; Leisola, M.S.A.; Fiechter, A. *FEMS Microbiol. Lett.* **1986**, *35*, 33.
70. Wariishi, H.; Gold, M.H. *FEBS Lett.* **1989**, *243*, 165.
71. Razal, R.A. Ph. D. thesis **1990** (Virginia Polytechnic Institute and State University, Virginia, U.S.A.).
72. Chua, M.G.S.; Choi, S.; Kirk, T.K. *Holzforschung*, **1983**, *37*, 55.

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Chapter 20

Enzymatic Lignin Degradation

An Extracurricular View

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Unprecedented reputability was conferred upon the field of lignin biodegradation in 1983 by the reported isolation from the white-rot fungus *Phanerochaete chrysosporium* of an extracellular lignin peroxidase. Yet this enzyme polymerizes lignin preparations and low molecular weight phenols *in vitro*, and lignin peroxidase activity does not seem to be a prerequisite for lignin degradation *in vivo* after all. Nevertheless introduction of the enzyme into *P. chrysosporium* cultures accelerates the overall lignin biodegradative process. The dilemma might find resolution in a simple working hypothesis: the function of lignin peroxidase could lie chiefly in the polymerization of potentially toxic phenolic compounds into molecular species sufficiently large that they can no longer penetrate through the fungal cell wall; concomitantly the enzyme may introduce into the macromolecular lignin fabric functional groups that render the polymeric structure more susceptible towards subsequent degradation by another enzyme. The polymerization of lignins in the presence of lignin peroxidase arises from the coupling of phenoxy radicals derived from free phenolic moieties; consequently fungal laccases and Mn^{II}-dependent peroxidases would be expected to engender similar effects. It would appear, therefore, that the enzyme responsible for lignin depolymerization *in vivo* has eluded identification.

Among the naturally occurring polymers, lignins are second only to cellulose in abundance, and in terms of energy content they might actually be the most abundant. They are found as cell-wall components in all dry-land arborescent and herbaceous plants. Native lignin macromolecules are generally thought to be constituted by an essentially random proportionate distribution of ten different linkages between *p*-hydroxyphenylpropane units (1). They are not uncommonly described as complicated aromatic polymers that "glue" woody plant cells together and afford some measure of protection against microbial attack.

In gymnosperms, the primary lignin precursors are the two monolignols coniferyl and *p*-coumaryl alcohols, while in angiosperms sinapyl alcohol is also a participant in

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the biosynthetic pathway (2). On the other hand, the lignins of grasses and cereals, in addition to units derived from the three primary monolignol precursors, contain some covalently bound *p*-hydroxycinnamic (*viz.* *p*-coumaric and ferulic) acids (3). There are, furthermore, significant variations among lignins, in their monomer composition and frequency of interunit linkages, with cell type (4) and morphological region of the plant cell wall (5, 6).

Lignins, then, as a group of abundant biopolymers embodying some significant diversity, occupy a pivotal position in the carbon cycle of the biosphere. It is indeed hardly surprising that the subject of lignin biodegradation has commanded attention for a considerable period of time (7). The quest for the biochemical determinants of lignin decomposition in nature has been remarkably fruitful during the past twelve years. The isolation, reported in 1983 from the white-rot fungus *Phanerochaete chrysosporium*, of an enzyme considered to exhibit ligninolytic activity represented a turning point in the field. The intensive research effort prompted by this claim constitutes a fascinating story of such breadth that its many facets cannot be fully documented in the space permitted by a brief appreciation of its substance. The work itself, which has been carried out predominantly by nine different groups in the United States, Japan, Sweden, Switzerland, France and England, is unquestionably of a high order. Yet profoundly important questions remain. The most central of these is the focus of the present article.

Lignin Degradation by Hydroxyl Radicals

It was surmised in 1980 (8) that highly reactive species derived from oxygen, rather than direct enzyme action, might be responsible for the initial depolymerizing transformations opening onto the pathways through which white-rot fungi convert lignins to CO₂. A correlation was soon discovered between ligninolytic activity and the production of H₂O₂ by *P. chrysosporium* cell extracts (9); affirmation of a causal connection received support from the inhibitory effect of catalase towards lignin biodegradation by the microorganism (10, 11).

The species invoked as primarily responsible for the initial depolymerization of lignins were hydroxyl radicals formed by the single-electron reduction of H₂O₂ (9, 10). The plausibility of the working hypothesis was reinforced by their effect upon a dehydropolymerizate from [2-¹⁴C]coniferyl alcohol: as shown in Figure 1, essentially complete degradation of such a 17 gL⁻¹ preparation was achieved with the (Fenton's reagent) hydroxyl radical generating system 1 M H₂O₂/10mM FeSO₄ (12). Indeed hydroxyl radical scavengers were found to inhibit lignin biodegradation by *P. chrysosporium* (10). In August 1983, however, the first reports about a lignin degrading enzyme from *P. chrysosporium* were published (13, 14). The impact on the field was overwhelming.

Lignin Peroxidase

The original accounts about lignin peroxidase (13, 14) seemed to indicate that the enzyme was capable of degrading two quite different kinds of lignin preparation. In the presence of concentrated extracellular *P. chrysosporium* culture fluid containing 0.05 gL⁻¹ protein with 0.2 mM H₂O₂ at pH 3.0, 22% of a ¹⁴C-methylated aqueous acetone extract from spruce (*Picea engelmannii* Parry) wood appeared to undergo depolymerization in 1 h at 37° C (Figure 2). The enzymatic activity responsible for the effect was assigned to a single polyacrylamide gel electrophoretic band because veratraldehyde was liberated, albeit at a severely attenuated rate, from the purified spruce wood extract

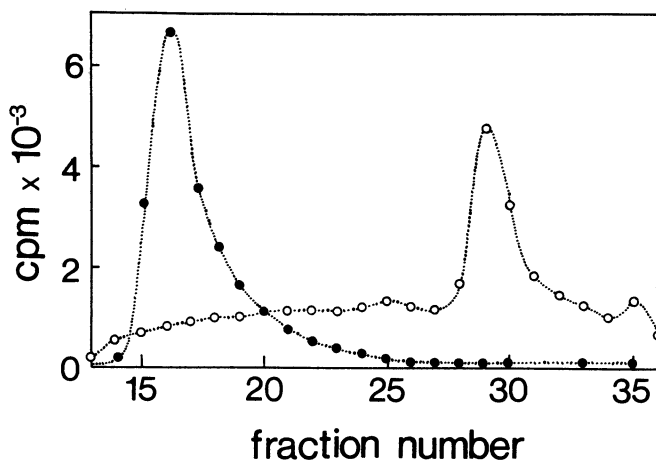


Figure 1. Degradation (○) of 17 gL^{-1} dehydropolymerizate (●) from $[2\text{-}^{14}\text{C}]$ coniferyl alcohol by hydroxyl radicals generated from $1 \text{ M H}_2\text{O}_2/10 \text{ mM FeSO}_4$. Sphadex LH20/DMF elution profiles adapted and redrawn from reference 12.

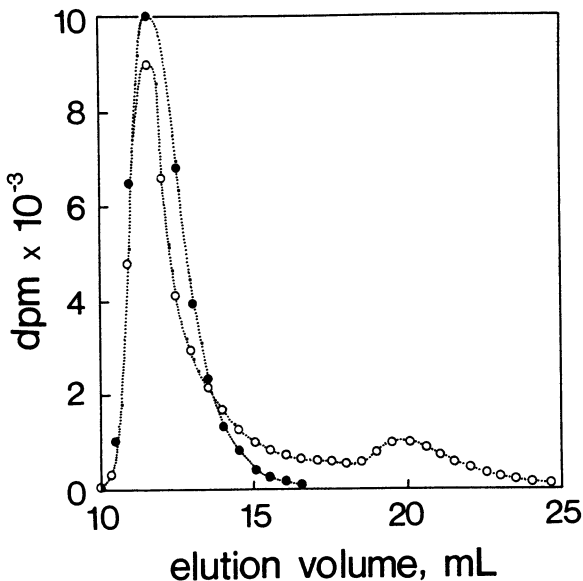


Figure 2. Degradation (○) of ^{14}C -methylated aqueous acetone spruce wood extract (●) in presence of concentrated extracellular *P. chrysosporium* culture fluid at pH 3.0 containing 0.05 gL^{-1} protein with $0.2 \text{ mM H}_2\text{O}_2$ during 1 h at 37°C . Sphadex LH20/DMF elution profiles adapted and redrawn from reference 13.

(13). Alternatively employing glucose oxidase (0.02 units mL⁻¹)/glucose (3 mM)/dioxygen as a means of producing H₂O₂, at pH 4.5 the enzymes in the extracellular medium of *P. chrysosporium* cultures engendered 10% degradation during 18 h at 37°C (14) of 0.17 mg dehydropolymerizate from [*ring*-¹⁴C]coniferyl alcohol (15).

Although originally viewed as an oxygenase (16), the enzyme thought to catalyze these transformations subsequently revealed itself as falling within the peroxidase family (17-20). The scope of the work devoted to characterizing the lignin peroxidase from *P. chrysosporium* has been truly spectacular. Fifteen different heme-containing lignin peroxidase species have been purified from the extracellular medium of the microorganism (21), and of these the major form (that with an isoelectric point of 4.65) has been crystallized (22). Even the gene encoding the predominant lignin peroxidase isozyme—denoted "H8" (23)—has been cloned and sequenced (24, 25).

The noteworthiness of lignin peroxidase from *P. chrysosporium* lies in its ability to oxidize aromatic compounds without free phenolic hydroxyl groups to the corresponding cation radicals (26-28); such intermediates derived from appropriately substituted phenylpropanoid structures may undergo C_α-C_β cleavage. On the other hand, enzymes confined to phenol oxidase behavior, such as horseradish peroxidase (29), facilitate single-electron transfers from *p*-hydroxyphenylpropanoid moieties to yield phenoxy radical intermediates that could either incur C_α-arene cleavage or become coupled to one another forming new intermolecular covalent bonds (30). Consequently the redox potential of lignin peroxidase, in its suitably oxidized "compound I" form (19), is greater than that of horseradish peroxidase but evidently less than that of chloroperoxidase (31).

Be that as it may, only three years after the original claims that lignin depolymerization *in vivo* is under the direct enzymatic control of lignin peroxidase, a very different picture began to emerge. It was disclosed that 0.8 units mL⁻¹ lignin peroxidase (as a concentrated extracellular solution from a carbon-limited *P. chrysosporium* culture medium) engenders the net polymerization of 1 mg mL⁻¹ alkali-isolated straw lignin at pH 4.0 in the presence of 0.54 μmole H₂O₂ portions added at 2 hourly intervals (32). Curiously, the introduction of 1.2 μmole mL⁻¹ veratryl alcohol (3,4-dimethoxybenzyl alcohol) enhanced the effect considerably (Figure 3). A much more rapid rate yet was observed for the polymerization of 1 mg mL⁻¹ milled wood spruce lignin under these conditions (32). Furthermore, four lignin peroxidase fractions, separated from the extracellular enzyme solution through isoelectric focusing, were found to polymerize the milled wood lignin sample in a like manner.

The alkali straw lignin and milled wood spruce lignin (32) are structurally more closely related to the native biopolymer than the preparations originally investigated (13, 14), and it now seems reasonably clear that *in vitro* lignin peroxidase causes net polymerization rather than degradation of lignin derivatives. Indeed some evidence had previously surfaced insinuating that the polymerization of lignins may also occur in intact *P. chrysosporium* cultures. Thus, with a pine kraft lignin fraction, a close and sustained interaction has been observed to develop between the mycelium and a small subset of components; among these as time progressed, the proportion of the highest molecular weight species first increased appreciably before becoming somewhat attenuated and approaching a stable value (33).

Lignin Biodegradation by *P. chrysosporium*: A Conceivable Rationale

P. chrysosporium cultures themselves do, of course, engender lignin degradation even though lignin peroxidase *in vitro* brings about lignin polymerization. That lignin

biodegradation is nevertheless *influenced* by lignin peroxidase in some manner has been substantiated by a centrally important study which appeared in 1988 (34). Mycelial pellets from nitrogen-limited *P. chrysosporium* cultures were carefully washed to remove all extracellular lignin peroxidase and the veratryl alcohol previously added to induce ligninolytic activity. The ensuing cultures (50 mL fungal pellets concentrated 4-fold) retained their competence to degrade HCl/dioxane-isolated ^{14}C -labeled straw lignin in the presence of glucose oxidase (2.5 units) at an appreciable rate even though no lignin peroxidase activity could be detected in the medium (34). Introduction of lignin peroxidase (2 units— $\mu\text{mole min}^{-1}$ veratryl alcohol oxidation) into the system enhanced lignin biodegradation by a factor of 4, while the simultaneous inclusion of 75 μmole veratryl alcohol accelerated the rate by a further factor of 1.5 (Figure 4).

At first sight, the simplest explanation for the absence of polymerization *in vivo* is that the cation radical intermediates formed from lignin components through electron transfer to lignin peroxidase may react along more than one pathway: rapid metabolism of the low molecular weight products by *P. chrysosporium* would tend to favor net degradation of lignin preparations at the expense of their polymerization (35). Whatever the case, there must be other enzymes and/or cofactors contributing to the ligninolytic system of *P. chrysosporium*.

These considerations have prompted the formulation of a provisional rationale for the ligninolytic processes elicited by *P. chrysosporium*. The scheme itself is an extension of a suggestion devised to account for the fate of veratryl alcohol in *P. chrysosporium* cultures (34).

That there could be a connection between *P. chrysosporium* mediated veratryl alcohol degradation and lignin biodegradation was first pointed out in 1981 (36). Veratryl alcohol is actually a secondary metabolite of *P. chrysosporium* (37), the reduction of veratraldehyde possibly being the last step in its biosynthesis (36). *In vitro*, veratryl alcohol is oxidized by lignin peroxidase to veratraldehyde (the predominant product), two quinones, and two lactones resulting from aromatic ring cleavage, as shown in Figure 5 (38). In nitrogen limited cultures, the quinone products are degraded by *P. chrysosporium* at about the same rate as veratryl alcohol itself (34), but veratraldehyde is quantitatively *reduced* to veratryl alcohol, which thereupon undergoes degradation (39). The reductive transformation could be controlled by an intracellular NADP-dependent aryl alcohol oxidoreductase which has been partially purified from *P. chrysosporium* cell extracts (34).

These findings led to the proposition that the veratryl alcohol is degraded *via* the quinone intermediates (Figure 5) to CO_2 through a series of transformations involving lignin peroxidase, perhydroxy radicals and the NADP-dependent aryl alcohol oxidoreductase. Veratraldehyde, the *major* product of lignin peroxidase catalyzed veratryl alcohol oxidation, is rapidly reduced *back* to veratryl alcohol; it is the further metabolism of the side products of the oxidative process, *viz.* the quinones and lactones, that drives the overall transformation towards completion (34).

An interesting feature of the suggested scheme may be found in the manner in which quinone ring cleavage is envisaged to take place. The oxidoreductase reduces the quinones first to hydroquinones, which are then oxidized by the lignin peroxidase to the corresponding phenoxy radicals; these in turn may *either* be oxidized to quinones *or* react with perhydroxy radicals giving intermediates leading towards ring opening. The ring opened products (*e.g.* muconic acid derivatives) would be further degraded by *P. chrysosporium* through to CO_2 (40).

The foregoing working hypothesis has been adapted to provide a rationale that might account for lignin biodegradation by *P. chrysosporium* (Figure 6). Single-electron

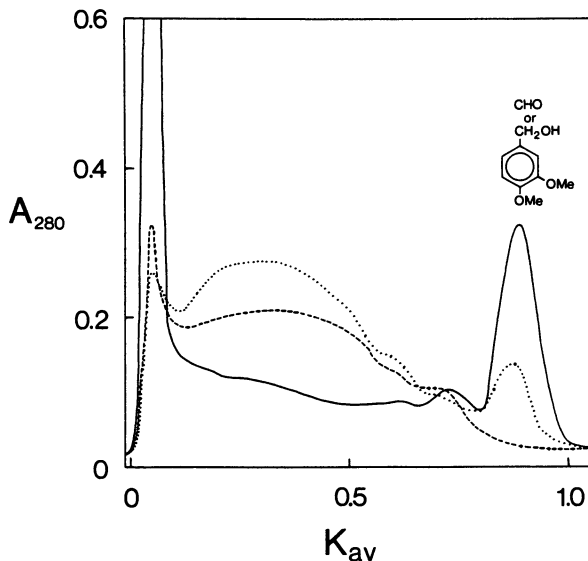


Figure 3. Polymerization of 1 mg mL^{-1} alkali-isolated straw lignin (dotted line) by $0.8 \text{ units mL}^{-1}$ lignin peroxidase (as concentrated extracellular *P. chrysosporium* culture fluid) at pH 4.0 in the absence (broken line) and presence (solid line) of $1.2 \text{ } \mu\text{mole mL}^{-1}$ veratryl alcohol; $0.54 \text{ } \mu\text{mole mL}^{-1}$ H_2O_2 added at 2 hourly intervals for 24 h. Sephadex G75/ aqueous 0.5% (w/v) NaOH elution profiles adapted and redrawn from reference 32.

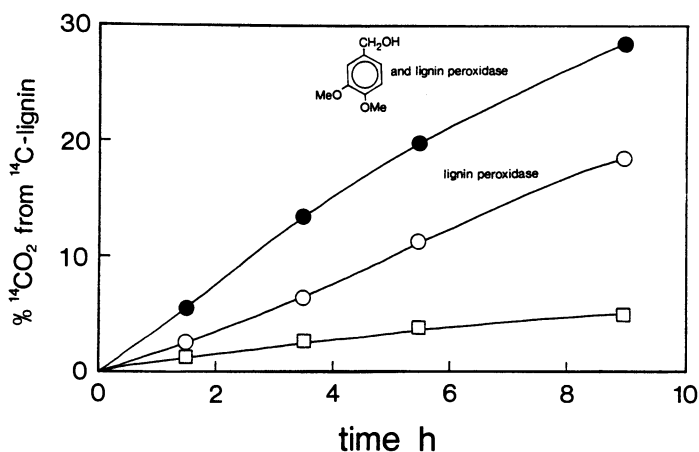


Figure 4. Biodegradation of HCl/dioxane-isolated ^{14}C -labeled straw lignin by 50 mL washed *P. chrysosporium* mycelial pellets (\square) in the presence of 2.5 units glucose oxidase; accelerative effect engendered by 2 units lignin peroxidase in absence (\circ) and presence (\bullet) of $75 \text{ } \mu\text{mole}$ veratryl alcohol. Adapted and redrawn from reference 34.

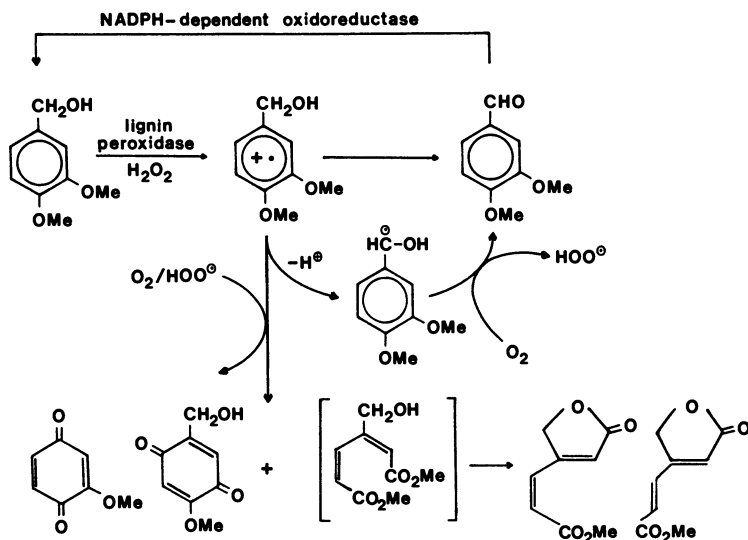


Figure 5. Plausible pathway for *P. chrysosporium* mediated veratryl alcohol degradation. Adapted and redrawn from reference 34.

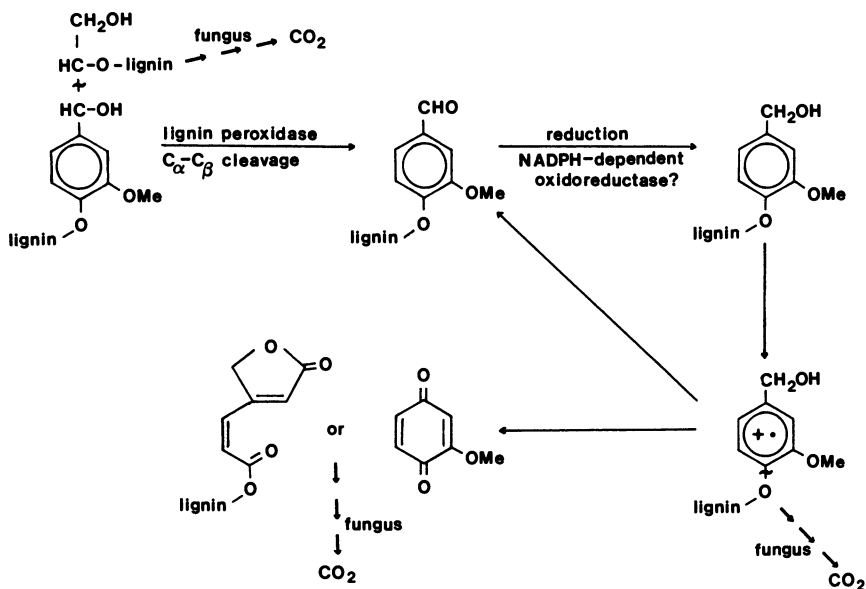


Figure 6. Plausible pathway for lignin biodegradation by *P. chrysosporium* exemplified for the β -O-4' interunit linkage. Adapted and redrawn from reference 34.

transfer from a nonphenolic lignin monomer residue to lignin peroxidase would yield the corresponding cation radical. Thereupon C_α - C_β cleavage could lead to depolymerization with the concomitant formation of an aromatic aldehyde moiety which would be *less* susceptible to subsequent single-electron oxidation. If such an aldehyde were part of a low molecular weight (dimeric, trimeric, oligomeric) lignin fragment, it could be transported across the cell membrane into a hypha of *P. chrysosporium* and reduced by the NADP-dependent aryl alcohol oxidoreductase to the corresponding alcohol. The resulting benzyl alcohol moiety could be oxidized by lignin peroxidase to the cation radical, which might then react with water to liberate a methoxy substituted quinone (Figure 6). This quinone would in turn be metabolized further by *P. chrysosporium* to CO_2 (34).

There is actually some circumstantial evidence in favor of the view that reductive processes may play a part in lignin biodegradation by *P. chrysosporium*. For example, following C_β - C_γ cleavage in β -*O*-4' substructures where C_α - C_β cleavage is precluded, the occurrence of reductive β -aryl ether cleavage may be inferred from the appearance of monomeric aromatic lignin degradation products possessing β -methyl groups (41). Indeed a range of monomeric and dimeric aromatic aldehydes and acids have been reduced to the corresponding alcohols by ligninolytic *P. chrysosporium* cultures (39, 42-44). Furthermore, the quinones (Figure 5) resulting from lignin peroxidase catalyzed oxidation of veratryl alcohol are also reduced by *P. chrysosporium* to the corresponding hydroquinones (45).

The foregoing working hypothesis, then, envisages lignin biodegradation to be facilitated by a sequence of alternating oxidative and reductive events involving, respectively, lignin peroxidase and a suitable oxidoreductase. The viability of such a scheme should be demonstrable through investigations of the two enzymes acting concertedly *in vitro*. No report along these lines has hitherto appeared for the aryl alcohol oxidoreductase, but the effect of cellobiose:quinone oxidoreductase (46, 47) has been explored (48) since it had been previously suggested that the enzyme might be able to reduce phenoxy radicals as well as quinones (47). Nevertheless cellobiose:quinone oxidoreductase was found to be without effect upon lignin peroxidase catalyzed phenoxy radical formation from acetosyringone (48); furthermore, it had no influence upon the apparent product size distribution arising from lignin peroxidase catalyzed polymerization of either guaiacol or a dehydropolymerizate from coniferyl alcohol (48).

Reservations about Lignin Peroxidase Participation in Lignin Biodegradation

Studies with model compounds embodying the predominant β -*O*-4' interunit linkage in lignins have shown that lignin peroxidase catalyzed C_α - C_β cleavage in the arylglycerol- β -aryl ether substructure leads to the liberation of a phenolic moiety (30, 49). These phenolic degradation products together with the phenolic units already present in lignins (1) would be expected to undergo polymerization at the hands of lignin peroxidase (48). Furthermore, *P. chrysosporium* is quite capable of degrading lignin preparations even when all extracellular lignin peroxidase activity has been washed from the culture medium (34).

Invoking lignin peroxidase participation under such conditions would require that the enzyme be either intracellularly confined (34) or membrane bound. However, when extracellular lignin peroxidase levels are completely suppressed by 40 ppm Mn^{II} concentrations, the rate of lignin biodegradation by *P. chrysosporium* remains appreciable (50), being attenuated only 7.6-fold from that prevailing under partially

optimized culture conditions (*vide infra*: **Other Enzymes**, Table I). It is hardly likely for manganese regulation of lignin peroxidase activity to operate by affecting the intracellular confinement or membrane binding of the enzyme.

Alternatively, it could be argued that the persistence of lignin biodegradation after the *P. chrysosporium* pellet cultures have been washed (34) is facilitated by subsequent excretion of lignin peroxidase from the hyphae. If so, the corresponding rate of $^{14}\text{CO}_2$ evolution from the ^{14}C -labeled straw lignin substrate would be expected to increase with time. Yet, as shown in Figure 4, this was not found to be the case. Consequently, it seems quite clear that lignin peroxidase activity is not obligatory for lignin depolymerization to occur *in vivo*.

N.G. Lewis contrived the witty notion that it should be possible to conscript lignin peroxidase for dehydrogenatively polymerizing monolignols into macromolecular lignin-like entities *in vitro* (51). Thus, to a solution containing lignin peroxidase (~ 57 units— $\mu\text{mole min}^{-1}$ guaiacol oxidation) and, as a putative stabilizer, veratryl alcohol (1.1 mM) at pH 4.0 (64 mL initial volume) under N_2 , $[2\text{-}^{13}\text{C}]$ coniferyl alcohol (3.5 $\mu\text{mole h}^{-1}$) and H_2O_2 (4 $\mu\text{mole h}^{-1}$), in separate solutions at pH 4.0, were added over a 48 h period (114 mL final volume). After centrifuging the mixture, the supernatant was treated with H_2O_2 (8.3 $\mu\text{mole h}^{-1}$) at pH 4.0 for another 24 h. The yield of the two dehydropolymerizate fractions together was quantitative. Of the combined product, a portion (10 mg) in aqueous 22% dioxane (ultimate solution composition, 45.7 mL final volume) was subjected to the further action of lignin peroxidase (~ 18 units) for 20 h in the presence of H_2O_2 (0.264 mmole total).

The interunit linkages in the dehydropolymerizate resulting from the action of lignin peroxidase/ H_2O_2 on $[2\text{-}^{13}\text{C}]$ coniferyl alcohol for the first 72 h period are manifested in the signals of the corresponding ^{13}C NMR spectrum (Figure 7a). These closely match the resonances from the dehydropolymerizate obtained through the more traditional means of incubating $[2\text{-}^{13}\text{C}]$ coniferyl alcohol with horseradish peroxidase/ H_2O_2 (Figure 7b). Similar results with $[1\text{-}^{13}\text{C}]$ and $[3\text{-}^{13}\text{C}]$ coniferyl alcohol confirmed that the oxidations by H_2O_2 of the monolignol, catalyzed respectively by lignin peroxidase and horseradish peroxidase, yield synthetic lignin-like dehydropolymerizates which are more or less identical to one another (51).

The effective molecular weight distributions in aqueous 0.10 M NaOH of the dehydropolymerizates formed during the first and second stages of exposure to lignin peroxidase activity are eloquently portrayed in Figure 8. Preincubation in aqueous 0.10 M NaOH had little effect upon the relative proportions of species eluting at different retention volumes from Sephadex G100. An increase in the populations of higher molecular weight entities is clearly engendered by further action of lignin peroxidase upon the dehydropolymerizate initially formed from $[2\text{-}^{13}\text{C}]$ coniferyl alcohol (51).

Such findings are quite consistent with the polymerization of alkali-isolated straw and milled wood spruce lignin preparations (32) encountered at the hands of extracellular *P. chrysosporium* culture fluid and purified lignin peroxidase isozymes (*vide supra*: **Lignin Peroxidase**). Evidently the 10% degradation originally reported for a dehydropolymerizate from $[\text{ring-}^{14}\text{C}]$ coniferyl alcohol in the presence of an extracellular *P. chrysosporium* culture medium (14) is exceptional; unfortunately no data were disclosed in support of this claim (14).

Nevertheless, the partial ($\sim 22\%$) depolymerization of a ^{14}C -methylated aqueous acetone extract from spruce wood, reported in the other of the first two accounts about lignin peroxidase (13), was subsequently confirmed (52). Yet extended exposure at pH 3.0 to extracellular *P. chrysosporium* culture fluid, or purified lignin peroxidase, with H_2O_2 failed to reduce the degree of polymerization below that initially achieved, even

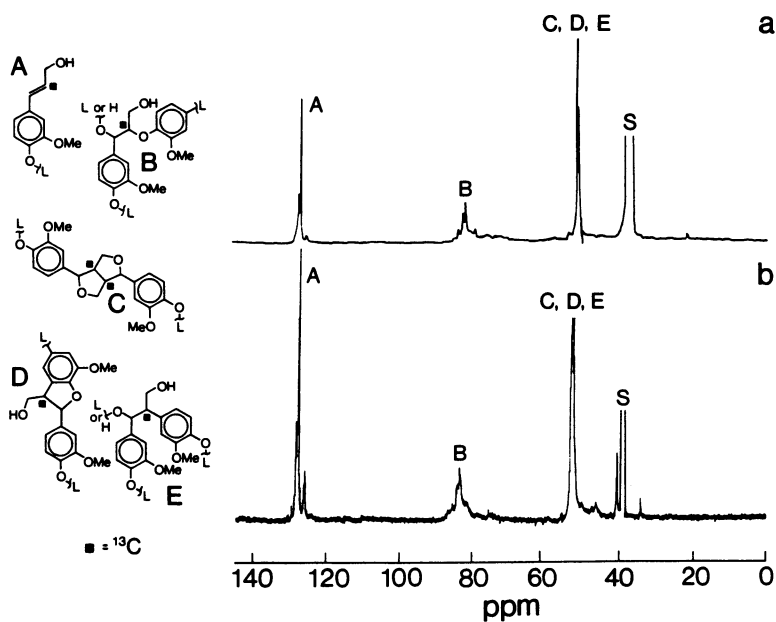


Figure 7. ^{13}C NMR solution spectra of dehydrogenative polymerizates formed from $[2\text{-}^{13}\text{C}]$ coniferyl alcohol with H_2O_2 in the presence of (a) lignin peroxidase at pH 4.0 and (b) horseradish peroxidase at pH 6.5 (51). S denotes solvent while A-E refer to substructural assignments depicted on left.

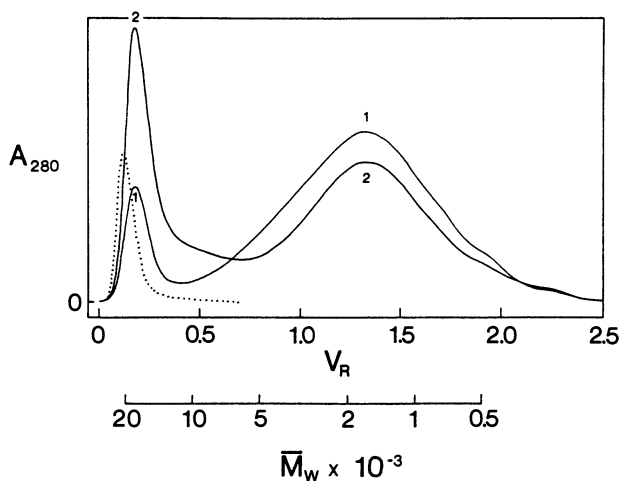


Figure 8. Dehydrogenative polymerization (1) of $[2\text{-}^{13}\text{C}]$ coniferyl alcohol with H_2O_2 at the hands of lignin peroxidase during 72 h at pH 4.0; effect of further enzyme action (2) upon dehydropolymerizate for subsequent 20 h period in the presence of H_2O_2 (51). Sephadex G100/aqueous 0.10 M NaOH elution profiles monitored at 280 nm (dotted line: blue dextran).

when veratryl alcohol was present. Comparative experiments witnessed the polymerization of a dehydropolymerizate from [*ring*-¹⁴C]coniferyl alcohol and a lignin sample from wheat straw under these conditions (52).

Moreover, no ¹³C NMR spectral changes were detected as a consequence of treating dehydropolymerizates from [1-¹³C], [2-¹³C] and [3-¹³C]coniferyl alcohol, respectively, or a dehydrogenative copolymer of *p*-[*ring*-4'-¹³C]coumaryl alcohol and coniferyl alcohol, at pH 3.0 with extracellular *P. chrysosporium* culture fluid, or purified lignin peroxidase, in the presence of H₂O₂; nor was the outcome affected by prior methylation of the substrates (52). Thus the result originally encountered with the purified spruce wood extract (13) is not representative of polymeric lignin-like preparations at all.

T. Umezawa has probed the effect of lignin peroxidase upon a dehydrogenative copolymer of 4-ethoxy-3-methoxyphenylglycerol- β -syringaresinol and coniferyl alcohol, where the trimeric residues were appended in a 1:6 (trimer:monomer) molar ratio to the macromolecular chains derived from the monolignol (53, 54). The formation of products resulting from C _{α} -C _{β} , β -O-4' and aromatic ring cleavage in the 4-ethoxy-3-methoxyphenylglycerol- β -syringyl ether moieties was accelerated precipitously by introducing veratryl alcohol into the H₂O₂-containing pH 3.0 tartrate buffer wherein indications of such lignin peroxidase catalyzed transformations were being sought (54).

The total molar yield of cleavage products detected was substantially lower than that from 4-ethoxy-3-methoxyphenylglycerol- β -(2',6'-dimethoxyphenyl) ether under the same conditions (Figure 9). The difference could, *a priori*, arise from the aromatic hydroxyl groups on the polymer chains formed from coniferyl alcohol: phenolic residues would be anticipated to undergo single electron oxidation more readily than their alkoxy counterparts. Yet upon ethylation (diazoethane) of the copolymer, contrary to expectation the yields of cleavage products were actually diminished (Figure 9). Regrettably the molecular weight distributions for the dehydrogenative copolymer and its ethylated derivative after exposure to the enzyme were not determined. Unanswered, therefore, was the question of whether the result resembled those from the action of lignin peroxidase on alkali-isolated straw and milled wood spruce lignin (32), or on the dehydropolymerizate from coniferyl alcohol alone (52), where the *net* effects were ones of polymerization (*cf.* Figure 3).

Viewed as a whole, then, the evidence presently available does *not* support the contention that lignin peroxidase is a key enzyme for the series of transformations bringing about lignin biodegradation in nature. Yet its presence can actually accelerate the degradation of lignins *in vivo* (34). Consequently the formulation of some provisional ideas about its possible functions would certainly be desirable.

Nitrogen starvation (55), carbon starvation (56) and sulfur starvation (56) have variously been reported to trigger biosynthesis of the ligninolytic system in *P. chrysosporium*, while a number of components have been purported to act as inducers of ligninolysis, *viz.* lignin through its biodegradation products (57, 58), lignin model compounds (57), veratryl alcohol (57, 58) and also oxygen (59). Accordingly lignins appear to be degraded by the microorganism only during secondary metabolism when primary growth has ceased because of the depletion of some nutrient. Yet the ligninolytic system can be synthesized in the *absence* of lignin during this secondary metabolic phase (55): veratryl alcohol, itself a secondary metabolite of *P. chrysosporium*, apparently induces lignin peroxidase, as do lignin biodegradation products also (57, 58). However, the enhanced enzymatic activity does *not always* increase the net rate of ligninolysis (57). At the very least, this finding indicates that

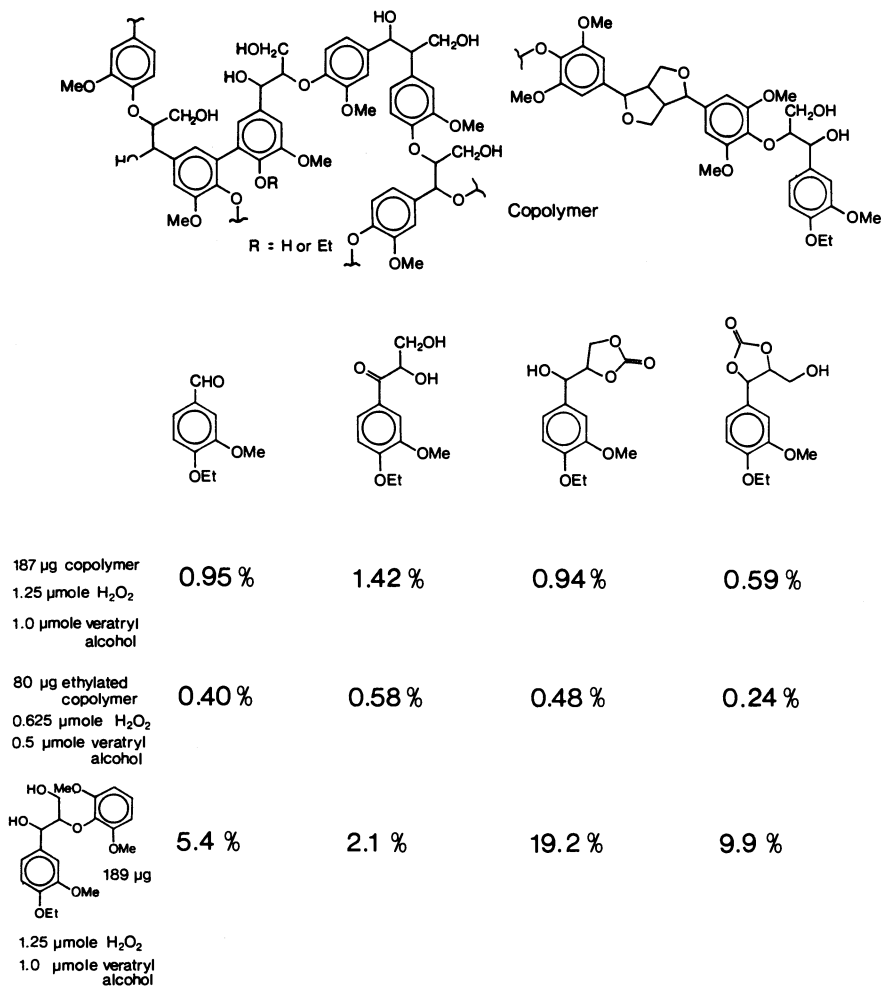


Figure 9. Molar yields, obtained after 20 minutes at pH 3.0 with 0.13 units mL^{-1} lignin peroxidase in the presence of H_2O_2 and veratryl alcohol, of $\text{C}_\alpha\text{-C}_\beta$, $\beta\text{-O-4}'$ and aromatic ring cleavage products from 4-ethoxy-3-methoxyphenylglycerol- β -syringyl ether moieties in dehydrogenative copolymer of coniferyl alcohol and 4-ethoxy-3-methoxyphenylglycerol- β -syringaresinol (54).

lignin peroxidase does not participate in the rate-limiting step of the ligninolytic system. But the observation that lignin degradation *does* occur in the complete absence of detectable lignin peroxidase activity (34, 50) can only be taken to imply that the enzyme is *not directly* involved in ligninolysis at all.

Provisional Roles for Lignin Peroxidase

What functions, then, could lignin peroxidase fulfill as far as *P. chrysosporium* is concerned? An answer to this question must, at the time of writing, involve some degree of speculation, but a coherent working hypothesis should be valuable in providing direction for future research. The most likely role for lignin peroxidase would be as an enzymatic detoxifying agent acting upon phenolic compounds in a manner analogous (but not mechanistically nor operationally identical) to the laccase from *Rhizoctonia praticola* (60). Concomitantly and inevitably, lignin peroxidase will tend to polymerize lignin components possessing free phenolic moieties. It was indeed suggested long ago that white-rot fungi would be expected to excrete enzymes capable of detoxifying the lower molecular weight phenolic compounds released from lignins during their microbiological decomposition (61, 62).

Moreover, the ability of lignin peroxidase also to oxidize nonphenolic units in lignins may confer an additional benefit upon *P. chrysosporium*. When aromatic hydroxyl groups are inaccessible or absent, new functionalities may be introduced into the macromolecular lignin fabric which render the polymeric structure more susceptible towards subsequent degradation *by another enzyme*. For example, the introduction of a carbonyl group at the α' -position in a nonphenolic β -O-4' ether moiety could bring about such an effect: the rate of nucleophilic displacement of the aryloxy group from the β -position could, as a result, be enhanced (Figure 10) by more than an order of magnitude (63). Further oxidation would not, on the other hand, occur: nonphenolic aromatic rings with α -carbonyl substituents are not substrates for lignin peroxidase (64).

The appearance of α -carbonyl moieties has been manifested through the action *in vitro* of lignin peroxidase on nonphenolic dimeric lignin model compounds (64), but not on polymeric lignin preparations possessing aromatic hydroxyl groups (52). Nevertheless it has been found that heavily degraded lignins isolated from spruce (*Picea engelmannii* Parry) wood, decayed to a 50% weight loss by *Coriolus versicolor*, contain 0.17 (per monomer residue) carbonyl groups α - to the aromatic rings (65). In this connection it should be mentioned that the white-rot fungus *C. versicolor* also excretes significant levels of lignin peroxidase (66).

Experimental Confirmation for the Roles of Lignin Peroxidase

Support for the working hypothesis which has begun to emerge may be discerned among lignin fractions purified from spruce wood chips that had been partially degraded by *P. chrysosporium* to a 20% loss in lignin content (67). Middle molecular weight acidic and phenolic fractions with very similar molecular weight distributions were isolated from the methanol extract of the substrate. The acidic fraction was distinguished by carbonyl and carboxyl group frequencies (per benzene ring) of 0.14 and 0.35, respectively (68), but possibly a lower aromatic hydroxyl content than the phenolic fraction (67). A substantially higher molecular weight acidic fraction, but no phenolic counterpart, could be secured from the subsequent aqueous 96% dioxane extract (Figure 11).

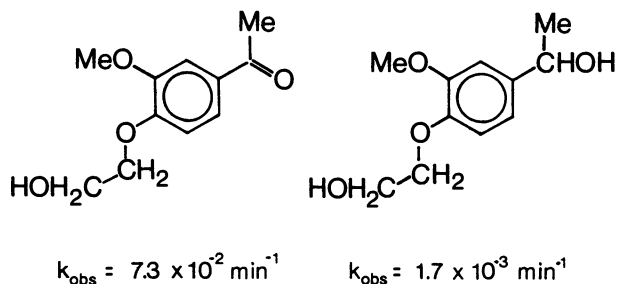


Figure 10. Enhancement of alkyl aryl ether cleavage resulting from introduction of carbonyl group into *para* position of aromatic ring. Unimolecular rate coefficients for $4.8 \times 10^{-3} \text{ M}$ reactants in aqueous 30% ethyleneglycol monomethyl ether containing $0.53 - 0.54 \text{ M}$ hydroxide and $0.08 - 0.09 \text{ M}$ bisulfide at 170°C (63).

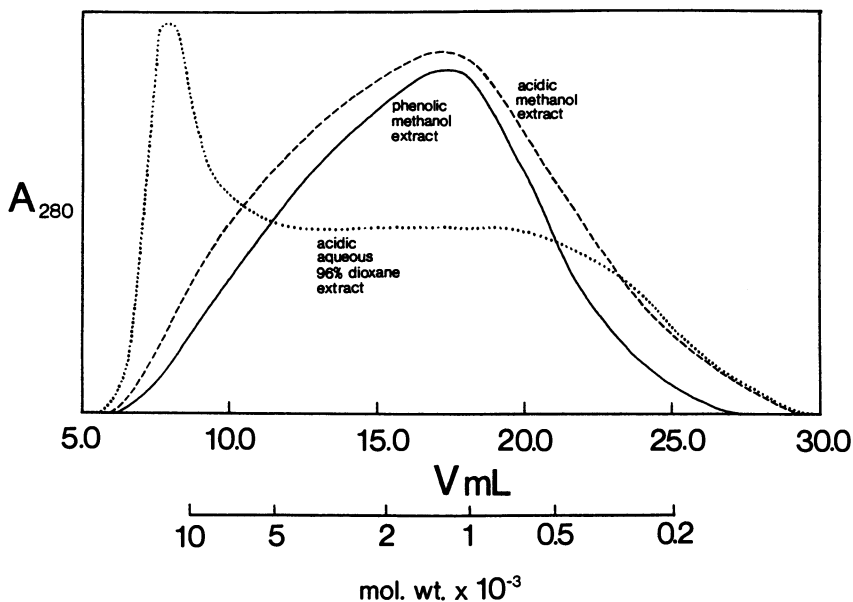


Figure 11. Molecular weight distributions of middle molecular weight phenolic (solid line) and acidic (broken line) fractions from methanol extract, and higher molecular weight acidic (dotted line) fraction from subsequent aqueous 96% dioxane extract, of spruce wood chips degraded by *P. chrysosporium* to 20% loss in lignin content. Sephadex LH60/0.10 M LiCl-DMF profiles adapted and redrawn from reference 67.

The identities of the ^{13}C NMR signals from the fungally degraded phenolic lignin fraction were predominantly similar to those from spruce milled wood lignin (68). Although containing, *inter alia*, decidedly significant numbers of carbonyl and carboxyl groups, the acidic fractions had been affected only to a small extent by aromatic ring cleavage (67, 68). A telling aspect of the comparison between the two acidic fractions, however, lies in the observation that their differences were confined primarily to the molecular weight distributions (67).

The most economical explanation for these findings lies in the realization that the middle molecular weight phenolic, middle molecular weight acidic, and the higher molecular weight acidic fractions represent consecutive stages in the modification of spruce lignin by *P. chrysosporium*. Such a sequence of events would fulfill the expectation that the microorganism should excrete an enzyme capable of detoxifying the lower molecular weight phenolic compounds released from lignins during their fungal decomposition (61, 62). One way of accomplishing the task would be by polymerizing these species into molecular entities sufficiently large that they can no longer penetrate through the hyphal cell wall.

Lignin peroxidase is admirably suited to playing such a role. The enzyme is capable of cleaving $\text{C}_\alpha\text{-C}_\beta$ bonds (64, 69) and aromatic rings (70) in a variety of nonphenolic dimeric lignin model compounds by catalyzing the formation of corresponding cation radical intermediates (20, 28, 71). However, single-electron oxidation of phenols to phenoxy radicals would be a considerably more facile process, and it was actually realized at a relatively early stage that the phenolic moieties liberated as a consequence of homolytic $\text{C}_\alpha\text{-C}_\beta$ cleavage in arylglycerol β -aryl ether model compounds would be polymerized at the hands of lignin peroxidase (30, 48, 49). Indeed, while $\text{C}_\alpha\text{-C}_\beta$ cleavage and phenoxy radical coupling have opposing effects upon their molecular weight distributions, lignin preparations undergo *net polymerization* through the action of lignin peroxidase *in vitro* (32, 52).

In this connection it is worth emphasizing that the transformations taking place, after initial lignin peroxidase catalyzed cation radical formation from appropriate nonphenolic lignin model compounds, can exhibit remarkable sensitivity even to certain substitution patterns within the molecular framework quite distant from the atomic centers directly affected. For example, in the presence of lignin peroxidase at pH 3.0, 1-(3',4'-dimethoxyphenyl)-2-(4"-methoxyphenoxy)propane-1,3-diol primarily experiences $\text{C}_\alpha\text{-C}_\beta$ cleavage to liberate veratraldehyde, while only traces of 1-(3',4'-dimethoxyphenyl)-3-hydroxy-2-(4"-methoxyphenoxy)propan-1-one can be detected among the products (64). On the other hand, the 2"-methoxyphenoxy isomer of the starting material, which is structurally more closely representative of native lignins, is converted to veratraldehyde and the 1-propanone oxidation product, respectively, in very similar yields (Figure 12).

Such contrasts are of more than passing interest. It has been convincingly documented (72) that oxidation of the benzylic hydroxyl in 1-(4'-ethoxy-3'-methoxyphenyl)-2-(2"-methoxyphenoxy)propane-1,3-diol to a carbonyl group markedly *retards* metabolism of this nonphenolic β -O-4' ether lignin model compound, but that selective introduction of α -carbonyl groups *enhances* the depolymerization of a spruce lignin preparation by *P. chrysosporium* cultures (Figure 13). The apparent contradiction is properly resolved in the observation that the presence of a carbonyl group at the α' -position in a nonphenolic β -O-4' ether moiety can increase the rate of nucleophilic displacement of the aroxy group from the β -position by more than an order of magnitude (63).

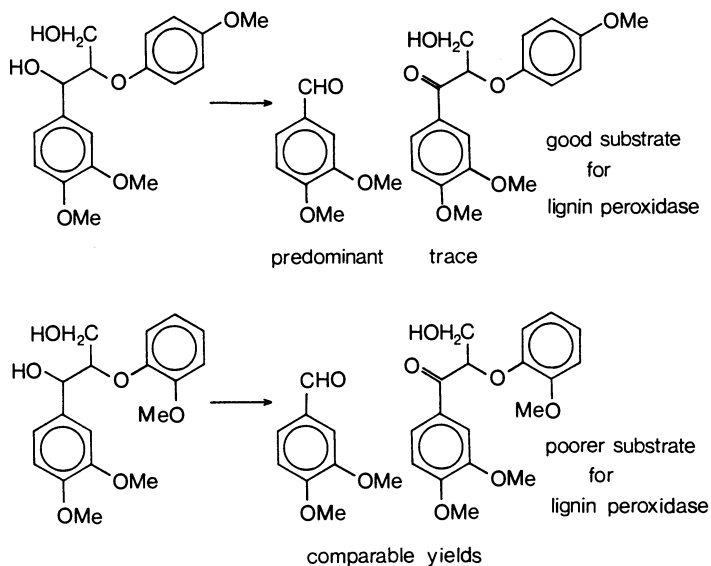


Figure 12. Relative proportions of C_{α} - C_{β} cleavage and C_{α} -oxidation in two dimeric nonphenolic β - O -4' ether compounds (0.2 M) engendered at pH 3.0 by 0.41 units mL^{-1} lignin peroxidase with 0.8 M H_2O_2 (64).

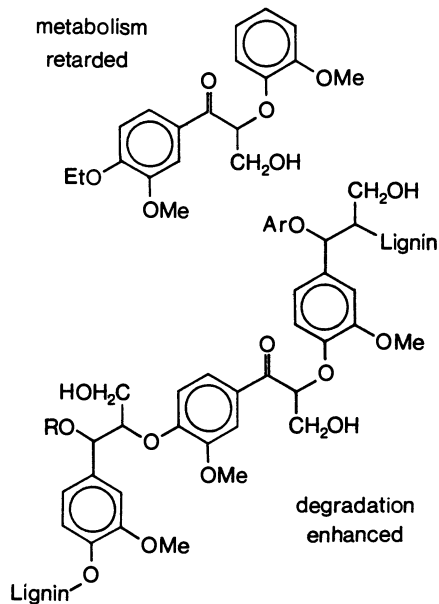


Figure 13. Contrasting effects upon their transformations by *P. chrysosporium* of introducing α -carbonyl groups into a dimeric nonphenolic β - O -4' ether compound and the lignin macromolecule itself (72).

Herein must reside the central insight into how lignin depolymerization can be accelerated indirectly by lignin peroxidase *in vivo*. As well as detoxifying the extracellular fungal environment, lignin peroxidase may be quite capable of introducing into the lignin fabric functional groups which render the polymeric structure more susceptible towards degradation by *another enzyme*.

Other Enzymes

What, then, might the identity be of the cardinal enzyme facilitating lignin biodegradation? Certainly extracellular enzymes other than lignin peroxidase are excreted by *P. chrysosporium*. For example, a demethylase (73) and six Mn^{II}-dependent peroxidases—all probably the products of a single gene—have been documented (74-6). In nitrogen-limited *P. chrysosporium* cultures, lignin peroxidase attains maximal activity after the highest Mn^{II}-dependent peroxidase concentrations are observed (76), but the reasons for the expression of these two types of extracellular peroxidases and their relationships to lignin depolymerization have not been established.

It should be mentioned that the Mn^{II}-dependent peroxidases act by oxidizing Mn^{II} to Mn^{III} (74, 77). Interestingly, at pH 3.0 with pyrophosphate as a chelator, Mn^{III} has been found to oxidize milled wood spruce lignin through a process that is sharply enhanced by reduced glutathione (78). The mechanism is thought to involve oxidation of the thiol by Mn^{III} to a thiyl radical, which in turn can abstract an electron from the α - or α' -position of a suitable nonphenolic β -O-4' substructure (79). Either of the alternative benzyl radicals may undergo C₈-O cleavage if the decomposition is not preempted by loss of a second electron leading to carbonyl group formation. Nevertheless, extracellular thiols are unlikely to play a significant role in lignin biodegradation by white-rot basidiomycetous fungi (79).

When thiols are absent, Mn^{II}-dependent peroxidase isozyme I from *P. chrysosporium* cannot oxidize the nonphenolic β -1 linked lignin model dimer 1-(3',4'-diethoxyphenyl)-2-(4"-methoxyphenyl)propane-1,3-diol (80). The corresponding 1-(3',5'-dimethoxy-4'-hydroxyphenyl) compound, on the other hand, does transfer a single electron to enzyme-generated Mn^{III}. Because coupling of the resulting species is hindered, subsequent transformations of the phenoxy radical were found primarily to engender C_α-oxidation and C_α-arene cleavage in a ~3:1 ratio (80). However, since they can catalyze the formation of polymeric products from unhindered phenols (81), the Mn^{II}-dependent peroxidases would be expected to cause a *net* polymerization of lignins (82) in a manner not so different from that encountered with lignin peroxidases (32, 52).

The concentrations of Mn^{II} in culture media have been reported to regulate the extracellular lignin peroxidase and Mn^{II}-dependent peroxidase activities expressed by a number of white-rot fungi (50). Such effects are illustrated for *P. chrysosporium* and *Lentinula edodes* in Table I. At high Mn^{II} levels, the specific activities of Mn^{II}-dependent peroxidase were high for both microorganisms, while those of lignin peroxidase fell below detectable limits. The corresponding rate of lignin biodegradation was fast for *L. edodes* (83) but, although still appreciable, attenuated by a factor of 7.6 (from partially optimized culture conditions) for *P. chrysosporium* (50).

When Mn^{II} levels were low, conversely, the specific activities of Mn^{II}-dependent peroxidase were low for both microorganisms, while those of lignin peroxidase were high (Table I). For *L. edodes* under these conditions, the lignin biodegradation rate was reduced 16-fold (83) in the face of a greater specific lignin peroxidase activity than optimally expressed by *P. chrysosporium* (50)!

Evidently lignin peroxidase furthers lignin biodegradation by *L. edodes* much less, even, than Mn^{II}-dependent peroxidase promotes lignin biodegradation by *P. chrysosporium*. Thus the findings summarized in Table I substantiate that neither lignin peroxidase nor Mn^{II}-dependent peroxidase necessarily accelerates the degradation of lignins *in vivo*. The implication seems again to be that the process is critically dependent upon another extracellular enzyme altogether.

Three laccase preparations (I, II and III) were isolated from the extracellular culture medium of *Coriolus versicolor* by consecutive fractionation through Sephadex G50 and DEAE Sephadex A25 (84). The laccase III preparation at pH 4.0 reduced the apparent molecular weight of a lignin-derived fraction that had been obtained by eluting the water-soluble extract of a cellulase treated ezomatsu wood residue through

Table I. Correlation of Manganese Regulated Lignin Peroxidase and Mn^{II}-dependent Peroxidase Activities with Lignin Biodegradation for *Phanerochaete chrysosporium* and *Lentinula edodes*^a

	<i>Phanerochaete chrysosporium</i> BKM-F-1767 (ATCC 24725)			
	high Mn ^{II} level		low Mn ^{II} level	
	40 ppm	10 ppm	0.32 ppm	0.01 ppm
specific Mn ^{II} -dependent peroxidase activity ^b	1308 [6] ^c	—	51 [8] ^c	—
specific lignin peroxidase activity ^b	0	—	150 [7] ^c	—
cumulative % ¹⁴ C ₂ O ₂ released from ¹⁴ C-DHP	5.1% [3.3-10.7] ^d	—	38.7% [3.3-10.7] ^d	—
	<i>Lentinula edodes</i> RA-3-2E (ATCC 48085)			
	high Mn ^{II} level		low Mn ^{II} level	
	40 ppm	10 ppm	0.32 ppm	0.01 ppm
specific Mn ^{II} -dependent peroxidase activity ^b	691 [9] ^c	—	33 [9] ^c	—
specific lignin peroxidase activity ^b	0	0	180 [9] ^c	—
cumulative % ¹⁴ C ₂ O ₂ released from ¹⁴ C-DHP	—	23.5% ^e [5-41] ^d	—	1.5% ^e [5-41] ^d

^aCulture media partially optimized for *P. chrysosporium* (50) unless otherwise indicated. ^bnmol/min. measured at room temperature per mg dry mycelial weight for vanillylacetone as a Mn^{II}-dependent peroxidase substrate and veratryl alcohol as a lignin peroxidase substrate; 0 denotes activity below detectable limit while — indicates assay not performed. ^cTime, at 39°C for *P. chrysosporium* or at 30°C for *L. edodes*, after inoculation when maximum activity appears (days). ^dInterval over which ¹⁴C₂O₂ evolution from dehydrogenative polymerizate (DHP) of [*ring-U-¹⁴C]coniferyl alcohol is measured (days after inoculation). ^eCulture media partially optimized for *L. edodes* at 22°C (83).*

Sephadex G10 with water (85). Depolymerization (Figure 14) was no less extensive than that brought about by the action of concentrated extracellular *P. chrysosporium* culture fluid upon a methylated aqueous acetone spruce wood extract in the presence of H_2O_2 at pH 3.0 (13).

The laccase III preparation from *C. versicolor* was separated by isoelectric focusing into three fractions IIIa, IIIb and IIIc (86). Two-dimensional electrophoresis of IIIc revealed a single phenol oxidase component detected by guaiacol staining, although silver staining did disclose the presence of a second protein. At pH 4.0, fraction IIIc primarily engendered C_α -arene cleavage in the phenolic β -O-4' lignin model dimer syringylglycerol- β -syringyl ether (86), while syringylglycerol- β -guaiacyl ether experienced predominantly C_α - C_β cleavage at the hands of laccase III (87). It appears, however, that nonphenolic compounds are not appreciably degraded by the laccases from *C. versicolor* (88).

A seemingly extracellular (perhaps membrane-bound) NAD-dependent oxidoreductase enzyme system was isolated over 20 years ago (89) from *Poria subacida* cultures that cleaved the aryloxy moiety from veratrylglycerol- β -guaiacyl ether (Figure 15). Concomitant demethylation of the veratryl *p*-methoxy group occurred so that guaiacylglycerol and guaiacol were formed in the presence of NADH (90). The 30% maximal yield of guaiacol from the enzyme catalyzed transformation was at the time rationalized in terms of putative stereospecificity towards one (*erythro* or *threo*) of the substrate's diastereomers (89). Unfortunately no reference to the effect of the enzyme system upon the depolymerization of lignins has been reported.

The significance of the result lay in the fact that a structural representative of the most frequent interunit linkage in the lignin macromolecule can be cleaved enzymatically through a *non-oxidative* process: no products arising from the polymerization of the guaiacol liberated were detected. In contrast, guaiacol has not been found among the reaction products arising from lignin peroxidase catalyzed single-electron oxidation and subsequent C_α - C_β cleavage of veratrylglycerol- β -guaiacyl ether (49, 64): upon release, the phenol is rapidly polymerized to colored components.

Conclusions

The case for the *direct* involvement of lignin peroxidase in *P. chrysosporium* effectuated lignin biodegradation is dispelled by four observations: (i) detectable lignin peroxidase activity is *not* a prerequisite for ligninolysis by the microorganism (34); (ii) lignin peroxidase alone *in vitro* polymerizes rather than depolymerizes lignin preparations (32, 52); (iii) *nonoxidatively* depolymerized lignin fractions can be isolated from wood that has been partially degraded by *P. chrysosporium* (67, 68); (iv) elevated levels of lignin peroxidase activity are correlated with *slower* rates of lignin biodegradation in *L. edodes* cultures (50, 83). Nevertheless, lignin peroxidase may be capable of introducing functional groups into some of the lignin monomer residues that would result in enhanced susceptibility of the macromolecular structure towards subsequent depolymerization (72) by *another* enzyme. The quest for the *key* enzyme, upon which the decomposition of lignins in nature ultimately rests, promises to be as fascinating as the journey which led to the original discovery of lignin peroxidase in 1983.

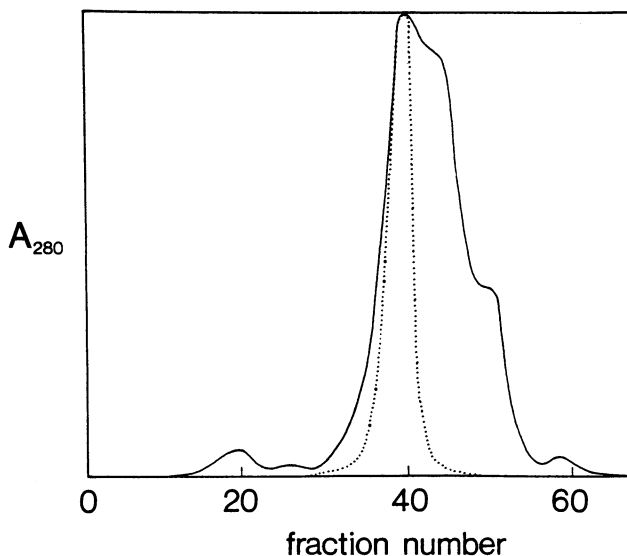


Figure 14. Change (solid line) in apparent molecular weight distribution of water-soluble extract from a cellulase-treated ezomatsu wood residue (dotted line) at pH 4.0 brought about by the laccase III preparation from *C. versicolor*. Sephadex G10/H₂O elution profiles adapted and redrawn from reference 85.

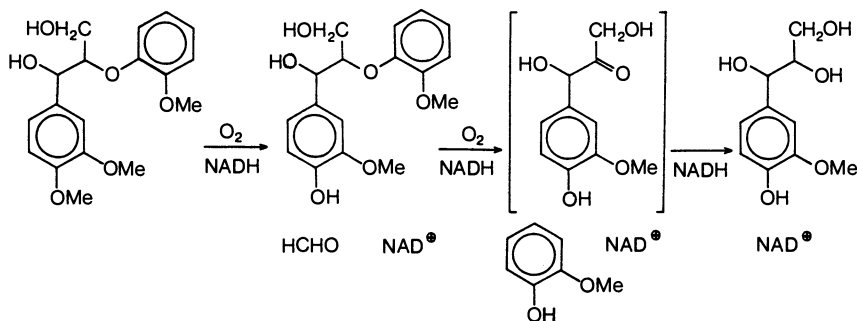


Figure 15. Hypothetical scheme for the transformations experienced by veratrylglycerol- β -guaiacyl ether at the hands of a seemingly extracellular NAD-dependent oxidoreductase system from *Poria subacida* (90).

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Literature Cited

1. Sakakibara, A. *Wood Sci. Technol.* **1980**, *14*, 89-100.
2. Grisebach, H. In *The Biochemistry of Plants: 7. Secondary Plant Products*; Conn, E.E., Ed.; Academic Press: New York, 1981; pp 457-78.
3. Scalbert, A.; Monties, B.; Lallemand, J.-Y.; Guittet, E.; Rolando, C. *Phytochemistry* **1985**, *24*, 1359-62.
4. Musha, Y.; Goring, D.A.I. *Wood Sci. Technol.* **1975**, *9*, 45-58.
5. Terashima, N.; Tomimura, Y.; Araki, H. *Mokuzai Gakkaishi* **1979**, *25*, 595-9.
6. Goring, D.A.I. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: New York, 1985; pp 161-207.
7. *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*; Kirk, T.K., Higuchi, T., Chang, H.-m., Eds.; CRC Press: Boca Raton, 1980; vol. I and II.
8. Hall, P.L. *Enzyme Microb. Technol.* **1980**, *2*, 170-6.
9. Forney, L.J.; Reddy, C.A.; Tien, M.; Aust, S.D. *J. Biol. Chem.* **1982**, *257*, 11455-62.
10. Kutsuki, H.; Gold, M.H. *Biochem. Biophys. Res. Commun.* **1982**, *109*, 320-7.
11. Faison, B.D.; Kirk, T.K. *Appl. Environ. Microbiol.* **1983**, *46*, 1140-5.
12. Gold, M.H.; Kutsuki, H.; Morgan, M.A.; Kuhn, R.M. *Proc. Internat. Symp. Wood Pulp. Chem.* **1983**, *4*, 165-8.
13. Tien, M.; Kirk, T.K. *Science* **1983**, *221*, 661-3.
14. Glenn, J.K.; Morgan, M.A.; Mayfield, M.B.; Kuwahara, M.; Gold, M.H. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 1077-83.
15. Gold, M.H.; Mayfield, M.B.; Cheng, T.M.; Krisnangkura, K.; Shimada, M.; Enoki, A.; Glenn, J.K. *Arch. Microbiol.* **1982**, *132*, 115-22.
16. Kirk, T.K.; Shimada, M. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: New York, 1985; pp 579-605.
17. Kuila, D.; Tien, M.; Fee, J.A.; Ondrias, M.R. *Biochemistry* **1985**, *24*, 3394-7.
18. Harvey, P.J.; Schoemaker, H.E.; Bowen, R.M.; Palmer, J.M. *FEBS Lett.* **1985**, *183*, 13-6.
19. Renganathan, V.; Gold, M.H. *Biochemistry* **1986**, *25*, 1626-31.
20. Hammel, K.E.; Kalyanaraman, B.; Kirk, T.K. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 3708-12.
21. Leisola, M.S.A.; Kozulic, B.; Meussdoerffer, F.; Fiechter, A. *J. Biol. Chem.* **1987**, *262*, 419-24.
22. Troller, J.; Smit, J.D.G.; Leisola, M.S.A.; Kallen, J.; Winterhalter, K.H.; Fiechter, A. *Biotechnology* **1988**, *6*, 571-3.
23. Kirk, T.K.; Croan, S.; Tien, M.; Murtagh, K.E.; Farrell, R.L. *Enzyme Microb. Technol.* **1986**, *8*, 27-32.
24. Tien, M.; Tu, C.-P.D. *Nature* **1987**, *326*, 520-3.
25. Smith, T.L.; Schalch, H.; Gaskell, J.; Covert, S.; Cullen, D. *Nucleic Acids Res.* **1988**, *16*, 1219.

26. Gold, M.H.; Kuwahara, M.; Chiu, A.A.; Glenn, J.K. *Arch. Biochem. Biophys.* **1984**, *234*, 352-62.
27. Schoemaker, H.E.; Harvey, P.J.; Bowen, R.M.; Palmer, J.M. *FEBS Lett.* **1985**, *183*, 7-12.
28. Kersten, P.J.; Tien, M.; Kalyanaraman, B.; Kirk, T.K. *J. Biol. Chem.* **1985**, *260*, 2609-12.
29. Dunford, H.B. *Adv. Inorg. Biochem.* **1982**, *4*, 41-68.
30. Higuchi, T. *Wood Res. (Kyoto Univ.)* **1986**, *73*, 58-81.
31. Renganathan, V.; Miki, K.; Gold, M.H. *Biochemistry* **1987**, *26*, 5127-32.
32. Haemmerli, S.D.; Leisola, M.S.A.; Fiechter, A. *FEMS Microbiol. Lett.* **1986**, *35*, 33-6.
33. Janshekar, H.; Brown, C.; Haltmeier, Th.; Leisola, M.; Fiechter, A. *Arch. Microbiol.* **1982**, *132*, 14-21.
34. Leisola, M.S.A.; Haemmerli, S.D.; Waldner, R.; Schoemaker, H.E.; Schmidt, H.W.H.; Fiechter, A. *Cellulose Chem. Technol.* **1988**, *22*, 267-77.
35. Schoemaker, H.E.; Leisola, M.S.A. *Proc. 31st IUPAC Congress 1987, Section 4*, 267-80.
36. Shimada, M.; Nakatsubo, F.; Kirk, T.K.; Higuchi, T. *Arch. Microbiol.* **1981**, *129*, 321-4.
37. Lundquist, K.; Kirk, T.K. *Phytochemistry* **1978**, *17*, 1676.
38. Haemmerli, S.D.; Schoemaker, H.E.; Schmidt, H.W.H.; Leisola, M.S.A. *FEBS Lett.* **1987**, *220*, 149-54.
39. Leisola, M.S.A.; Fiechter, A. In *Advances in Biotechnological Processes: 5*; Mizrahi, A., van Wezel, A.L., Eds.; Liss: New York, 1985; pp 59-89.
40. Ander, P.; Eriksson, K.-E.; Yu, H.-s. *Arch. Microbiol.* **1983**, *136*, 1-6.
41. Chen, C.-L.; Chang, H.-m. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: New York, 1985; pp 535-56.
42. Ander, P.; Hatakka, A.; Eriksson, K.-E. *Arch. Microbiol.* **1980**, *125*, 189-202.
43. Nakatsubo, F.; Kirk, T.K.; Shimada, M.; Higuchi, T. *Arch. Microbiol.* **1981**, *128*, 416-20.
44. Enoki, A.; Goldsby, G.P.; Krisnangkura, K.; Gold, M.H. *FEMS Microbiol. Lett.* **1981**, *10*, 373-7.
45. Schoemaker, H.E.; Meijer, E.M.; Leisola, M.S.A.; Haemmerli, S.D.; Waldner, R.; Sanglard, D.; Schmidt, H.W.H. In *Biogenesis and Biodegradation of Plant Cell Wall Polymers*; Lewis, N.G., Paice, M.G., Eds.; ACS Symp. Ser. **1989**, *399*, 454-71.
46. Westermark, U.; Eriksson, K.-E. *Acta Chem. Scand. B* **1974**, *28*, 204-8.
47. Westermark, U.; Eriksson, K.-E. *Acta Chem. Scand. B* **1974**, *28*, 209-14.
48. Odier, E.; Mozuch, M.D.; Kalyanaraman, B.; Kirk, T.K. *Biochimie* **1988**, *70*, 847-52.
49. Tien, M.; Kirk, T.K. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 2280-4.
50. Bonnarme, P.; Jeffries, T.W. *Appl. Environ. Microbiol.* **1990**, *56*, 210-7.
51. Sarkanen, S.; Razal, R.A.; Piccarriello, T.; Yamamoto, E.; Lewis, N.G. *J. Biol. Chem.* **1991**, *266*, in press.
52. Kern, H.W.; Haider, K.; Pool, W.; de Leeuw, J.W.; Ernst, L. *Holzforschung* **1989**, *43*, 375-84.
53. Umezawa, T.; Higuchi, T. *FEBS Lett.* **1989**, *242*, 325-9.
54. Umezawa, T.; Higuchi, T. *Mokuzai Gakkaishi* **1989**, *35*, 1014-20.
55. Keyser, P.; Kirk, T.K.; Zeikus, J.G. *J. Bacteriol.* **1978**, *135*, 790-7.
56. Jeffries, T.W.; Choi, S.; Kirk, T.K. *Appl. Environ. Microbiol.* **1981**, *42*, 290-6.
57. Faison, B.D.; Kirk, T.K. *Appl. Environ. Microbiol.* **1985**, *49*, 299-304.

58. Faison, B.D.; Kirk, T.K.; Farrell, R.L. *Appl. Environ. Microbiol.* **1986**, *52*, 251-4.
59. Bar-Lev, S.S.; Kirk, T.K. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 373-8.
60. Bollag, J.-M.; Shuttleworth, K.L.; Anderson, D.H. *Appl. Environ. Microbiol.* **1988**, *54*, 3086-91.
61. Gierer, J.; Opara, A.E. *Acta Chem. Scand.* **1973**, *27*, 2909-22.
62. Lyr, H. *Nature* **1962**, *195*, 289-90.
63. Gierer, J.; Ljunggren, S. *Svensk Papperstidn.* **1979**, *82*, 71-81.
64. Kirk, T.K.; Tien, M.; Kersten, P.J.; Mozuch, M.D.; Kalyanaraman, B. *Biochem. J.* **1986**, *236*, 279-87.
65. Kirk, T.K.; Chang, H.-m. *Holzforschung* **1975**, *29*, 56-64.
66. Jönsson, L.; Karlsson, O.; Lundquist, K.; Nyman, P.O. *FEBS Lett.* **1989**, *247*, 143-6.
67. Chua, M.G.S.; Chen, C.-L.; Chang, H.-m. *Holzforschung* **1982**, *36*, 165-72.
68. Robert, D.; Chen, C.-L. *Holzforschung* **1989**, *43*, 323-32.
69. Renganathan, V.; Miki, K.; Gold, M.H. *Arch. Biochem. Biophys.* **1985**, *241*, 304-14.
70. Umezawa, T. *Wood Res. (Kyoto Univ.)* **1988**, *75*, 21-79.
71. Hammel, K.E.; Tien, M.; Kalyanaraman, B.; Kirk, T.K. *J. Biol. Chem.* **1985**, *260*, 8348-53.
72. Fenn, P.; Kirk, T.K. *J. Wood Chem. Technol.* **1984**, *4*, 131-48.
73. Huynh, V.-B.; Crawford, R.L. *FEMS Microbiol. Lett.* **1985**, *28*, 119-23.
74. Glenn, J.K.; Gold, M.H. *Arch. Biochem. Biophys.* **1985**, *242*, 329-41.
75. Paszczyński, A.; Huynh, V.-B.; Crawford, R. *Arch. Biochem. Biophys.* **1986**, *244*, 750-65.
76. Leisola, M.S.A.; Kozulic, B.; Meussdoerffer, F.; Fiechter, A. *J. Biol. Chem.* **1987**, *262*, 419-24.
77. Glenn, J.K.; Akileswaran, L.; Gold, M.H. *Arch. Biochem. Biophys.* **1986**, *251*, 688-96.
78. Forrester, I.T.; Grabski, A.C.; Burgess, R.R.; Leatham, G.F. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 992-9.
79. Wariishi, H.; Valli, K.; Renganathan, V.; Gold, M.H. *J. Biol. Chem.* **1989**, *264*, 14185-91.
80. Wariishi, H.; Valli, K.; Gold, M.H. *Biochemistry* **1989**, *28*, 6017-23.
81. Paszczyński, A.; Huynh, V.-B.; Crawford, R. *FEMS Microbiol. Lett.* **1985**, *29*, 37-41.
82. Kirk, T.K. In *Biochemistry and Genetics of Cellulose Degradation*; Aubert, J.-P., Beguin, P., Millet, J., Eds.; Academic Press: New York, 1988; pp 315-32.
83. Leatham, G.F. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 51-8.
84. Morohoshi, N.; Wariishi, H.; Muraio, C.; Nagai, T.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 218-25.
85. Morohoshi, N.; Fujita, K.; Wariishi, H.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 310-5.
86. Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 495-502.
87. Wariishi, H.; Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 892-8.
88. Kawai, S.; Umezawa, T.; Shimada, M.; Higuchi, T.; Koide, K.; Nishida, T.; Morohoshi, N.; Haraguchi, T. *Mokuzai Gakkaishi* **1987**, *33*, 792-7.
89. Fukuzumi, T.; Takatuka, H.; Minami, K. *Arch. Biochem. Biophys.* **1969**, *129*, 396-403.
90. Fukuzumi, T. In *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*; Kirk, T.K., Higuchi, T., Chang, H.-m., Eds.; CRC Press: Boca Raton, 1980; vol. II, pp 73-94.

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Chapter 21

Lignin–Carbohydrate Complexes from Poplar Wood

Isolation and Enzymatic Degradation

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Four distinct lignin-carbohydrate complexes (LCCs) were isolated from a thermal hydrolysate of *Populus deltoides* wood by a preparative sequence involving extraction with ethyl acetate, ethyl alcohol, and exhaustive fractionation on a variety of gel filtration media. Purified LCCs presented peaks of superimposable carbohydrate content and ultraviolet absorption when subjected to gel filtration chromatography wherein each given species eluted within the included gel volume. While LCCs of apparent high molecular size were typified by high ultra-violet absorption, lower total carbohydrate, and critical micelle concentrations in the range of 20 to 35 micrograms dry weight, LCCs of apparent low molecular weight possessed less ultraviolet absorbance, significantly more carbohydrate, and critical micelle concentrations that were 10-fold greater on average than the larger sized species. All LCC preparations were highly *O*-acetylated, and contained varying proportions of glucose, xylose, mannose, and uronic acids. Treatment of purified LCCs with a variety of enzymes including acetyl xylan esterase, xylanase, β -mannanase, and β -glucosidase independently and in combination, while not producing monomeric (carbohydrate) components, generated products of altered gel permeation and critical micelle concentration properties.

Lignin-carbohydrate complexes (LCCs), covalent associations of lignin polymers with hemicellulose moieties, are typified by their extreme stability to physico-chemical and possibly to biological agents. Since the first description of LCCs by Björkman (1), many studies to characterize the linkages between carbohydrate and lignin components have been conducted. The task has been formidable in that the drastic conditions used in sufficiently degrading the cell wall network to facilitate LCC isolation can hydrolyze some lignin-carbohydrate linkages, while allowing other new linkage to be formed (2,3). Further complexity arises from the diversity

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of hemicellulose which may be composed of varying amounts of xylan, glucomannan, galactoglucomannan, and arabinan and which differs with the source of plant cell wall material (4,5). LCCs have been isolated from milled wood lignin and purified by alkaline treatment and/or differential extraction with organic solvents (4,6,7,8), and from rumen fluids (9,10,11). In these studies, even LCCs from a single source exhibited variable molecular weights, lignin:carbohydrate ratios, and hydrophobicity emphasizing that a major deterrent in LCC characterization arises from the difficulty in isolating homogeneous entities.

In recent years, enzymic modification/removal of lignin from LCCs has received increased attention because of potential applications in the pulp and paper industry, waste treatments, fuel products, and animal feeds. While preliminary studies suggested that lignin peroxidases do not result in lignin removal (12), and likely result in lignin polymerization (13), use of hemicellulolytic enzymes has enhanced chemical pulp bleaching processes (14), with concomitant improvements in pulp beatability (15) and tear strength (12). However, chemical and enzymic treatments to remove the blockage by lignin of carbohydrate hydrolysis by rumen enzymes (16,17) have not been fully successful. Isolation, purification, and characterization of *P. deltooides* LCCs and the effect of hemicellulases upon these materials are reported below.

Materials and Methods

Preparation of LCCs. An aqueous 10% (w/v) suspension of *P. deltooides* wood (particle size <0.5mm) was passed through a high shear valve and plug flow reactor combination for a total residence time of 2 minutes at 217°C. The reaction was quenched by flash discharge to atmospheric pressure, and the hemicellulose starting material was separated from the treated residue by centrifugation at 10,000 x g for 15 minutes. Following concentration of the filtrate by evaporation under reduced pressure, the hemicellulose starting material was lyophilized.

Gel Permeation Chromatography. Samples were filtered on columns of Bio-Gel P-6DG (Bio-Rad Laboratories), and columns of Sephadex G-10, G-25, and G-50 (Pharmacia Corp.) using deionized water as eluant. Gel filtration properties are expressed in terms of the distribution coefficient K_{av} calculated from the relationship $K_{av} = (V_e - V_o) / (V_t - V_o)$ where V_e is the volume at which a component elutes, V_o is the void volume, and V_t is the total volume of the system. Blue dextran 2,000 and xylose were used to determine V_o and V_t respectively.

Enzyme Treatment of LCCs. Purified *Bacillus circulans* xylanase (specific activity, 110 units/mg protein), purified *Coriolus versicolor* β -mannanase (specific activity 179 units/mg protein), purified *Thermoascus aurantiacus* β -glucosidase (specific activity, 150 units/mg protein), and purified *Schizophyllum commune* acetyl xylan esterase (AXE; specific activity, 180 units/mg protein) were used. Unit activity for xylanase and β -mannanase was defined as the amount of enzyme catalyzing the release of 1 μ mol reducing power per minute at 50°C and pH 6.0 using oat spelts

xylan (Sigma) and konjacu root glucomannan substrates, respectively. Unit activity for acetyl xylan esterase and β -glucosidase was defined as the amount of enzyme catalyzing the release of 1 μ mol acetic acid or *p*-nitrophenol per minute at 50°C and pH 6.0 using larch wood acetyl xylan and *p*-nitrophenyl- β -glucopyranoside (Sigma) substrates, respectively.

Purified LCCs (10 mg dry weight) were incubated at 50°C for 3 h with 50 units of each enzyme alone and in specified combinations in a total volume of 1.0 mL. Reaction mixtures contained a final concentration of 20 mM potassium phosphate buffer, pH 6.0. Enzyme digests were then fractionated on columns of Sephadex G-50 or Sephadex G-10 as described above.

Analytical Techniques. Total carbohydrate and reducing groups were estimated by the phenol-sulfuric acid technique (18) and the Somogyi modification (19) of the Nelson technique, respectively. Neutral sugars were quantitated following trifluoroacetic acid hydrolysis (20), by hplc on either a Bio-Rad HPX-8P or three HPX-8H column(s) in series using water or 10 mM sulfuric acid, respectively, as eluants. Uronic acids were estimated with the *m*-hydroxydiphenyl reagent (21). Acetyl groups were quantitated by hplc after alkaline hydrolysis (22). Lignin was determined with acetyl bromide reagent (23) using ethylene glycol soluble lignin from *P. deltoides* as standard. Critical micelle concentration (cmc) was determined using the interaction of pinocyanol chloride (Sigma) with increasing concentrations of LCCs as previously described (24). The concentration at which rapid change in absorption occurs at 547 and 598 nm was defined as the critical micelle concentration. Elemental analyses were performed on a Perkin-Elmer 240C Elemental Analyzer.

Spectroscopy. Fourier transform infrared (FTIR) analysis was performed as follows: 5 to 7 mg of sample was mixed with KBr in a grinder and the sample having approximately 1 to 2% (w/v) of LCC was mounted in the diffuse reflectance accessory (DRIFT) of a Nicolet DB-5 FTIR spectrometer. Four hundred scans were accumulated at a resolution of 4 cm^{-1} . Absorptions for CO_2 and H_2O were subtracted and baseline correction applied to eliminate the ramping effect of the DRIFT technique. ^{13}C -Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker CXP-180 spectrometer operating at a resonance frequency of 45.267 MHz. Spinning speed was 3.5 kHz. CPMAS measurements were run at an ambient temperature (297° K) with a 1 H decoupling field of 60 kHz. Typically 3,600 scans (sweep width setting of 20 kHz) were taken with a delay time of 2 seconds between acquisition cycles. Contact time was 2 ms. Free induction decays were 512 words, which were zero filled to 4 k prior to Fourier transformation. Electron spin resonance (ESR) spectra were recorded and measured with a Varian E-12 spectrometer, a Systron-Donner frequency counter, and a Bruker D35M gaussmeter. For the range 100-300 K, temperature control inside the rectangular resonant cavity was achieved with a stream of pre-cooled N_2 and a proportional controller.

Results

LCC Purification. The sequence for LCC purification and component yield appears in Figure 1. The lyophilized hemicellulose starting material (40 g as an aqueous 10% w/v suspension) underwent two cycles of extraction with equal volumes of ethyl acetate. Resultant aqueous (i.e., ethyl acetate insoluble) material was sequentially subjected to two cycles of precipitation at 4°C with 40 volumes of 95% ethanol, evaporation under reduced pressure to remove ethanol, and lyophilization. Water-resuspended precipitate (6 g) was fractionated according to the scheme illustrated in Figure 1. Fractionation strategies involved successive re-chromatography of materials on permeation gels of greater exclusion limits until components having superimposable A_{280} and carbohydrate content eluted in the included gel volume. For this and all subsequent filtration steps, pooled fractions were concentrated by lyophilization, and dried samples were dissolved with water at concentrations of not less than 25 mg/mL dry weight. Isolation of components having superimposable A_{280} and carbohydrate content was dependent upon maintenance of high sample concentrations and avoidance of ionic environments. After initial fractionation of higher and lower molecular weight fractions "A" and "B" using Bio-Gel P-6 DG (Fig 2a), fractions were pooled as indicated. Further representative fractionations of low molecular weight LCC material into the key components B1, B2, and B3, as well as the penultimate fractionation of ARB1 on Sephadex G-50 generating LCC 1, and the penultimate fractionation generating LCCs 2 and 3 are illustrated in Figures 2b, 2c, and 2d respectively. For LCC 4 (Figure 1), alternate filtration on a gel of lower exclusion limit was necessary to remove non-carbohydrate associated A_{280} material.

Characterization of LCCs. Properties of the four LCCs appear in Table I.

Table I. Properties of *Populus deltoides* Lignin-carbohydrate Complexes

Property	Lignin-carbohydrate Complex			
	1	2	3	4
K_{av}^a	0.100	0.067	0.685	0.039
cmc ^b ($\mu\text{g/mL}$)	20-30	25-35	250-350	260-320
A_{280} (units/mg)	13.40	17.7	1.00	1.60
Carbohydrate ($\mu\text{g/mg}$)	204	188	748	398
Lignin ($\mu\text{g/mg}$)	925 ^c	893 ^c	223	383

^a All K_{av} values were obtained using Sephadex G-50 gel filtration media with the exception of LCC 4 where Sephadex G-10 was employed.

^bcmc; critical micelle concentration.

^cValues for lignin content were beyond the range of the assay.

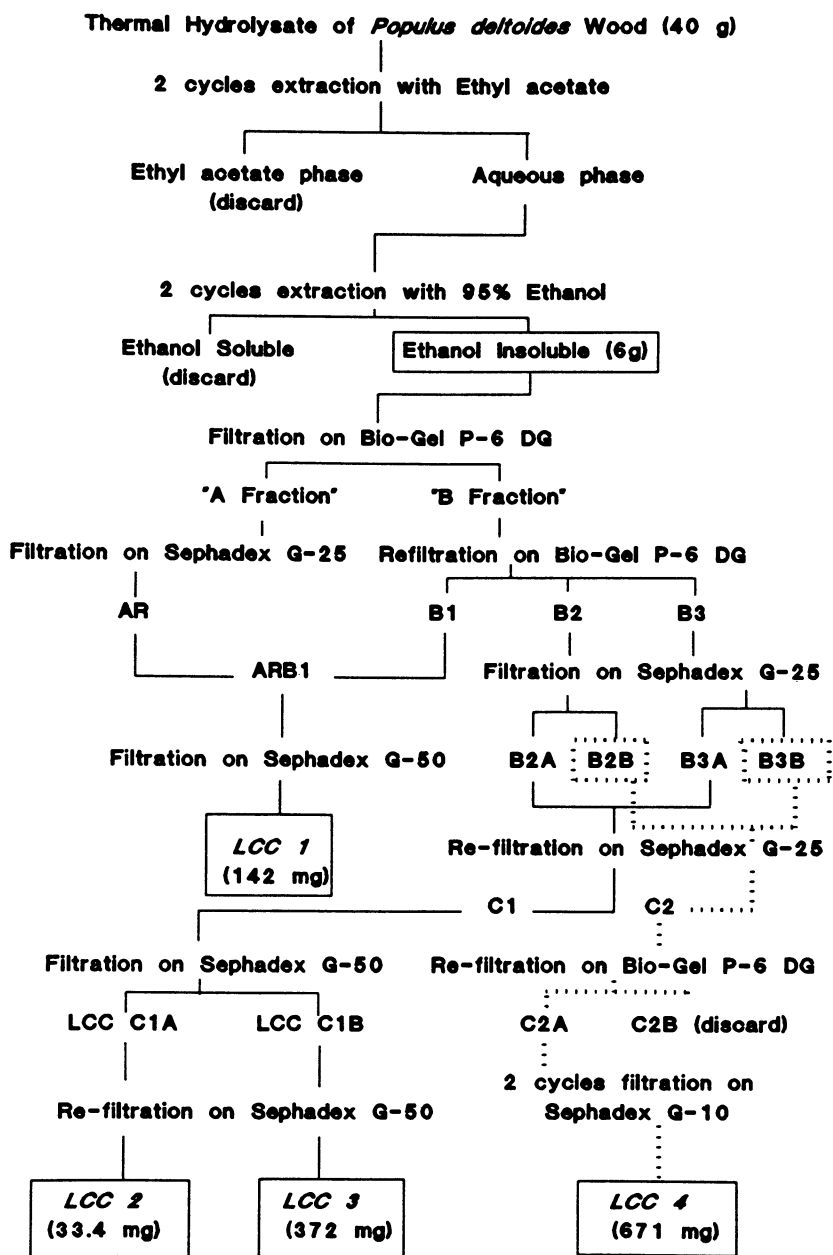


Figure 1. Scheme used for the purification of *Populus deltoides* LCCs.

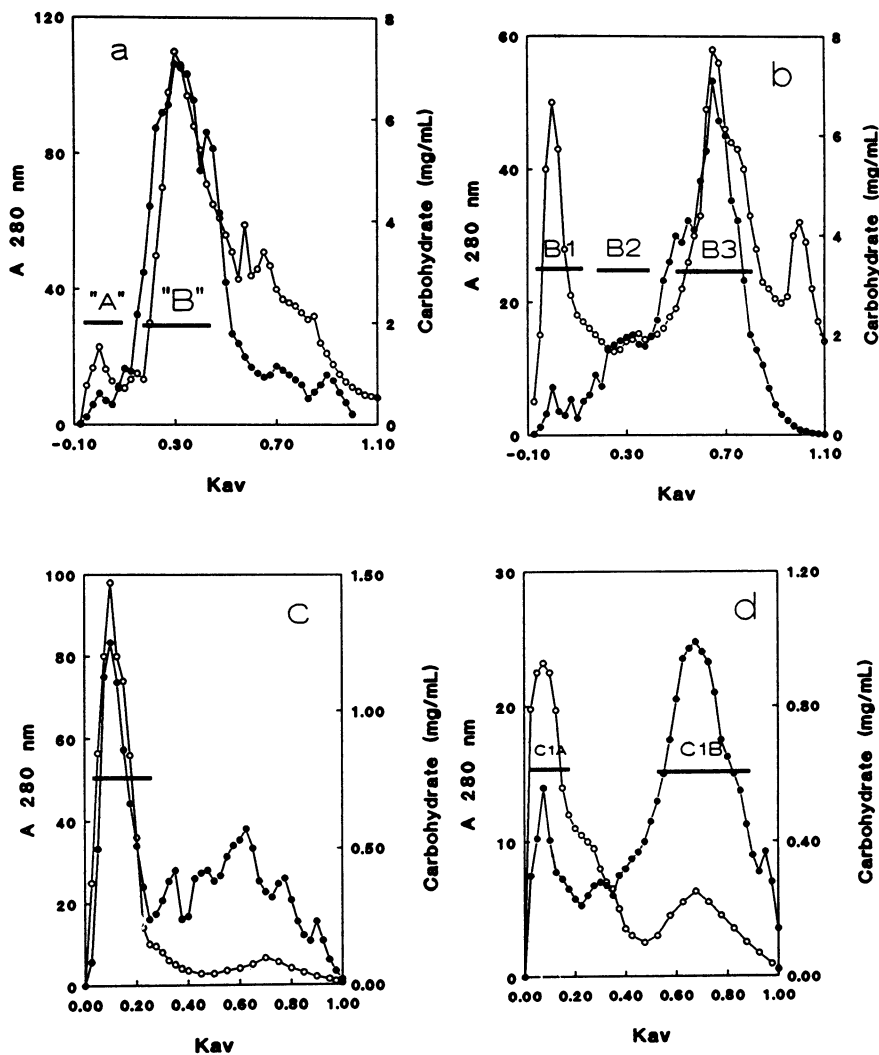


Figure 2. Representative fractionations employed in the purification of *Populus deltoides* LCCs. a, initial fractionation of high ("A") and low ("B") molecular weight components through Bio-Gel P-6; b, re-fractionation of "B" through Bio-Gel P-6; c, penultimate fractionation of ARB1 on Sephadex G-50; d, penultimate fractionation of C1 components on Sephadex G-50. A_{280} , (○---○); carbohydrate, (●---●).

The least abundant moieties, LCCs 1 and 2, were relatively high molecular weight species having lower cmc values than the more abundant, smaller LCCs 3 and 4, which exhibited about 10-fold higher cmc values.

Elemental analyses of the thermal hydrolysate, ethanol insoluble material, and purified LCCs appear in Table II. The ash content of starting material was highly conserved in the ethanol insoluble fraction, and particularly in LCCs 1 and 2. After adjusting for ash content, contents of C, H, and O were comparable in the four LCC preparations.

Table II. Elemental Analysis of *Populus deltoides* LCC Components^a

Sample	C	H	N	O ^b	Ash
Thermal hydrolysate	44.89	5.82	2.79	41.94	4.56
Ethanol precipitate	34.90	4.21	0.47	37.87	22.55
LCC 1	29.47	3.31	1.12	66.10	31.50
LCC 2	29.43	3.63	1.67	39.00	26.27
LCC 3	40.95	5.21	0.19	53.65	1.91
LCC 4	33.47	5.21	0.37	60.56	11.65

^aData are expressed as percentages of dry weight.

^bOxygen was calculated by difference.

Carbohydrate/*O*-acetyl content of *P. deltoides* LCCs appears in Table III. All purified LCCs were *O*-acetylated moieties containing varying amounts of glucose, mannose, xylose, and uronic acids. Relative to xylose, the highest levels of uronic acid and *O*-acetyl content were encountered in LCCs 1 and 2. LCC 1 was the sole moiety to contain galactose. Xylose was the most abundant sugar found in both LCCs 3 and 4, both of which also contained nearly equimolar amounts of glucose and mannose.

FTIR spectra of various *P. deltoides* preparations in the frequency range of 800 to 1,900 cm⁻¹ appear in Figure 3. Spectra were examined to 4,000 cm⁻¹, but no essential differences were encountered with the exception of LCC 3 which presented a prominent C-H band at approximately 2,900 cm⁻¹. Spectra for LCCs 1 and 3 were the most distinct. LCC 1 displayed a prominent diffuse band in the 1,500 - 1,700 cm⁻¹ region possibly associated with either aromatic ring stretches or conjugated keto-carbonyl groups. LCC 3 possessed a prominent peak at 1,736 cm⁻¹ apparently associated with xylan carbonyl stretching and a 1,047 cm⁻¹ band ascribed to the C-O stretch in hemicelluloses. The 987 cm⁻¹ hemicellulose stretch was more clearly evident in LCC 3 spectra. All four preparations exhibited absorption increases in the 800-850 cm⁻¹ regions ascribed to aromatic band absorption. A small peak at 875 cm⁻¹ present in LCCs 1 and 3 was assigned to lignin.

CP-MAS ^{13}C NMR spectra of LCCs 3 and 4 appear in Figure 4a and 4b respectively. LCC 3 displayed a prominent resonance at approximately 174 ppm which was assigned to the acetyl group in acetyl xylan and which confirmed the assignment of the $1,736\text{ cm}^{-1}$ FTIR absorption peak. LCC 4 presented a broad resonance in this region with major peaks at 183 and 176 ppm, and shoulders at 171 and 169 ppm. Both spectra presented peaks associated with the C-1 of sugars centered on 103-104 ppm, and peaks associated with the secondary hydroxyl groups on carbons centered on 75 ppm and primary hydroxyl groups at about 65 ppm. LCC 3 spectra clearly contained much more carbon with primary hydroxyl groups and had much more methyl- CH_3 signal at 22 ppm than did LCC 4 spectra.

Table III. Chemical Composition of *Populus deltoides* Lignin-carbohydrate Complexes

LCC	Component	$\mu\text{mol/mg}$	Molar Ratio
1	Glucose	0.050	0.38
	Mannose	0.027	0.20
	Xylose	0.133	1.00
	Galactose	0.039	0.29
	Uronic acids	0.233	2.40
	O-acetyl	0.320	1.75
2	Glucose	0.061	0.46
	Mannose	0.022	0.17
	Xylose	0.153	1.00
	Uronic acids	0.144	0.94
3	O-acetyl	0.133	0.87
	Glucose	0.206	0.09
	Mannose	0.222	0.10
	Xylose	2.300	1.00
	Uronic acids	0.480	0.21
4	O-acetyl	1.580	0.69
	Glucose	0.211	0.21
	Mannose	0.211	0.21
	Xylose	0.993	1.00
	Uronic acids	0.356	0.36
	O-acetyl	0.400	0.40

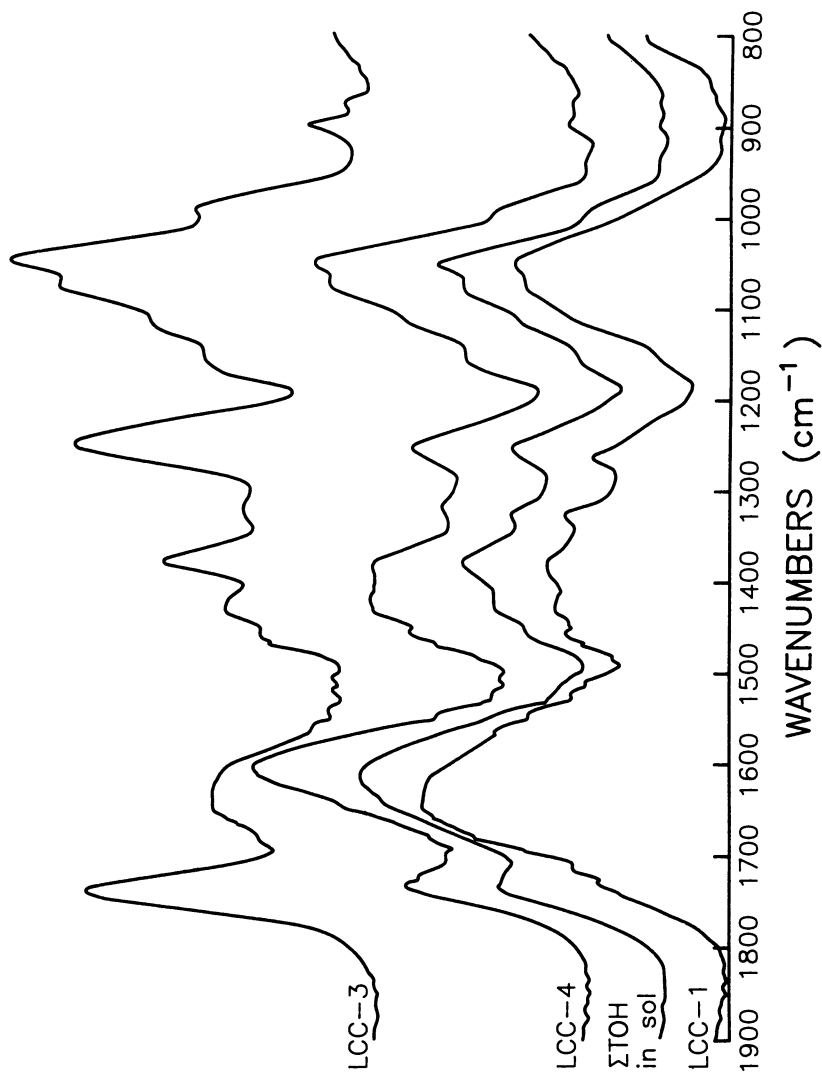


Figure 3. FTIR spectra of *Populus deltoides* LCCs 1, 3, and 4, and the ethanol-insoluble fraction used in their preparation.

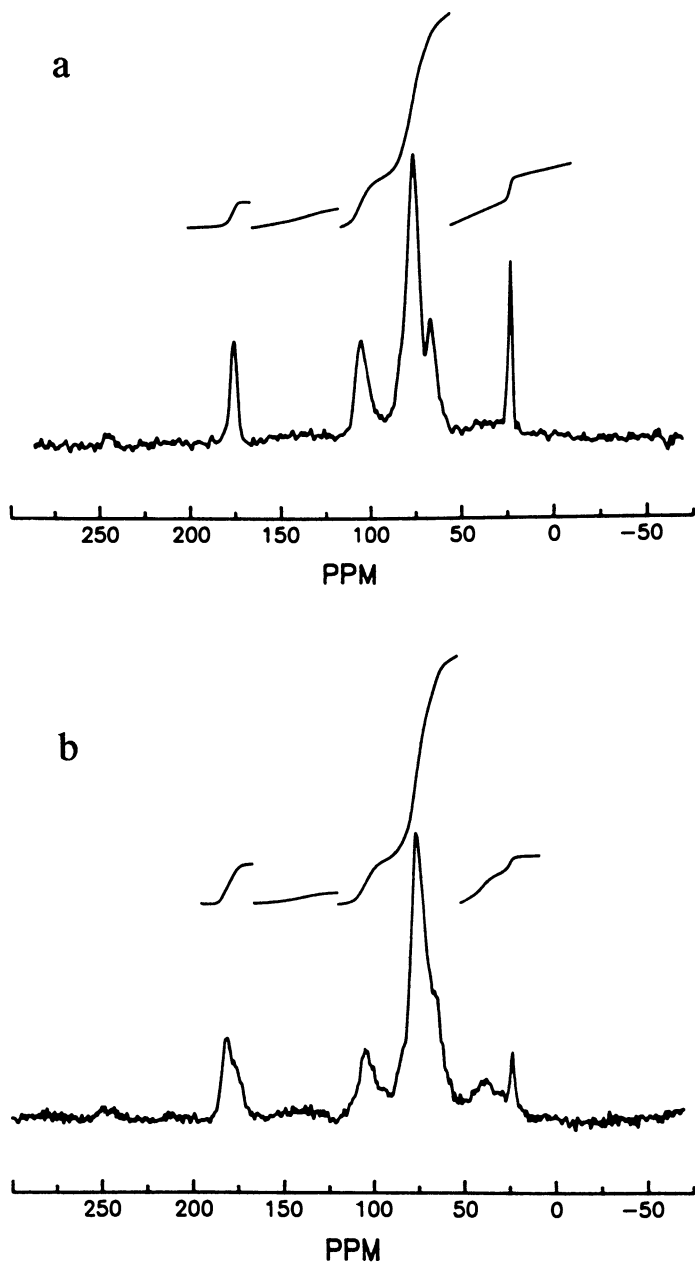


Figure 4. CP-MAS ^{13}C NMR spectra of *Populus deltoides* LCCs 3 (a), and 4 (b).

ESR spectra of LCCs 3 and 4 appear in Figure 5. A strong free radical component at $g = 2.0045$ observed in the spectrum of LCC 3 (Figure 5a) was evident but diminished in the spectrum of LCC 4 (Figure 5b). Relative spins (Mn^{2+}) of the thermal hydrolysate, LCC 3 and LCC 4 were 16, <5, and 100 respectively. Independent estimation of the quantity of manganese ions in LCC 4 generated an value of 10^{16} ions/gram. These data indicate that LCC 4 which is obtained in about 2% yield from the thermal hydrolysate concentrates manganese relative to LCC 3 which is obtained in about 1% yield.

Enzyme Treatment of *P. deltoides* LCCs. Initial experiments indicated that when LCC1 was treated with xylanase or β -mannanase in the absence of AXE, there was no detectable release of reducing groups. While xylanase or β -mannanase digestion of LCCs 3 and 4 mediated reducing group release, approximately 3-fold higher levels were observed upon co-incubation with AXE. For this reason, AXE was used in all further enzyme digests unless otherwise stated. Amounts of reducing power released by various enzyme treatments appears in Table IV.

Table IV. Effect of Enzymes on *Populus deltoides* LCCs

LCC	Treatment (All included AXE)	Reducing Power	
		$\mu\text{g}/\text{mg}$ LCC	% of Total Carbohydrate
1	Xylanase	53.5	26.2
	β -Mannanase	62.1	30.4
	Xylanase + β -mannanase	50.5	24.8
	β -Glucosidase	32.8	16.1
	Xylanase + β -glucosidase	25.0	12.3
3	Xylanase	155.8	20.8
	β -Mannanase	62.9	8.4
	Xylanase + β -mannanase	166.0	22.2
	β -Glucosidase	71.9	9.6
	Xylanase + β -glucosidase	79.2	10.6
4	Xylanase	59.4	14.9
	β -Mannanase	44.9	11.3
	Xylanase + β -mannanase	67.2	16.9
	β -Glucosidase	53.1	13.3
	Xylanase + β -glucosidase	62.8	15.8

Independent of the enzyme treatment, there was only limited LCC degradation, highest levels being observed with LCC 1. Most digests using enzyme combinations exhibited lower levels of reducing power than did digests with single enzymes.

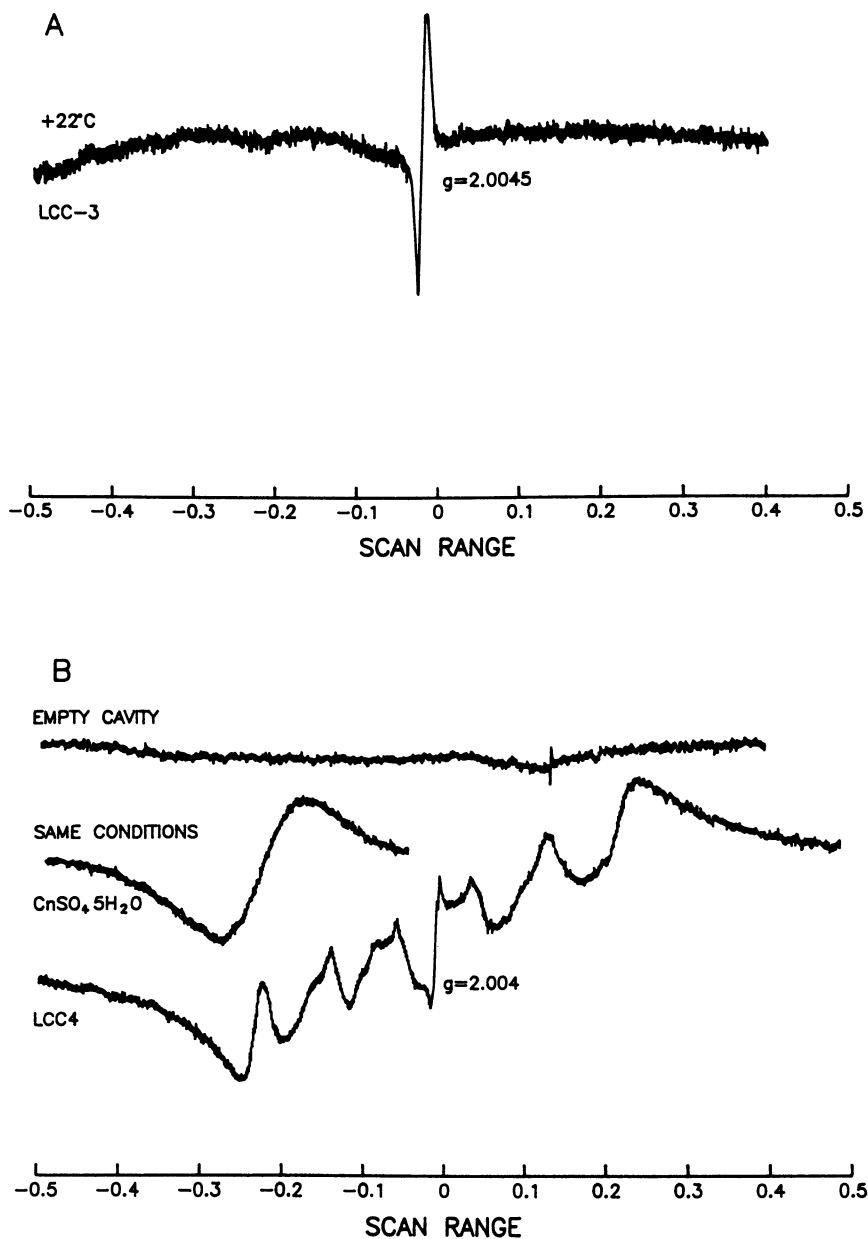


Figure 5. ESR spectra of *Populus deltoides* LCC preparations. LCC 3 (A); LCC 4 (B).

Elution profiles of purified and enzyme treated LCCs 1,3, and 4 appear in Figures 6, 7, and 8, respectively. For each treated LCC, independent of the enzyme(s) used, elution profiles were qualitatively identical. In enzyme digests of LCCs 1 and 3, while there was loss of congruence of A_{280} and carbohydrate content, there was little evidence for the production of significant amounts of low molecular weight material. Enzyme treatment of LCC 1 ($K_{av} = 0.1$, cmc = 20 - 30 $\mu\text{g/mL}$), for example, generated a major product ($K_{av} = 0.08$; cmc = 80 - 100 $\mu\text{g/mL}$) of decreased carbohydrate content. Digestion of LCC 3 ($K_{av} = 0.685$; cmc = 250-350 $\mu\text{g/mL}$; Figure 7a) with xylanase and AXE generated elution profiles having a predominantly A_{280} peak ($K_{av} = 0.03$; cmc = 80 - 100 $\mu\text{g/mL}$) and a predominantly carbohydrate-containing peak ($K_{av} = 0.74$; cmc = 85 - 95 $\mu\text{g/mL}$; Figure 7b). The same enzyme digests of LCC 4 ($K_{av} = 0.039$; cmc = 260 - 320 $\mu\text{g/mL}$; Figure 8a) resulted in profiles containing a major A_{280} carbohydrate-containing peak of $K_{av} = 0.56$, cmc = 100 - 120 $\mu\text{g/mL}$. In no instance was there evidence for enzyme catalyzed monosaccharide production.

Discussion

The sequence to which the *P. deltooides* thermal hydrolysate was subjected generated four LCCs distinguishable by gel permeation behaviour, carbohydrate and *O*-acetyl content, critical micelle concentration, and content of A_{280} . While carbohydrate compositions of these LCCs were comparable with those described for LCCs isolated from the woods of birch, bald cypress (25), beech (26), and bamboo (27), *P. deltooides* LCCs contained significantly higher levels of uronic acids and *O*-acetyl groups and were smaller moieties, ostensibly because of the initial thermal hydrolysis used in their isolation. Coupled with chemical analyses, FTIR, and CP-MAS ^{13}C NMR spectrometric data indicated that the carbohydrate components are likely derived from *O*-acetyl-4-*O*-methylglucuronyl-*D*-xylan and (*O*-acetyl)- β -glycomannan fragments. Although carbohydrate peaks were clearly present in FTIR and ^{13}C NMR spectra, the instrumental techniques were not definitive with respect to lignin/aromatic components. While FTIR analysis indicated the presence of aromatic nuclei, anticipated aromatic resonances in the 90-135 ppm region, syringyl/guaiacyl resonances in the 145-160 ppm region, or aryl methoxyl resonances in the 55-57 ppm region of CP-MAS ^{13}C NMR were not so evident. A suggestion of peaks in the anticipated regions of LCC 4 ^{13}C NMR spectra prompted a search for free radicals by ESR on the basis that their association with aromatic nuclei might interfere with NMR measurements. With LCC 3, ESR analysis indicated the presence of a strong free radical signal, which if associated with aromatic nuclei could interfere with their detection in NMR spectra. LCC 4 exhibited a significant concentration of manganese ions which could also result in less defined NMR spectra in aromatic regions. The high ash content of LCCs 1,2, and 4 was at variance with their K_{av} properties and the fact that at no point during their purification were they exposed to sources of ion contamination. Therefore, high ash levels, which in at least one case are associated with Mn^{2+} content, appear to be reflections of the complexing ability, (attributable in some degree to the uronic acid content) of the LCCs. Inability of

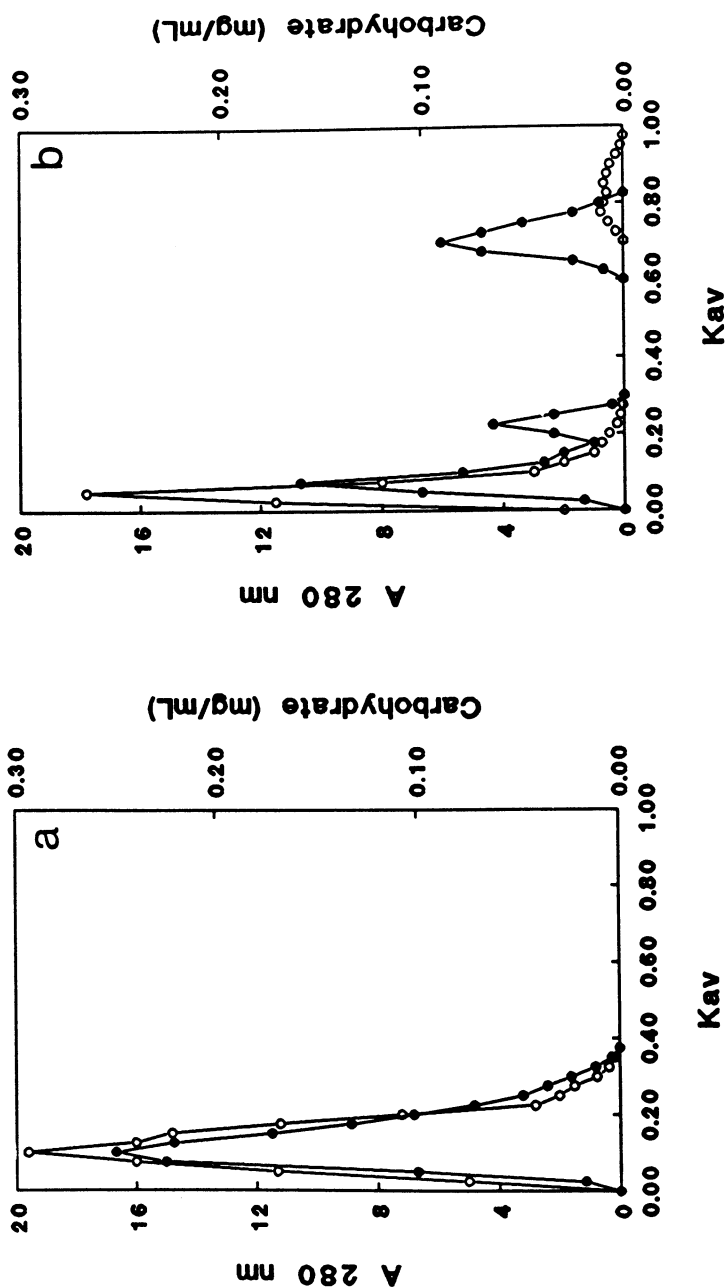


Figure 6. Elution profiles of LCC 1 from Sephadex G-50. Untreated LCC 1 (a); Xylanase + AXE digest of LCC 1 (b). A₂₈₀ (○—○); carbohydrate, (●—●).

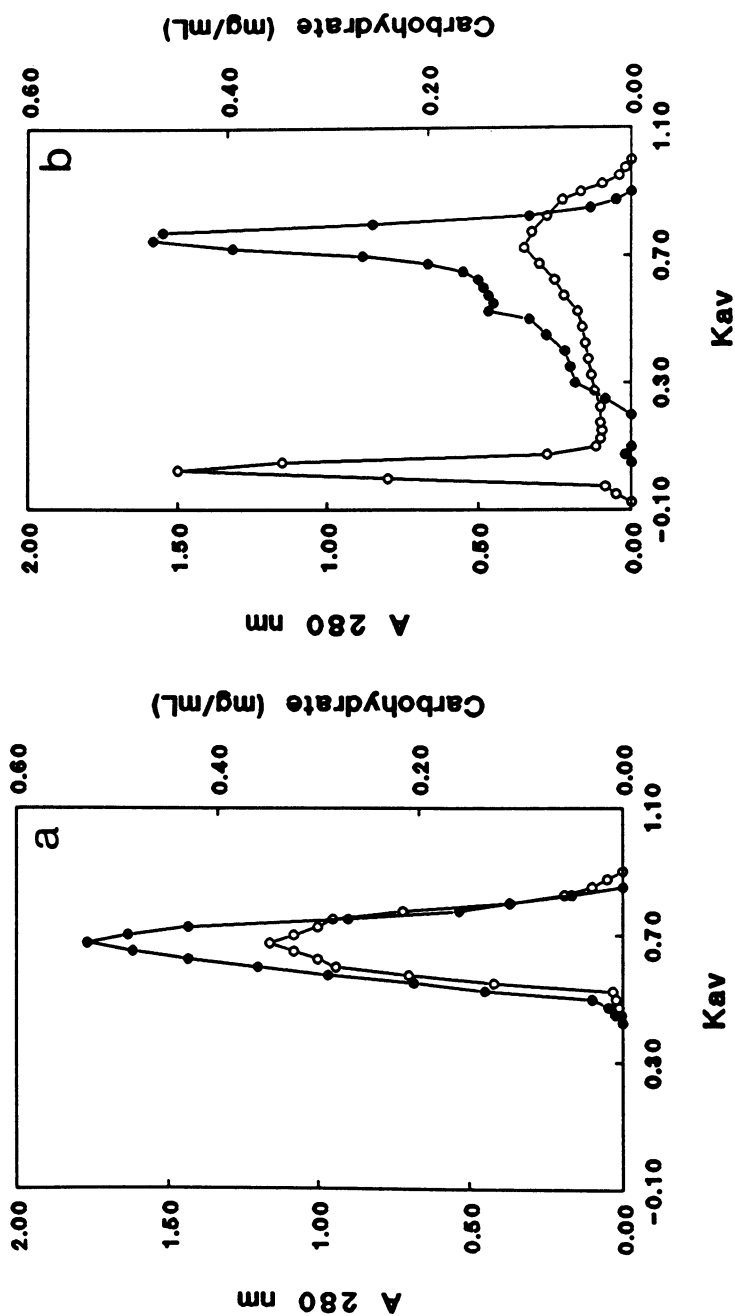


Figure 7. Elution profiles of LCC 3 from Sephadex G-50. Untreated LCC 3 (a); Xylanase + AXE digest of LCC 3 (b). A_{280} , (○—○); carbohydrate, (●—●).

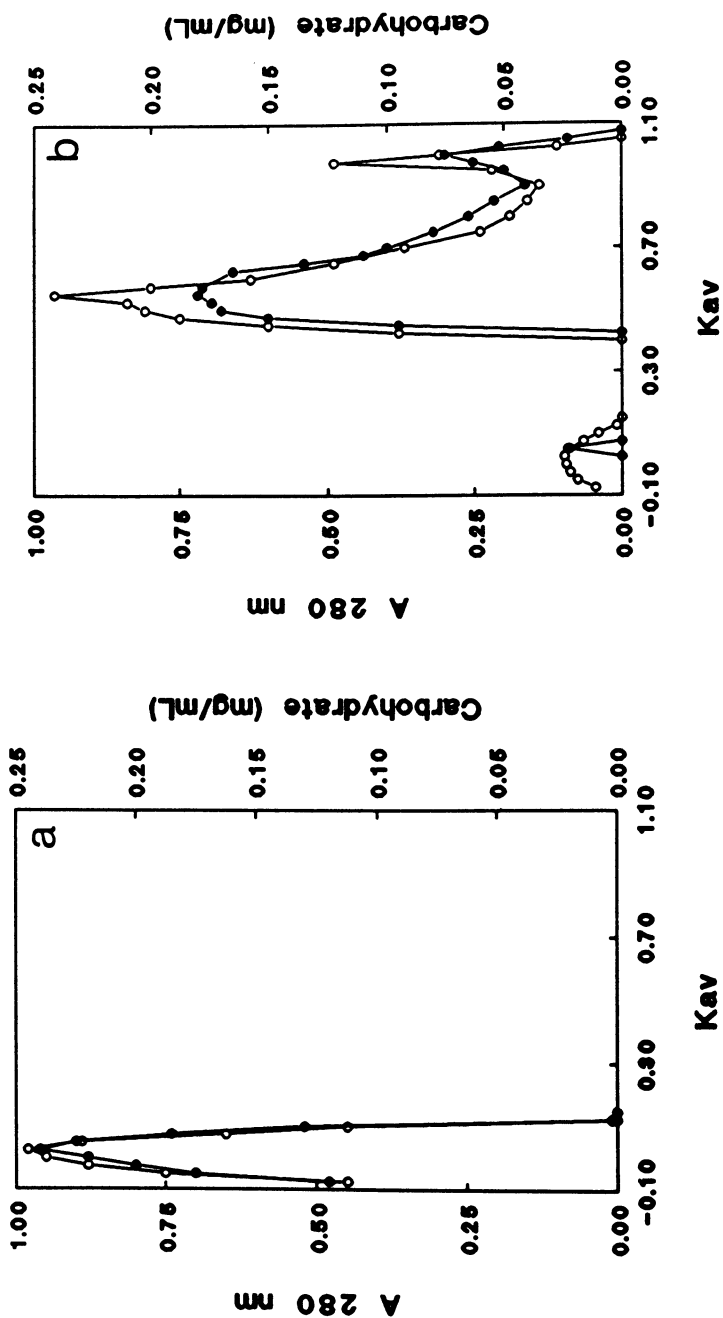


Figure 8. Elution profiles of LCC 4 from Sephadex G-10. Untreated LCC 4 (a); Xylanase + AXE + β -mannanase digest of LCC 4 (b). A_{280} (○—○); carbohydrate, (●—●).

^{13}C NMR to confirm the aromatic component of LCCs from graminaceous sources has been previously documented (10).

That lignin or lignin-like components exist in the *P. deltooides* moieties designated as LCCs is supported by the following: all purified moieties exhibited strong ultraviolet absorption and high lignin content as estimated the acetyl bromide method. LCCs 1 and 2 were so highly reactive in this lignin assay as to deliver abberantly high values (Table I). Moreover, like the lignin-rich *Pinus densiflora* LCC reacted with pinocyanol chloride (24), all purified *P. deltooides* preparations presented evidence of micelle formation, a condition mediated by interaction of hydrophilic carbohydrate and hydrophobic lignin in aqueous environments.

Overall, enzymic treatment of purified *P. deltooides* LCCs conserved lignin as measured by A_{280} . With LCC 1 (Figure 6), enzyme treatment produced a small increase in the K_{av} of the major A_{280} peak and some dissociation of carbohydrate-containing material. A more dramatic effect was seen with LCC 3 (Figure 7). Elution profiles of untreated LCC 3 exhibited a congruent A_{280} , carbohydrate-containing peak of $K_{av} = 0.685$. However, in elution profiles of enzyme-treated LCC 3, highest A_{280} was associated with a peak ($K_{av} = 0.05$) which contained but traces of carbohydrate while highest carbohydrate content was non-congruently associated with A_{280} in a peak of $K_{av} = 0.74$. With LCC 4 (Figure 8), enzyme treatment while not producing a significant dissociation of A_{280} and carbohydrate content, did result in a large increase of K_{av} from 0.039 to 0.56.

While the results of this study do not confirm the existence of covalent linkages between the lignin and carbohydrate components of *P. deltooides* LCCs, conservation of high molecular weight lignin and lack of monomeric carbohydrate generation under any tested polysaccharidase treatment infers that lignin protects the carbohydrate component(s) of LCCs from extensive enzymic breakdown. The high potential for ion binding most likely attributable to the high uronic acid content of the LCCs almost certainly affects the conformation of the complex and may play a large role in rendering them refractory to enzymic degradation.

Literature Cited

1. Björkman, A. *Ind. Eng. Chem.* 1957, 49, 1395.
2. Iverson, T.; Wannström, S. *Holzforschung* 1986, 40, 19.
3. Josleau, J.-P.; and Kesraoui, R. *Holzforschung* 1986, 40, 163.
4. Azuma, J.; Takahashi, N.; Koshijima, T. *Carbohydr. Res.* 1981, 93, 91.
5. Koshijima, T.; Watanabe, T.; Azuma, J. *Chem. Lett.* 1984, 1737.
6. Eriksson, Ö.; Goring, D.; Lindgren, B. *Wood Sci. Technol.* 1980, 14, 267.
7. Brice, R.E.; Morrison, E.; *Carbohydr. Res.* 1982, 101, 93.
8. Lundquist, K.R.; Simonsson, R.; Tingsvik, F. *Svensk Papperstidn* 1983, R44.
9. Neilson, M.J.; Richards, G.N. *Carbohydr. Res.* 1982, 104, 1.
10. Nordkvist, E.; Graham, H.; Åman, P. *J. Sci. Food Agric.* 1989, 48, 311.
11. Conchie, J.; Hay, A.J.; Lomax, J.A. *Carbohydr. Res.* 1988, 177, 127.
12. Viikari, L.; Ranua, M.; Kantelinen, A.; Linko, M.; Sundquist, J. *Proc. Internatl. Symp. Wood Pulp. Chem.* 1987, 1, 151.

13. Haemmerli, S.D.; Leisola, M.S.; Fiechter, A. *FEMS Microbiol. Lett.* 1986, 35,33.
14. Jurasek, L.; Paice, M.G. *Proc.1988 Tappi Bleaching Conference*, 1988, p.11.
15. Noe, P.; Chevalier, J.; Mora, F.; Comtat, J. *J. Wood Chem. Technol.* 1986, 6, 167.
16. Dietrichs, H.; Sinner, M.; Puls, J. *Holzforschung* 1978, 32, 193.
17. Milstein, O.; Vered, Y.; Sharma, A.; Gressel, J.; Flowers, H.M. *Appl. Environ. Microbiol.*, 1983, 46, 55.
18. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F., *Anal. Chem.* 1956, 28, 350.
19. Somogyi, M. J. *Biol. Chem.* 1952, 195,19.
20. Morrison, I.M. *Phytochem.* 1988, 27, 1097.
21. Blumenkrantz, N.; Asboe-Hansen, G. *Anal. Biochem.* 1973, 54, 484.
22. Biely, P.; MacKenzie, C.R.; Puls, J.; Schneider, H. *Bio/Technol.* 1986, 4, 731.
23. Johnson, D.B.; Moore, W.E.; Zank, L.C. *Tappi* 1961, 44, 793.
24. Yaku, F.; Tsuji, S.; Koshijima, T. *Holzforschung* 1979, 33, 54.
25. Azuma, J.; Tetsuo, K. *Methods in Enzymology* 1988, 161, 12.
26. Takahashi, N.; Koshijima, T. *Wood Res.* 1987, 74, 1.
27. Azuma, J.; Nomura, T.; Koshijima, T. *Agric. Biol. Chem.* 1985, 49, 2661.

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Chapter 22

Cellulase

Insights through Recombinant DNA Approaches

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Cellulases are inherently interesting. Induction of such complex multiple synergistic components is efficiently accomplished though the substrate is extracellular and insoluble. Their study has been focused historically on applied aspects, initially on means of inhibiting their degradative action, with recent emphasis on obtaining enhanced yields/activities to promote efficient hydrolysis of cellulose to glucose syrups. Cloning of cellulases has opened new vistas for basic study. It facilitates purification of the individual components, a major advance in a notoriously difficult arena. It has aided development of the concept of the triple (binding, hinge and active site) domains of cellulase, besides allowing their further characterization via site specific mutagenesis. Evolutionary relationships have been clarified. The rDNA methodology also directly aids the application of cellulases through improvement and/or hyperproduction of specific components, thereby allowing them to be mixed optimally for routine and new applications.

Cellulases are central to the world's carbon cycle and energy flow as a result of cellulose being the world's most abundant natural polymer. Production guesstimates are in the range of 1×10^{11} tons per year. This abundant, but recalcitrant structural component is recycled through the action of cellulases. Cellulases also engender economic interest as they are both prime agents of decay and yet also a potential key to conversion of waste biomass (agricultural, food and forestry residues) into fermentation feedstocks (1). Furthermore, they are crucial in cell wall synthesis of plants and fungi, facilitating morphogenic development by permitting selective intercalation of cellulose building blocks within the wall.

The study of cellulases initially focussed on means of inhibiting their activities in connection with man's desire to prevent decay of cellulosic materials - from timber to tents. More recently when energy oil supplies were restricted in the 1970's, interest in cellulase surged with a variety of proposals to reduce the energy deficit through use of cellulosic residues as an energy resource. In general, these scenarios envisaged conversion of biomass, especially waste biomass, to glucose and thence by fermentation to oxychemicals, e.g., ethanol as both a liquid transportation fuel and also an octane enhancer.

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As a result of these studies, major advances in our understanding of cellulases occurred over the last decade. The previously somewhat restricted study of a few fungi (e.g., *Trichoderma reesei*, *Phanerochaete chrysosporium*) was greatly enlarged to include other fungi, besides bacterial and plant systems. Even so, the surface has just been scratched as there must be myriads of cryptic cellulolytic species in forest litter and agricultural soils that have been ignored because they do not produce copious amounts of readily recoverable extracellular enzymes. One major finding is the confirmation that cell-bound cellulases (cellulosomes) are efficient and occur widely (2,3). General screening has also resulted in the discovery of cellulases with alkaline pH optima. These latter have been shown to act effectively as adjuncts in laundry detergents and are already on the market in Japan (see Horikoshi, this symposium).

Molecular and biochemical studies have inspired novel ideas on the mechanisms of how a cellulase enzyme attacks a large insoluble substrate (Grohmann et al., this symposium). Such concepts in conjunction with molecular biological advances have advanced the idea of a cellulase being comprised of three facets; an active site domain, a hinge region and a cellulose binding zone. It has been proposed that 25 known cellulase gene sequences fit into six natural families based on comparison of DNA sequences and hydrophobic cluster analyses (2,4). Such analyses are significant in relation to practical application and evolutionary perspective. We believe that considerably greater insight can be gained from this type of analysis. With this in mind we present a comparison of cellulases based on domain analysis and multiple sequence alignments especially with regard to the cellulase of *Microbispora bispora* currently under study in our laboratory (5).

Domain Analysis

Analyses of the cellulases have concentrated mainly on comparison of groups of enzymes based upon both their overall structure, as well as their primary sequence. These analyses have shown these enzymes generally to be made up of three distinct regions; the catalytic domain, the cellulose binding domain, and a linker region also known as the hinge region. An example sequence, the *Microbispora bispora* endoglucanase is shown in Figure 1. Interestingly, some sequences contain two or more binding regions, each separated by a linker domain (6).

We have recently undertaken a reconsideration of cellulase sequences by analyzing each domain of the sequence separately. Here we describe initial observations and methodologies we are developing for the analysis and comparison of cellulase domains. Novel methods of classification will give greater insight into theoretical, evolutionary and practical approaches to the analysis of cellulase.

The analysis of cellulase sequences is facilitated by first dividing the sequences into each of the three predominant domains; the catalytic, binding and linking domains. The organization of some cellulase genes is such that the catalytic domain at the amino terminus is followed by the linking domain and the binding domain, e.g., Fig. 1. However, several cellulases are characterized by a carboxy terminal catalytic domain, with the binding and linking domains located at the amino terminal end.

Cellulase sequences can readily be subdivided into the representative domains by sequence homology. We decided on initial groupings based upon a FastA analysis of each sequence, using 30 cellulase sequences as the database. The FastA analysis is based upon homology or similarity comparisons as developed by Lipman and Pearson (7,8), and implemented by the Genetics Computer Group sequence analysis package (9). Each of the catalytic and binding domains were then edited out of the primary sequence, using homology to several known catalytic and binding domains and a series of homology searches were run with each sequence. The linker or hinge region was used as the dividing region when deciding domain boundaries, as this sequence is readily observed by its repeating P/S/T motif. The P/S/T motif is characterized by a repeating pattern of proline-serine or proline-threonine residues which separate the binding and

catalytic domains of most cellulases. Grouping of domains into families was based solely on observed sequence similarity, and multiple sequence alignments (10) were performed on each family to confirm the family relatedness.

Catalytic Domains

An analysis of catalytic domains results in some distinctive family groupings. These groupings are listed in Table 1. The striking characteristic of these groups is that they all share significant sequence similarity when they are analyzed using only their homologous domains. The inclusion of the often non-homologous hinge and binding domains tends to dilute the similarity observed.

The catalytic domain sequences do not seem to be strongly, or at least solely influenced by phylogeny. For example, we see in Family 1 the presence of both bacterial and fungal cellulase sequences. This family has a different relationship between its members than do Families 4 and 5, which are comprised of members of a single species. However, we do notice that cellulase sequences from a particular organism can be distributed into several different families.

Multiple sequence alignments of each family highlight specific, and distinct features in each group. These features may be sufficient to describe both the similarity between the members of a group, and to discriminate them from the members of other groups. We are currently developing methods that may be able to provide the functionality to make these determinations. A representative sequence alignment of the catalytic domain Family 1 is shown in Figure 2. Such alignments are very useful in the determination of key residues that may be involved in the conserved enzymatic activities of these subunits.

Binding Domains

The binding domains can also be classified into families on the basis of sequence homology (Table 2). However, this relationship is not the same as that of the catalytic domain (c.f. Tables 1 and 2), suggesting that perhaps these two domains have evolved separately and have only recently been united.

The relationships observed in the substrate binding domain seem to be most strongly influenced by the relationship present between the organisms themselves. This is in contrast to the catalytic domains where we noted that the relationships are not strongly influenced by phylogeny. This finding suggests the possibility that cellulase domains have evolved separately. One particular example that is notable, is the substrate binding domains of the *P. fluorescens* CMCase and the xylanase from the same organism. These two enzymes are active against quite distinct substrates, namely highly crystalline cellulose and amorphous xylan, yet their binding domains share a high level of similarity. This level of similarity is missing from the catalytic domains of these enzymes. These data suggest that substrate binding may be a more generalizable function, and that the evolution of these domains may be different than for the catalytic domains.

Again, multiple sequence alignments can help us to elucidate the key residues involved in the cellulose substrate binding function of these domains. Figure 3 shows just such an alignment, using the sequences of the members of the binding domain Family 1. This multiple sequence alignment is particularly interesting as the *Pseudomonas* domains share very significant sequence homology with each other, as well as the other substrate binding domains, while binding different substrates.

Linker Domain

The linker region, sometimes referred to as the PSbox or hinge region, has a very different type of conservation than do the other domains. In fact, these domains can

Table 1. Cellulase catalytic domain families, organized by similarity scores as determined by successive FastA searches¹

FAMILY 1

Cellulomonas fimi Endoglucanase A (11)
Microbispora bispora Endoglucanase I (5)
Streptomyces sp. Cellulase A (12)
Trichoderma reesei Cellobiohydrolase II (13)

FAMILY 2

Bacillus sp. Cellulase (14)
Bacillus sp. Cellulase A (14)
Bacillus sp. Cellulase B (15)
Bacillus sp. Cellulase C (16)
Bacillus subtilis Cellulase (17)
Bacillus subtilis Cellulase B (18)
Clostridium acetobutylicum Cellulase (19)
Erwinia chrysanthemi Cellulase Z (20)

FAMILY 3

Clostridium thermocellum Cellulase D (21)
Pseudomonas fluorescens CMCCase (22)

FAMILY 4

Clostridium thermocellum Cellulase A (23)
Clostridium thermocellum Cellulase B (24)
Clostridium thermocellum Cellulase D (21)

FAMILY 5

Trichoderma reesei Cellulase (25)
Trichoderma reesei Cellulase A (26)
Trichoderma reesei Exocellobiohydrolase I (27)

¹Each of the representative members of a family demonstrated a similarity score of at least 5 standard deviations from the mean score when the search was made using the PIR database, release 24 (28).

Table 2. Cellulase binding domain families, organized by similarity scores (see Table 1)

FAMILY 1

Cellulomonas fimi Endoglucanase A (11)
Cellulomonas fimi Exoglucanase (29)
Microbispora bispora Endoglucanase I (5)
Pseudomonas fluorescens CMCase (22)
Pseudomonas fluorescens Xylanase A (30)

FAMILY 2

Bacillus sp. Cellulase (14)
Bacillus sp. Cellulase A (14)

FAMILY 3

Clostridium thermocellum Cellulase A (23)
Clostridium thermocellum Cellulase B (24)
Clostridium thermocellum Cellulase D (21)
Clostridium thermocellum Cellulase E (31)
Clostridium thermocellum Cellulase X (31)
Clostridium thermocellum Xylanase Z (32)

FAMILY 4

Trichoderma reesei Cellulase A (26)
Trichoderma reesei Cellulase (25)
Trichoderma reesei Cellobiohydrolase II (13)
Trichoderma reesei Cellobiohydrolase I (33)
Phanerochaete chrysosporium Exocellobiohydrolase I (34)

only be very loosely organized by homology as they only seem to preserve specific characteristics rather than distinct sequences. At this point, no real determination of the families can be made. Such a determination awaits the development of a method for comparing very diverse sequences.

Summary

The classification of cellulase sequences into domains allows the further recognition of distinct sequences that characterize each family. To make these sequences evident, one must use multiple sequence alignments of the members of each individual family. We have concentrated on the *Microbispora bispora* endoglucanase, and present some initial multiple sequence alignments of the catalytic and binding domain families of which it is a member.

A domain analysis of the cellulase sequences illuminates some striking relationships between these diverse enzymes. The domains of many of these genes have apparently either evolved separately, or have experienced some sort of directed evolution. The presence of strong relationships between binding domains within a species, with the classification of the catalytic domains into different families, suggests the former hypothesis. It does appear that cellulase gene evolution may be taking place piecemeal. That is, the binding domains may have evolved independently of the catalytic domains, with the distinct possibility that they were recruited subsequent to the development of the catalytic domains.

The linker or hinge domain may simply be a region of protein sequence that efficiently links the divergent catalytic and binding domains. We have observed that in many multi domain proteins there are one or several proline residues, often associated with threonine or serine, in the region between the domains. This could be a common method of domain association that links distinctly separate domains and thus facilitates their coordinate evolution and development into a contiguous protein sequence. That would explain the observation of both amino terminal catalytic domains, as in the *M. bispora* endoglucanase, as well as carboxy terminal catalytic domains, as in the *T. reesei* CBH II, existing within the same catalytic domain family.

The results of this analysis are obviously preliminary. We present them in the hope that we can stimulate both discussion and research in the area of cellulase domain analysis. It is our intention to fully develop the sequence analysis of these domains, and to develop logical criteria for family membership within each of the domain families.

Acknowledgments

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Literature Cited

1. Glick, B. R.; Pasternak, J. J. *Biotech. Adv.* 1989, 7, 361-386.
2. Beguin, P. *Ann. Rev. Microbiol.* 1990, 24, 219-248
3. Robson, L. M.; Chambliss, G. H. *Enzyme Microbiol. Technol.* 1989, 11, 626-644.
4. Henrissat, B.; Claeyssens, M.; Tomme, P.; Lemesle, L.; Mornon, J.P. *Gene* 1989, 81, 83-95.
5. Yablonsky, M. D.; Elliston, K. O.; Eveleigh, D. E. In *Enzyme systems for lignocellulose degradation*; Coughlan, M. P., Ed.; Elsevier Applied Science: London, UK, 1989; pp 73-83.

6. Owolabi, J. B.; Beguin, P.; Kilburn, D. G.; Miller, R. C. J.; Warren, R. A. J. *Appl. Environ. Microbiol.* 1988, 54, 518-523.
7. Pearson, W. E.; Lipman, D. J. *Proc. Nat. Acad. Sci.* 1988, 85, 2444-2448.
8. Lipman, D. J.; Pearson, W. R. *Science* 1985, 227, 1435-1441.
9. Devereux, J.; Haeberli, P.; Smithies, O. *Nucleic Acids Res.* 1984, 12(1), 387-395.
10. Higgins, D. G.; Sharp, P. M. *Computer applications in the biosciences* 1989, 5, 151-153.
11. Wong, W. K. R.; Gerhard, B.; Guo, Z. M.; Kilburn, D. G.; Anthony, R.; Warren, J.; Miller, R. C. J. *Gene* 1986, 44, 315-324.
12. Nakai, R.; Horinouchi, S.; Beppu, T. *Gene* 1988, 65, 229-238.
13. Teeri, T. T.; Lehtovaara, P. K. S.; Salovuori, I.; Knowles, J. *Gene* 1987, 51, 43-52.
14. Fukumori, F.; Sashihara, N.; Kudo, T.; Horikoshi, K. *J. Bacteriol.* 1986, 168, 479-485.
15. Fukumori, F.; Kudo, T.; Narahashi, Y.; Horikoshi, K. *J. Gen. Microbiol.* 1986, 132, 2329-2335.
16. Fukumori, F.; Kudo, T.; Sashihara, N.; Nagata, Y.; Ito, K.; Horikoshi, K. *Gene* 1989, 76, 289-298.
17. Robson, L. M.; Chambliss, G. H. *J. Bacteriol.* 1987, 169, 2017-2025.
18. MacKay, R. M.; Lo, A.; Willick, G.; Zuker, M.; Baird, S.; Dove, M.; Moranelli, F.; Seligy, V. *Nucleic Acids Res.* 1986, 14, 9159-9170.
19. Zappe, H.; Jones, W. A.; Jones, D. T.; Woods, D. R. *Appl. Environ. Microbiol.* 1988, 54, 1289-1292.
20. Guiseppi, A.; Cami, B.; Aymeric, J. L.; Ball, G.; Creuzet, N. *Mol. Microbiol.* 1988, 2, 159-164.
21. Joliff, G.; Beguin, P.; Aubert, J. P. *Nucleic Acids Res.* 1986, 14, 8605-8613.
22. Hall, J.; Gilbert, H. *J. Mol. Gen. Genetics* 1988, 213, 112-117.
23. Beguin, P.; Cornet, P.; Aubert, J. P. *J. Bacteriol.* 1985, 162, 102-105.
24. Grepinet, O.; Beguin, P. *Nucleic Acids Res.* 1986, 14, 1791-1799.
25. Penttilae, M.; Lehtovaara, P.; Nevalainen, H.; Bhikhabhai, R.; Knowles, J. *Gene* 1986, 45, 253-263.
26. Van Arsdell, J. N.; Kwok, S.; Schweickart, V. L.; Ladner, M. B.; Gelfand, D. H.; Innis, M. A. *Bio/Technology* 1987, 5, 60-64.
27. Shoemaker, S.; Schweickart, V.; Ladner, M.; Gelfand, D.; Kwok, S.; Myambo, K.; Innis, M. *Bio/Technology* 1983, 1, 691-696.

28. George, D. G.; Barker, W. C.; Hunt, L. T. *Nucleic Acids Res.* 1986, 14, 11-15.
29. O'Neill, G.; Goh, S. H.; Warren, R. A. J.; Kilburn, D. G.; Miller, R. C., Jr. *Gene* 1986, 44, 325-330.
30. Hall, J.; Hazlewood, G. P.; Huskisson, N. S.; Durrant, A. J.; Gilbert, H. J. *Mol Microbiol.* 1989, 3, 1211-1219.
31. Hall, J.; Hazlewood, G. P.; Barker, P. J.; Gilbert, H. J. *Gene* 1988, 69, 29-38.
32. Grepinet, O.; Chebrou, M.-C.; Beguin, P. J. *Bacteriol.* 1988, 170, 4582-4588.
33. Teeri, T. T.; Lehtovaara, P.; Kauppinen, S.; Salovuori, I.; Knowles, J. *Gene* 1987, 51, 43-52.
34. Sims, P.; James, C.; Broda, P. *Gene* 1988, 74, 411-422.

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Chapter 23

Structure of Cellulolytic Enzymes

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The structures of two cellobiohydrolases (E.C.3.2.1.91) from *Trichoderma reesei* (CBH I, CBH II) were determined by small angle X-ray scattering (SAXS) analysis. Both enzymes have a tadpole-like shape with an isotropic head and a long flexible tail. The CBH I molecule has a length of 18.0 nm, the head has a maximum diameter of 2.09 nm; CBH II has a length of 21.5 nm with a head of 2.10 nm. The SAXS data are discussed in relation to recent X-ray diffraction studies and other known structural properties (amino acid sequences, disulfide linkages, glycosylation, active center localization, cellulose-binding sites) of the enzymes.

Cellulases are highly specialized fungal or bacterial enzymes catalyzing the hydrolysis of beta-1,4-glycosidic bonds in crystalline and amorphous cellulose, soluble celldextrins and some derivatives such as carboxymethyl cellulose, hydroxyethyl cellulose and chromophoric glycosides of celldextrins (for review see ref. 1). Considerable progress has recently been made in the purification of these enzymes (for review see ref. 1), the determination of their substrate specificity and kinetic properties 2,3, gene analysis and cloning 4 as well as in their structural and functional characterization 5. All evidences available point to a two domain organization of these cellulolytic enzymes: a catalytic (hydrolytic) domain (core) and a separate cellulose binding domain. In most sequenced cellulases 80 to 90 % of all amino acids are located in the core part which thus contains the major mass of the cellulase protein. Such an organization has been demonstrated unambiguously for four cellulases (CBH I, CBH II, EG I and EG III) from *Trichoderma reesei* 5-7, and two enzymes (CenA and Cex) from *Cellulomonas fimi* 8. It was tentatively deduced for several other cellulases e.g. those from *Clostridium thermocellum* 9.

Analysis of a large number of genes coding for cellulases clearly showed that most of the cellulase proteins are in fact products of individual genes. For example, cbh1, cbh2, eg11, eg13 were identified and sequenced from *Trichoderma reesei* (for review see 4); more than fifteen cellulase genes were found in *Clostridium thermocellum* and five of them were sequenced 9. The comparison of the amino acid sequences suggests that cellulases usually show no or only weak sequence homology in the catalytic domain. Exceptions are CBH I and EG I of *T. reesei* with 50% sequence homology in the head domain. Comparisons of secondary structure using more elaborate methods such as hydrophobic cluster analysis have established the existence of at least six structural cell-

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ulase families composed of enzymes from widely different organisms, but showing strong homologies within each family 10. Most cellulases also contain a 30 to 60 amino acid long sequence rich in proline and highly glycosylated threonine and serine residues. This region is frequently called block B 4 or PT-box 8. Adjacent to block B the so called "cellulose binding domain" (block A) is found. It contains about 30 to 100 amino acids. The sequence in this block A appears to be highly conserved in cellulases from the same organism. For example, in *T. reesei* the enzymes CBH I, CBH II, EG I and EG III show 70% sequence homology in block A consisting of 30 amino acids. Two cellulases from *C. thermocellum* (EG A and EG B) show an identical reiterated sequence of 24 amino acids in block A which is preceded in both enzymes by a domain rich in proline, threonine and serine. The repeated sequence of 24 amino acids is also found in a third *C. thermocellum* cellulase (EG D) but here the proline-rich sequence is missing 9. Similarly in two cellulases from *Cellulomonas fimi* a highly conserved block A is apparent, adjacent again to a PT box 8. The contiguous AB regions, i.e. the tail domains, are located at either the carboxy- or at the amino terminus of the polypeptide chain. In the first case the sequence of the three blocks from the N- to the C-terminus is therefore "core-B-A", in the latter case "A-B-core". It seems reasonable to assume that this interchanged position of the B and A part was mediated in evolution by gene shuffling.

For *Trichoderma reesei* the cellulases most thoroughly investigated with respect to their structural properties are CBH I and CBH II and to a lesser extent the two endoglucanases (EG I and EG III) 11 (Table I). For most other cellulases structural characterizations are confined to the amino acid sequence as deduced from the base sequence of the respective genes and to sequence related investigations such as hydrophobic cluster analysis 10 or computer aided molecular modelling and homology

TABLE I: Analysis performed on *T. reesei* enzymes

Enzyme	amino acids	domain sequence	analysis performed
CBH I	497	core-BA	aa sequence in part from protein and in full from gene (cbh1), number and location of SS bridges, region of O-glycosylation, types of carbohydrate, papain cleavage site, hydrophobic cluster analysis, computer model of active site, 2D-NMR on a synthetic tail fragment, SAXS on whole CBH I, head domain and xylan/CBH I complex
CBH II	447	ABB'-core	aa sequence in part from protein and in full from gene (cbh2), number of SS bridges, region of O-glycosylation, types of carbohydrate, papain cleavage site, hydrophobic cluster analysis, SAXS on whole CBH II and head domain, three dimensional structure of head by X-ray diffraction
EG I	437	core-BA	aa sequence in part from protein and in full from gene (eg11), hydrophobic cluster analysis, SAXS not successful
EG III	397	AB-core	aa sequence in part from protein and in full from gene (eg13), high mannose content 47 mole/mole enzyme, hydrophobic cluster analysis

comparison. One should however keep in mind that such investigations provide only approximations of the structural and functional organization. Only X-ray diffraction analysis of crystalline cellulases with resolution in the 0.1 – 0.2 nm range would allow precise determination of the tertiary conformation and the spatial position of the amino acid residues. The core domains of CBH I and CBH II have been crystallized 11,12 and very recently, the fully resolved three-dimensional structure of the CBH II core was published 13. EGD from *Clostridium thermocellum* cloned in *E. coli* has also been crystallized 14.

Small-Angle X-Ray Scattering

Small-angle X-ray scattering (SAXS) has been used since quite some time for the determination of the structure of macromolecules in solution (for a review see 14). Also for non-crystallizing proteins some structural details can be obtained and since measurements can be performed under more or less "natural" conditions in aqueous solutions and various influences on the shape of the molecule can be studied (e.g. effects of ligand binding). Colleagues from the Institute of Physical Chemistry at the University of Graz have a long standing tradition and experience in using this technique. In fact most of the pioneering work in theory and practice (Kratky camera) and the development of this method have been performed in this Institute under the leadership of Prof. Dr. O. Kratky (For review see 16).

Several parameters, e. g. the radius of gyration (R_g) and the maximal diameter (D_{max}), of the molecule are available directly from the scattering function $I(h)$ or from the so-called distance distribution function $p(r)$ which may be obtained by Fourier transformation of the $I(h)$ function 17. The distance distribution function $p(r)$ gives some preliminary information concerning the shape of the measured molecule. Computer-aided modelling to simulate the distance distribution and scattering functions with models made up of small spheres can be used to reveal further structural details by trial and error. Models for the shape of the molecule are generated in this way until their $p(r)$ and $I(h)$ functions closely match the experimental data 16. The computed model which fits best to the experimental functions is taken as representative for the shape of the investigated molecule.

The resolution of SAXS depends on the form of the molecule and is on average between 0.2 and 0.5 nm. The main difference from high resolution X-ray diffraction is that SAXS can resolve only details of the tertiary and quaternary structure of the molecule and thus give information about the molecular shape but not on the secondary structure.

The principle of the method is briefly as follows: A series of at least four concentrations (ranging from 5 – 50 mg/ml) are measured successively for several hours each. During the measurement of each SAXS curve the scattered radiation is recorded for a range of angles between nearly zero and 3 to 5 angular degrees with respect to the primary beam. The measurement can be performed at any temperature that is suited to the needs of the sample. After completion of the measurements the data set is extrapolated to zero concentration and Fourier transformed. The results are the de-smear scattering and distance distribution functions. The average amount of sample needed for a complete SAXS analysis is 30 to 50 mg of an at least 95% pure sample.

Structure of Cellobiohydrolase I from *T. reesei*

Cellobiohydrolase I (CBH I, 1,4- β -D-glucan-cellobiohydrolase, E.C. 3.2.1.91) is the main protein (ca. 60%) of the cellulase complex produced by *T. reesei* strains. CBH I hydrolyses crystalline cellulose, acid swollen cellulose and 4-methylumbelliferyl-cellodextrins by cleaving off the terminal cellobiose unit from the non reducing end of the chain. It operates with retention of configuration in the reaction products 19,20. The abundance of this enzyme and its stability has facilitated its purification to homogeneity

by various chromatographic methods in quantities up to several hundred milligrams, amounts sufficient for detailed biochemical and physicochemical studies.

Large parts of its amino acid sequence had already been determined in 1984 ²¹ and this had later been confirmed by sequencing the corresponding gene *cbh1* ^{22,23}. CBH I consists of 497 amino acid residues: 33 Asp, 23 Asn, 57 Thr, 56 Ser, 23 Glu, 19 Gln, 28 Pro, 63 Gly, 29 Ala, 23 Val, 24 Cys, 6 Met, 11 Ile, 26 Leu, 24 Tyr, 15 Phe, 13 Lys, 5 His, 9 Arg, 9 Trp. The degree of glycosylation (5 to 10%) depends on the mutant and probably also on the culture conditions. Identified sugar residues include 16 mannose, 6 glucose and 5 galactose units per CBH I molecule ²⁴. Upon SDS polyacrylamide electrophoresis and native polyacrylamide electrophoresis homogeneity is proven and the molecular mass found by different methods is between 59 and 68 kDa ²⁴.

The CBH I, prepared from *Trichoderma reesei* MCG 77 and purified by chromatofocusing exhibited a molecular mass (SDS-PAGE) of 59 ± 1.5 kDa and its isoelectric point was 3.6. After initial SAXS measurements were performed on this sample, results surprisingly pointed to an unusual shape. The scattering curve indeed suggested that the molecule has a rather large head and a flexible tail. It was first thought that this might be an artifact caused by impurities (e.g. peptides) in the preparation or some other unknown factors. CBH I was therefore prepared again from a batch of another fermentation, and no impurities could be detected by SDS-PAGE or FPLC on a Mono Q column or in the analytical ultracentrifuge. The SAXS measurements gave again nearly the same result as obtained with the first CBH I preparation (Figure 1) and we were therefore convinced that CBH I has an unusual "tadpole" shape. The model published in 1986 ²⁴ together with the two distance distribution functions $p(r)$ experimentally measured for these two CBH I preparations and the $p(r)$ curve calculated for the tadpole model is shown in Figure 1. The dimensions of the molecule are given in Table II.

This publication ²⁴ started a series of collaborative efforts which confirmed and significantly extended our knowledge about the three-dimensional shape of cellobiohydrolases. A CBH I prepared from the *T. reesei* strain VTT D-80133 by affinity chromatography ²⁵ revealed by SAXS molecular dimensions which are very close to those previously measured (Figure 2). The model derived from the scattering curves again showed a tadpole structure with a total length of 18.0 nm. About two-thirds of this length is confined to the tail. This time more distinct structural details could be observed, e.g. the collar-like part in the tail probably representing the region of glycosylation (block B).

The presence of two structural domains in CBH I has been postulated previously based on the amino acid sequence and the location of disulfide bridges ²⁶ and this was later confirmed by limited proteolytic studies ²⁷. Treatment with papain cleaves the polypeptide chain of CBH I at about residue 430 leading to a small (10 kDa) C-terminal glycoprotein which strongly binds to cellulose and a large 54 kDa N-terminal core protein which contains the active center. It could be shown by SAXS ²⁵ that this core protein is the head domain of the CBH I molecule (Figure 3). The molecular dimensions of the cleaved head part isolated from the papain digest by affinity chromatography coincide with the dimensions of the head seen in the intact molecule (Table II).

Such a polypeptide chain folding into two distinct domains could be correlated with the amino acid sequence. The N-terminus of the polypeptide chain is located at an as yet unknown site of the head part and starts with the sequence Pyr.Glu-Ser-Ala-Cys-Thr-, the C-terminal region is located at the end of the tail and has the sequence -Pro-Tyr-Tyr-Ser-Gln-Cys-Leu. From the total of 497 amino acids about 430 are located in the head part, where the chain is partially ordered in β -structures. Ten disulfide bridges give stability and rigidity to this part of the molecule ²⁴. The bridges interconnect the cysteine residues 4-50, 19-25, 61-71(?), 67-72(?), 138-397, 172-209(?), 176-210(?), 230-256, 238-243, and 261-331. The exact position of some

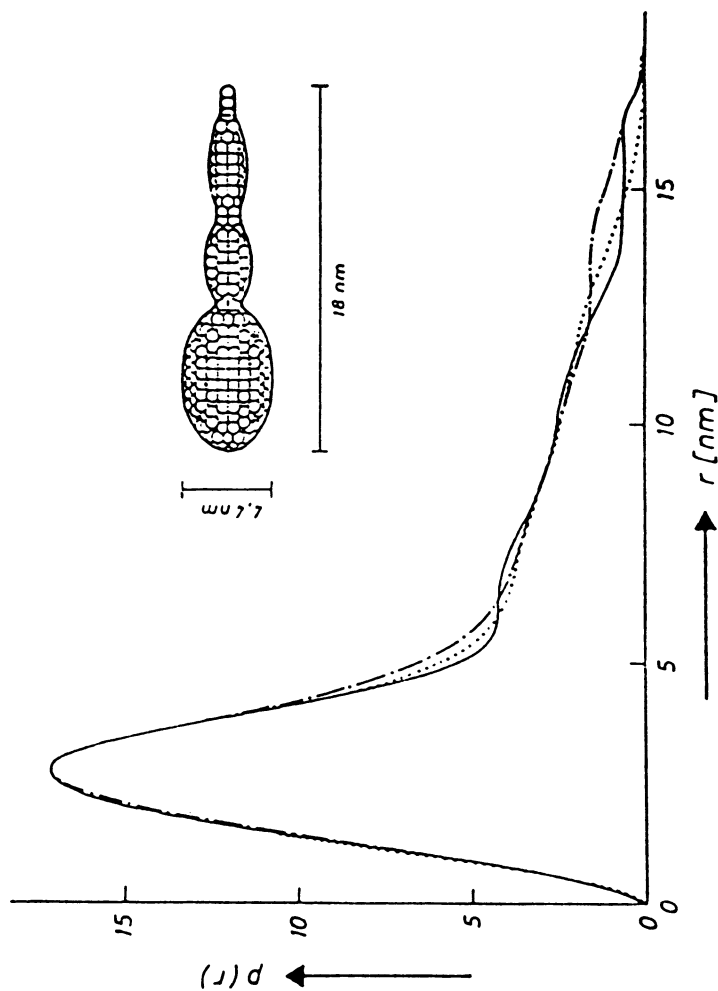


Figure 1. Model of CBH I deduced from SAXS measurements proposed 1986 (from ref. 24 with permission of the authors). The distance distribution function $p(r)$ was determined for two different CBH I preparations (—; - - - - -), the dotted line (· · · · ·) giving the $p(r)$ function calculated for the model.

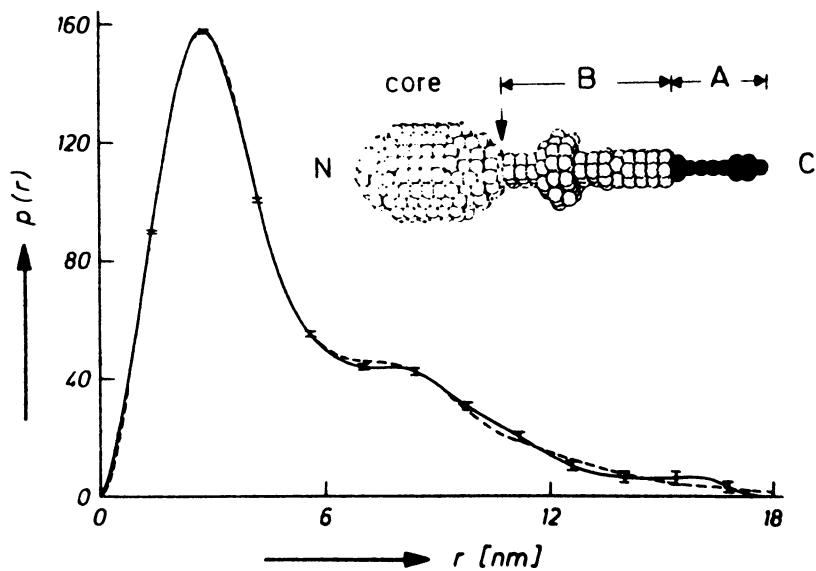


Figure 2. Model of CBH I deduced from a second series of SAXS measurements (redrawn from ref. 25 with permission).

The sequence of the domains from the N-terminus to the C-terminus is C-B-A. The arrow indicates the site papain cleavage.

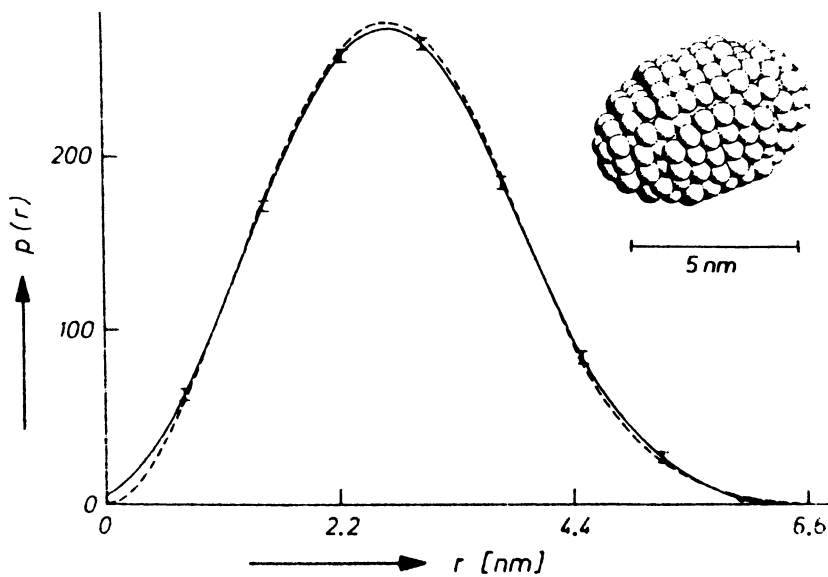


Figure 3. SAXS-based model for CBH I core protein (Reproduced with permission from ref. 25; Copyright Springer 1988).

The core protein was prepared by limited papain proteolysis from the intact enzyme preparation shown in Fig. 2

local bridges is yet unknown. It should be noted that two SS-bridges connect regions which are 70 (261 to 331) and 259 (138 to 397) amino acid residues distant from each other. Such non-local bridges could contribute significantly to the compactness of the head part of the molecule. After the amino acid at position 429 (Thr) the protein chain probably exits from the main body of the head and the sequence which follows is (430) Gly-Asn-Pro-Ser-Glu-Gly-Asn-Pro-Pro-Gly-Gly-Asn-Pro (442). In analogy to other structurally characterized proteins the Gly-Asn-Pro - boxes can be seen as a hinge region which makes a flexible connection between the head and the remaining tail part. That this region has a flexible connection to the head was already proposed in the SAXS study from the variability of the distance distribution function seen in the tail part of the molecule 21. The following sequence contains 22 amino acids (443 to 464) rich in threonine (8 residues) and serine (3 residues) which probably reflects the region of the O-glycosylated block B. The last stretch of the tail comprises 33 amino acids (465 to 497) and contains two SS bridges which span from residue 469 to 486 and from 480 to 496 26. This last part of the tail domain (block A) contains binding site(s) for cellulose. In the tadpole model the region of glycosylation is probably the bulgy, collar-like part of the tail. If so, the connecting piece to the head should be the hinge region and the part following the bulge should be the cellulose binding domain A. In summary, the tail of CBH I consists of 67 amino acid residues, the first part (hinge region and glycosylated region, block B) contains 34 residues, the second part (cellulose binding region, block A) consists of 33 amino acid residues.

The C-terminal polypeptide block A (residues 462-497) has also been synthesized and its three dimensional structure in solution (90% H₂O/10%D₂O) was determined by two-dimensional NMR-spectroscopy with a 600 MHz instrument 28. According to this analysis the peptide has a wedge-like shape with dimensions 3 x 1.8 x 1.0 nm. This wedge probably represents the last part in the SAXS model which has a length of about 4 nm and a maximum diameter of 1.4 nm. The principle element of the secondary structure in this part is the β -conformation. Three antiparallel short β -sheets composed of residues 466 to 470 (β_1), 485 to 489 (β_2) and 493 to 496 (β_3) were identified. The sites which could be responsible for binding to cellulose were not identified but the NMR data indicate that one face of the wedge is flat and hydrophilic (4 tyrosine residues) whereas the other face is hydrophobic. Both structures could potentially interact with the cellulose chain on the surface of the crystallites. Some evidence has been advanced for the fact that tyrosine residues play a part in the binding phenomenon 5.

The core protein prepared by papain cleavage is enzymatically active, which proves that the active center, where the β -1,4-glycosidic bonds are cleaved, is in the head domain of the protein. The amino acids of the active site have not yet been identified with certainty. From chemical modification experiments with more or less COOH-group specific reagents (carbodiimide, Woodward's reagent K) it seems now rather well established that a carboxylate group, probably a glutamic acid residue, plays a crucial role in catalytic activity 29. Glutamic acid has also been identified as a part of the catalytic domain in several lysozymes, e.g. human milk, duck egg white, hen egg white or T4 phage lysozyme. In T4 phage lysozyme glutamic acid 11 and aspartic acid 20 are catalytically active residues and it has been proposed that in CBH I catalytically active residues are either glutamic acid 64 and aspartic acid 74 30 or glutamic acid 126 29 and aspartic acid 130 29.

SAXS investigations have also been presented which suggest that CBH I can undergo some kind of induced fit with a change of shape if it interacts with a ligand 32. The insoluble cellulose itself cannot be used for such studies since it would disturb the scattering experiment. Instead of cellulose a water soluble xylan (*Rhodymenia palmata*) with an average molecular weight of 3000 to 5000 was used. This xylan interacts very strongly with the intact CBH I and its core, most probably by binding to the active (hydrolytic) center and, to a lesser extent, to the cellulose binding domain. SAXS measurements of CBH I in the presence of a twofold molar excess of xylan revealed

that the binding of xylan to the head leads to a significant elongation of the particle, in particular in the tail domain (Table II). From computer aided simulation studies it was suggested that the xylan binding site (which eventually might be found to be the catalytic center of the enzyme) is located at the surface of the head near the tail part.

Table II: Molecular dimensions of *T. reesei* cellobiohydrolases and their core as determined by SAXS

Rg = radius of gyration in nm \pm 5%, D_{max} = maximum distance in nm \pm 5%, n.d.: not determined

	whole enzyme			core		
	Rg	D _{max}	shape	Rg	D _{max}	shape
CBH I ^a	4.20	18.0	tadpole	n.d.	n.d.	
CBH I ^b	4.27	18.0	tadpole	2.09	6.5	ellipsoid
CBH II ^c	5.40	21.5	tadpole	2.10	6.0	ellipsoid
CBH I/xylan ^d	5.18	22.0	tadpole	2.06	5.8	ellipsoid

a) MCG 77, 24; b) QM 9414, (VTT-D-80133); 25 c) QM 9414 31;

d) QM 9414 in presence of *Rhodymenia palmata* xylan (molar ratio 2.0 to 2.5) 32.

Structure of Cellobiohydrolase II from *T. reesei*

The second cellobiohydrolase (E.C. 3.2.1.91) excreted by *T. reesei* was termed CBH II. This enzyme also produces mainly cellobiose with minor amounts of glucose and celotriose from crystalline or amorphous cellulose. Its specific activity as measured with Avicel is about twice as high as this of CBH I. CBH II operates with inversion of configuration as opposed to the reaction mechanism valid for CBH I 19,20. Immunologically, the CBH II protein is clearly distinct from that of CBH I 33 and it is proven that both enzymes are encoded by different genes, i.e. *cbh1* and *cbh2* 7. The functional role of these enzymes in cellulose hydrolysis is not yet clear but it appears that CBH II acts synergistically in a cooperative mode with CBH I (for review see 1). Recent evidence point to a possible "loose complex" formation between both enzymes occurring in solution prior to cellulose adsorption 34.

The amino acid sequence has been partly determined on the purified protein and was fully deduced from the sequenced gene 7,35. According to this analysis the mature CBH II protein sequence is 447 amino acids long, the numbers of amino acid residues deduced from the cDNA sequence per CBH II molecule being as follows: 56 Ala, 13 Arg, 30 Asn, 21 Asp, 12 Cys, 21 Gln, 8 Glu, 39 Gly, 4 His, 15 Ile, 32 Leu, 10 Lys, 4 Met, 12 Phe, 31 Pro, 46 Ser, 34 Thr, 12 Try, 21 Tyr, 26 Val. At the N-terminus the chain begins with Pyr.Glu-Ala-Cys-Ser-Ser-Val-Thr... and ends at the C-terminus with ...Thr-Asn-Ala-Asn-Pro-Ser-Phe-Leu.

For the SAXS studies a CBH II sample was prepared by affinity chromatography from *T. reesei* QM 9414 to give the enzyme in a homogeneous form 27. In SDS-PAGE the protein had a size of 58 kDa and the isoelectric point was 4.9. Glycosylation was estimated as 8 to 18 % 36. The molar absorptivity at 280 nm was 75 000 M⁻¹cm⁻¹. To obtain the core protein partial proteolytic hydrolysis with papain was per-

formed, similarly as in the case of CBH I studies 27. The core exhibited a size of 45 kDa (SDS-PAGE) and an isoelectric point of 4.4. Figures 4 and 5 show the distance distribution functions measured by SAXS for the intact CBH II and its core protein together with the models which best fit these curves 31. The dimensions of the molecule are given in Table II. Although the three-dimensional shapes of the CBH I and CBH II molecules appear to be very similar, some important differences are evident. For example CBH II has a somewhat smaller head (by about 10%) but its tail is significantly longer (by about 35%). In the CBH II molecule the tail part shows a long symmetrical part (light and dark grey in Figure 4), which probably represents the regions of block B and B'. In the amino acid sequence this region is rich in threonine and serine and it can be assumed that it is the region of O-glycosylation. In CBH II the glycosylation does not lead to the characteristic collar-like structure seen in CBH I.

In contrast to CBH I the N-terminus of the protein chain of CBH II coincides with the tail, whereas the C-terminus represents the core. Based on the sequence and homologies with CBH I, the CBH II molecule is organized in four domains, A, B, B' and the core, where ABB' represents the tail linked to the head part. A is the terminal part consisting of 38 amino acids with 6 cysteine residues located at positions 3, 10, 20, 21, 27 and 37. These probably form three local SS bridges 7. The exact position of these bridges has not yet been determined. The domain A is the black part in the model of Figure 4. Following A is a sequence (block B) of 24 amino acids (39-62) rich in serine (9 residues) and threonine (5 residues). This sequence is followed by block B' consisting of 26 amino acids (63-89) again rich in serine (6 residues) and threonine (6 residues). The amino acid sequences in block B and B' are highly conserved. A distinct hinge region like that found in CBH I is not found in the B' block although this region is rich in proline (5 residues) but a typical hinge region with the Gly-Asn-Pro sequence (amino acid 90-92) does connect the tail part (i.e. B') with the main body of the protein.

It can be assumed that the amino acids following this hinge region (Val 93 to Leu 447) are part of the head domain. The point of papain cleavage is at amino acid 82 27. The core part of the polypeptide chain is mainly folded in β -sheets (34 %) and to a lesser extent (15 %) arranged in α -helical structures 7. In contrast with CBH I the core of CBH II possesses only 2 disulfide bridges (176-235; 368-415) and four free sulfhydryl groups. Similarly to CBH I carboxyl functions are involved in the active center (Asp 175 and Glu 184) 28.

Very recently 13 the three dimensional structure of the CBH II core was fully determined by X-ray diffraction. The polypeptide chain is folded in α -helices and β -strands (α , β -protein with a central β -barrel built up by seven parallel strands. Six of the β -strands are linked by α -helices. Near the C-terminus of the enzyme is a tunnel with dimensions well suited to take up a single cellulose chain. Two aspartic acid residues (175 and 221) are probably involved in the active center.

Conclusions

Cellulases apparently share a common structural organization which is characterized by a central core containing the catalytically active domain and a tail containing a highly glycosylated region (block B) and a cellulose binding domain (block A). Region A may serve as an anchor which strongly binds to cellulose and thereby loosens the rigid cellulose structure; this could facilitate the accessibility to the glycosidic bond of the active center in the core. The hydrolysis of β -1,4-glycosidic bonds probably proceeds by a general acid catalyzed mechanism, mediated by COOH groups of glutamic and aspartic acid residues, either through a double displacement mechanism (CBH I) or by single displacement (CBH II).

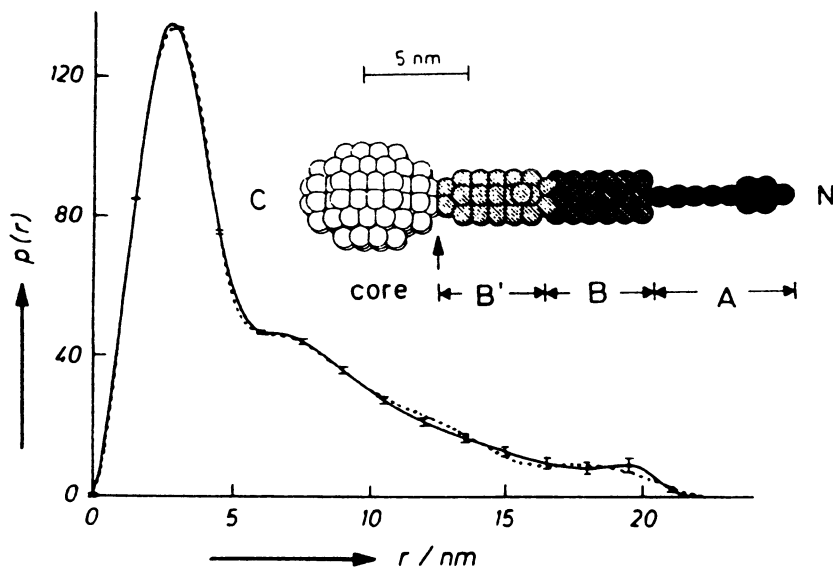


Figure 4: SAXS-based model of CBH II (redrawn from ref. 31 with permission) Note that differently to CBH I the C-terminus of the chain is at the core domain and the N-terminus at the tail domain. The sequence of the domains from the N-terminus to the C-terminus is therefore A-B-B'-C. B' is a repeat of B with strong sequence homologies. The arrow indicates the papain cleavage site.

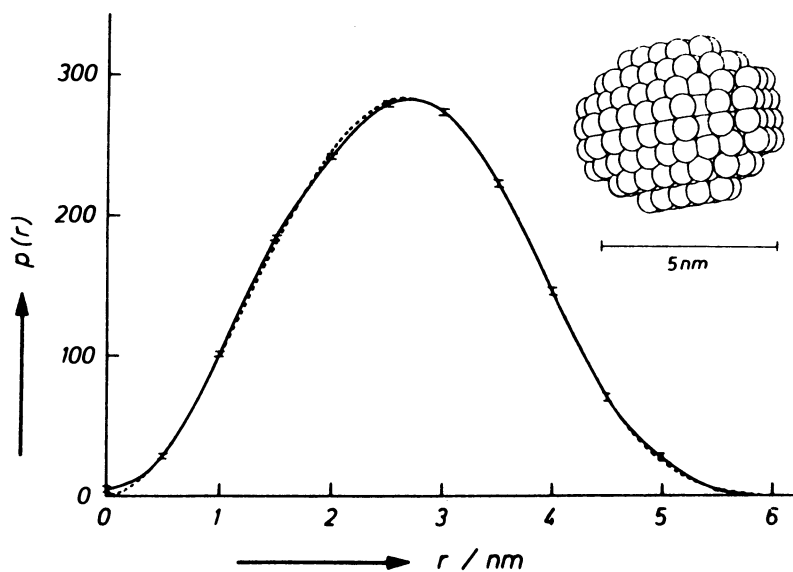


Figure 5: SAXS-based model for the CBH II core protein (reproduced with permission from ref. 31; Copyright Academic Press 1988). The core protein was prepared by limited papain proteolysis from the intact molecule shown in Fig. 4.

Literature Cited

1. Enari, T.-M.; Niku-Paavola, M.-L.; Wood, T. M. *CRC Critical Reviews on Biotechnology* 1987, 5, 67-87.
2. Beguin, P.; Millet, J.; Grepinet, O.; Navarro, A.; Juy, M.; Amit, A.; Poljak, R.; Aubert, J.-P. In *FEMS Symp. 43: Biochemistry and Genetics of Cellulose Degradation*; Aubert, J.P.; Beguin, P.; Millet, J., Eds.; Academic Press, London, UK, 1988, pp 267-282.
3. Claeysens, M.; van Tilbeurgh, H.; Tomme, P.; Wood T. M.; McCrae, S. *Biochem. J.* 1989, 261, 819-825.
4. Knowles, J.; Lehtovaara, P.; Teeri, T. *Trends Biotechnol.* 1987, 5, 255-261.
5. Claeysens, M.; Tomme, P. In *Enzyme systems for lignocellulose degradation*; Coughlan, M. P., Ed.; London, UK, 1989, pp 37-49.
6. Penttilä, M.; Lehtovaara, P.; Nevalainen, H.; Bhikhabhai, R.; Knowles, J. *Gene* 1986, 45, 253-263.
7. Teeri, T.; Lehtovaara, P.; Kauppinen, S.; Salovuori, I.; Knowles, J. *Gene* 1987, 51, 43-52.
8. Ong, E.; Greenwood, J. M.; Gilkes, N. R.; Kilburn, D. G.; Miller Jr, R. C.; Warren, A. J. *Trends Biotechnol.* 1989, 7, 239-243.
9. Beguin, P.; Millet, J.; Chauvaux, S.; Yagüe, E.; Tomme, P.; Aubert, J.-P. In *Enzyme systems for lignocellulose degradation*; Coughlan, M. P., Ed.; London, UK, 1989, pp 57-72.
10. Henrissat, B.; Claeysens, M.; Tomme, P.; Lemesle, L.; Mornon, J. P. *Gene* 1989, 81, 83-95.
11. Bergfors, T.; Rouvinen, J.; Lehtovaara, P.; Caldentey, X.; Tomme, P.; Claeysens, M.; Pettersson, G.; Teeri, T.; Knowles, J.; Jones, T. A. *J. Mol. Biol.* 1989, 209, 167-169.
12. Pettersson, G. *personal communication*.
13. Rouvinen, J.; Bergfors, T.; Teeri, T.; Knowles, J. K.C.; Jones, T. A. *Science* 1990, 249, 380-386.
14. Joliff, G.; Beguin, P.; Juy, M.; Millet, J.; Ryter, A.; Poljak, R.; Aubert, J.-P. *BioTechnology* 1986, 4, 896-900.
15. Pilz, I.; Glatter, O.; Kratky, O. *Meth. Enzymol.* 1979, 61, 148-249.
16. Kratky, O.; Pilz, I. *Quarterly Reviews of Biophysics* 1978, II, 39-70.
17. *Small Angle X-ray Scattering*; Glatter, O.; Kratky, O., Eds.; Academic Press, London, UK, 1982.
18. Glatter, O. *A. Phys. Austr.* 1980, 52, 243-256.
19. Knowles, J.; Lehtovaara, P.; Murray, M.; Sinnott, M. L. *J. Chem. Soc. Chem. Commun.* 1988, 21, 1401-1402.
20. Claeysens, M.; Tomme, P.; Brewer, C. F.; Hehre, E. J. *FEBS Lett.* 1990, 263, 89-92.
21. Fägerstam, L. G.; Pettersson, G.; Engström, J. A. *FEBS Lett.* 1984, 167, 309-315.
22. Teeri, T.; Salovuori, L.; Knowles, J. *BioTechnology* 1983, Oct., 696-699.
23. Shoemaker, S.; Schweichart, V.; Ladner, M.; Gelfand, D.; Kwok, S.; Myambo, K.; Innis, M. *BioTechnology* 1983, Oct., 691-696.
24. Schmuck, M.; Pilz, I.; Hayn, M.; Esterbauer, H. *Biotechnol. Lett.* 1986, 8, 397-402.
25. Abuja, P. M.; Schmuck, M.; Pilz, I.; Claeysens, M.; Esterbauer, H. *Eur. Biophys. J.* 1988, 15, 339-342.
26. Bhikhabhai, R.; Pettersson, G. *Biochem. J.* 1984, 222, 729-736.
27. Tomme, P.; van Tilbeurgh, H.; Pettersson, G.; van Damme, J.; Vandekerckhove, J.; Knowles, J.; Teeri, T.; Claeysens, M. *Eur. J. Biochem.* 1988, 170, 575-581.
28. Kraulis, P. J.; Clore, M.; Nilges, M.; Jones, T. A.; Pettersson, G.; Knowles, J.; Gronenborn, A. M. *Biochemistry* 1989, 28, 7241-7257.
29. Tomme, P.; Claeysens, M. *FEBS Lett.* 1989, 243, 239-243.

30. Knowles, J.; Teeri, T. T.; Lehtovaara, P.; Penttilä, M.; Saloheimo, M. *FEMS Symp. 43: Biochemistry and Genetics of Cellulose Degradation* 1988, 153–169.
31. Abuja, P. M.; Pilz, I.; Claeysens, M.; Tomme, P. *Biochem. Biophys. Res. Commun.* 1988, 156, 180–185.
32. Abuja, P. M.; Pilz, I.; Tomme, P.; Claeysens, M. *Biochem. Biophys. Res. Commun.* 1989, 169, 615–623.
33. Mischak, H.; Hofer, F.; Messner, R.; Weissinger, E.; Hayn, M.; Tomme, P.; Esterbauer, H.; Kuchler, E.; Claeysens, M.; Kubicek, C. P. *Biochim. Biophys. Acta* 1989, 990, 1–7.
34. Tomme, P.; Heriban, V.; Claeysens, M. *Biotechnol. Lett.* 1990, 12, 525–530.
35. Chen, C. M.; Gritzali, M.; Stafford, D. W. *Bio/Technology* 1987, 5, 274–278.
36. Teeri, T. *The cellulolytic enzyme system of Trichoderma reesei*; Doctoral Thesis; VTT Publications 38; Technical Research Center, Espoo, FL, 1987

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Chapter 24

Thermal Unfolding of *Trichoderma reesei* CBH I

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Purified cellobiohydrolase I (CBH I) was subjected to thermal denaturation analysis using differential scanning calorimetry (DSC). As many as three endothermic structural transitions were detected during heating of the native enzyme from 20°C to 75°C at a range of pH values from 4.80 to 8.34. DSC analyses of the separated proteolytic fragments representing the catalytic "core" and cellulose-binding "tail" regions of the CBH I molecule revealed that all three of the transitions arose from the core region. Two of these transitions were inferred as the deconvoluted constituent peaks of an asymmetrical peak that appeared at 64°C at pH 4.8 but was shifted to 33.4°C as the pH was increased to 8.34. A much smaller third transition at 55°C appeared to be much less pH-dependent, but was observed only when the two major transitions were shifted to temperatures substantially lower than 55°C (as at pH 7.5 or above). Cellobiose at 100 mM dramatically stabilized the CBH I molecule, shifting the position of the major, two-component peak from 64°C to 72°C at pH 4.8, and from 33.4°C to 51.9°C at pH 8.34.

Differential scanning calorimetry (DSC) is a powerful and very general method of studying the structural organization of proteins as it relates to thermal stability (1-9). The generality of the method is due to the fact that it detects any temperature-dependent folding/unfolding process that proceeds with a significant uptake or release of heat. Unlike methods depending on changes in intrinsic fluorescence (10), DSC does not depend on the convenient location of reporter groups within the molecule. To a much greater extent than circular dichroism methods, it is capable of detecting changes in protein structure that affect primarily the interactions between structural domains, with relatively little effect on the structure of the individual domains themselves and thus relatively little effect on the overall secondary-structure composition of the molecule (9).

Like most other approaches to structural analysis of proteins, however, DSC is most valuable when it is not the only source of structural information concerning a given protein. DSC can provide evidence concerning the number of domains, or "cooperative unfolding units" into which a protein is divided, the stabilities of the individual domains,

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and the stabilizing interactions, if any, between the various unfolding units. This "thermodynamic structure" will be much more interesting and useful if the thermally induced transitions measured by DSC, and the structural units inferred from them, can be assigned to specific regions of a physical structure known from other methods.

Cellobiohydrolase I (CBH I) from *Trichoderma reesei* is one of four well-characterized enzymes -- two endoglucanases (EG I and EG II) and two cellobiohydrolases (CBH I and CBH II) -- that are minimally required for efficient depolymerization of cellulose by the cellulase system of this organism (11-17). Because of the importance of CBH I in this system, an unusually large body of information has been collected concerning the physical structure of this enzyme. The amino-acid sequence has been determined, both directly (18) and from the DNA sequence (19). The disulfide-bond patterns have been described (18) and significant sequence homologies have been discovered between the C-terminal segment of CBH I and peptide-chain segments found at either the N-terminal or C-terminal of other *T. reesei* enzymes that are known to bind to and hydrolyze crystalline cellulose (15,16). Small-angle X-ray scattering (SAXS) studies have revealed that the CBH I molecule is tadpole-shaped, with an extended tail section connected to an ellipsoidal "head", or core, section through a flexible and heavily glycosylated "hinge" region (20,21). Of great importance for the interpretation of this structural picture is the development of a papain-cleavage procedure capable of splitting the CBH I molecule very specifically into a head or core segment containing the catalytic site for hydrolysis of β -1,4-glycosidic bonds, and a tail fragment containing most of the glycosylation of the protein along with an extreme C-terminal segment capable of binding tightly to crystalline cellulose (22). SAXS studies using a macromolecular xylan inhibitor of CBH I as a marker for the active site have suggested that the catalytic site is located in the core region very near the point of attachment of the tail segment (23). The kinetics of the catalytically active core fragment have been investigated against both crystalline cellulose and small soluble substrates (22), and the binding of the separated tail fragment to crystalline cellulose has been studied, both with the proteolytically-derived fragment itself, and with a 36-residue synthetic C-terminal cellulose-binding region ("block A") (24). The solution structure of the synthetic cellulose-binding region has been determined by means of two-dimensional NMR (25), and crystals of the catalytic-domain proteolytic fragment have been obtained (26), holding out the promise of an X-ray crystallographic picture of this part of the molecule. DSC analysis requires relatively large quantities of purified protein; for this reason it was of no small importance to us that (i) CBH I is the most abundantly produced of the enzymes in the *T. reesei* cellulase mixture (27), and (ii) a general purification approach has been outlined (27).

Because of the availability of this remarkably extensive physical picture of the CBH I molecule (as well as the potential availability in adequate quantities of the purified enzyme itself), DSC studies can provide valuable insights into structure-function relationships for this important cellulase. We present here a comparison of DSC analyses of the intact CBH I molecule with those of the isolated catalytic-domain and tail-region proteolytic fragments.

Materials and Methods

CBH I Purification. The purification procedure developed in this study followed the general size exclusion chromatography/anion exchange chromatography protocol described by Shoemaker for the purification of CBH I (27). The enzyme utilized in this study was purified from a commercial Genencor 150L cellulase preparation, reportedly a high-productivity *T. reesei* mutant strain. Aliquots of Genencor 150L were concentrated and dialyzed against 50 mM sodium acetate buffer pH 5.0 with 100 mM NaCl (buffer A) using an Amicon (Danvers, MA) model DC-2 Hollow Fiber

Ultraconcentrator equipped with dual H1P10-20 cartridges. The large capacity of this system permitted the processing of 10 g lots (total protein, BioRad Protein assay, Richmond, CA) of the Genencor preparation for subsequent purification. Initial stages of the purification were performed on a BioPilot column chromatography system (Pharmacia LKB Biotechnology, Pleasant Hill, CA). All chromatography columns and packing materials were from Pharmacia. Size exclusion chromatography (SEC) of the ultrafiltered cellulase preparation was conducted with a 11.3 cm x 90 cm BioProcess column packed with Sephacryl S-200 HR gel at a flow rate of buffer A of 10 mL/min. Approximately 1 g of total protein was loaded each run. The CBH I positive fractions from each chromatographic step were identified immunologically with an ImmunoBlot assay kit (BioRad) and CBH I-specific monoclonal antibodies (28). The monoclonal antibodies were a gift from R. Ellis at Colorado State University. The CBH I immunopositive fractions were pooled, concentrated, and dialyzed against 20 mM BisTris pH 5.8 buffer for ion exchange chromatography (IEC). A 6 mm x 20 cm column packed with Q Sepharose Fast Flow gel was used for this step. This column was subjected to a linear gradient from 0 to 1 M NaCl in 20 mM BisTris pH 5.8 buffer at a flow rate of 10 mL/min. CBH I immuno-positive fractions, found to elute at a salt concentration of 0.3 M NaCl, were pooled and concentrated to approximately 1.25 mg/mL. A final high-performance SEC (HPSEC) step was required to obtain a single electrophoretic species by sodium dodecylsulfate gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with the Pharmacia PhastSystem using PhastGel 8%-25% crosslinked gradient gel and SDS buffer strips. This step was conducted using two Pharmacia Superose 6 & 12 (HR 10/30) columns connected in series. Buffer A was used at a flow rate of 0.5 mL/min. This column series was calibrated with the Gel Filtration chromatography standards kit from Pharmacia. Approximately 200 mg of CBH I was obtained from 50 mL of the crude Genencor product.

Papain Cleavage of CBH I. The CBH I-core and -tail peptide fractions were produced by proteolytic cleavage with papain (Boehringer Mannheim lot #11277523-67). The reaction conditions utilized were slightly modified from those given by van Tilbeurgh et al. (29). The reactions were done in 50 mM ammonium acetate pH 6.2 buffer. Molar ratios of CBH I (MW 67 kDa)/papain (MW 23 kDa) ranged from 6/1 to 8/1. These reactions were conducted at 22°C for 4 to 6 h. The CBH I-core and -tail fragments of CBH I from these hydrolysates were separated from the unreacted native CBH I by HPSEC with the Pharmacia Superose 6 & 12 columns described above.

Instrumentation for Differential Scanning Microcalorimetry. Denaturation thermograms were obtained using a Microcal MC-2 Scanning Calorimeter (Microcal, Northampton, MA), interfaced through a DT 2801 A/D converter to an IBM PC-XT microcomputer. Instrument control and data acquisition were by means of the DA-2 software package (Microcal). The sample cell capacity is 1.130 mL, and runs were made with an overpressure of 30 psig (N₂), at scan rates of either 0.5 or 1.0 degrees per minute.

Sample Preparation. Protein samples were concentrated and exchanged into the buffer to be used in each DSC run by extensive diafiltration against the buffer using Amicon stirred cells and ultraconcentration membranes with 45 psig nitrogen pressure. For samples of native CBH I and of the core fragment of CBH I, the ultrafiltration membrane used was the polysulfone PM-10 (nominal cut-off, 10 kDa); the considerably smaller (approx. 10 kDa) tail fragment of CBH I was concentrated and diafiltered over a cellulose acetate YC-05 membrane (nominal MW cut-off, 500 Da).

The protein sample was then degassed under aspirator vacuum for a minimum of 20 min, with stirring, in order to remove excess dissolved gas forced into the solution during the diafiltration step. A sample of diafiltration buffer, to be used in filling the reference cell of the calorimeter, was given identical treatment. Buffers used were 50 mM acetate (pH 4.80), 43.4 mM 2-[N-morpholino]ethanesulfonic acid (MES) (pH 6.32),

47.6 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (pH 7.50), and 52.3 mM Bicine (pH 8.35). All pH adjustments were made at 20°C; the buffer concentrations listed were chosen to yield an ionic strength of 0.0262 M to match that of the pH 4.8 acetate buffer.

Protein Concentration Determinations. After filling of the calorimeter cells with protein solution (sample cell) and diafiltration buffer (reference cell), the protein concentration of the sample as loaded was determined spectrophotometrically. For intact CBH I and its core fragment, the extinction coefficient used was 1.42 (g/L)^{-1} at 280 nm (30). Because the tail fragment of CBH I (approximately residues 430-496) contains four tyrosine residues, but no tryptophan or phenylalanine residues (19,22), the spectrum of this fragment is essentially that of tyrosine. Accordingly, the concentration of this fragment was determined by measuring the molar concentration of tyrosine residues spectrophotometrically (molar extinction taken as that of the N-acetyl methyl ester in water, equal to $1.420 \times 10^3 \text{ cm}^{-1}$ at 274.6 nm [31]) and then dividing by a factor of 4 to obtain the molar concentration of the peptide.

Deconvolution Analysis of DSC. Data analysis was carried out using the DECONV section of the DA-2 software package. This software, which is based on the deconvolution procedure of Freire and Biltonen (4), allows deconvolution of differential heat-capacity peaks either as the result of simple addition of multiple independent transitions or as the result of more complex mathematical processes representing the combination of transitions that interact in such a way that an obligatory reaction sequence is imposed (sequential transitions).

Each thermogram was normalized on scan rate, the corresponding (scan-rate-normalized) buffer-buffer baseline was subtracted, and the differential heat capacity values were divided by the number of moles of protein or peptide in the sample, to yield ordinate values in terms of $\text{calories mol}^{-1}\text{deg}^{-1}$. The resulting files were then analyzed using the deconvolution software.

DSC Nomenclature. The term T_m as used here is not a "melting temperature," but is instead standard DSC usage for the temperature at which the maximum differential heat capacity is observed during the denaturation of a protein sample. (" T_{max} " would be a more extended expression of this term.) For a two-state (single-transition) process, T_m approximates, but is not identical to, the value of T_d or $T_{1/2}$, the temperature at which the transition is half-complete. ΔH° is the calorimetrically measured molar enthalpy of denaturation, represented by the area under the endothermic peak, divided by the number of moles of protein in the sample. The van't Hoff, or effective, enthalpy is a measure of the sharpness of the endothermic peak, or an *inverse* measure of the temperature range over which the transition occurs. For a denaturation that can be represented as a two-state, single-transition process, $\Delta H_{vH} = \Delta H^\circ$. Values of $\Delta H_{vH}/\Delta H^\circ$ significantly less than unity are generally taken as indicating that a given denaturation process involves more than two significantly populated states and thus more than two transitions. Detailed discussions of these terms can be found in references 2-7.

Results

Purification of CBH I and CBH I Fragments. The purified CBH I preparation eluted at a volume from HPSEC that corresponded to an apparent molecular weight of 70 kDa. Interestingly, the core protein prepared by papain cleavage eluted at an earlier volume than did the native enzyme on the HPSEC system (see Figure 1). This fragment was, however, shown to be of a smaller molecular weight (approximately 57 kDa) by reduced SDS-PAGE on the Pharmacia PhastSystem (see Figure 2). The apparent molecular weight of the native CBH I was 68 kDa by SDS-PAGE. These values agree

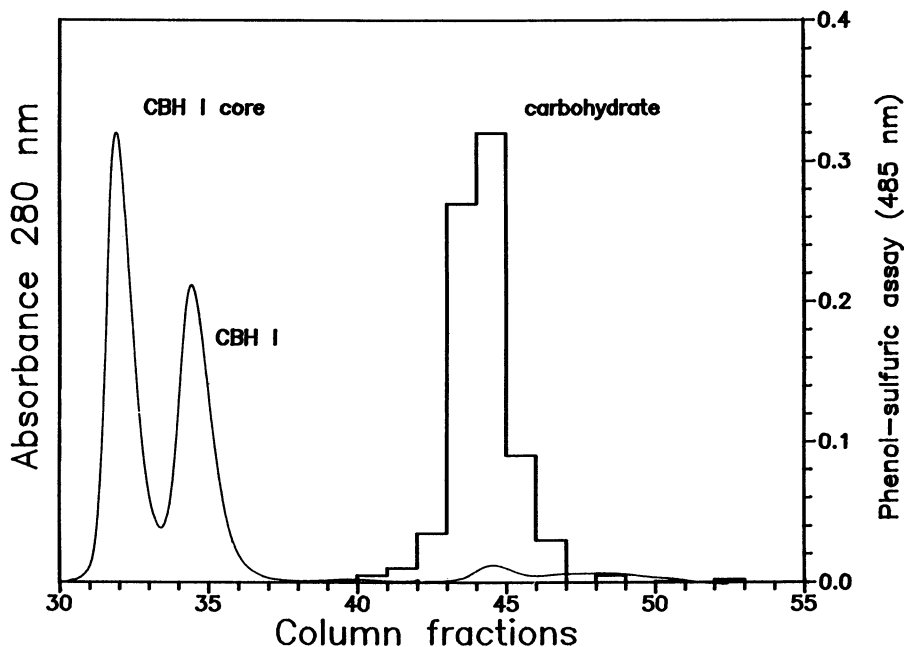


Figure 1. Elution profile of CBH I-papain reaction products on two Superose HPSEC columns (HR 6 & 12) connected in series. The elution of the tail fragment was confirmed using the phenol-sulfuric acid assay for carbohydrate.

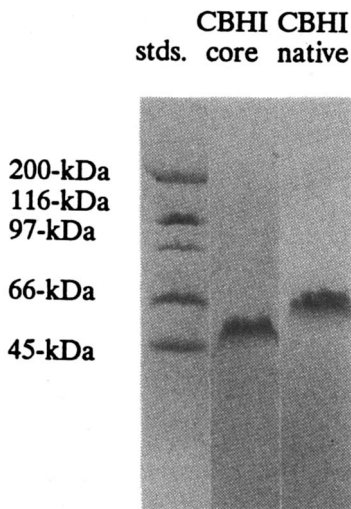


Figure 2. SDS-polyacrylamide gel electrophoresis of the purified CBH I and CBH I core fragment using the Pharmacia PhastSystem.

well with those found by Tomme et al. (22). The elution of the CBH I core at a lesser volume than expected may indicate an altered, if not expanded, structure after papain hydrolysis, thus yielding a larger hydrodynamic radius. The narrow band width of the core fraction (similar to that for native CBH I) indicates that this fragment is monodisperse. Specific activity of the purified enzyme, assayed at 50°C against 4.0 mM *p*-nitrophenyl- β -D-cellobioside (pH 5.0 in 10 mM acetate, 0.1 M NaCl), was 46.3 nanomoles substrate hydrolyzed per minute per milligram of protein.

DSC Analysis. At pH 4.80 in 50 mM acetate, the thermal denaturation of *T. reesei* CBH I is expressed as a single, asymmetrical endotherm (Figure 3A, upper curve) with T_m at 64.05°C, calorimetric enthalpy (ΔH°) equal to 398 kcal/mol and effective, or van't Hoff enthalpy (ΔH_{vH}) of denaturation equal to 173 kcal/mol (Table I). The overall process appears to be irreversible, in that no endotherms are observed upon cooling and rescanning of the sample (data not shown). No scan-rate dependence of T_m is observed, however, upon comparing runs at 60°C/h and 30°C/h. Moreover, as shown by Figure 4, heat-uptake for the denaturation can be modeled quite well by a reversible, equilibrium process involving two transitions with T_m at 61.38°C and 64.36°C and enthalpies of approximately 180 kcal/mol and 235 kcal/mol, respectively. In this model, the two constituent transitions are each assumed to represent a two-state process ($\Delta H_{\text{vH}} = \Delta H^\circ$), and it is further assumed that any given protein molecule cannot undergo the second transition until it has undergone the first transition, which in this case is the transition with the lower T_m (i.e., these are sequential transitions). The results of similar deconvolutions of samples scanned under other conditions are presented in Table I.

DSC analyses of the separated proteolytic fragments representing the core and tail regions of the CBH I molecule (Figure 3A) reveal that at pH 4.80 in 50 mM acetate, the thermogram of the core segment essentially reproduces that of the intact molecule under these conditions, whereas the thermogram of the tail fragment has no detectable endotherms in the temperature region between 12°C and 95°C. (The scan of the tail fragment extended beyond the region shown in the figure.) The virtual identity of the peaks observed near 64°C for both CBH I and its core fragment is shown clearly in Figure 5, in which symbols representing the data for CBH I are co-plotted with a line connecting the points (no symbols) for the core fragment.

DSC runs carried out on CBH I at an initial pH of 8.34 (Figure 3C) reveal an asymmetrical peak with a shape similar to that observed at pH 4.80, but with a markedly reduced amplitude and with T_m shifted down the temperature scale by more than 30 degrees, to approximately 33.4°C. In addition, at pH 8.34, the thermogram for intact CBH I shows a broad, low peak centered near 55°C. Thermograms of the core fragment at this high pH (Figure 3C) show the same shift of the principal peak to lower temperature as is seen for the intact enzyme, and also show the low, broad peak centered near 55°C. At an intermediate pH (7.50, Figure 3B), intact CBH I again shows two peaks, but the principal peak is not shifted as far down the temperature scale as at pH 8.34, and is not completely resolved from the low peak at 55°C. This latter peak (55°C) appears, at least over this pH range, to be much less sensitive to pH than is the principal, two-component peak. At pH 6.32 (scan not shown) intact CBH I shows only a single, asymmetrical peak, again similar to that observed at pH 4.80, but with T_m at 57.95°C, approximately 6 degrees lower than the T_m measured at pH 4.8. Since at pH 6.32 the principal peak covers the region on the thermogram in which the "low, broad peak" would appear, were it present at the same position as at pH 7.5 and 8.34, it is not possible to say with any assurance whether this peak is present at pH 6.32 as well. The 50° - 58°C regions of some scans at pH 4.8, such as the one chosen for illustration in Figure 3A, may be interpreted as indicating the presence of a broad, low 55°C peak overlapping the 64°C principal peak; other scans under the same conditions give no such indication. In practice, small nonreproducibilities in even the most carefully run baselines make it extremely difficult to identify broad, low peaks substantially overlapped by much larger peaks. When the principal, two-component peak is displaced

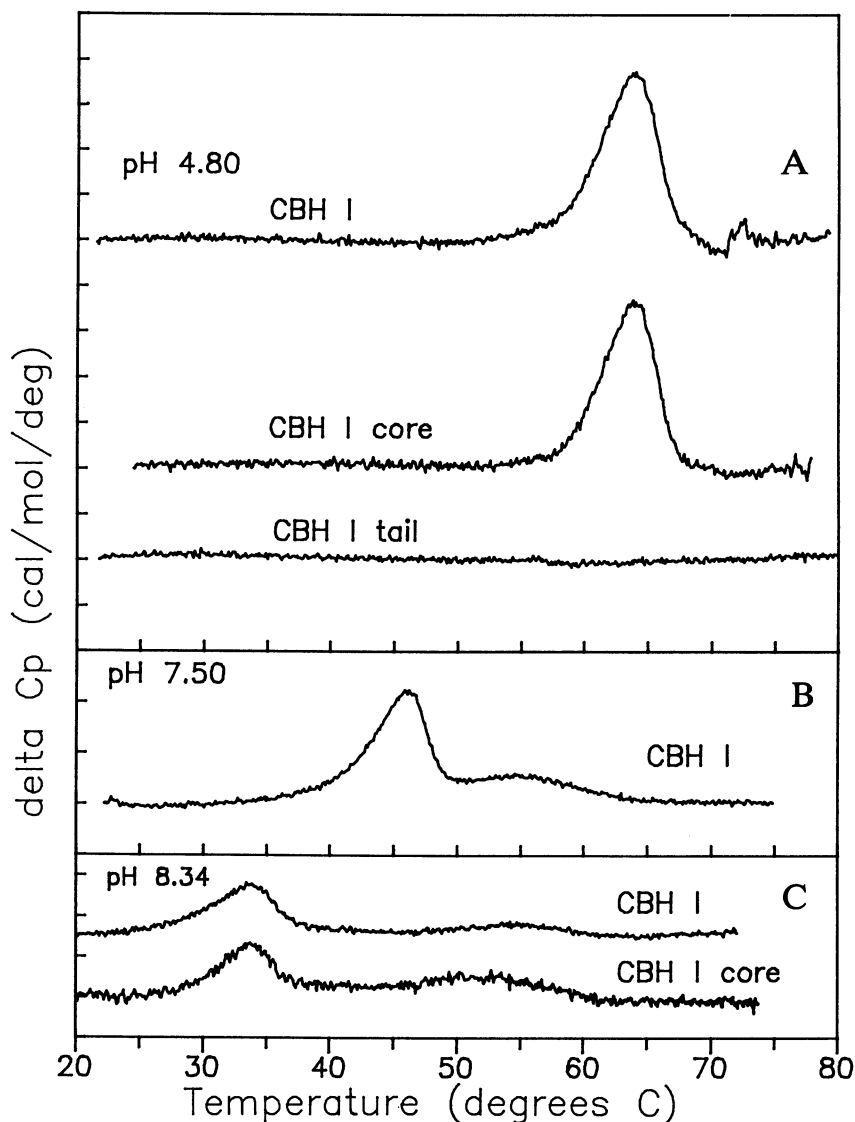


Figure 3. DSC thermograms of native CBH I and of fragments produced by limited proteolysis. For native CBH I and the core fragment, protein concentrations were in the range from 0.91 to 1.31 mg/ml. The concentration of the tail fragment was 0.41 mg/mL. Scan rate was 30°C/hr. Each division on the ordinate represents 20,000 cal/mol/deg.

Table I. Deconvolution of the Principal, pH-Dependent Endotherm for CBH I and Its Core Fragment

Sample analyzed	pH	T_m (°C)	Overall parameters ^a ΔH°	ΔH_{vH}	$\Delta H_{vH}/\Delta H^\circ$	Deconvoluted component peaks ^{b,c} $T_{m1}(\Delta H^\circ)$	$T_{m2}(\Delta H^\circ)$
CBH I	4.80	64.0	398	173	0.435	61.7 (180)	64.3 (235)
	6.32	58.0	354	183	0.517	55.7 (139)	58.0 (218)
	7.50	46.2	266	133	0.489	43.6 (106)	46.2 (160)
	8.34	33.4	168	111	0.661	30.4 (68)	33.6 (114)
CBH I core	4.80	63.9	381	172	0.450	61.5 (167)	64.3 (225)
	8.34	33.2	203	155	0.760	32.8 (77)	33.4 (138)
CBH I + 1 mM CB	4.80	65.7	384	167	0.435	63.0 (170)	66.0 (224)
CBH I + 100 mM CB	4.80	72.0	390	153	0.392	68.7 (162)	72.2 (227)
CBH I + 10 mM CB	8.34	43.3	186	118	0.630	41.8 (64)	42.6 (111)
CBH I + 100 mM CB	8.34	51.9	181	149	0.820	50.8 (70)	51.3 (131)

^aValues of ΔH are given in kcal/mol. ^bA sequential relationship between the transitions is assumed (see Materials and Methods), the transition with lower T_m occurring first. ^c $\Delta H_{vH} = \Delta H^\circ$. CB = cellobiose.

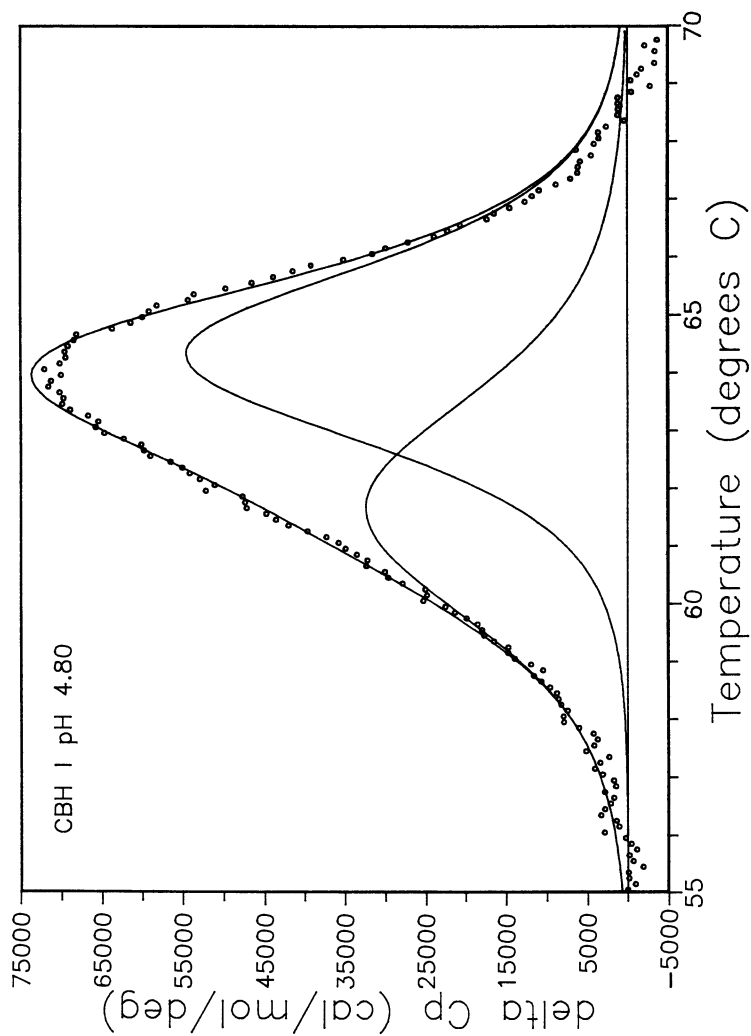


Figure 4. Deconvolution of the denaturational endotherm for native CBH I at pH 4.80. Circles represent experimental values for differential heat capacity; the solid curves represent the overall best fit model and the two sequential component transitions that contribute to the overall fit (See text).

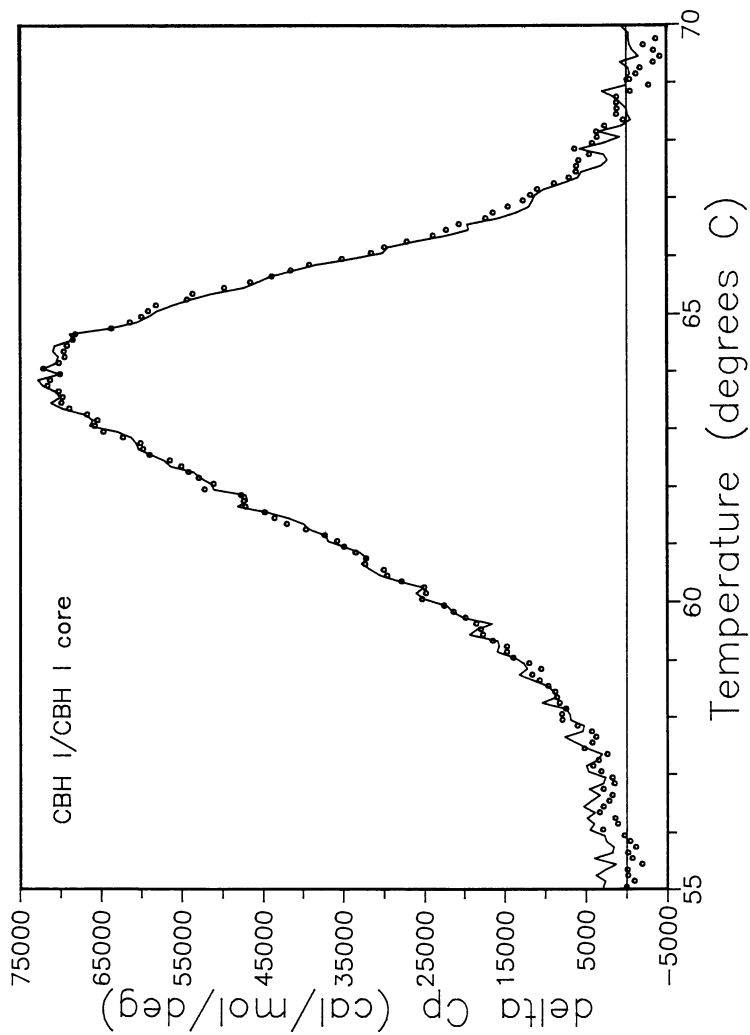


Figure 5. Co-plot of the denaturational endotherms measured at pH 4.8 for native CBH I (open circles, no connecting lines) and the core fragment (line connecting data points, no symbols).

to higher temperatures at pH 4.80 in the presence of high concentrations of the competitive inhibitor cellobiose (see below), the cleared 50°-60°C region of the baseline shows no indication of a peak at 55°C.

Calorimetric Reversibility of the Transitions. As stated above, the calorimetric events observed at pH 4.80 during heating of CBH I to 75°-80°C are irreversible in that when the sample is cooled and subsequently rescanned, no endotherms are observed. When a sample at pH 6.32 is scanned over this same temperature range, again no peaks are observed upon cooling and rescanning. For samples scanned to 75°C at pH 7.5 and pH 8.34, however, there is partial, but quite significant, reversibility in that rescans of the samples show peaks at the same temperatures, and of the same shapes, but of reduced amplitudes (data not shown). For the pH values tested, and at the ionic strength (0.026 M) employed in these studies, the greatest reversibility was attained at pH 7.5. A sample scanned a total of four times still showed quite recognizable peaks during the fourth scan, with the principal peak having at each scan approximately 70% of the area measured during the scan immediately preceding. The percentage of reversibility was smaller at pH 8.34, with the rescanned peak reduced to approximately 30% the area of the peak observed in the first scan. It is worth noting that when a CBH I sample at pH 7.5 is scanned to 85°C rather than to 75°C, no endotherms at all are observed during a second scan.

Simple visual observation of the samples upon removal from the calorimeter cell after the experiments revealed that varying degrees of coagulation had taken place in some of the samples during the experimental cycle. At sample loadings in the neighborhood of 1.0 mg/mL, the samples scanned at pH 8.34 and pH 7.5 were clear upon removal from the cell. The pH 6.32 samples were somewhat milky in appearance, and the pH 4.8 samples were virtually opaque.

Figure 6 depicts the relationship between the specific enthalpy of denaturation measured for CBH I at each of the pH values used in this study, and the T_m of the principal peak at that pH. The straight line represents a least-squares best fit to the four experimental data points and the empirically derived intersection point (Reference 2, see Discussion) in the upper right corner. All the values determined in the absence of cellobiose, both those at pH values at which the denaturation exhibits a substantial degree of overall reversibility, and those at which the overall process is completely irreversible, are in reasonably good agreement with the linear relationship.

Stabilization by Cellobiose. Figure 6 also shows that when different concentrations of the competitive inhibitor cellobiose are present in the DSC samples at pH 4.8 and pH 8.34, the principal denaturation peaks are displaced to higher temperatures, as indicated by the higher T_m values shown. The ΔH° values, however, do not follow the trend of increasing enthalpy with increasing T_m that is seen for the data in the absence of cellobiose. Instead, the peak areas in the presence of cellobiose are essentially the same as for the peaks appearing at lower temperatures at these pH values in the absence of cellobiose.

Discussion

Calorimetric Deconvolution Models and the Reversibility or Irreversibility of Overall Denaturation Processes. The deconvolution procedures used to analyze the thermograms presented in this study are based on equilibrium models, even though the overall denaturation process seen over a cycle of heating to a temperature above T_m and then cooling to below T_m is, depending on the pH, either completely or partially irreversible. There is ample precedent in the literature for the application of equilibrium models in such cases, however. Convincing evidence has been presented

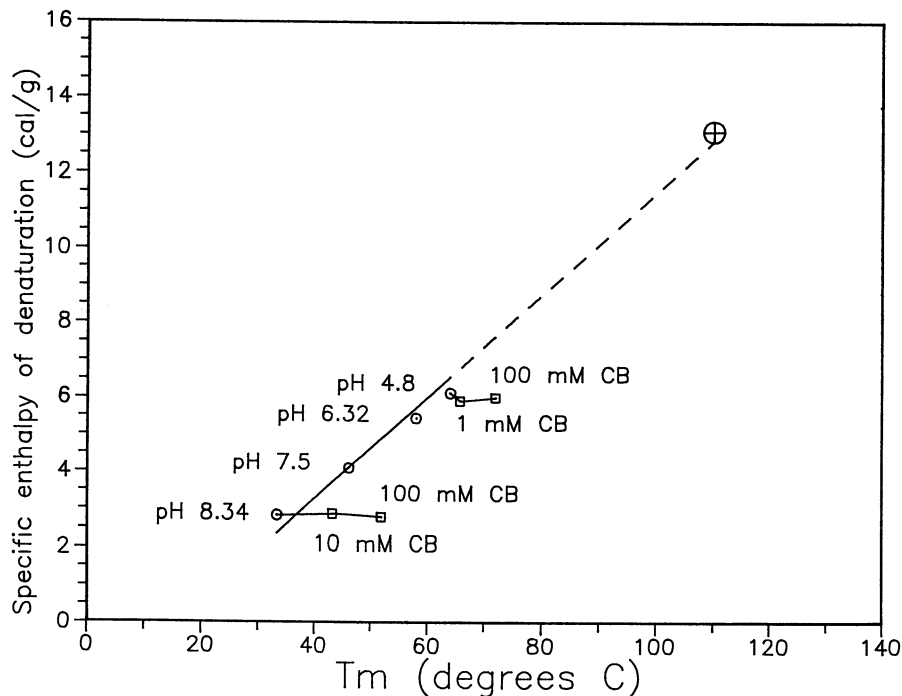


Figure 6. Specific enthalpy of denaturation for native CBH I, plotted as a function of the overall T_m observed as the enzyme molecule is progressively destabilized by increasing the pH. Dot-centered circles represent the specific enthalpy in the absence of cellobiose; the straight line is a linear least-squares best fit to these data points, plus the empirically derived intersection point (reference 2, see Discussion) represented by the crossed circle at upper right. The squares represent enthalpies measured at pH 4.80 and pH 8.34 in the presence of the indicated concentrations of cellobiose.

that the DSC data for many apparently irreversible protein denaturations can be represented quite well by equilibrium models (5,32-34), as long as the results do not show any dependence of T_m on scan rate. In these cases the actual unfolding process may be reversible, and the overall irreversibility may be due to other processes that occur subsequent to the unfolding, as shown in equation 1,



in which an unfolded form of the protein (U) is in equilibrium with the native form (N), but can also be converted by an irreversible process to the permanently denatured form, D. In the case of our results, one likely candidate for the irreversible post-unfolding step is intramolecular aggregation of the unfolded protein molecules, since the samples at pH 4.8 and pH 6.32 are visibly coagulated after the runs. Frequently, one or more fairly sharp, small exotherms (negative peaks), sometimes followed by or interspersed with endotherms of similar dimensions, are observed in the temperature region immediately above a major unfolding peak. An example of this is seen in the thermogram for CBH I in Figure 3A. These small "baseline disturbances," which are somewhat repeatable from run to run in the sense that they tend to occur in the same temperature range, but are not reproducible in terms of amplitude or exact fine structure, may be the result of coagulation of unfolded protein molecules, followed by disruption of the aggregates upon further heating. The negative sign of some of these fluctuations suggests an exothermic process such as coagulation; the sharpness of the fluctuations may reflect the multimolecular nature of such processes. Another possible explanation for the overall irreversibility is suggested by published observations that proteins can undergo covalent modification when heated to 80°C or above (35-37). There may be a connection between such modifications and the fact that the significant reversibility observed for the denaturation of CBH I (pH 7.5) on scanning to 75°C is completely destroyed when the sample is scanned to 85°C instead.

Localization of DSC Transitions Within the CBH I Molecule. Small-angle X-ray scattering studies (38), partial proteolysis studies (22), and the combination of the two (20) have shown that the CBH I molecule is roughly tadpole-shaped, with the catalytic site located in the larger (approx. 55 kDa) core region of the molecule. The site that binds to crystalline cellulose is located in the considerably smaller (10 kDa) wedge-shaped (25) tail region, which is connected to the core region by a flexible, heavily glycosylated hinge segment of the peptide chain.

In view of the known bilobed structure of CBH I, our finding that the thermal denaturation of CBH I at pH 4.8 (Figure 3A) could readily be modeled as the result of two transitions of unequal enthalpy (Figure 4) immediately suggested the possibility that the larger and smaller transitions might represent, respectively, the unfolding of the core and tail regions of the molecule. When the CBH I molecule was subjected to limited proteolysis and the isolated core and tail fragments were analyzed separately by DSC at pH 4.8, however, it was found that the asymmetric endotherm observed for the intact enzyme was almost exactly reproduced by DSC of the core fragment, whereas scanning of the tail fragment produced no detectable endotherms at all. Both of the proposed contributing transitions must therefore be assigned to the core region.

In an attempt to dissect the overall endothermic envelope observed for the intact enzyme and the core fragment, both the core and the intact enzyme were studied by DSC at increasing pH values. In the case of two or more domains of a protein molecule that unfold independently of each other, but have thermal stabilities similar enough that the two independent transitions overlap at a given pH, it may be that the stabilities of the two domains are sufficiently different functions of pH that a change in pH will move the two peaks to different positions on the temperature scale. In an alternate

possibility, two **transitions** that actually have substantially different intrinsic T_m values may appear as a merged peak under certain solution conditions, because the domain with lower intrinsic T_m is significantly stabilized by interactions with the more stable domain (8). In this case, a large change in solution pH might break the interactions at the interface between the domains, thus allowing the two domains to unfold independently, each at its own intrinsic T_m (8,9).

Varying the pH over the range from 4.8 to 8.34 does dramatically affect the thermal stability both of the intact enzyme and of the core fragment, decreasing T_m for the principal peak of both by some 30 degrees. At pH 7.50 and 8.34 a smaller, relatively pH-independent peak is resolved from the principal peak for both protein native protein and core, but the principal peak retains the same type of asymmetry it exhibits at pH 4.8 and 6.32, and is still best deconvoluted with the assumption of two transitions. Thermal denaturation of the core region of CBH I would appear to involve at least two, and probably three, different transitions. If the large, asymmetrical peak observed for the intact enzyme and for the core is indeed composed of two interacting transitions, a shift in pH of 3.5 units is apparently not sufficient to uncouple the transitions.

Another reason for varying the pH in these experiments is that increasing the pH to values farther removed from the isoelectric point of the protein may reduce the tendency of unfolded molecules to aggregate, thereby permitting demonstration of the reversibility of the unfolding step and facilitating the interpretation of the data. From the results it would appear that this possibility is realized. Privalov and coworkers (2,6), in analyzing the results of their own investigations and those of other laboratories into the denaturations of a number of small, globular proteins, have delineated an empirical relationship between the magnitude of the enthalpy of denaturation and the T_m value of the denaturation. According to their analysis, when the T_m and denaturational enthalpy values for a given globular protein are varied (as by changing the pH), the enthalpy increases linearly with increases in T_m . For the same values of T_m in the experimentally accessible range, different proteins will have different values of denaturational enthalpy, but the slopes of the linear plots for the different proteins differ in such a way (plots for proteins with smaller enthalpies at low values of T_m having larger positive slopes) that all of the plots, if extended linearly to high temperatures, appear to intersect at approximately 110°C, with a specific enthalpy value of approximately 13.1 cal/g (2). Recent experimental and theoretical developments indicate that the plots become nonlinear above 80°C, with intersection at higher temperature and lower enthalpy value than that described above, but the linear approximation -- and its apparent intersection near 110°C -- still provide a good fit to the data between 0°C and 80°C (6). The (110°C, 13.1 cal/g) point is the "empirically derived intersection point" plotted in Figure 6. The linear relationship between denaturational enthalpy and T_m was initially derived using proteins and conditions for which there was substantial reversibility of the overall denaturation process and for which the observed transitions were well explained by two-state (single-transition) models (2). The first condition does not apply to some of the data in Figure 6, and all the data are best explained by models involving multiple transitions, but the good fit of the data to the type of linear relationship observed for reversible-equilibrium denaturations suggests that interpretation of our data in terms of equilibrium models is justified.

We have interpreted the principal denaturation peaks in our data in terms of multiple sequential transitions, but in fact most of the peaks in the data base can be fitted almost as well by a model involving two independent transitions. For endotherm envelopes of this size and degree of asymmetry, the differences between the fits obtained from sequential and independent models are too small to allow a choice of one or the other based simply on goodness of fit. Our provisional preference for the sequential model is based on other considerations, one being the failure of a 3.5-unit

change in pH to separate the component peaks that are proposed to produce the overall envelope. From the standpoint of effective catalyst design, it would be reasonable to expect that two domains of an enzyme would have similar thermal stability limits near the pH of optimal activity. There would not seem to be as great a driving force, however, for the evolutionary selection of domain structures with near-identical thermal stabilities at pH values far removed from the region at which the enzyme is active. Unless the thermal stabilities of two independent domains happen to vary with pH in nearly identical manners over a fairly wide pH-range, adjusting the pH to the extremes would be expected to drive the T_m values apart. Another reason for our preference in models is simply the information provided by SAXS studies concerning the geometry of the CBH I molecule. The core region appears to be a fairly compact ellipsoidal mass (21,38), with no evidence of domains that are well separated as are the core and tail regions. Figure 7 shows schematically a picture of the CBH I molecule that we suggest on the basis of our DSC results, combined with the results of other workers that we have discussed above. The molecule is presented as being composed of well-separated, and minimally interacting, core and tail regions, with the core region further divided into two structural domains (C and C') that are calorimetrically distinguishable, but are in such intimate contact with each other that their denaturations are strongly coupled.

Stabilization by Cellobiose. The series of DSC scans in the presence of cellobiose was another experiment intended to shed some light on the interaction, or lack thereof, between different parts of the core region. If a potent competitive inhibitor such as cellobiose (39) binds to a site that is completely contained within one domain that unfolds independently of any other domain, the binding of the inhibitor would be expected to stabilize only that domain. Conversely, if the binding site is located in a domain that interacts strongly with another, or if the binding site is located in a cleft between two domains, with each domain contributing some groups that interact directly with the inhibitor, then binding of the inhibitor would be expected to stabilize both of the domains.

What we observe, both at the pH value (4.80) chosen to be near the activity optimum for the enzyme and at the value (8.34) chosen to produce substantial pH-stress, is that in the presence of cellobiose the enzyme has markedly higher T_m values, but the overall shape of the denaturation envelope is very similar to that observed in the absence of the inhibitor. In addition, the overall ΔH° values in the presence of even quite high concentrations of inhibitor are very close to those observed at lower temperatures in the absence of inhibitor, rather than resembling the values that the linear regression of Figure 6 would seem to imply for denaturation processes at these elevated temperatures.

Perhaps the simplest approach to explaining these results is based on the assumption that in the equilibrium depicted in equation 2,



the enzyme-inhibitor complex EI is not significantly unfolded even at temperatures somewhat higher than the high-temperature edge of the apparent unfolding envelope observed in the presence of the inhibitor. Using this assumption, and the integrated form of the van't Hoff equation for the temperature dependence of an equilibrium constant (equation 3) (40),

$$\ln (K_{T_1}/K_{T_2}) = (\Delta H/R) (1/T_2 - 1/T_1) \quad (3)$$

it can be shown by setting T_1 equal to T_d (the temperature for half-completion of the unfolding, which for a single-transition process is fairly closely approximated by T_m) and imposing the requirement that half of the total enzyme present must be unfolded (i.e., $[EI] + [E] = [U]$), that the apparent T_d will be displaced to the higher temperature T_2 , as given by equation 4.

$$T_2 = T_1 \Delta H^\circ / [\Delta H^\circ - RT_1 \ln(1 + I/K_i)] \quad (4)$$

In equations 3 and 4, the temperatures are expressed in degrees Kelvin; I is the concentration of the competitive inhibitor, and K_i the dissociation constant for the enzyme-inhibitor (EI) complex at the temperature T_2 .

In this formulation, the strong resemblance, both in peak shape and peak area, of the denaturation profiles in the presence of inhibitor to those in the absence of inhibitor (Figure 6, Table I) is explained in terms of the conclusion that the denaturation peaks represent in both cases the same thermodynamic event, i.e., the unfolding of the free enzyme, uncomplexed by inhibitor. The endothermic peaks observed in the presence of inhibitor are thus considered to be simply images of the peaks seen in absence of inhibitor, displaced along the temperature axis according to the general principle described by equation 4. The T_m values observed in the presence of cellobiose therefore serve as approximations only of apparent T_d values derived from the values for free enzyme. The T_m values for the EI complex have not, according to this analysis, been observed in the results presented here. The T_m values observed at the highest cellobiose concentration (0.1 M) used here do, however, indicate lower limits for the actual values for the CBH I/cellobiose complex.

The analysis presented above for the effect of cellobiose on CBH I thermal stability must be considered preliminary, in that equation 3, and therefore also equation 4, which is derived from equation 3, apply strictly only to single-transition processes. Equation 4 does, however, serve well to illustrate the general type of peak-displacement being proposed here. Exact numerical application of such an equation would require accurate estimates, not presently available, for such quantities as the K_i values for native CBH I and cellobiose at pH values far removed from the activity optimum, and/or at temperatures at which the native enzyme, in the absence of the inhibitor, does not exist in measurable proportions. Qualitatively, however, we believe that the analysis presented above can serve as a useful conceptual basis, or springboard, for further investigations of catalytic-domain structure and function.

Conclusions

We have adopted as our working hypothesis a model in which thermal denaturation of the core region of CBH I in the pH range 4.80 - 8.34 and at ionic strength 0.026 M involves three distinguishable endothermic transitions, one of which is relatively independent of pH and appears to be uncoupled from the other two at pH 7.50 and pH 8.34, the other two of which (the proposed constituent transitions of the pH-dependent asymmetric "principal peak" of the CBH I thermograms) appear to be tightly coupled over the entire pH range studied. Results presented for the stabilization of the core region by cellobiose indicate that this potent competitive inhibitor may serve as a powerful probe in DSC studies of the relationship between the stability of the core

region as a whole and the maintenance of the structure of the cellobiose-binding region of the catalytic site, and by extension, of the catalytic site as a whole. The fact that the cellulose-binding tail region of the CBH I molecule, which has been shown by NMR studies to have a well-defined structure in solution, does not show any measurable endothermic transitions in the temperature range from 12°C to 95°C is thought-provoking. Studies utilizing other instrumental techniques are planned to address the question of the temperature-dependence of the conformation of the tail fragment over this range.

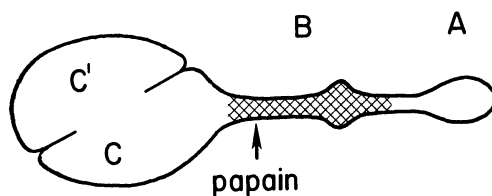


Figure 7. Postulated structure of the CBH I molecule, based on binding and catalysis (22,24) and SAXS (20,21) studies, plus the calorimetric results presented in this paper. The core and tail regions are described as having minimal interactions in terms of structural stabilization; the two domains of the core region, however, interact very strongly (See Discussion).

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Literature Cited

1. Jackson, W.M.; Brandts, J.F. *Biochem.* **1970**, *9*, 2294-2301.
2. Privalov, P.L.; Khechinashvili, N.N. *J. Mol. Biol.* **1974**, *86*, 665-684.
3. Pfeil, W.; Privalov, P.L. *Biophys. Chem.* **1976**, *4*, 23-32.
4. Biltonen, R.L.; Freire, E. *CRC Crit. Rev. Biochem.* **1978**, *5*, 85-124.
5. Sturtevant, J.M. *Ann. Rev. Phys. Chem.* **1987**, *38*, 463-88.
6. Privalov, P.L.; Potekhin, S.A. *Methods Enzymol.* **1986**, *131*, 4-51.
7. Privalov, P.L.; Gill, S.J. *Advan. Protein Chem.* **1988**, *39*, 191-234.
8. Brandts, J.F.; Hu, C.Q.; Lin, L.N.; Mas, M.T. *Biochem.* **1989**, *28*, 8588-8596.
9. Bailey, J.M.; Lin, L.-N.; Brandts, J.F.; Mas, M.T. *J. Protein Chem.* **1990**, *9*, 59-67.
10. Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, NY; **1983**.
11. Fagerstam, L.G.; Pettersson, L.G. *FEBS Lett.* **1980**, *119*, 97-100.
12. Shoemaker, S.; Watt, K.; Tsitovsky, G.; Cox, R. *Bio/Technology* **1983**, *1*, 691-696.
13. Wood, T.M.; McCrae, S.I. *Biochem. J.* **1986**, *234*, 93-99.
14. Henrissat, B.; Driguez, H.; Viet, C.; Schulein, M. *Bio/Technology* **1985**, *3*, 722-726.
15. Knowles, J.; Lehtovara, P.; Teeri, T. *Trends Biotechnol.* **1987**, *5*, 255-261.

16. Knowles, J.; Teeri, T.T.; Lehtovaara, P.; Penntila, M.; Saloheimo, M. In *Biochemistry and Genetics of Cellulose Degradation*; Aubert, J.-P.; Beguin, P.; Millet, J., Eds.; Academic Press: London, 1988; pp 153-169.
17. Woodward, J.; Hayes, M.K.; Lee, N.E. *Bio/Technology* 1988, 6, 301-304.
18. Bhikhabhai, R.; Pettersson, G. *Biochem. J.* 1984, 222, 729-736.
19. Shoemaker, S.; Schweickart, V.; Ladner, M.; Gelfand, D.; Kwok, S.; Myambo, K.; Innis, M. *Bio/Technology* 1983, 1, 691-696.
20. Abuja, P.M.; Schmuck, M.; Pilz, I.; Claeysens, M.; Esterbauer, H. *Eur. Biophys. J.* 1988, 15, 339-342.
21. Abuja, P.M.; Pilz, I.; Tomme, P.; Claeysens, M. *Biochem. Biophys. Res. Commun.* 1988, 156, 180-185.
22. Tomme, P.; van Tilbeurgh, H.; Pettersson, G.; van Damme, J.; Vandekerckhove, J.; Knowles, J.; Teeri, T.; Claeysens, M. *Eur. J. Biochem.* 1988, 170, 575-581.
23. Abuja, P.M.; Pilz, I.; Tomme, P.; Claeysens, M. *Biochem. Biophys. Res. Commun.* 1989, 169, 615-623.
24. Johansson, G.; Stahlberg, J.; Lindeberg, G.; Engstrom, A.; Pettersson, G. *FEBS Lett.* 1989, 243, 389-393.
25. Kraulis, P.J.; Clore, M.; Nilges, M.; Jones, T.A.; Pettersson, G.; Knowles, J.; Gronenborn, A.M., *Biochem.* 1989, 28, 7241-7257.
26. Teeri, T.T.; Jones, A.; Kraulis, P.; Rouvinen, J.; Penttila, M.; Harkki, A.; Nevalainen, H.; Vanhanen, S.; Saloheimo, M.; Knowles, J.K.C. In *Trichoderma reesei Cellulases: Biochemistry, Genetics, Physiology and Application*; Kubicek, C.P.; Eveleigh, D.E.; Esterbauer, H.; Steiner, W.; Kubicek-Pranz, E.M., Eds.; The Royal Society of Chemistry: Cambridge, UK, 1990; pp 156-167.
27. Shoemaker, S.; Watt, K.; Tsitovsky, G.; Cox, R. *Bio/Technology* 1983, 1, 687-690.
28. Nieves, R.A.; Himmel, M.E.; Todd, R.J.; Ellis, R.P. *Appl. Environ. Microbiol.* 1990, 56, 1103-1108.
29. van Tilbeurgh, H.; Tomme, P.; Claeysens, M.; Bhikhabhai, R.; Pettersson, G. *FEBS Lett.* 1986, 204, 223-227.
30. Gum, E.K.; Brown, R.D. *Biochim. Biophys. Acta* 1976, 446, 371-386.
31. Bailey, J.E., Ph.D. Thesis, London University, In *Handbook of Biochemistry -- Selected Data for Molecular Biology*; Sober, H.A., Ed.; Chemical Rubber Co.: Cleveland, OH, 1968; p. B-74.
32. Edge, V.; Allewell, N.M.; Sturtevant, J.M. *Biochem.* 1985, 24, 5899-5906.
33. Manly, S.P.; Matthews, K.S.; Sturtevant, J.M. *Biochem.* 1985, 24, 3842-3846.
34. Hu, C.Q.; Sturtevant, J.M. *Biochem.* 1987, 26, 178-182.
35. Ahern, T.J.; Klivanov, A.M. *Science* 1985, 228, 1280-1284.
36. Tomazic, S.J.; Klivanov, A.M. *J. Biol. Chem.* 1988, 263, 3086-3091.
37. Tomazic, S.J.; Klivanov, A.M. *J. Biol. Chem.* 1988, 263, 3092-3096.
38. Schmuck, M.; Pilz, I.; Hayn, M.; Esterbauer, H. *Biotechnol. Lett.* 1986, 8, 397-402.
39. Claeysens, M.; van Tilbeurgh, H.; Tomme, P.; Wood, T.M.; McCrae, S.I. *Biochem. J.* 1989, 261, 819-825.
40. Moore, W.J. *Physical Chemistry*; 3rd Edition, Prentice-Hall: London, 1962; p. 181.

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Chapter 25

Bacterial Cellulases

Regulation of Synthesis

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Bacteria represent a promising source for the production of industrial enzymes. Bacterial cellulases are an especially interesting case in point. Many thermophilic bacterial species produce cellulases that are stable and active at high temperature, resistant to proteolytic attack, and stable to mechanical and chemical denaturation. However, cellulase productivities in bacteria are notoriously low compared to other microbial sources. In this paper bacterial enzyme production systems will be discussed with a focus on comparisons of the productivities of known bacterial cellulase producers. In an attempt to draw conclusions concerning the regulation of cellulase synthesis in bacterial systems, a tentative model for regulation in *Acidothermus cellulolyticus* has been developed.

Successful utilization of cellulose materials, an abundant renewable resource, depends on the economically feasible process for cellulase production, good pretreatment methods for cellulosics, efficient microbial and enzymatic hydrolysis of cellulose to low molecular weight products, and appropriate chemical and/or biological conversion of the hydrolysis products to other useful products which can be applied as liquid fuels, chemical feedstocks, and food materials. A major hindrance to the industrial enzymatic hydrolysis is the high cost of cellulase, which has been estimated to be up to 60% of the total operating costs. Improvement of cellulase production has focused on selection of hypercellulolytic strains, optimization of fermentation conditions, and development of the most effective mode of operation (batch, continuous, fed-batch, etc.). The selection criteria for suitable cellulase sources are: fast-growing microorganisms, the strain with high cellulase yields and high specific activity, and enzyme systems with high thermostability.

The degradation of cellulose by bacterial systems occurs both aerobically and anaerobically. The aerobic bacteria include *Acidothermus cellulolyticus*, *Cellulomonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Cellvibrio* sp., *Cytophaga* sp., *Microbispora* sp., and *Thermomonospora* sp. The anaerobic bacteria are *Acetivibrio cellulolyticus*, *Bacteroides* sp., *Clostridium* sp., *Ruminococcus* sp., and *Micromonospora* sp. This review will cover some mesophilic and thermophilic cellulolytic bacteria, some of which have been reviewed by Ljungdahl and Eriksson (1), Coughlan and Ljungdahl (2), and Robson and Chambliss (3).

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According to studies on fungal cellulolytic enzymes, the model of crystalline cellulose hydrolysis requires synergistic action of at least three cellulase components: endo-1,4- β -glucanase (carboxymethyl cellulase), exo-1,4- β -glucanase (often a cellobiohydrolase) and β -glucosidase (cellobiase) (4,5). All cellulolytic bacteria synthesize endoglucanase and either β -glucosidase (6,7) or cellobiose phosphorylase (8) or a combination of the two (9). Both aerobic and anaerobic bacteria synthesize cellobiose phosphorylase that catalyzes cellobiose to glucose and glucose-1-phosphate. As some of these organisms do not produce a β -glucosidase, it seems reasonable to assume that the phosphorylases provide a means of metabolizing cellulose degradation products and of diminishing the inhibitory effect of cellobiose on cellulase activity (10).

Extracellular cellulases in bacteria have been purified from a number of bacterial species, including *Micromonospora bispora* (11), *Cytophaga* sp. (12), *Streptomyces flavogriseus* (13), *Cellulomonas uda* (14), *Clostridium stercorarium* (15), *Acetivibrio cellulolyticus* (16), and *Ruminococcus albus* (17). Cellulolytic enzymes of *C. thermocellum* are organized into a distinct multiunit complex which have been coined "cellulosome" by Lamed et al. (18). Scanning electron microscopic evidence by Lamed et al. (19,20) demonstrated a clear correlation between cellulolytic activity and the appearance of protuberance-like structures on the bacterial cell surface. This correlation has been tentatively extended over a wide range of physiological and evolutionary boundaries.

Cellulolytic bacteria can be found which produce only cell-bound cellulase such as *Cytophaga* (12), only cell-free cellulase, such as *Cellvibrio vulgaris* (21), *Bacillus* sp. (22), *Clostridium* sp. (23), *Acetivibrio cellulolyticus* (24), and *Thermoactinomyces* (25,26), and both cell-bound and cell-free cellulase such as *Pseudomonas* (27), *Bacteroides succinogenes* (28), and *Cellvibrio fulvus* (29). However, the location of cellulase in bacteria is also dependent upon the environments in which the bacteria are grown and the age of the culture (29,27).

The synthesis of cellulases is regulated by induction and catabolite repression mechanisms. Cellulases are induced by soluble derivatives from cellulose or several other low molecular weight carbohydrates, but enzyme synthesis is repressed by the presence of glucose or other readily metabolized sugars. Evidence supporting this conclusion has been obtained for bacteria, e.g. *Acetivibrio cellulolyticus* (30), *Acidothermus cellulolyticus* (31), *Cellulomonas uda* (32), and *Thermomonospora* (33,34,35). The known inducers for cellulase synthesis are cellulose, cellulose derivatives, cellobiose, sophorose, and lactose. The effects of cellobiose, lactose, and sophorose on cellulase synthesis of bacterial systems are significantly different from fungal systems. We shall now review the properties of cellulase synthesis regulation systems in four genera of anaerobic and four genera of aerobic bacteria, for which repression/induction data have been published.

Anaerobic Bacteria

The cellulolytic systems of anaerobic bacteria have been reviewed recently by Coughlan and Ljungdahl (2). We shall concentrate on the transcription regulation systems of *Acetivibrio cellulolyticus*, *Bacteroides* sp., *C. thermocellum* and *Ruminococcus* sp.

***Acetivibrio cellulolyticus*.** This bacterium is an obligate anaerobe, and grows at pH 6.5 - 7.7 at temperatures from 20 to 40°C (36). Of about 30 polysaccharides and sugars tested, *A. cellulolyticus* differs from most cellulolytic organisms in its ability to utilize only cellulose, cellobiose, and salicin for growth. Endoglucanase and exoglucanase formation by this organism in microcrystalline cellulose containing medium were optimum at pH 5.0 and 50°C. The cellulase activity increased progressively during the exponential growth phase and corresponded to the extent of growth (24). Both exo- and endoglucanase activities were shown to be regulated by induction and catabolite repression (30). Only cellobiose and salicin stimulated endoglucanase synthesis; salicin was approximately half as effective as cellobiose.

***Bacteroides cellulosolvens* and *Bacteroides succinogenes*.** *Bacteroides cellulosolvens* was isolated from sewage sludge (37). This organism is a non-spore-forming, mesophilic anaerobe, capable of growing in a synthetic medium containing cellulose or cellobiose as the sole carbon source. It does not require yeast extract for growth and does not utilize glucose or xylose as a carbon source. Laboratory cultures for enzyme production were maintained at 37°C in medium containing 20 g/L CaCO₃ in an atmosphere of N₂-CO₂ (80%-20%) (38). *B. cellulosolvens* produced extracellular and cell-associated cellulases, possessed endoglucanase, exoglucanase, and xylanase activities and degraded filter paper *in situ*. However, this enzyme system lacked β -glucosidase activity and cellobiose was converted to glucose-1-phosphate and glucose by the action of cellobiose phosphorylase (38).

B. succinogenes is a predominant rumen cellulolytic bacterium (39); it grows optimally at between pH 6 and pH 7 and temperature 37°C and 40°C and can ferment cellulose, cellobiose, glucose, starch, dextrin, maltose, and trehalose to succinate and acetate as the main products; carbon dioxide is fixed during growth of *B. succinogenes* S-85 (40). The conditions used for production of enzyme for characterization studies were pH 6.5 and 39°C. Cells grown on either cellobiose or glucose exhibited cell-bound exoglucanase and cellobiase activities. The cellobiase, which was largely cell-associated, appeared to be constitutive. The only hydrolysis product was glucose, therefore the enzyme cleaving cellobiose appeared to be a β -1,4-glucosidase rather than a cellobiose phosphorylase. An enzyme, cellodextrinase, which released the cellobiose group from p-nitrophenyl cellobioside was also isolated from the periplasmic space of *B. succinogenes* grown on microcrystalline crystalline cellulose (41). The function of cellodextrinase presumably is to hydrolyze cellodextrins, which enter the periplasmic space, to cellobiose and glucose for transport into the cell. The endoglucanase and cellobiosidase activities coded for by the cel gene of *B. succinogenes* have been expressed and secreted into the periplasmic space in *E. coli* (19).

***Clostridium thermocellum*.** Several related clostridial species are thermophilic, cellulolytic bacteria which grow at pH 7.0, 60°C and ferment cellulose directly to ethanol and organic acids (42). All *C. thermocellum* and *C. thermocelluloaseum* strains are able to ferment cellulose and cellobiose. Although *C. thermocellum* degrades xylan, the resulting xylose and xylobiose are not fermented and they accumulate in the broth (43). McBee reported that no growth of *C. thermocellum* occurred on glucose, fructose, arabinose, lactose, salicin, sorbitol, or mannitol (44). Ng et al. reported growth on cellulose and cellobiose but not on glucose or xylose (45). Johnson et al. proved that *C. thermocellum* can utilize cellulose, cellobiose, glucose, fructose, and sorbitol as carbon sources (46). Whereas *C. thermohydrosulfuricum* catabolized cellobiose with cellobiase and hexokinase enzymes, but *C. thermocellum* utilized cellobiose phosphorylase and phosphoglucomutase (47). A cellodextrin phosphorylase was also present in *C. thermocellum*, which phosphorylates cellodextrins with formation of α -D-glucose-1-phosphate and preservation of the glucosidic bond energy (48). The cellulolytic enzymes and β -glucosidase have been found to be constitutive and extracellular enzymes in *C. thermocellum*. *C. thermocellum* also produced a cell-associated β -glucosidase, which is capable of hydrolyzing cellobiose to glucose. Since β -glucosidase K_m for cellobiose of the β -glucosidase is about 10 times greater than that of the cellobiose phosphorylase (43).

***Ruminococcus albus* and *Ruminococcus flavefaciens*.** These bacteria are important cellulose-degraders found in the rumen of cattle and sheep (2). Most isolated strains ferment cellulose and xylan and all ferment cellobiose. Fermentation of glucose and some other carbohydrates depends on the particular strain. *R. flavefaciens* and *B. succinogenes* can ferment the highly ordered crystalline cellulosic substrates but *R. albus* cannot. No evidence has been found for extracellular cellulase production by *R. albus*, but Ohmiya et al. purified cellobiosidase from this culture (17). Laboratory growth of *R. albus* has been conducted at pH 7.0 and 37°C.

R. flavefaciens cells, during growth in pure culture, released cellulase, endoglucanase, and xylanase into the culture fluid. This microorganism hydrolyzed cellulose to yield only cellobiose as a product (49). It had been reported that a cellobiose phosphorylase and glucokinase were present in *R. flavefaciens* (8). The *Ruminococcus* cellulase system was repressed by disaccharides such as cellobiose, sucrose, and lactose (50).

Aerobic Bacteria

We shall examine transcription regulation systems of *Acidothermus cellulolyticus*, *Cellvibrio* sp., *Cellulomonas* sp., and *Thermomonospora* sp.

Acidothermus cellulolyticus. *A. cellulolyticus*, was isolated from the acidic hot springs of northern Yellowstone National Park, Wyoming (51). This thermophilic, acidophilic, aerobic, cellulolytic bacterium can grow on several carbohydrates, including glucose, cellobiose, sucrose, xylose, and cellulose (51,31). It grew at 37 to 65°C, with the optimum growth temperature of 55°C. The pH range for growth was 3.5 to 7, with an optimum pH of 5. Cellulolytic enzymes were produced when the cells were grown on cellobiose and/or cellulose. The cellulolytic activity was secreted mainly during the late log and early stationary phases of growth. The cellulase enzyme complexes can completely degrade microcrystalline cellulose; this indicates that the full component of cellulolytic enzymes is being secreted (51). The products of cellulose hydrolysis are D-glucose and D-cellobiose.

The cellulase production, cell growth, and cellobiose degradation rates of batch culture in 2.5, 5.0, 7.5, and 10.0 g/L of cellobiose as a substrate were studied by Shiang et al. (31). The maximum filter paper cellulase activities (0.021 to 0.069 U/mL) and volumetric productivities (between 0.92 and 2.49 U/L·h) were proportional to the concentration of cellobiose. Preliminary work showed that the better cellulase yields in batch culture were achieved using a mixed substrate system containing cellobiose and Solka Floc. A cellulase concentration of 0.105 U/mL was achieved in 70 h, which was three times greater than that using 5 g/L cellobiose alone. The substrate system using sucrose and Solka Floc produced 0.110 U/ml in 50 h. Of the sugars added to Solka Floc cellulose fermentations, D-cellobiose, D-glucose, D-fructose, D-xylose, and sucrose stimulated cellulolytic enzyme production, but only D-cellobiose and D-xylose were effective cellulase inducers.

Regulation of cellulase synthesis by induction and catabolite repression in *A. cellulolyticus* was also studied (52). Various compounds such as L-sorbose, cyclic AMP (cAMP), L-glucose, 2-deoxyglucose (2-DG), glucose-1-phosphate (G-1-P), sophorose, salicin, sugar alcohols, and isopropylthiogluconide (IPTGlu) were added along with Solka Floc to improve extracellular cellulase formation by the culture. When cAMP was added exogenously to *A. cellulolyticus* cultures in the concentration range of 10 to 200 mg/L, cellulase yields were increased with the increasing levels of cAMP (Figure 1). The enzyme production rates were identical between 35 and 50 h using four different levels of cAMP; cell growth was not affected. L-Sorbose, L-glucose, 2-DG, G-1-P, sophorose, IPTGlu, and sugar alcohols enhanced cellulase activity production, but the onset time and the time requirement to reach the maximum enzyme activity were different with each condition. All these substances functioned as moderators in a proposed cellulase synthesis model. On the basis of the experimental results, only cellobiose, xylose, sophorose, and unknown soluble derivatives from cellulose were considered as inducers. A tentative regulatory mechanism of cellulase synthesis is proposed, in which repressor, inducer, cAMP, and moderator were all involved in controlling the rate and the yield of enzyme production (52).

Cellvibrio gilvus. This organism grows well on cellobiose, cellodextrins, and cellulose, but less well on glucose; conditions for optimized growth or enzyme production were not

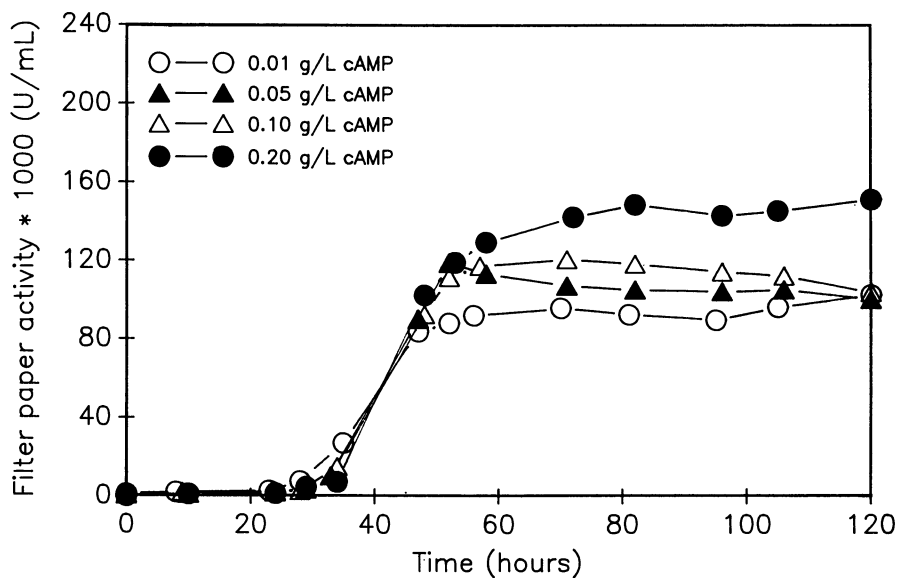


Figure 1. Time course of cellulase activity accumulation in *A. cellulolyticus* cultures containing 15 g/L Solka Floc and various concentrations of cAMP.

found. This species has been reported by Carpenter and Barnett, to possess an inducible cellulase (53), but Storvick et al. described *C. gilvus* as constitutive for at least four electrophoretically distinct endo-1,4- β -glucanases, which degrade cellulose to cellobiose and some cellotriose (54). Growth conditions for this organism are pH 7.0 and 30°C. In the study of Breuil and Kushner, addition of higher concentrations (0.5%) of CMC to cellulose (filter paper) caused a good stimulation of both growth and cellulase formation (55). Glycerol addition did not interfere with cellulase formation, whereas both cellobiose and glucose repressed cellulase formation in the presence of cellulose. The higher the concentration of cellobiose added in the medium, the longer the delay in the appearance of cellulase. Physical contact between cells and cellulose fibers was necessary for both cellulase induction and growth stimulation (55). *C. gilvus* only contained a cellobiose phosphorylase which has been purified by Sasaki et al. (56). This enzyme does not phosphorylate cellotriose or higher cellodextrins. It has been suggested that cellotriose and higher cellodextrins are metabolized by transglucosylation to yield cellobiose which is then cleaved by cellobiose phosphorylase (57, 58).

***Cellulomonas uda*, *Cellulomonas fimi*, and *Cellulomonas flavigena*.** In a study of Stoppok et al., endo-1,4-glucanases of *C. uda* were found to be extracellular, but a very small amount of cell-bound endoglucanase was considered to be the basal level of the cells (32). Endoglucanase synthesis in *C. uda* can be induced by small amounts of cellobiose, as is the case for another *Cellulomonas* species described by Stewart and Leatherwood (59). Sophorose was not effective at all as either an inducer or repressor of endoglucanase formation in *C. uda*. Catabolite repression of endoglucanase formation occurred in the presence of glucose (32). β -Glucosidases were formed constitutively and were found to be cell bound regardless of the carbon source. No cytoplasmic glucosidase activity could be measured in *C. uda*. Possibly, *C. uda* possesses cellobiose phosphorylase and cellodextrin phosphorylase (32). Stewart and Leatherwood also reported that catabolite repression of cellulase synthesis occurred in *Cellulomonas* sp. when moderate levels of glucose were added to the medium. Cellobiose acted as a competitive inhibitor of catabolite repression and an inducer of cellulase synthesis (59).

Langsford et al. reported that *Cellulomonas fimi* culture supernatants contained cellulase and proteinase activities, for which there appeared to be a relationship. Glucose repressed the synthesis of both activities and cellulose induced both (60). Adding cellulose to *Cellulomonas* sp. (NRCC 2406) cultures stimulated growth and improved production of cellulases (61). Optimum conditions for growth and cellulase production were pH 6.5 and 30°C. The addition of glucose in the presence of cellulose inhibited growth. Several species of *Cellulomonas* have cellobiose phosphorylase.

Addition of glucose and cellobiose activated the growth of *Cellulomonas flavigena* ATCC 482 on CMC, but both repressed cellulase formation (62). The same phenomenon was observed with addition of lactose, sodium acetate, mannose, glycerol, fructose, and galactose. The cellulase formation in *C. flavigena* was enhanced at dissolved oxygen levels and yeast extract concentrations which are not optimal for growth. Restriction in ATP synthesis and growth of this culture also resulted in increased cellulase formation (62). As proposed by Hulme and Stranks, limiting the metabolism of cellulolytic microorganisms, may be the main mechanism for induction of cellulase synthesis (63).

***Thermomonospora curvata* and *Thermomonospora fusca*.** A few mycelium-producing and spore-forming bacteria have been investigated with regard to cellulolytic ability. *Thermomonospora* sp. is a thermophilic actinomycete with the optimum growth temperatures between 55 to 60°C. Stutzenberger identified *T. curvata* (64) and optimized cellulase production by using a minimal medium (65). Ground cotton fibers supported the highest cellulase production compared to other soluble and insoluble carbohydrates. Cellobiose induced only ca. 30% as much endoglucanase as ground cotton in *T. curvata*, whereas in *T. fusca*, cellobiose appeared to be as good an inducer

as cellulose (35). The levels of cAMP during cellulase induction and repression in *T. curvata* were studied (66,35,67). A catabolite repression-resist mutant of this microorganism was obtained (33). Cellulase biosynthesis of this mutant was undiminished by glucose, 2-deoxyglucose, or α -methyl glucoside, which are potent repressors in the wild type. Intracellular cyclic AMP levels were higher in the mutant in both the absence and the presence of repressors. During growth of *T. curvata* on glucose, specific cellulase activity and cAMP levels were 0.03 U/mg dry wt. (endoglucanase units) and 2 pmol/mg dry wt., respectively. These values increased to about 6 and 25, respectively, with growth on cellulose (67). Detectable endoglucanase production ceased when cAMP levels dropped below 10 pmole/mg dry wt. Cellobiose (0.2 g/L) also caused a sharp decrease in cAMP levels and repressed endoglucanase production when added to cellulose-grown cultures.

Cellulose degradation by *T. curvata* was affected by both cellulase inducer (cellobiose) and cellulase repressor (glucose) (33). The addition of glucose repressed endoglucanase production; production resumed only after the repressor was consumed. In contrast, the generation of glucose during growth on cellobiose had no effect on the continuous uptake of cellobiose from the culture fluid. Glucose was not utilized until the cellobiose concentration, dropped below 3 mM (~ 1 g/L) (68). Cellobiose was not cleaved by extracellular β -glucosidase and was not transported as glucose. No evidence of cellobiose phosphorylase or a cellobiose-specific phosphoenolpyruvate-phosphotransferase system was observed (11). Possibly, the hydrolysis of cellobiose to glucose during fermentation was due to intracellular β -glucosidase.

The results of Moreira et al. showed that *T. fusca* (formerly *Thermoactinomyces* sp., strain YX) can grow on glucose, cellobiose, and cellulose (35). Glucose was an effective repressor of cellulase synthesis, and cellobiose, microcrystalline cellulose, and Solka Floc induced high levels of enzyme activity. Furthermore, a mathematical model for the cellulose fermentation was developed based on mechanistic considerations of cellulase synthesis and enzymatic cellulose hydrolysis (35). In *T. fusca*, endocellulase synthesis varied over a 100-fold range depending on the carbon source used. The study of Lin and Wilson showed that the variation was caused by two regulatory mechanisms: an induction mechanism that increased endoglucanase synthesis rate about 20-fold and a growth rate-dependent repression mechanism that changed the synthesis rate over a 6-fold range in both induced and noninduced cells (34). Endocellulase synthesis of *T. fusca* was induced by cellulose, cellobiose, or celloedextrin, and derepression of enzyme synthesis was achieved by limiting the source of either carbon, nitrogen, or phosphorus.

A summary of the key information has been compiled on the anaerobic and aerobic bacteria discussed above. Comparison of the substrates for growth of these organisms (Table I) show that all utilize cellobiose and various forms of cellulose. The two species belonging to *Bacteroides* have different specificity for substrates, while those for *Ruminococcus*, *Cellulomonas* and *Thermomonospora* were the same. Table I also allows comparison of the behavior of the 13 species of cellulolytic bacteria toward cellobiose. More variability is noted in this regard and no correlation between induction/repression can be made with the mechanism of cellobiose degradation.

Comparison with Fungal Systems

The cellulase systems that have been investigated in greatest detail have been derived from mesophilic fungi; the most studied strains are *Trichoderma reesei* and its mutants. Major limitations of cellulases from mesophilic fungi are low-temperature stability and low rate of enzyme production. Thermophilic bacteria grow rapidly and produce thermostable cellulase but generally have not yielded high productivities. Cellulase productivities of *Thermomonospora fusca* N-35, *Acetivibrio cellulolyticus* and *Cellulomonas* (NRCC 2406) are comparable to those of the *Trichoderma* mutants. Comparisons of cellulase activities and productivities of these and other mesophilic and thermophilic bacteria are shown in Table II.

Table II. Cellulase Production by Some Mesophilic and Thermophilic Microorganisms

Organism	Incubation temperature (°C)	Cellulosic substrate	Cellulase activity (FPU ^a /mL)	Cellulase productivity (FPU ^a /L.h)	Ref.
<i>Trichoderma reesei</i> QM 6a	28	6% cotton	5.00	15.00	73
<i>Trichoderma reesei</i> QM 9414	28	6% cotton	10.00	30.00	73
<i>Trichoderma reesei</i> Rut-C30	28	6% cotton	14.00	42.00	73
<i>Trichoderma reesei</i> L27	28	8% Avicel	18.00	94.00	74
<i>Sporotrichum thermophile</i>	45	1% wheat straw	0.07	1.46	75
<i>Thielavia terrestris</i> ATCC 26917	52	1% Solka Floc	0.11	3.43	76
<i>Clostridium thermocellum</i>	60	1% Solka Floc	0.14	1.94	77
<i>Thermomonospora fusca</i>	55	1% Avicel	0.15	5.10	35
<i>Thermomonospora fusca</i> mutant N-35	55	1% Avicel	0.33	27.50	78
<i>Thermomonospora curvata</i>	53	cellulose	0.10	1.39	66
<i>Thermomonospora curvata</i> mutant F	53	cellulose	0.18	2.50	66
<i>Thermomonospora curvata</i>	53	8% cotton	0.26	3.61	33
<i>Thermomonospora curvata</i> mutant G-11	53	8% cotton	0.29	4.03	33
<i>Acidothermus cellulolyticus</i>	55	1.5% Solka Floc and 0.5% sucrose	0.16	3.31	b
<i>Acetivibrio cellulolyticus</i>	37	0.5% Avicel	3.36	46.67	24
<i>Cellulomonas</i> (NRCC 2406)	30	1% Solka Floc	7.00	72.92	61

^aInternational filter paper cellulase activity unit. ^bShiang, M.; Linden, J.C.; Mohagheghi, A.; Grohmann, K.; Himmel, M.E. *Appl. Microbiol. Technol.*, submitted.

Regulation of Cellulase Synthesis

The biosynthesis of cellulase is stringently regulated by a variety of factors in wild strains, which makes cellulase production and utilization costly on a commercial scale. Bacteria exhibit both positive and negative control of protein synthesis. An example of negative control in prokaryotes is the lactose operon and of positive control, the arabinose operon, both found in *E. coli*. Regulation of catabolite repressible enzymes in bacteria is through an additional control involving the levels of cAMP in the cell. At present, there is some disagreement as to whether cAMP is involved in controlling cellulase synthesis. The experimental results presented by Suzuki (79) suggested that it was not involved in the case of *Pseudomonas fluorescens* var. *cellulosa*. Stutzenberger conclusively showed cAMP to be involved with regulation of cellulase synthesis by *Thermomonospora* (80). Goksoyr and Eriksen suggested the possibility that ATP rather than cAMP may be the small molecular regulator in some instances (81). On the contrary, Wood et al. showed that the addition of cAMP exogenously increased cellulase synthesis in toluene-treated *T. curvata* (67). Moreover, *T. curvata* catabolite repression-resistant mutants which expressed cellulase at elevated rates had two to three times the intracellular cAMP content of the parent strain (67). In *T. fusca*, the repression effect

was partially relieved when 5 mM cAMP was added concomitantly with the repressing carbon source; however, cAMP never completely abolished repression (34).

The cyclic AMP receptor protein (CRP), to which cAMP must be complexed for release from catabolite repression, has two distinct domains: the N-terminal portion binds to cAMP, and the carboxyl terminal end binds to DNA. Exactly how glucose affects the intracellular levels of cAMP is still being investigated. One important theory suggests that the repression mechanism is strongly linked with the phosphoenolpyruvate: sugar phosphotransferase transport system (PTS) (82). In the absence of glucose or any other repressive sugar, the phosphorylated HPr protein transfers its phosphate moiety to a regulatory protein (RPr). The RPr-P then, either by allosteric interaction or by phosphorylation, transforms an inactive adenylate cyclase into an active form. This enzyme catalyzes the conversion of ATP into cAMP. In the presence of glucose, the HPr~P phosphorylates enzyme III of the PTS system which, in turn, phosphorylates the sugar upon transport through the cell membrane. Therefore, in the case of catabolite repression, adenylate cyclase remains in the inactive form and the cAMP concentration remains low.

Regulation Models of Cellulase Synthesis

Gong and Tsao (6) had proposed a unified model for the regulation of cellulase synthesis. This postulated mechanism of regulation took into account the importance of glucosidase in the regulation of glucose and also of cellobiose levels within the cells. In this model, cellobiose and glucose represent the limiting cellulolytic products of the basal cellulase. Cellobiose serves as a carbon source and a potential inducer. Active transport of cellobiose across the cell membrane is achieved by an adaptive transport enzyme system. Both constitutive and adaptive glucosidases are present as a membrane bound enzyme, which would hydrolyze part of cellobiose to glucose. After cellobiose enters the cells, the intracellular cellobiose could become the active inducer and react with repressor protein to initiate induction. Alternatively, the potential inducer could be hydrolyzed by intracellular glucosidase resulting in the accumulation of glucose and the repression of cellulase synthesis. The affinity of the active inducer for the repressor protein determines the efficiency of induction. Induction of cellulase synthesis occurs when the repressor protein has been inactivated by the active inducer and transcription and translation follow.

Stutzenberger described the regulation of microbial cellulolytic activity at the biosynthetic, secretory, and catalytic levels in detail (80). This model for the regulation of cellulase biosynthesis shared many features in common with the well-studied ara operon in *E. coli*. In the ara operon, the regulator protein functions as a repressor in the absence of inducer (arabinose) and as an activator in its presence; the regulatory protein is not synthesized constitutively. In the cellulase operon, it is now generally accepted that a low level of constitutive enzymes degrades their exogenous substrates, and the resultant low-molecular-weight products enter the cell and induce further synthesis of extracellular enzymes (22).

In Stutzenberger's regulation model of cellulase synthesis, control of β -glucosidase biosynthesis is not included. The operon is an activator-controlled inducible system requiring the cooperative binding of two regulatory protein to the control region for maximal transcription. One regulatory protein, called CAM for cellulase activator molecule, requires formation of a complex with inducer in order to bind the specific control region on the DNA. The other regulatory protein is CAP (referred to CRP above) which requires cAMP for binding to DNA. When neither CAM nor CAP are in their complexed states, cellulase operon expression occurs at a very low rate (basal level) due to the inability of RNA polymerase to initiate transcription in their absence. Under conditions of carbon limitation in the absence of inducer, cAMP levels increase sufficiently to create complexes with CAP which then bind weakly to the control region and cause a detectable but less than maximal increase in expression. Under conditions

in which inducer is provided at growth-limiting rates, CAM and CAP bind in a cooperative manner to the control region; their simultaneous presence allows maximal transcription by RNA polymerase to provide coordinate expression of the extracellular cellulolytic complex.

Model Development for *A. cellulolyticus*

The effect on cellulase production of various compounds when added along with Solka Floc to *A. cellulolyticus* was studied by Shiang (52). For instance, Figure 1 shows enzyme production rates at different concentrations of cAMP to be nearly identical during the 35 to 50 h fermentation period. This may be due to limiting rates of permeability of the cAMP into the cell regardless of extracellular concentration. However, increasing amounts of cAMP addition increased final cellulase yields proportionally. The cAMP-specific stimulation of cellulase production in *A. cellulolyticus* indicates that the synthesis of cellulase may be regulated to some extent by intracellular cAMP levels.

Addition of glucose-1-phosphate increased cellulase yields and shortened the time required to reach maximum FPA (Figure 2). Enzyme yields and productivities using 0.5 g/L G-1-P and 15 g/L Solka Floc were almost three times as great as using the same concentration of Solka Floc alone. The maximum enzyme concentration produced after 71 h of inoculation was 0.201 U/mL and the volumetric and specific cellulase productivities were 2.83 U/L·h and 0.57 U/g dry wt·h, respectively. The significantly enhanced cellulase production by G-1-P may be a result of transport system phenomena or enzyme induction. One might postulate the following two processes. First, in the presence of G-1-P, the phosphorylated protein HPr in PTS transfers its phosphate moiety to a regulatory protein (RPr). The RPr-P then transforms an inactive adenylate cyclase into an active form. Thus, ATP is converted to cAMP which complexes a cAMP receptor protein (termed as CRP or CAP) to form CRP·cAMP complex. This complex binds to the promoter, enhances RNA polymerase binding to the operon promoter and initiation of transcription. Secondly, if G-1-P were a phosphorolysis product of cellobiose by the cellobiose phosphorylase reaction, G-1-P may be acting as a basic inducer molecule to turn on cellulase enzyme synthesis in this culture.

In the *A. cellulolyticus* system, comparisons of maximum cellulase activity, time to maximum FPA, as well as volumetric and specific enzyme productivities with various substances added to Solka Floc are summarized in Table III. The values are reported relative to values obtained using 15 g/L Solka Floc as the sole substrate. Most of the addition components to Solka Floc fermentations enhanced cellulase yields; exceptions were mannose, L-arabinose, and IPTGal. The highest relative enzyme activities (309 and 308%) were achieved using G-1-P and salicin, respectively. Supplementation of cellobiose, sucrose, L-glucose, cAMP, sophorose, xylose, and IPTGlu significantly reduced the time required to reach maximum FPA. The greatest volumetric enzyme productivity improvement, approximately 4.5 fold (446%), occurred using 0.2 g/L L-glucose. Moreover, adding 0.25 g/L IPTGlu increased the relative specific enzyme productivity by 400%.

Four categories of effectors (additives to the Solka Floc fermentations) can be classified by comparing relative maximum enzyme concentration and relative time required to reach the maximum FPA, to the chemical structures of these supplementing compounds.

The first group is comprised that of compounds which potentially could be degraded to form G-1-P by phosphorolysis by beta-linkage specific enzymes. They include IPTGlu, cellobiose, sophorose, salicin, and sucrose. Addition of these compounds, or exogenous G-1-P, to Solka Floc fermentations improved maximum cellulase yields from 171 to 309%, and the time period for enzyme synthesis was reduced from 95 to 59% compared with using Solka Floc only.

The second group of effectors is made up of compounds which potentially could be transported by mechanisms other than the PTS presumably used for D-glucose

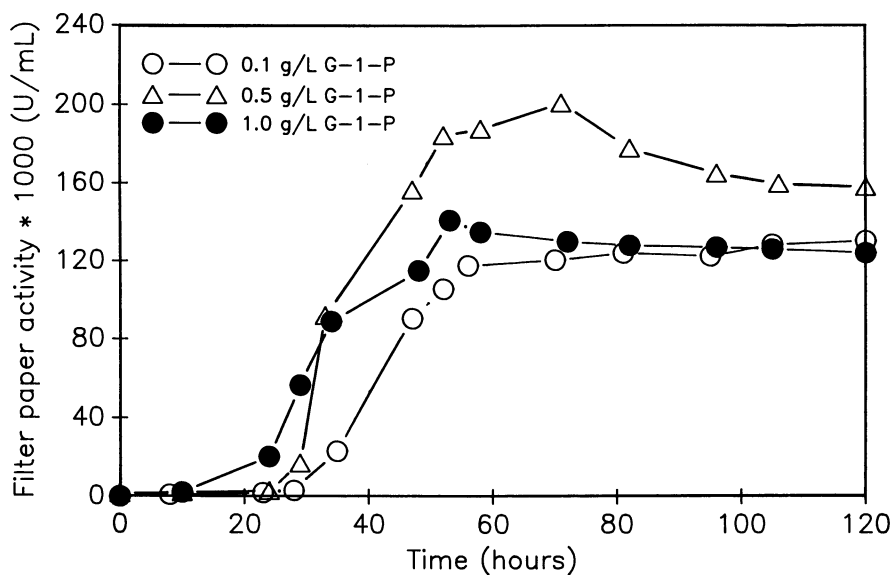


Figure 2. Time course of cellulase activity accumulation in *A. cellulolyticus* cultures containing 15 g/L Solka Floc and various concentrations of glucose-1-phosphate.

Table III. Comparison of Cellulase Production Parameters Obtained Using Various Substance Addition with Parameters Obtained Using Solka Floc Only

Concentrations of effectors added to 15 g/L Solka Floc fermentations	Relative maximum enzyme conc. (%)	Relative time to maximum FPA (%)	Relative volumetric enzyme productivity (%)	Relative specific enzyme productivity (%)
Control: 15 g/L SF	100	100	100	100
2.5 g/L cellobiose	252	65	386	253
5.0 g/L glucose	206	106	195	137
5.0 g/L sucrose	245	59	414	247
1.0 g/L xylose	178	69	259	226
0.2 g/L L-glucose	258	58	446	379
50 mg/L cAMP	183	64	286	268
0.5 g/L G-1-P	309	88	354	300
0.2 g/L 2-deoxyglucose	257	95	271	258
0.2 g/L sophorose	171	69	248	263
1.0 g/L L-sorbose	257	89	290	305
2.5 g/L maltose	162	143	114	105
2.5 g/L fructose	154	111	139	116
2.5 g/L mannose	69	81	85	79
2.5 g/L L-arabinose	58	52	113	111
2.5 g/L glycerol	132	123	108	111
2.5 g/L mannitol	254	101	251	205
2.5 g/L sorbitol	249	130	121	111
2.5 g/L IPTGlu	234	59	396	400
2.5 g/L IPTGal	89	148	61	74
1.0 g/L methyl glucoside	215	148	146	174
1.0 g/L salicin	308	95	325	274

transport. Supplementation of these compounds increased cellulase production (178 to 258%) and reduced fermentation time (95 to 58%). These are D-xylose, L-sorbose, L-glucose, and 2-deoxyglucose. Both the first and second groups may be associated with high concentrations of cAMP. The level of cAMP in the cells is determined by the activities of the enzyme, adenylate cyclase. Adenylate cyclase is membrane-bound, and its activity is high only if components of the sugar transport system are phosphorylated, which would be the case in the absence of sugars transported by the PTS mechanism.

The third group is that of compounds which may potentially be transported by the PTS and inhibit cAMP production. Cellulase synthesis is initiated after these compounds are consumed for cell growth. This group includes D-glucose, D-fructose, maltose, mannitol, glycerol, sorbitol, and β -methyl glucoside. The presence of these compounds in Solka Floc fermentations, enhanced enzyme yields (132 to 254%) but the time required to complete cellulase synthesis took longer (106 to 148%) than the control.

The fourth group of effectors are compounds that inhibit cellulase production and/or cell growth. They are L-arabinose, D-mannose, lactose, and IPTGal.

In accordance with the derivation of an expression for the regulation of the lac operon by Yagil and Yagil (83), the relationships discussed above between the relative rates of enzyme synthesis, α , and effector concentration, E, were evaluated. From

equilibrium analysis of binding repressor molecules to the operator, the following expression:

$$\log (\alpha / (1-\alpha) - \alpha_b) = \pm n \log [E] + \log \alpha_b \pm \log K_1$$

permits linear plotting of experimental data and the evaluation of three quantities: n , the number of effector molecules combining with a repressor molecule, K_1 , the dissociation constant of this interaction and K_2/R_t (designated α_b), the ratio of the repressor-operator dissociation constant to total repressor concentration. It is assumed that α_b represents the basal level of cellulose expression. The affinity of the effectors to the repressor is represented by values of α , which are measured as the ratio of specific enzyme productivities ($U/g \cdot h$) to the maximum specific productivity observed in these studies.

$$\alpha = (\text{sp. product.}) / (\text{sp. product.})_{\max}$$

The rate of enzyme synthesis is proportional to the number of operators free to be transcribed.

The subscript "max" denotes specific enzyme productivity at full induction.

From the experimental results using the various effectors with *A. cellulolyticus*, the (sp. product.)_{max} obtained was 0.76 $U/g \cdot h$ using 0.25 g/L IPTGlu with Solka Floc, and the specific productivity at the basal level was 0.19 $U/g \cdot h$ using Solka Floc alone. Relationships between effector concentration and the rate of cellulase synthesis are shown in Figure 3. A positive slope indicates that the binding of effector molecules with repressor molecules to relieve the repressed state of the cellulase gene. The negative slope suggests that binding of effector molecules interact with repressor molecules to cause repression.

Although sufficient data has not been accumulated for statistical regression analysis, the effect of cellobiose, glucose, cAMP, G-1-P, and sophorose on the rate of cellulase synthesis may be considered. At concentrations of cellobiose above 5 g/L, a straight line with a negative slope suggests that cellobiose molecules bind with repressor molecules to cause repression. The three data points available for glucose are seen to behave in a manner similar to those for cellobiose. Both sophorose and G-1-P in Solka Floc fermentations have positive slopes. Supplementation of cAMP at concentrations less than 50 mg/L gives a positive slope, but a negative slope was obtained with the greater levels of cAMP. The nature of these results may be a function of the convention used to calculate specific productivity, which considers the time frame to reach maximum enzyme levels. Hence, it appears that an effector may act both as inducer and repressor, or in other words, as a moderator of gene product expression.

Based on models from Gong and Tsao (6), and Stutzenberger (80), and the results derived for *A. cellulolyticus*, the tentative mechanism for the regulation of cellulase synthesis derived by Shiang (52) is shown in Figure 4. The new mechanism presumes that the operon consists of R, M, P, O, and S regions. The product of the gene R is an inactive repressor protein, and the M gene codes for CAM protein. P, O, and S represent promoter, operator, and cellulase structure genes, respectively. Both inactive repressor protein and CAM requires complex formation with repressor and inducer to form active repressor molecule and active inducer molecule, respectively. Therefore, active repressor molecule will bind the operator that blocks the transcription process, and the active inducer is able to bind the promoter to initiate transcription by RNA polymerase.

The induction of this operon responds to the intracellular concentration of cAMP, which is determined by the carbon source available to the cell. When cells are grown on cellobiose or cellulose that do not inhibit adenylate cyclase, cAMP is made in sufficient quantities for induction of cellulase. On the contrary, when cells are grown on glucose or other readily metabolized carbohydrates that do inhibit adenylate cyclase,

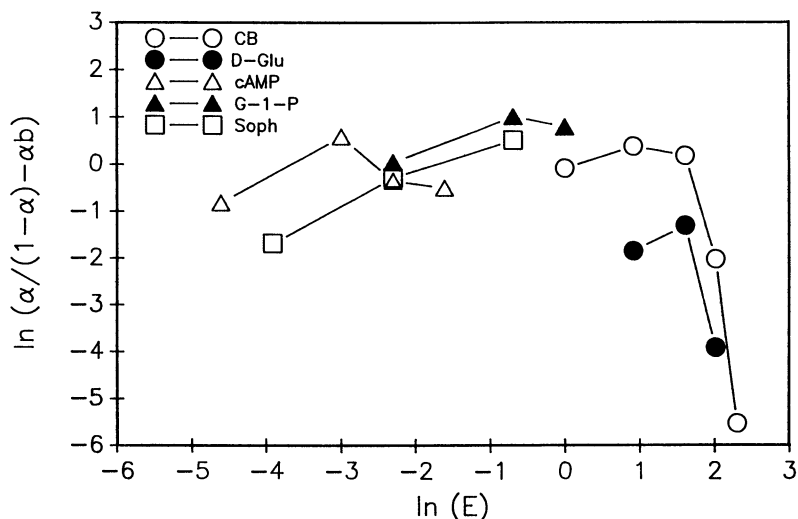


Figure 3. Relationship between concentrations of cellobiose, D-glucose, cAMP, glucose-1-phosphate and sophorose and rate of cellulase synthesis according to Yagil and Yagil (82).

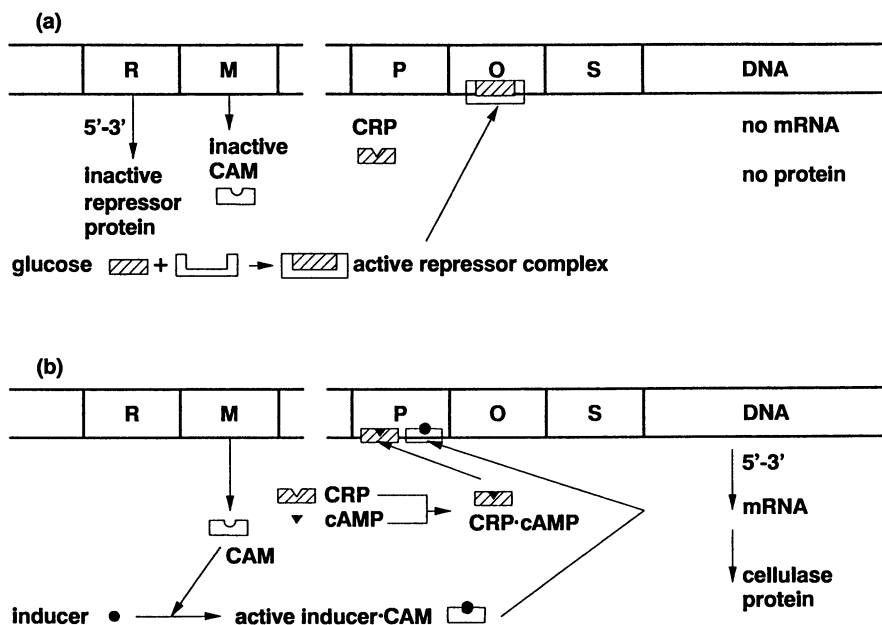


Figure 4. Proposed model for induced synthesis of cellulase enzyme (a) in the presence of repressor and (b) in the presence of inducer, cAMP and modulator.

very little cAMP is available to permit induction of cellulase. However, once the inhibitory carbon source is exhausted, that is when the cell is maximally derepressed for adenylate cyclase, the cell is capable of producing large amounts of cAMP. Thus, the possible mechanism of cellulase induction as described in Figure 4a is incomplete.

Figure 4b now accounts for the involvement of CRP•cAMP and active inducer•CAM complexes. When neither CAM nor CRP are in their complexed states, cellulase expression occurs at a very low basal level (constitutive). Using effectors from the first and second groups, cAMP levels increase to create CRP•cAMP complexes which bind to the promoter region, increase the affinity of RNA polymerase at P, and partially turn on the cellulase synthesis. In the presence of inducers, large amounts of CAM are produced analogous to the formation of protein C in the ara operon. Any of the types of effectors may then bind to CAM, and both active inducer•CAM and CRP•cAMP complexes bind the promoter cooperatively. Their simultaneous presence allows maximal transcription for the expression of the cellulase enzymes. In *A. cellulolyticus*, different levels of glucose, sucrose, cellobiose and other effectors appear to stimulate CAM production at various rates. In this manner, the initiation of and rate of cellulase synthesis makes each substrate system unique.

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Literature Cited

1. Ljungdahl, L.G.; Eriksson, K.-E. *Adv. Microbial. Ecol.* **1985**, 237-299.
2. Coughlan, M.P.; Ljungdahl, L.G. In *Biochemistry and Genetics of Cellulose Degradation*; Academic Press: New York, NY, 1988; pp 11-30.
3. Robson, L.M.; Chambliss, G.H. *Enzyme Microb. Technol.* **1989**, 11, 626.
4. Eriksson, K.-E. *Adv. Chem. Ser.* **1969**, 95, 83-104.
5. Wood, T.M. *Biochem. Soc. Trans.* **1985**, 13, 407-410.
6. Gong, C.-S.; Tsao, G.T. In *Cellulase biosynthesis and regulation*; Academic Press, Inc., New York, 1979; pp 111-140.
7. Ohmiya, K.; Shimizu, Kurachi, Y.; Shimizu, S. *J. Bacteriol.* **1985**, 161, 432-434
8. Ayers, W.A. *J. Bacteriol.* **1958**, 76, 515-517.
9. Park, W.S.; Ryu, D.D.Y. *J. Ferment. Technol.* **1983**, 61, 563-571.
10. Coughlan, M.P. *Biotechn. Gen. Engin. Rev.* **1985**, 3, 39-109.
11. Bartley, T.; Waldron, C.; Eveleigh, D. *Appl. Biochem. Biotechnol.* **1984**, 9, 337.
12. Chang, W.T.H.; Thayer, D.W. *Can. J. Microbiol.* **1977**, 23, 1285-1292.
13. MacKenzie, C.R.; Bilous, D.; Johnson, K.G. *Can. J. Microbiol.* **1984**, 30, 1171-1178.
14. Nakamura, K.; Kitamura, K. *J. Ferment. Technol.* **1983**, 61, 379-382.
15. Creuzet, N.; Berenger, J.-F.; Frixon, C. *FEMS Microbiol. Lett.* **1983**, 20, 347-350.
16. Saddler, J.N.; Khan, A.W. *Can. J. Microbiol.* **1980**, 26, 760-765.
17. Ohmiya, K.; Shimizu, M.; Taya, M.; Shimizu, S. *J. Bacteriol.* **1982**, 150, 407-409.
18. Lamed, R.; Setter, E.; Kenig, R. *Biotechnol. Bioeng. Symp.* **1983**, 13, 163-181.
19. Lamed, R.; Bayer, E.A. In *Biochemistry and Genetics of Cellulose Degradation*; Academic Press: New York, NY, 1988; pp 101-106.
20. Lamed, R.; Naimark, J.; Morgenstern, E.; Bayer, E.A. *J. Bacteriol.* **1987**, 169, 3792-3800.
21. Oberkotter, L.V.; Rosenberg, F.A. *Appl. Environ. Microbiol.* **1978**, 36, 205-209.

22. Priest, F.G. *Rev.* 1977, 41, 711-753.
23. Lee, B.H.; Blackburn, T.H. *Appl. Microbiol.* 1975, 30, 346-353.
24. Saddler, J.N.; Khan, A.W. *Can. J. Microbiol.* 1980, 26, 760-765.
25. Hagerdal, B.G.R.; Ferchak, J.D.; Pye, E.K. *Appl. Environ. Microbiol.* 1978, 36, 606-612.
26. Hagerdal, B.G.R.; Harris, H.; Pye, E.K. *Biotechnol. Bioeng.* 1979, 21, 345-355.
27. Yamane, K.; Yoshikawa, T.; Suzuki, H.; Nisizawa, K. *J. Biochem.* 1971, 69, 771-780.
28. Groleau, D.; Forsberg, C.W. *Can. J. Microbiol.* 1981, 27, 517-530.
29. Berg, B. *Can. J. Microbiol.* 1975, 21, 51-57.
30. Saddler, J.N.; Khan, A.W.; Martin, S.M. *Microbios.* 1980, 28, 97-106.
31. Shiang, M.; Linden, J.C.; Mohagheghi, A.; Rivard, C.J.; Grohmann, K.; Himmel, M.E. *Appl. Biochem. Biotechnol.* 1990, 24/25, 223-235
32. Stoppok, W.; Rapp, P.; Wagner, F. *Appl. Environ. Microbiol.* 1982, 44, 44-53.
33. Fennington, G.; Neubauer, D.; Stutzenberger, F. *Appl. Environ. Microbiol.* 1984, 47, 201-204.
34. Lin, E.; Wilson, D.B. *Appl. Environ. Microbiol.* 1987, 53, 1352-1357.
35. Moreira, A.R.; Phillips, J.A.; Humphrey, A.E. *Biotechnol. Bioeng.* 1981, 23, 1339-1347.
36. Saddler, J.N.; Khan, A.W. *Can. J. Microbiol.* 1979, 25, 1427-1432.
37. Murray, W.D.; Sowden, L.C.; Colvin, J.R. *Int. J. Syst. Bacteriol.* 1984, 34, 185-187.
38. Giuliano, C.; Khan, A.W. *Appl. Environ. Microbiol.* 1984, 48, 446-448.
39. Halliwell, G.J. *Gen. Microbiol.* 1963, 32, 441-448.
40. Cato, E.P.; Moore, W.E.C.; Bryant, M.P. *Int. J. Syst. Bacteriol.* 1978, 28, 491-495.
41. Huang, L.; Forsberg, C.W. *Appl. Environ. Microbiol.* 1987, 53, 1034-1041.
42. Duong, T.-V.C.; Johnson, E.A.; Demain, A.L. In *Topics in Enzyme and Fermentation Biotechnology*; A. Wiseman, Ed.; Ellis Horwood: Chichester, UK, 1983, Vol. 7; pp 156-195.
43. Gordon, J. Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, Massachusetts, 1981.
44. McBee, R.H. *J. Bacteriol.* 1954, 57, 505-506.
45. Ng, T.K.; Weimer, P.J.; Zeikus, J.G. *Arch. Microbiol.* 1977, 114, 1-7.
46. Johnson, E.A.; Bouchot, F.; Demain, A.L. *J. Gen. Microbiol.* 1985, 131, 2303-2308.
47. Ng, T.K.; Zeikus, J.G. *J. Bact.* 1982, 150, 1391-1399.
48. Sheth, K.; Alexander, J.K. *J. Biol. Chem.* 1969, 244, 457-464.
49. Pettipher, G.L.; Latham, M.J. *J. Gen. Microbiol.* 1979, 110, 21-27.
50. Fusee, M.C.; Leatherwood, J.M. *Can. J. Microbiol.* 1971, 18, 347-353.
51. Mohagheghi, A.; Grohmann, K.; Himmel, M.E.; Leighton, L.; Updegraff, D.M. *International J. System. Bacteriol.* 1986, 36, 435-443.
52. Shiang, M. Ph.D. Dissertation, Colorado State University, Fort Collins, Colorado, 1990.
53. Carpenter, A.S.; Barnett, L.B. *Arch. Biochem. Biophys.* 1967, 122, 1-7.
54. Storvick, W.O.; Cole, F.E.; King, K.W. *Biochem.* 1963, 2, 1106-1110.
55. Breuil, C.; Kushner, D.J. *Can. J. Microbiol.* 1976, 22, 1776-1781.
56. Sasaki, T.; Tanaka, T.; Nakagawa, S.; Kainuma, K. *Biochem. J.* 1983, 209, 803-807.
57. King, K.W.; Vessal, M.I. *Adv. Chem. Series* 1969, 95, 7-25.
58. Shafer, M.L.; King, K.W. *J. Bacteriol.* 1965, 113-116.
59. Stewart, B.J.; Leatherwood, J.M. *J. Bacteriol.* 1976, 128, 609-615.
60. Langsford, M.L.; Gilkes, N.R.; Wakarchuk, W.W.; Kilburn, D.G.; Miller, Jr., R.C.; Warren, R.A.J. *J. Gen. Microbiol.* 1984, 130, 1367-1376.
61. Vladut-Talor, M.; Kauri, T.; Kushner, D.J. *Arch. Microbiol.* 1986, 144, 191-195.
62. Kolankuya, N. *Hacettepe Bull. Nat. Sci. Eng.* 1980, 9, 1-10.
63. Hulme, M.A.; Stranks, D.W. *Nature* 1970, 226, 469-470.
64. Stutzenberger, F.J. *Appl. Microbiol.* 1971, 22, 147-152.
65. Stutzenberger, F.J. *Appl. Microbiol.* 1972, 24, 77-82.

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66. Fennington, G.; Neubauer, D.; Stutzenberger, F. *Biotechnol. Bioeng.* **1983**, *25*, 2271-2276.
67. Wood, W.E.; Neubauer, D.G.; Stutzenberger, F.J. *J. Bacteriol.* **1984**, *160*, 1047-1054.
68. Bernier, R.; Stutzenberger, F. *Appl. Environ. Microbiol.* **1987**, *53*, 1743-1747.
69. Alexander, J.K. *J. Biol. Chem.* **1968**, *243*, 2899-2904.
70. Schafer, M.L.; King, K.W. *J. Bacteriol.* **1965**, *89*, 113-116.
71. Schimz, K.-L.; Broll, B.; John, B. *Arch. Microbiol.* **1983**, *135*, 241-249.
72. Groleau, D.; Forsberg, C.W. *Can. J. Microbiol.* **1983**, *27*, 504-517.
73. Ryu, D.D.Y.; Mandels, M. *Enzyme Microb. Technol.* **1980**, *2*, 91-102.
74. Shoemaker, S.P.; Raymond, J.C.; Bruner, R. In *Trends in the Biology for Fermentations for Fuels and Chemicals*; Hollaender, A.; Rabson, R.; Rogers, P.; San Pietro, A.; Valentine, R.; Wolfe, R., Eds.; Plenum Press: New York, NY, 1981; pp 89-109.
75. Creese, E. M.E.Sc. Thesis, Univ. Western Ontario, London, Ontario, Canada, **1983**.
76. Skinner, W.A.; Tokuyama, F. *U.S. Patent* 4,081,328, **1978**.
77. Saddler, J.N.; Chan, M.K.H. *Euro. J. Appl. Microbiol. Biotechnol.* **1982**, *16*, 99.
78. Meyer, H.; Humphrey, A.E. *Adv. Microbiol. Ecol.* **1982**, *5*, 237-299.
79. Suzuki, H. In *Symposium on Enzymatic Hydrolysis of Cellulose*; Bailey, M.; Enari, T.M.; Linko, M., Eds.; SITRA: Helsinki, 1975; pp 115-169.
80. Stutzenberger, F. *Annual Reports of Ferment. Proc.* **1985**, *8*, 111-154.
81. Goksoyr, J.; Eriksen, J. In *Microbial Enzymes and Bioconversion*; Rose, A., Ed.; Academic Press: London, England, 1980, Vol. 5; pp 283-330.
82. Botsford, J.L. *Microbiol. Rev.* **1981**, *45*, 620-642.
83. Yagil, G.; Yagil, E. *Biophys. J.* **1971**, *11*, 11-21.

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Chapter 26

Cellulomonas fimi β -1,4-Glucanases

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The basic structural and functional organization of four β -1, 4-glucanases from the cellulolytic bacterium *Cellulomonas fimi* has been established. Endoglucanase A (CenA) and an exoglucanase (Cex) each contain two independent structural and functional domains: a catalytic domain and an amino- or carboxyl-terminal cellulose-binding domain (CBD). The CBDs show significant sequence identity and similar structures are evident in glycanases from other bacteria. Endoglucanases B and C (CenB and CenC, respectively) are large (>100 kDa) enzymes composed of multiple domains. A CBD is found at the carboxyl-terminus of CenB. Both enzymes contain units of unknown function comprised of repeated blocks of amino acids. Conspicuous linker sequences occur at the junctions of the domains in all four enzymes and resemble sequences seen in other bacterial glycanases. The CBDs of CenA and Cex have been fused to heterologous proteins by genetic manipulation. Examples are presented to illustrate the potential of such fusions for protein immobilization or purification.

Structural & Functional Organization of *Cellulomonas fimi* β -1, 4-Glucanases

It is now apparent that many bacterial cellulases are composed of two or more structural and functional units or domains and it has been suggested that such enzymes arose by a process of domain shuffling (1). The domains may be catalytic or non-catalytic and their occurrence is often indicated in the primary protein structure by an intervening

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sequence rich in proline and hydroxyamino acids. The catalytic domains have recently been classified into families on the basis of sequence similarities and hydrophobic cluster analysis (2,3). The non-catalytic domains comprise a more diverse group and include the cellulose-binding domains (CBDs) as well as elements of unknown function (3). Four β -1, 4-glucanases from *C. fimi* have been studied by us in some detail and their structural and functional organization illustrates many of these features, as summarized below.

Endoglucanase A (CenA) and Exoglucanase (Cex). Two β -1, 4-glucanases, CenA and Cex, are major extracellular enzymes produced when *C. fimi* is grown on microcrystalline cellulose. Both are glycoproteins which react with concanavalin A (4). Their apparent molecular masses, determined by SDS-polyacrylamide gel electrophoresis, are 53.0 and 49.3 kDa, respectively (5). The genes encoding CenA and Cex have been cloned and expressed in *Escherichia coli* (6) and their DNA sequences have been determined (7,8). The lower apparent molecular masses of the cloned gene products (48.7 and 47.3 kDa, respectively) reflect the absence of glycosyl substitution.

CenA hydrolyzes carboxymethylcellulose randomly; Cex shows a preference for the hydrolysis of terminal linkages (4). Both enzymes also hydrolyze and bind tightly to insoluble cellulosic substrates (4,5,9,10). The end-products of cellulose hydrolysis for both enzymes are predominantly cellobiose and cellotriose. The basic structural and functional organization of CenA and Cex has been established and is represented in Figure 1. The primary structure of mature CenA is divided into two regions by the sequence: (PT)₄ T(PT)₇, the Pro-Thr box. Digestion of *ng*CenA (the non-glycosylated form of the CenA synthesized in *Escherichia coli* from recombinant *C. fimi* DNA) by a *C. fimi* protease releases a stable 'core' peptide comprising the carboxyl-terminal region (amino acids 135 - 443). This peptide retains enzymatic activity towards several cellulosic substrates but no longer binds to cellulose. The binding function is contained in the corresponding amino-terminal fragment (amino acids 1 - 135). Therefore, CenA is a bifunctional protein comprising a carboxyl-terminal catalytic domain joined to an amino-terminal CBD by the Pro-Thr box. Cex is similarly organized but the arrangement of its domains is reversed (5).

Small-angle X-ray scattering analyses have shown CenA is tadpole-shaped (Figure 2): the catalytic domain forms the head region and the CBD and Pro-Thr box forms the extended tail region (11). The model contains an angle of 135° between the long axes of the head and tail regions. The overall dimensions of the molecule are comparable to the similarly shaped cellobiohydrolases I & II (CBH I and II) from *Trichoderma reesei* (12).

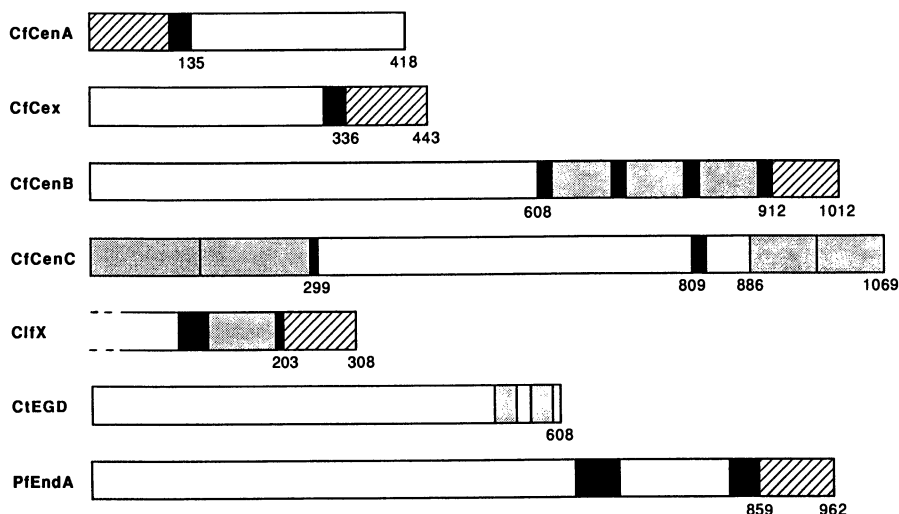


Figure 1. The organization of catalytic and non-catalytic domains in cellulases from *C. fimi* and other bacteria. CfCenA, B and C, and CfCex are the endo- and exo- β -1, 4-glucanases of *C. fimi*. ClfX is a translated open reading frame from *Cellulomonas flavigena* (29). CtEGD and PfEndA are endo- β -1, 4-glucanases from *Clostridium thermocellum* and *Pseudomonas fluorescens*, respectively (30,31). The primary structures are drawn approximately to scale and are numbered from the amino terminus of the mature protein; ClfX is numbered from the start of the open reading frame. Unshaded areas represent catalytic domains, cross-hatched areas indicate cellulose-binding domains, repeated blocks of amino acids are stippled, and black areas represent linker regions.

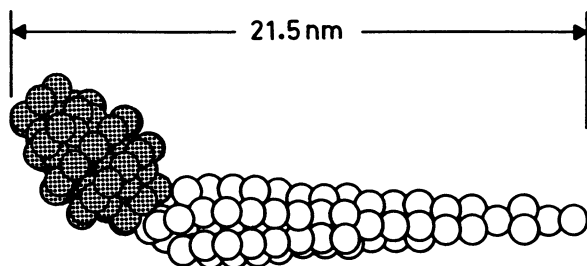


Figure 2. Model of CenA (non-glycosylated form) derived from small-angle X-ray scattering analyses. The structure of the catalytic domain (shaded region) was resolved by a separate scattering analysis of the isolated 'core' peptide (see text for details). Adapted from reference 11.

Catalytic Domains. The catalytic domain of CenA has been classified in family B (2); this family also contains *T. reesei* CBH II. CenA and CBH II both catalyse hydrolysis of β -1, 4-glucosidic bonds with inversion of anomeric carbon configuration (13,14). Inversion indicates a single displacement reaction mechanism. It is not yet known whether all members of family B are inverting enzymes or, more generally, whether all members of any given cellulase family share a common stereospecificity. Cex belongs to family F (2) and catalyses hydrolysis with retention of configuration (double displacement reaction) (13) but the stereospecificities of other members of family F have not yet been reported. The other members of family F are xylanases (2,3) and significant xylanase activity was also reported for Cex (4). Such relaxed substrate specificity is not unprecedented; several β -1, 4-glucanases with activity towards xylan, chitosan and other glycans have been described (e.g. refs. 15 and 16). For a cloned gene product, such results are unequivocal since contamination by other glycanases can be avoided.

Cellulose-binding Domains. The CBDs of CenA and Cex show significant (~ 50 %) sequence identity (1). Closely related sequences occur at the amino and carboxyl termini of several bacterial endo- β -1, 4-glucanases as well as at the amino terminus of a xylanase from *Pseudomonas fluorescens* (Figure 1). It is probable that each of these sequences represents a CBD, as recently demonstrated for endoglucanase B from *P. fluorescens* (17). Alignment of the CenA, Cex and other CBD sequences reveals that aromatic amino acids as well as Asn and Gly residues are strongly conserved (Figure 3). Moreover, two Cys residues, towards the amino and carboxyl ends, occur in all the CBD sequences except that from *Butyrivibrio*. The mechanism of binding is not yet known but a motif of four rather regularly spaced tryptophan residues (corresponding to CenA Trp¹⁴, Trp³⁴, Trp⁵⁰ and Trp⁶⁸, Figure 3) is strongly conserved in all the CBD sequences suggesting the involvement of these hydrophobic residues in the interaction with cellulose. It is noteworthy that certain tryptophan residues are also well conserved in the analogous starch-binding domains of amylases and related enzymes (18). Cellodextrins, as well as maltodextrins, inhibit the binding of an amylase starch-binding domain (Nikolov, Z.L., this volume); in contrast, there is presently no evidence for the interaction of CBDs with α -1, 4-glucose polymers. Proteolytic truncation of *ngCenA* showed that affinity for cellulose was retained by products missing up to 64 amino-proximal amino acids, although the strength of the interaction was lowered (10). At present, the functional significance of the binding to cellulose is not clear.

CBDs also occur at the amino or carboxyl termini of fungal cellulases (19) and the structure of the CBD for *T. reesei* CBH I has been resolved by 2-dimensional NMR (20). There is little sequence similarity between the bacterial and fungal CBDs although hydrophobic and

CfCenA	1	APGCRVDYAVTNOQWPGGFGANV-TITNLG-DPVSSWKLKDWYTAGQRIQQLWNG	52
CfCex	336	SGPAGCQVLGW-NQWNTGFTANV-TVKNTSSAPVDGWTLTFFSPSGQVTOQWSS	389
MbCela	353	QPPAGRACEATYALVNOQWPGGFAEV-TVKNTGSSPINGWTVQWTLFSGQSITQLWNG	409
ClfX	203	TGSCKVEYNASS-WNTGFTASV-RVNTGTTALNGWTLTFFPANGQTVQQGWSA	254
PfEndA	859	AASGGNQ--YVVTNOQWNGFTA-VIRVRNNGSSAINRWSVNSYSDGSRITNSWNA	912
PfEndB	30	AVCE--YRVTNEWGSFFTAS-IRITNNGSSITNGWSVSNWYTDGSRVTSWNA	78
PfXynA	1	QTATCS--YNIITNEWNTGYTGD-ITITNRGSSAINGWSVNWQYAT-NRLSSWNA	51
BfEnd	451	VSGALK-AEYTI-NNWGSYGQV-LIKVKNDASRVDGWTLKIKSEV-KIDSSWCY	502
Consensus		A C Y TNQWN GFTA V TV N GSS INGWTL W Y GQRITQSWNA	
TREGIII	161	GCLSLGAYCIVDIHNYARWNGGIIGQG-P-TNAQFTSL--WSQLASKYA-SQS-RVMFG	214
CfCenA	53	TASTNGQVSVTSLPWNGSIP TGGTASF GFNGS-WAGS-NPTPAFSLNGTTCGTVPT	109
CfCex	390	TVTQSGSAVTVRNAPWNGSIPAGGTAQGFNGS-HTGT-NAAPTAFSLNGTPTCTVG*	443
MbCela	410	DLSTSGNVTVRNVSWNGVNPAGGSTFGLGS-GTG--QL---SSSI---TCSAS*	456
ClfX	255	DWSQSGTTVTAKNAWNGSLAAGQTVDIGFNGA-HNET-NNKPAFTLNGATCTVG*	456
PfEndA	913	NVTGNN-PYAASALGWANIQPGQTAEFFGQTKGASRQV-PA-VT--GSVQQ*	308
PfEndB	79	GLSGAN-PYSATPVGWNTPSIPIGSSVEFGVQGN--GSSRAQVPAVT--GAIICGGG	131
PfXynA	52	NVSGSN-PYSASNLSWNGNIQPGQSVFQVKNKNGSAER-P-SV--GGSIICSGSVA	961
BfEnd	503	NIAEEGGYVITPMSWNSLSLEPSASVDFGIQGS---GS-IGT--SV--NISVQ*	547
Consensus		S SG V A WNGSIP G TV FGFQGS GS P SV NG C	
TREGIII	215	-IMNEPHDVNI-NT-WAATVQEVVTA-IRNAGATSQFISLPLGNDWQSAQAFISDG	266

Figure 3. Sequence alignments of the cellulose-binding domains and putative cellulose-binding domains of bacterial glucanases. CfCenA, B and C, and CfCex are the endo- and exo- β -1, 4-glucanases of *C. fimi*. ClfX is a translated open reading frame from *Cellulomonas flavigena* (29). MbCela is an endo- β -1, 4-glucanase from *Microbispora bispora* (32). PfEndA and B, and PfXynA are endo- β -1, 4-glucanases and a xylanase from *Pseudomonas fluorescens* (17,31,33). BfEnd is an endo- β -1, 4-glucanase from *Butyrivibrio fibrisolvens* (34). Also included is a related sequence from TrEG III, endoglucanase III of *Trichoderma reesei* (35). The consensus sequence shows amino acid residues common to four or more bacterial CBDs. The conserved Trp motif (see the text) and cysteine residues are shaded.

hydroxyamino acids show a similar pattern of distribution in both types (21). It is also of interest that *T. reesei* endoglucanase III (EG III, recently renamed EG II) contains a sequence (amino acids 161 - 266), outside of its CBD, with the conserved Trp motif of the bacterial CBDs (Figure 3); other consensus residues of the bacterial CBD are also represented. The significance of this sequence in EG III is not clear, but the amino acid cluster: -Asn-Glu-Pro- contained therein (amino acids 217 - 219) is common to some 16 bacterial and fungal cellulases and may be directly involved in catalysis (22).

Linker Regions. In both CenA and Cex a 20 - 23 amino acid sequence containing only proline and threonine residues (the Pro-Thr box) joins the CBD to the catalytic domain. Similar sequences, rich in proline and hydroxyamino acid residues, are evident in many of the other known cellulase primary structures (Table 1). Typically, these regions comprise 20 - 30 amino acids; exceptionally long sequences are found in some *P. fluorescens* glycanases. Analogous structures occur in unrelated enzymes e.g. the 2-oxo acid dehydrogenases of prokaryotes and eukaryotes (Table 1). They are believed to provide flexibility between, and critical spatial separation of, adjoining domains. In *ngCenA* and *ngCex* the amino and carboxyl junctions of the Pro-Thr box are particularly susceptible to proteolysis suggesting an exposed location. In the native, glycosylated forms of these enzymes, sugar substitution at nearby sites is assumed to provide a degree of protection (9).

Endoglucanase B (CenB) and Endoglucanase C (CenC). The gene encoding CenB was isolated from the original shotgun cloning of *C. fimi* genomic DNA (6). CenC was purified from *C. fimi* culture supernatant and its gene subsequently cloned using a DNA probe based on the determined amino terminus (23). Both enzymes are large (> 100 kDa) endo- β -1, 4-glucanases that bind to cellulose (23,24). The organization of their domains, deduced from recently determined primary structures, is shown in Figure 1.

Catalytic Domains. The catalytic domain of CenB (amino acids 1 - 608) shows 35% sequence identity with an endoglucanase from *Persea americana* (avocado); it has therefore been placed in family E, subfamily 2 (2,3). The CenC catalytic domain (amino acids 299-809) shows 28% sequence identity with *Clostridium thermocellum* endoglucanase D (EGD) and 43% identity with *P. fluorescens* endoglucanase A (End A), both members of family E, subfamily 1 (2,3).

Non-catalytic Domains. CenB contains a 100 amino acid carboxyl-terminal sequence with significant sequence identity to the other bacterial CBDs shown in Figure 1. Nevertheless, when this is

Table 1. Linker Sequences from Bacterial β -1, 4-Glucanases and other Proteins

Organism	Protein	Designation	Linker Sequence	Reference
<i>Cellulomonas fimi</i>	endoglucanase	CenA	PT ₂ S(PT) ₄ T(PT) ₇ VTPQPT	7
<i>Cellulomonas fimi</i>	exoglucanase	Cex	(PT) ₃ T(PT) ₃ T(PT) ₃ S	8
<i>Cellulomonas flavigena</i>		ClfX	PDPTDEPTEDPT(DDPT) ₅ EDPT	29
<i>Cellulomonas flavigena</i>		ClfA	PDPTD ₂ PTQDPTD ₂ PT	29
<i>Microbispora bispora</i>	endoglucanase	CelA	P ₂ TYSFSPFST(PS) ₃ QSDPGS(PS) ₃	32
<i>Caldocellum saccharolyticum</i>	bifunctional exo-endoglucanase	CelB	T ₂ S ₂ (PT) ₄ (VT) ₂ (PT) ₅ VTAT(PT) ₃ PVSTPAT	36
<i>Pseudomonas fluorescens</i>	endoglucanase	(i)	PAPTMTVAPTAT(PT) ₂ LSPTV(TP) ₂ APTQTAI(PT) ₂ LTPN(PT) ₂	31
		(ii)	S ₁₁ VPVS ₇ I ₂ PS ₆ IQPS ₆ MPS ₈ V ₂ AS ₅ VS	
<i>Clostridium thermocellum</i> <i>Bacillus</i> sp. strain N4	endoglucanase	CelE	S ₄ ASNINS ₁₂ AIVS ₅ V ₂ S ₆	37
	endoglucanase	CelB	PLVS(PT) ₃ LMPTFSPTVT	38
<i>Trichoderma reesei</i> Human Human	endoglucanase	EgIII	P ₂ SDFTP ₂ SDPDPGEPTP ₂ SDPGEYP	35
	immunoglobulin	IgA1	P ₂ SESPDP ₄ SEPE(PDPGE) ₃ PDPPT ₂ SDPEYP	
	2-oxo acid dehydrogenase complex	E2	PGAT ₂ IT ₂ STRP ₂ SGPT ₄ RA(1S) ₂ S ₂ TP ₂ TS ₂ PVPSTP ₂ TPSPSTP ₂ T PQVP ₃ TP ₃ VA ₂ VP ₂ TPQPLAPFAPSAPCPATPAGP	

removed by genetic deletion (24), the enzyme retains significant affinity for cellulose. This indicates the presence of an additional, amino-proximal CBD with a rather different primary structure to those so far identified. A triple repeat sequence occurs between the catalytic domain and the carboxyl-terminal CBD (Figure 1). Each repeated unit contains approximately 98 amino acids. These are separated from one another by linker sequences resembling the Pro-Thr boxes of CenA and Cex. It is interesting that the translated open reading frame ClfX from *Cellulomonas flavigena* contains a putative CBD at its carboxyl terminus (Figure 3) and that this is preceded by a single copy of a sequence with 50 % sequence identity to the CenB repeats (Figure 1).

The CenC catalytic domain is flanked at both its amino and carboxyl ends by double repeat sequences. Neither pair correspond to any other cellulase sequences reported to date. Double repeat structures occur in several endo- β -1, 4-glucanases from *C. thermocellum*, for example EGD (Figure 1), but these are much smaller than those of CenC. The functional significance of the repeated sequences is not yet clear in any of these examples. The conserved duplicated region of the *C. thermocellum* endoglucanases resembles the Ca^{++} -binding site of several Ca^{++} -binding proteins but deletion of this region from *C. thermocellum* endoglucanase D did not influence Ca^{++} -binding kinetics (25). Possible functions for the repeated regions of bacterial cellulases include their involvement in the protein-protein interaction required for enzyme complex formation. There is as yet no evidence for such complexes in *Cellulomonas* spp. but the association of several enzymes and other proteins into the cellulosome is now firmly established for the cellulolytic *Clostridia* (26).

Application of the *C. fimi* Cellulose-binding Domains

The construction of hybrid proteins containing bacterial CBDs may provide a cheap generic method for enzyme immobilization and/or purification using cellulosic matrices. The CBD can be fused at the amino or carboxyl terminus, as in the parent cellulase, to suit individual applications. We have constructed model fusion proteins using the *C. fimi* CBDs to demonstrate this potential.

TnphoA insertional mutagenesis was used to generate a series of CenA' PhoA fusions. Those polypeptides which contained the entire CBD_{CenA} were readily purified from *E. coli* periplasmic extracts by binding to filter paper (27). Similarly, CBD_{Cex} was fused to the carboxyl terminus of an *Agrobacterium* β -glucosidase (Abg), in this case by direct ligation of appropriate gene fragments (21,28). The Abg CBD_{Cex} hybrid could be purified from crude cell extracts by adsorption to a cellulose column. After extraneous proteins were washed off with phosphate-buffered 1 M NaCl, Abg CBD_{Cex} could be eluted with H₂O (28). Removal of the CBD from the hybrid would require further engineering: the introduction of a specific cleavage site at the fusion junction.

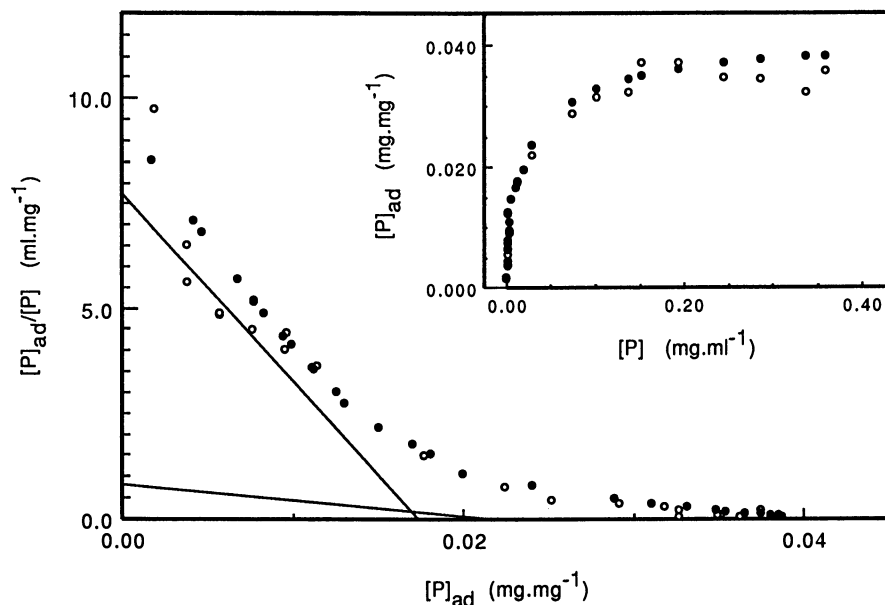


Figure 4. Adsorption of AbgCBD_{Cex} to microcrystalline cellulose. The main panel shows a Scatchard analysis of the experimental data (open circles). Data were resolved into two classes of binding interactions of relatively high and low affinity (straight lines). The respective adsorption parameters derived from these linear plots are given in the text. Closed circles show data obtained by substitution of these derived parameters into a Langmuir-type equation. The inset shows the same data plotted as an adsorption isotherm. (Reproduced with permission from reference 41. Copyright 1991 Butterworth Heinemann.)

The adsorption of Abg CBD_{Cex} to Avicel (microcrystalline cellulose) in 50 mM potassium phosphate, pH 7, at 4° is described by the adsorption isotherm shown in Figure 4 (inset). Abg itself has no significant affinity for Avicel. The adsorption data may be resolved by Scatchard analysis as a two-site model ($K_{d,1} = 1.5 \times 10^{-3}$ mg protein.ml⁻¹, $[P]_{ad, max,1} = 1.3 \times 10^{-2}$ mg protein.mg⁻¹ Avicel; $K_{d,2} = 5.2 \times 10^{-2}$ mg.ml⁻¹; $[P]_{ad, max,2} = 2.7 \times 10^{-2}$ mg.mg⁻¹) (Figure 4, main panel). Abg CBD_{Cex} shows quasi-irreversible adsorption to cellulosic substrates as illustrated in Figure 5. In this experiment identical columns containing chopped cotton fibers were loaded with approximately 1 mg Abg CBD_{Cex} per g cotton. The columns were continuously eluted at 37° or 50° with 50 mM potassium phosphate, pH7, containing *p*-nitrophenol- β -D-glucoside. Hydrolysis of the chromogenic substrate to *p*-nitrophenolate was determined from the absorption of the eluate at 400 nm. The fusion protein remained bound to the cotton matrix for at least 10 days during continuous operation at 37°. The enzyme was inactivated after 3 days at 50° (Figure 5) although there was no loss of protein from the column.

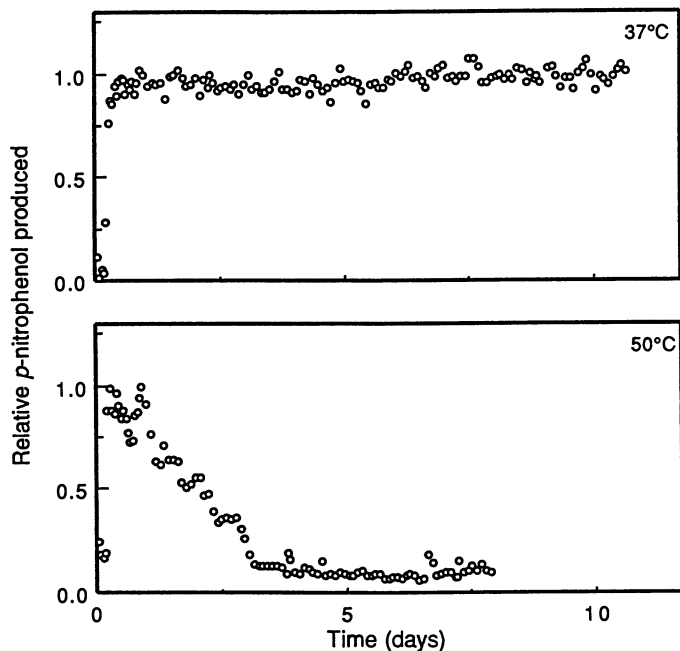


Figure 5. Performance of the fusion protein AbgCBD_{Cex} immobilized on a column containing chopped cotton fiber at 37° and 50°. (Reproduced with permission from reference 41. Copyright 1991 Butterworth Heinemann.)

Fusions to thermostable enzymes will allow us to evaluate adsorption at higher temperatures. When a column containing Abg-CBD_{Cex}, adsorbed to cellulose at pH 7.0, was eluted with an increasing or decreasing pH gradient (constant ionic strength), protein (enzymatically inactive) was eluted above pH 9, but there was no desorption evident at low pH. (Ong, E.; Gilkes, N.R.; Miller, R.C., Jr.; Warren, R.A.J.; Kilburn, D.G. *Enzyme Microb. Technol.*, in press.)

These results demonstrate several useful properties of the *C. fimi* CBDs for enzyme immobilization and purification. Presumably, the CBDs of other bacterial cellulases (Figure 3) can be used in similar ways. It remains to be seen whether these differ significantly in their affinity or capacity for adsorption; if so, certain CBDs may be preferable for specific purposes.

Acknowledgments. Our work on *C. fimi* cellulases has been generously supported by the Natural Sciences and Engineering Research Council of Canada.

Literature Cited

1. Warren, R.A.J.; Beck, C.F.; Gilkes, N.R.; Langsford, M.L.; Miller, R.C., Jr.; O'Neill, G.P.; Scheuffens, M.; Wong, W.K.R. *Proteins*. 1986, 1, 335-341.

2. Henrissat, B.M.; Claeysens, M.; Tomme, P.; Lemesle, L.; Mornon, J.-P. *Gene*. **1989**, *81*, 83-95.
3. Béguin, P. *Annu. Rev. Microbiol.* **1990**, *44*, 219-248.
4. Gilkes, N.R.; Langsford, M.L.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J. *J. Biol. Chem.* **1984**, *259*, 10455-10459.
5. Gilkes, N.R.; Warren, R.A.J.; Miller, R.C., Jr.; Kilburn, D.G. *J. Biol. Chem.* **1988**, *263*, 10401-10407.
6. Gilkes, N.R.; Kilburn, D.G.; Langsford, M.L.; Miller, R.C., Jr.; Wakarchuk, W.W.; Warren, R.A.J.; Whittle, D.J.; Wong, W.K.R. *J. Gen. Microbiol.* **1984**, *130*, 1377-1384.
7. Wong, W.K.R.; Gerhard, B.; Guo, Z.M.; Kilburn, D.G.; O'Neill, G.P.; Goh, S.H.; Warren, R.A.J.; Miller, R.C., Jr. *Gene*. **1986**, *44*, 315-324.
8. O'Neill, G.P.; Goh, S.H.; Warren, R.A.J.; Kilburn, D.G.; Miller, R.C., Jr. *Gene*. **1986**, *44*, 325-330.
9. Langsford, M.L.; Gilkes, N.R.; Singh, B.; Moser, B.; Miller, R.C. Jr.; Warren, R.A.J.; Kilburn, D.G. *FEBS Lett.* **1987**, *225*, 163-167.
10. Gilkes, N.R.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J. *J. Biol. Chem.* **1989**, *264*, 17802-17808.
11. Pilz, I.; Schwarz, E.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J.; Gilkes, N.R. *Biochem. J.* **1990**, *271*, 277-280.
12. Abuja, P.M.; Pilz, I.; Claeysens, M.; Tomme, P. *Biochem. Biophys. Res. Commun.* **1988**, *156*, 180-185.
13. Withers, S.G.; Dombroski, D.; Berven, L.A.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J.; Gilkes, N.R. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 487-494.
14. Knowles, T.K.C.; Lentovaara, P.; Murray, M.; Sinnott, M.L. *J. Chem. Soc. Chem. Commun.* **1988**, 1401-1402.
15. Shoemaker, S.; Watt, K.; Tsitovsky, G.; Cox, R. *Bio/technology* **1983**, *1*, 687-690.
16. Hedges, A.; Wolfe, R.S. *J. Bacteriol.* **1974**, *120*, 844-853.
17. Gilbert, H.J.; Hall, J.; Hazelwood, G.P.; Ferreira, L.M.A. *Molec. Microbiol.* **1990**, *4*, 759-767.
18. Svensson, B.; Jespersen, H.; Sierks, M.R.; Macgregor, E.A. *Biochem. J.* **1989**, *264*, 309-311.
19. Tomme, P.; van Tilbeurgh, H.; Pettersson, G.; Van Damme, J.; Vandekerckhove, J.; Knowles, J.; Teeri, T.; Claeysens, M. *Eur. J. Biochem.* **1988**, *170*, 575-581.
20. Kraulis, P.J.; Clore, G.M.; Nilges, M.; Jones, T.A.; Pettersson, G.; Knowles, J.; Gronenborn, A.M. *Biochemistry*. **1989**, *28*, 7241-7257.
21. Ong, E.; Greenwood, J.M.; Gilkes, N.R.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J. *Trends Biotechnol.* **1989**, *7*, 239-243.
22. Baird, S.D.; Hefford, M.A.; Johnson, D.A.; Sung, W.L.; Yaguchi, M.; Seligy, V.L. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 1035-1039.

23. Moser, B.; Gilkes, N.R.; Kilburn, D.G.; Warren, R.A.J.; Miller, R.C., Jr. *Appl. Environ. Microbiol.* **1989**, *55*, 2480-2487.
24. Owolabi, J.B.; Béguin, P.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J. *Appl. Environ. Microbiol.* **1988**, *54*, 518-523.
25. Chavaux, S.; Béguin, P.; Aubert, J.-P.; Bhat, K.M.; Gow, L.A.; Wood, T.M.; Bairoch, A. *Biochem. J.* **1990**, *265*, 261-265.
26. Bayer, E.A.; Kenig, R.; Lamed, R. *J. Bacteriol.* **1983**, *156*, 818-827.
27. Greenwood, J.M.; Gilkes, N.R.; Kilburn, D.G.; Miller, R.C., Jr.; Warren, R.A.J. *FEBS Lett.* **1989**, *244*, 127-131.
28. Ong, E.; Gilkes, N.R.; Warren, R.A.J.; Miller, R.C., Jr.; Kilburn, D.G., *Bio/technology* . **1989**, *7*, 604-607.
29. Al-Tawheed, A.R. *M.Sc. Thesis*, University of Dublin, **1988**.
30. Joliff, G.; Béguin, P.; Aubert, J.-P. *Nucleic Acids Res.* **1986**, *14*, 8605-8613.
31. Hall, J.; Gilbert, H.J. *Molec. Gen. Genet.* **1988**, *213*, 112-117.
32. Yablonsky, M.D.; Elliston, K.O.; Eveleigh, D.E. In *Enzyme Systems for Lignocellulose Degradation*; Coughlan, M.P., Ed.; Elsevier Applied Science: New York, **1989**; pp. 73-83.
33. Hall, J.; Hazelwood, G.P.; Huskisson, N.S.; Durrant, A.J.; Gilbert, H.J. *Molec. Microbiol.* **1989**, *3*, 1211-1219.
34. Berger, E.; Jones, W.A.; Jones, D.T.; Woods, D.R. *Molec. Gen. Genet.* **1989**, *219*, 193-198.
35. Saloheimo, M.; Lehtovaara, P.; Penttila, M.; Teeri, T.T.; Stahlberg, J.; Johansson, G.; Pettersson, G.; Claeysens, M.; Tomme, P.; Knowles, J.C. *Gene.* **1988**, *63*, 11-21.
36. Saul, D.J.; Williams, L.C.; Love, D.R.; Chamley, L.W.; Bergquist, P.L. *Nucleic Acids Res.* **1989**, *17*, 439.
37. Hall, J.; Hazelwood, P.J.; Barker, P.J.; Gilbert, H.J. *Gene.* **1988**, *69*, 29-39.
38. Fukumori, F.; Sashihara, N.; Kudo, T.; Horikoshi, K. *J. Bacteriol.* **1986**, *168*, 479-485.
39. Liu, Y.S.V.; Low, T.L.K.; Infante, A.; Putnam, F.W. *Science.* **1976**, *193*, 1017-1019.
40. Perham, R.N.; Packman, L.C. *Ann. N.Y. Acad. Sci.* **1989**, *573*, 1-20.
41. Ong, E.; Gilkes, N. R.; Miller, R. C., Jr.; Warren, R. A. J.; Kilburn, D. E. *Enzyme Microb. Technol.* **1991**, *13*, in press.

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Chapter 27

Comparison of Amylopullulanase to α -Amylase and Pullulanase

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An important step in enzymatic starch conversion technology is the use of thermostable liquefying α -amylase that works at 95-105°C. Various α -amylases (liquefying and saccharifying) from a variety of sources have different physicochemical properties and product patterns. Pullulanase, a starch debranching enzyme, is used to improve the efficiency of starch saccharification to glucose with glucoamylase or to maltose with β -amylase, and also for making various soluble branched cyclodextrins. Amylopullulanase is a new class of pullulanase that cleaves α -1,4 linkages of starch and produces mainly DP2-DP4 products. This enzyme from one source is stable and active at 90°C. The unique biochemical characteristics, mode of action and utility of these enzymes in starch conversion technology are described. Future directions of research include the development of low pH optimum and non-calcium requiring α -amylase, highly thermostable and environmentally compatible pullulanase, and new starch processing technology using amylopullulanase.

α -Amylase

Amylolytic enzymes are an important group of industrial enzymes. There are three major classes of amylolytic enzymes - endo-amylase (α -amylase), exo-amylase (β -amylase or glucoamylase) and debranching enzymes (pullulanase and isoamylase). α -Amylase hydrolyzes internal α -1,4 linkages of starch in an endo-fashion and produces a variety of oligosaccharides. It cannot act on α -1,6 linkages but can easily bypass them. So its prolonged action on starch produces α -limit dextrans (branched). There are two types of α -amylase - liquefying and saccharifying. The liquefying and saccharifying α -amylases differ in their mechanism of starch degradation. The saccharifying α -amylase generally produces an increase in reducing power over that

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of the liquefying enzyme. The α -amylase has been found in a variety of sources. Various α -amylases have different physicochemical properties and product patterns. Various sugar syrups are produced from starch mainly in a two-step process - liquefaction and saccharification. First, an aqueous slurry of starch (30-40% DS), is gelatinized (105°C, 5 min) and partially hydrolyzed at 95°C for 2 h by highly thermostable α -amylase to DE 5-10. The enzymatic starch liquefaction process was made possible by the use of highly thermostable α -amylase from *Bacillus licheniformis*. The optimum pH for the reaction is 6.0-6.5 and calcium (generally 50 ppm) is usually needed. Then the liquefied starch slurry is cooled down to 50-60°C and pH is adjusted to 4.0-5.5 depending on saccharolytic enzymes used for saccharification. The saccharification reaction takes a long time (48-72 h) to complete. The starch conversion processes and the use of α -amylase in it is shown in Figure 1.

Pullulanase

Pullulanase is an enzyme whose primary specificity is to hydrolyze α -1,6 linkages of pullulan. It is also a starch debranching enzyme which cleaves α -1,6 linkages of starch and produces linear dextrans. It is used with glucoamylase to yield greater than 95-96% glucose and with β -amylase to yield around 80-85% maltose. Glucoamylase is a saccharifying amylase that can hydrolyze both α -1,4 and α -1,6 linkages of starch but its action on α -1,6 linkages is relatively slow. β -Amylase, another saccharifying amylase, can neither hydrolyze α -1,6 linkages of starch nor bypass it and its prolonged action on starch produces β -limit dextrans. The efficiency of a saccharification reaction with glucoamylase or β -amylase can be improved by using a starch debranching enzyme that hydrolyzes the α -1,6 linkages giving linear dextrans of various chain lengths. The use of pullulanase in starch saccharification processes is shown in Figure 2. The advantages of using pullulanases in starch saccharification processes can be summarized as follows: (1) increases the glucose yield (about 2%) with glucoamylase; (2) increases the maltose yield (about 20-25%) with β -amylase; (3) reduces the saccharification time (to 48 h); (4) allows an increase in substrate concentration (to 40%, DS); and (5) allows a reduction in the use of glucoamylase (up to ~ 50%).

Amylopullulanase

Pullulan is a linear glucan of about 480 maltotriosyl units linked through α -1,6 glucosidic linkages. The α -1,6 linkages are considered to partially mimic the α -1,6 branch points of amylopectin, and pullulan has been widely employed as a model substrate for starch debranching enzymes (1). The degradation of pullulan can proceed by cleavage of α -1,6 linkages or any of the two α -1,4 linkages. The enzymes that hydrolyze pullulan can now be classified into five types. Table I summarizes the action of various pullulan degrading enzymes on pullulan and starch. Amylopullulanase is a new class of enzyme that hydrolyzes α -1,6 linkages of pullulan like normal pullulanase but unlike pullulanase, which cleaves only α -1,6 linkages of starch, this enzyme cleaves α -1,4 linkages of starch (3).

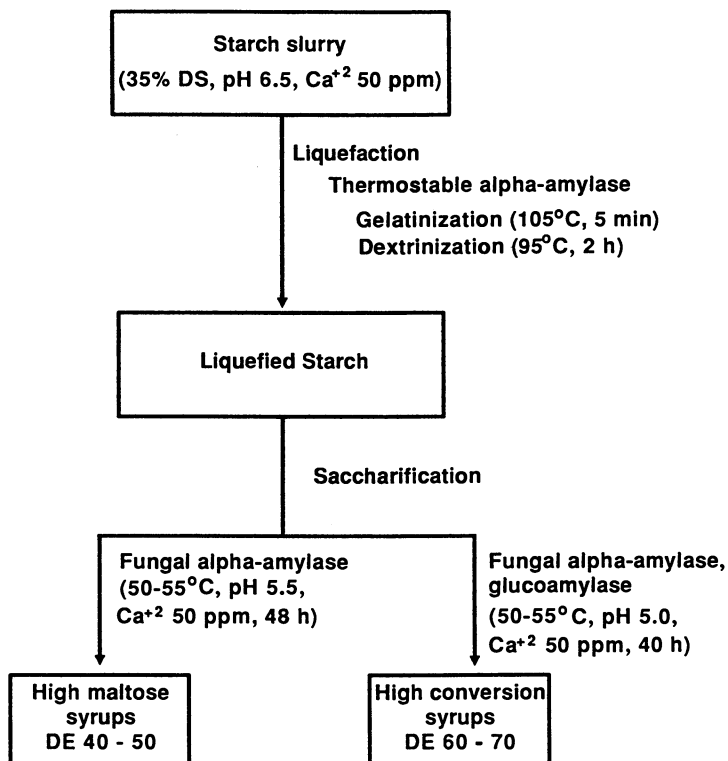


Figure 1. Starch bioprocessing using α -amylase.

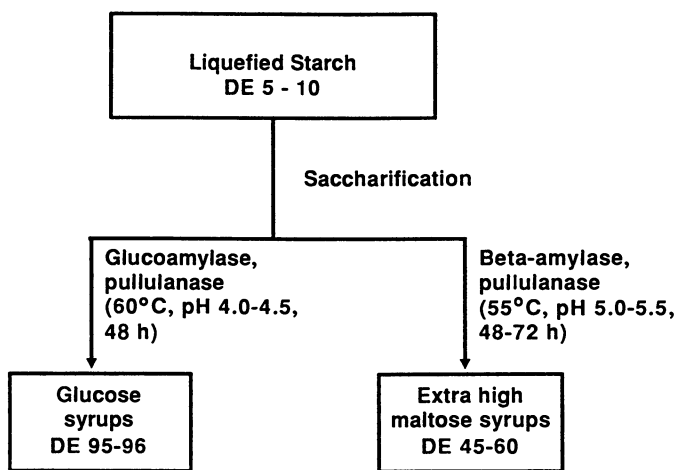


Figure 2. Use of pullulanase in starch saccharification processes.

Table I. Action of Pullulan Degrading Enzymes on Pullulan and Starch

Enzyme	Linkage Cleavage		Major End Product	
	Pullulan	Starch	Pullulan	Starch
Pullulanase (2)	α -1,6	α -1,6	Maltotriose	Linear dextrans
Amylopullulanase (3)	α -1,6	α -1,4	Maltotriose	DP2-DP4
Isopullulanase (4)	α -1,4	α -1,4	Isopanose	Not known
Neopullulanase (5, 6)	α -1,4	α -1,4	Panose	Maltose
Glucoamylase (7, 8)	α -1,4 and α -1,6	α -1,4 and α -1,6	Glucose	Glucose

Amylopullulanase from *Clostridium thermohydrosulfuricum* Strain 39E. Our group initiated a screening program for thermostable amylases from thermophiles. *Clostridium thermohydrosulfuricum* strain 39E (ATCC 33223) was isolated from Octopus Spring in Yellowstone National Park. The organism (an extreme thermoanaerobe) grows at 65°C and produces a cell-bound amylopullulanase activity (9). The synthesis of amylopullulanase in this organism was inducible and subject to catabolic repression (10). Catabolic repression resistant mutants were isolated which displayed improved starch metabolism features in terms of enhanced rates of growth, ethanol production and starch consumption. In chemostat cultures, both wild type and mutant strains produced amylopullulanase at high levels in starch limited chemostats but not in glucose or xylose limited chemostats. The enzyme was excreted into the medium when grown in continuous culture under substrate (maltose) limited condition (11).

The cell-bound amylopullulanase was solubilized with detergent and lipase. It was then purified to homogeneity by treatment with streptomycin sulfate and ammonium sulfate, and by DEAE-Sephacel, octyl-Sepharose and pullulan-Sepharose column chromatography (12). The final enzyme solution was purified 3511-fold over the crude enzyme extract with an overall recovery of 42% and had a specific activity of 481 units/mg protein. The average molecular weight of the enzyme was 136,500 determined by gel filtration on Sephacryl S-200 and SDS-PAGE, and it had an isoelectric point at pH 5.9. It was rich in acidic and hydrophobic amino acids. The purified enzyme was quite thermostable in the absence of substrate even up to 90°C with essentially no loss of activity in 30 min. However, the enzyme lost about 40% of its original activity at 95°C tested for 30 min. The optimum temperature for the action of the purified enzyme on pullulan was 90°C. However, the enzyme activity rapidly decreased on incubation at 95°C to only 38% of the maximal after 30 min. The enzyme was stable at pH 3.0-5.0 and was optimally active at pH 5.5. It produced only maltotriose and no panose or isopanose from pullulan.

In comparison with a pullulanase, the purified amylopullulanase displayed 3-fold higher specificity towards soluble starch and amylopectin and 7-fold higher specificity towards glycogen (12). The amylopullulanase hydrolyzed various high molecular weight starch substrates (Table II). Even the mammalian glycogen which

is hard for a pullulanase to attack was easily hydrolyzed by the enzyme. The final reaction products were DP2-DP4. This indicates that the pure amylopullulanase has unique mode of action and displays both " α -amylase" and "pullulanase" like activity. The action of amylopullulanase on low molecular weight oligosaccharides (maltotriose to maltoheptaose) was tested (Table III). The enzyme had no activity on maltotriose. Maltotetraose was a very poor substrate for the enzyme and maltose was not detected as its hydrolysis product. The other saccharides were hydrolyzed to maltotriose and residual sugar (i.e., glucose and maltose). Thus the minimum substrate requirement for its catalytic activity was a maltotriosyl unit. These results reflect an important deviation from pullulanase as no activity would have been shown towards any of these α -1,4 linked oligosaccharides by a pullulanase. The K_m (mg/ml) for pullulan (average MW 50,000) and low MW amylose (MW 4,100) were found to be 0.35 and 1.0, respectively. The apparent K_{cat} (min^{-1}) for pullulan was 16,000. Kinetic experiments on competitive inhibition with mixed substrates (pullulan and amylose) demonstrates that the single enzyme possesses an active site for cleavage of both α -1,6 and α -1,4-linked substrates (Figure 3).

Table II. Reaction Products of Amylopullulanase from *Clostridium thermohydrosulfuricum* Strain 39E on High MW Polysaccharides^a

Substrate	End Products					
	G_1	G_2	G_3	G_4	G_6	G_9
Pullulan	--	--	100	--	--	--
Amylose	--	37	47	16	--	--
Amylopectin	--	36	36	28	--	--
Soluble starch	--	39	39	22	--	--
Mammalian glycogen	--	22	47	31	--	--
Oyster glycogen	--	17	50	33	--	--

^aSolutions of 1% (w/v) pullulan, amylose, amylopectin, mammalian glycogen, oyster glycogen and soluble starch (pH 6.0) were incubated at 60°C with purified enzyme (0.05 U/ml). Samples were withdrawn after 216 hrs and heated at 100°C for 15 min for enzyme inactivation. The reaction products were analyzed by HPLC for sugars. (12). (Reprinted with permission from Ref. 13. Copyright 1990 Academic Press, Inc.)

The amylopullulanase from *C. thermohydrosulfuricum* strain 39E has been cloned and expressed in *Escherichia coli* (11).

Amylopullulanase from *Thermoanaerobacter* Strain B6A. Saha et al. (14) characterized an extracellular endo-acting amylopullulanase from *Thermoanaerobacter* strain B6A which was also isolated from a geothermal site in the USA. The enzyme was produced at high levels on a variety of carbon sources. It was purified to homo-

generity by DEAE-Sepharose CL-6B, gel filtration using HPLC and pullulan-Sepharose affinity column chromatography. It was a 450,000 MW protein with two equivalent subunits. The enzyme cleaved α -1,6 linkages of pullulan and produced multiple saccharides from cleavage of α -1,4 linkages in amylose. The amylopullulanase was stable up to 70°C (without substrate, 30 min incubation) and at pH 4.5-5.0. The optimum temperature and pH for the action of the enzyme were 75°C and 5.0, respectively. The K_m for pullulan and boiled soluble starch were 0.43 and 0.37 mg/ml, respectively. The final amylose hydrolyzate contained DP2 (44%), DP3 (33%), DP4 (17%) and higher saccharides (6%). The enzyme did not show any metal ion activation and the activity was inhibited by β - and γ -cyclodextrins but not by α -cyclodextrin.

Table III. Reaction Products of Amylopullulanase from *Clostridium thermohydrosulfuricum* Strain 39E on Low MW Oligosaccharides^a

Substrate	End Products					
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
Maltotriose	--	--	++	--	--	--
Maltotetraose ^b	++	--	++	++	--	--
Maltopentaose	--	++	++	--	--	--
Maltohexaose	--	--	++	--	--	--
Maltoheptaose	++	--	++	--	--	--

Major products observed are shown by the positive sign.

^aSolutions of 1% (w/v) maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were incubated at 60°C with purified enzyme (0.05 U/ml). Products were analyzed after 72 hours by HPTLC (Whatman HP-K). Plates were developed with n-BuOH:EtOH:H₂O (3:3:2, v/v) at 25°C and the products detected with a mixture of 0.2% (w/v) orcinol in MeOH and 20% H₂SO₄ in MeOH (1:1, v/v).

^bMaltotetraose was a very poor substrate for the enzyme and the products were observed only after long-term reaction. (Reprinted with permission from Ref. 13. Copyright 1990 Academic Press, Inc.)

Amylopullulanase from *Bacillus* Strain 3183. The extracellular amylopullulanase from the thermophilic *Bacillus* strain 3183 (ATCC 49341) was also purified by ammonium sulfate treatment, DEAE-Sepharose CL-6B, S-Sepharose, and octyl-Sepharose column chromatography and finally by preparative disc gel electrophoresis, and characterized (15). The purified enzyme also cleaved α -1,6 linkages of pullulan making only maltotriose, but it cleaved starch in α -1,4 linkages producing various saccharides (DP2 and higher). Unlike the two other amylopullulanase preparations, this enzyme activity was stimulated and stabilized by Ca⁺² (16).

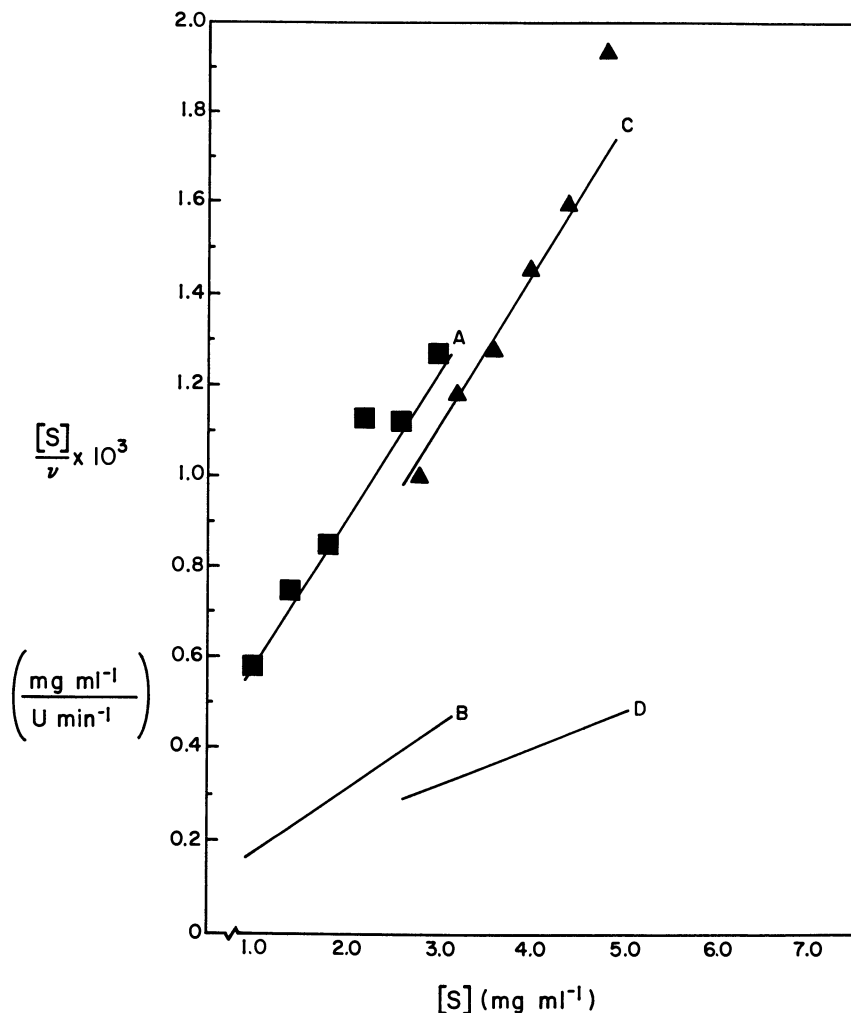


Figure 3. Kinetics of competitive inhibition of *Clostridium thermohydrosulfuricum* strain 39E purified amylopullulanase activity with mixed substrates. The solid lines A and C indicate the theoretical plots for competitive inhibition at amylose concentrations of 0.6 and 2.4 mg/ml, respectively. Lines B and D are the theoretical plots for the absence of inhibition at the same respective amylose concentrations. Pullulan was used at concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 mg/ml. For clarity, only two sets of data points were used in the above plot. (■) and (▲) are the practical data points obtained at 0.6 and 2.4 mg/ml amylose concentrations. All reaction mixtures contained 5% (v/v) dimethyl sulfoxide for solubility of amylose. $[S] = [A] + [P]$, where S is the total substrate concentration. A and P are the concentrations of amylose and pullulan, respectively. (Reproduced with permission from Ref. 13. Copyright 1990 Academic Press, Inc.)

A summary of some biochemical characteristics of amylopullulanase from the three sources is given in Table IV.

Table IV. Biochemical Characteristics of Amylopullulanase from Thermophiles

<i>Property</i>	<i>Clostridium thermohydro-sulfuricum Strain 39E (12, 13)</i>	<i>Thermo-anaerobacter Strain B6A (14)</i>	<i>Bacillus Strain 3183 (15, 16)</i>
Mol. weight (x 10 ⁴)	136.5	450	-
Optimum pH	5.0-5.5	5.0	6.0
pH stability	3.5-5.0	4.5-5.0	5.5-7.0
Optimum temp.	90°C	75°C	75°C
Thermostability	up to 90°C	up to 70°C	up to 70°C
Isoelectric point	pH 5.9	pH 4.5	-
Product			
from pullulan	maltotriose	maltotriose	maltotriose
from starch	DP2-DP4	DP2-DP4	DP2-DP4
Activator	-	-	calcium
Inhibitor			
Cyclodextrin (β - and γ -)	+	+	+

Amylopullulanase Type Activity from Other Sources. Amylopullulanase type activity has been demonstrated from *Thermoanaerobium* Tok 6-B1 (17, 18), *T. brockii* (19), *C. thermohydrosulfuricum* strain E101-69 (20, 21), *C. thermosulfurogenes* (22, 23), *Thermus* sp. AMD33 (24). The enzyme from *T. brockii* was cloned into *E. coli* and *B. subtilis* (19). The cloned enzyme could hydrolyze all of the α -1,6 glucosidic linkages in pullulan but it hydrolyzed α -1,4 and very few α -1,6 linkages in starch. The products of starch hydrolysis were various sized maltodextrins. The *Thermoanaerobium* strain Tok6-B1 enzyme hydrolyzed starch, amylopectin and amylose to yield predominantly maltose and maltotriose. Maltotetraose was slowly hydrolyzed to maltose (17). The enzyme action on maltooligosaccharides, containing seven or fewer glucose residues, occurred at α -1,4 linkages, two glucose residues away from a terminal glucose residue to give maltose as a product (18). It was also suggested that the hydrolysis of α -1,6 linkages of pullulan and α -1,4 linkages of amylose occurred at a common EDAC- [1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide] sensitive site. Melasniemi (20) reported that *C. thermohydrosulfuricum* strain E101-69 produced pullulanase and α -amylase, and suggested that the single protein had dual specificity and called it α -amylase-pullulanase. Later the enzyme was purified as two forms (I and II) from culture medium, by using gel filtration in 6 M-guanidine hydrochloride as a final step (21). Renatured α -amylase-pullulanase I and II had apparent MW of 370,000 \pm 85,000 and 330,000 \pm 85,000, respectively. Each

form appeared to be dimer of two similar subunits. They had similar amino acid composition, the same N-terminal amino acid sequence (Glu-Ile-Thr-Ala-Pro-Ala-Ile) and the same isoelectric point at pH 4.25. Both forms were glycoproteins having rhamnose, glucose, galactose and mannose, and neutral hexose content of 11-12%. The α -amylase-pullulanase gene from *C. thermohydrosulfuricum* strain E101-69 was cloned and expressed in *E. coli* (24). The cloned active α -amylase-pullulanase was separated into three peaks by gel filtration, each having α -amylase-pullulanase activity. More than 10 α -amylase-pullulanase specific polypeptides were revealed by immunoblotting after SDS-PAGE. The biggest active polypeptide had a MW of 165,000 and the smallest one had about 100,000 MW. The thermal stability of the enzyme was found to be the same as that of the extracellular enzyme produced by the host. However, the optimum temperature (80-85°C) of the cloned enzyme was 5°C lower than the native enzyme from the host. Thermostable *Thermus* sp. AMD33 amylopullulanase type enzyme (I and II) were purified to homogeneity (25). The MW and isoelectric points were determined as 135,000 (I and II) and 4.2 (I) and 4.3 (II), respectively. Takasaki (26) reported a pullulanase-amylase complex enzyme (MW 450,000) from *B. subtilis* that hydrolyzed α -1,6 linkages in pullulan and α -1,4 linkages in starch. Sata et al. (27) purified amylase-pullulanase enzyme produced by *B. circulans* F-2. The enzyme produced a series of maltooligosaccharides from amylose whereas it produced only maltotriose from pullulan. It was suggested that the enzyme possessed two active sites to hydrolyze α -1,4 and α -1,6 linkages with the same rate.

Potential Usefulness of Amylopullulanase in Biotechnology

The *Bacillus* strain 3183 amylopullulanase may not be useful as a true pullulanase (debranching enzyme) for use in the production of > 95% glucose syrups from liquefied starch because in combination with glucoamylase it did not increase the glucose yield or decrease the reaction time (14). The amylopullulanase from the two other organisms (*C. thermohydrosulfuricum* strain 39E and *Thermoanaerobacter* strain B6A) also did neither increase the glucose yield nor decrease the reaction time when used with glucoamylase (unpublished result). The amylopullulanase behaves like saccharifying α -amylase in their action pattern on starch. The amylopullulanase from *C. thermohydrosulfuricum* strain 39E is highly stable and active at 90°C and pH 5.0-5.5. It does not need calcium for activity. This enzyme may have potential for use directly, both in starch liquefaction (with or without α -amylase) and in saccharification processes (3). It may be useful for single step starch conversion into various maltodextrins (28). The amylopullulanase from the three sources may also be useful for making specialty corn syrups from liquefied starch (13-15).

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Literature cited

1. Plant, A. R.; Morgan, H. W.; Daniel, R. M. *Enzyme Microb. Technol.* **1986**, *26*, 668-672.
2. Bender, H.; Walenfels, K. *Biochem. Z.* **1961**, *334*, 79-95.
3. Saha, B. C.; Zeikus, J. G. *Trends Biotechnol.* **1989**, *7*, 234-239.
4. Sakano, Y.; Matsuda, N.; Kobayashi, T. *Agric. Biol. Chem.* **1971**, *35*, 971-973.
5. Kuriki, T.; Okada, S.; Imanaka, T. *J. Bacteriol.* **1988**, *170*, 1554-1559.
6. Shimizu, M.; Kanno, M.; Tamura, M.; Suekane, M. *Agric. Biol. Chem.* **1978**, *42*, 1681-1688.
7. Marshall, J. J. *Starch/Starke* **1975**, *27*, 377-383.
8. Saha, B. C.; Mitsue, T.; Ueda, S. *Starch/Starke* **1979**, *31*, 307-314.
9. Hyun, H. H.; Zeikus, J. G. *Appl. Environ. Microbiol.* **1985**, *49*, 1168-1173.
10. Hyun, H. H.; Zeikus, J. G. *J. Bacteriol.* **1985**, *164*, 1146-1152.
11. Mathupala, S. P.; Saha, B. C.; Zeikus, J. G. *Abstract of ASM Annual Meeting, Anaheim, CA, May, 1990*
12. Saha, B. C.; Mathupala, S. P.; Zeikus, J. G. *Biochem. J.* **1988**, *252*, 343-348.
13. Mathupala, S.; Saha, B. C.; Zeikus, J. G. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 126-132.
14. Saha, B. C.; Lamed, R.; Lee, Y.-Y.; Mathupala, S. P.; Zeikus, J. G. *Appl. Environ. Microbiol.* **1990**, *56*, 881-886.
15. Saha, B. C.; Shen, G.-J.; Srivastava, K. C.; LeCureux, L. W.; Zeikus, J. G. *Enzyme Microb. Technol.* **1989**, *11*, 760-764.
16. Shen, G.-J.; Srivastava, K. C.; Saha, B. C.; Zeikus, J. G. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 340-344.
17. Plant, A. R.; Clemens, R. M.; Daniel, R. M.; Morgan, H. W. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 427-433.
18. Plant, A. R.; Clemens, R. M.; Morgan, H. W.; Daniel, R. M. *Biochem. J.* **1987**, *246*, 537-541.
19. Coleman, R. D.; Yang, S.-S.; McAlister, M. P. *J. Bacteriol.* **1987**, *169*, 4302-4307.
20. Melasniemi, H. *Biochem. J.* **1987**, *246*, 193-197.
21. Melasniemi, H. *Biochem. J.* **1988**, *250*, 813-818.
22. Madi, E.; Antranikian, G.; Ohmiya, K.; Gottschalk, G. *Appl. Environ. Microbiol.* **1987**, *53*, 1161-1167.
23. Madi, E.; Antranikian, G. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 422-425.
24. Nakamura, N.; Sashihara, N.; Nagayama, H.; Horikoshi, K. *Starch/Starke* **1989**, *41*, 112-117.
25. Melasniemi, H.; Paloheimo, M. *J. Gen. Microbiol.* **1989**, *135*, 1755-1762.
26. Takasaki, Y. *Agric. Biol. Chem.* **1987**, *51*, 9-16.
27. Sata, H.; Umeda, M.; Kim, C.-H.; Taniguchi, H.; and Maruyama, Y. *Biochem. Biophys. Acta* **1989**, *991*, 388-394.
28. Saha, B. C.; Zeikus, J. G. *Enzyme Microb. Technol.* **1990**, *12*, 229-231.

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Chapter 28

Cyclodextrin Glucanotransferases Technology and Biocatalyst Design

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One of the challenges in the field of biotechnology is to learn how to design the catalytic activity of glucan biocatalysts to improve their stability, catalytic activity and product specificity. This overview presents recent developments in the area of cyclodextrin glucanotransferases (CGTase) and their product cyclodextrins (CD). A review of current CD/CGTase technology is presented, with extension to relate its importance on elucidating general glucosylase biocatalyst structural and functional relationships. A brief discussion of current research in genetic mutation of this class of biocatalysts to elucidate structural/functional features is also included.

Cyclodextrins (CDs) were first observed by Villiers (1) nearly a century ago. First thought to be a mysterious crystalline form of starch, the structure and chemistry of these unique cyclic glucan oligosaccharides have been elucidated through the contributions of Schardinger, Freudenburg, Cramer and French (2).

In the past decade, CDs have seen great industrial and commercial interest due their ability to form complexes with a variety of molecules (3), act as organic enzyme mimics (4) and provide enantiomeric chromatographic separations (5). In the last 5 years, CD manufacturing plants have been constructed in West Germany, Hungary, France, the U. S. and Japan. Current consumption of CDs in 1989 is estimated at 850 tons. Estimated consumption for 1995 is 5600 tons (Source: Consortium fur Electrochemische Industrie GmbH, Zielstattstrasse 20, 8000 Munich, West Germany 70). This growth is anticipated to dramatically increase as these materials continue to gain approval for use in pharmaceuticals, foods and fine chemicals.

As the second century of cyclodextrin research begins, attention is being focused on the biochemistry of CDs and their biocatalysts, cyclodextrin glucanotransferases (CGTases). Recent advances in protein structure and

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genetics have provided the tools to probe and alter the biocatalytic structure of CGTases and begin to understand their relationships to other amylolytic glycosylases. Current efforts in modification and alteration of CGTase structure have begun to yield critical information needed to "engineer" biocatalyst activity. These results, combined with information about carbohydrate conformation and structure, will eventually lead to the "engineering" of new glycosylase biocatalysts with significant industrial importance for the food, chemical, environmental and energy industries.

Properties of Cyclodextrins

Cyclodextrins (CDs) are cyclic molecules consisting of six, seven or eight glucose residues, designated α , β , and γ , respectively, linked by α -1,4 bonds (Figure 1). CDs are torus-shaped, with hydrophobic interiors and hydrophilic exteriors, due to the orientation of the glucose hydroxyl groups. This feature enables the cyclodextrin to form inclusion complexes with apolar compounds (6). CD cavity size and solubility are presented in Table I. Cyclodextrins can also be chemically modified to be immobilized, form polymers or add specific ligands (7) (8).

Table I. Properties of Cyclodextrins

CD type	α	β	γ
No. of glucoses	6	7	8
Cavity size (angstroms)	5.7	7.8	9.5
Solubility (gms/100 ml of water at 25 °C)	14.5	1.8	23.2

The primary advantage of CD complexation is to stabilize and protect sensitive host molecules, such as flavors, odors, or pharmaceuticals. CDs sharply reduce the volatility, chemical, thermal and photo reactivity of guest molecules. More recently, CDs have been used for separation of components in solution. For example, CDs can remove reactive components from fruit juices to prevent oxidation or eliminate bitterness. Attachment of CDs to chromatographic supports provides chiral separation, selective component removal and modified chemical reactivity. A number of modified and polymerized CD materials have gained acceptance as separation media (9).

Chemically modified-CDs have also proven useful as organic enzyme mimics (10). Modified CDs have catalyzed the de-esterification, hydrolysis, selective substitution, addition and isomerization of a variety of molecules (9). Advances in the chemical synthesis of CDs (11) and unusual "manno" CDs (12) promise novel new CD-like materials in the future.

A multitude of papers have been published describing the functionality of natural and modified CDs in complexing materials. Several reviews and publications provide current information on these complexes and their uses (13-15). Contemporary advances in cyclodextrin chemistry and new applications are available from continuing annual symposia (16).

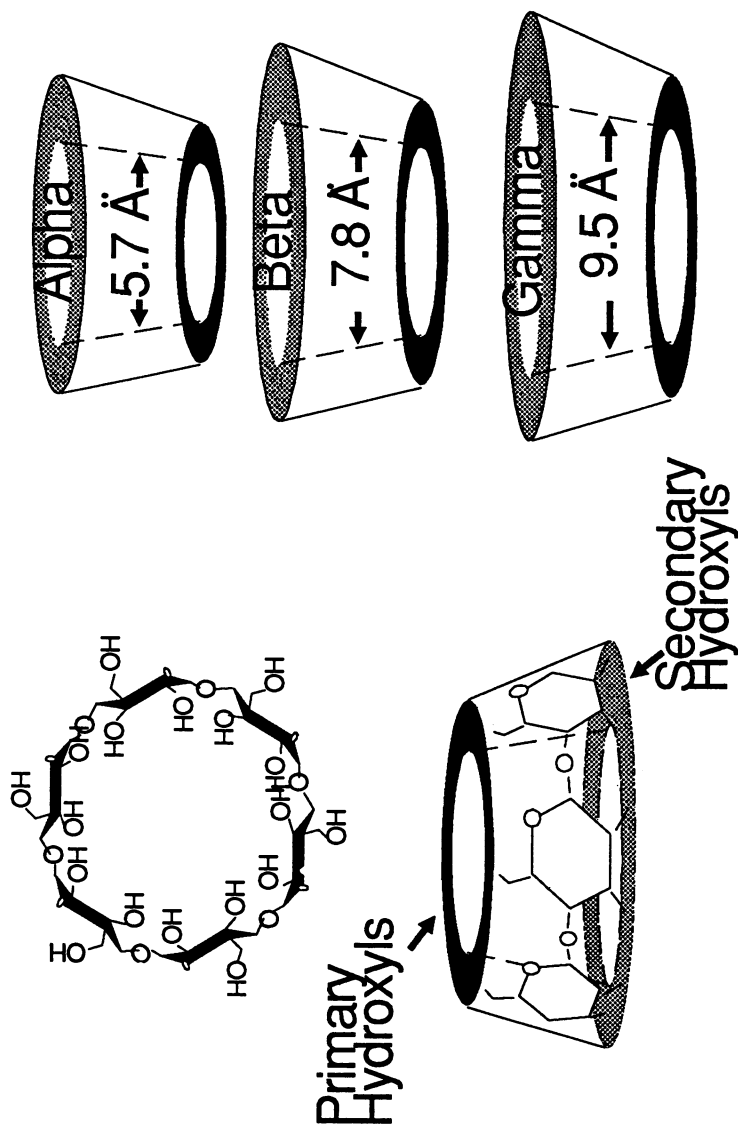


Figure 1. Structure of Cyclodextrins

Production of CDs

The production of CDs via enzymatic reaction with starch has been recently reviewed (17). CGTase is an extracellular protein and is usually isolated as a crude mixture from the medium. This crude protein is used directly for industrial fermentations. The basic process involves standard enzymatic fermentation, with careful attention to reaction temperature. All three CDs and some linear oligosaccharides are normally produced. Yields are highly dependent on the source of starch substrate. Potato starch is normally used or an extract of potato starch is often added to other starches (18-19). The potato starch component(s) responsible for stimulating CD formation have not been determined. Low starch concentrations (5%-10%) are normally used industrially. Published yields are in the 50% - 80% conversion range.

The distribution of α -, β - and γ -CDs is highly dependent on the origin of biocatalyst used (See section on Cyclodextrin Glucanotransferases). Product distributions may be altered by the addition of specific precipitants, such as aromatics and long chain alcohols (9, 20). Depending on molecular size, these precipitants preferentially complex with specific CD species and are removed from solution.

Since a mixture of CDs and linear oligosaccharides result from the enzymatic fermentation with starch, purification is needed to obtain specific CDs. Addition of aromatic organic solvents, such as trichloroethylene or toluene, quantitatively precipitate CDs, making them easy to separate from the remaining starch hydrolysate via centrifugation or filtration. The solvents are normally removed by steam distillation following separation. A non-solvent alternative separation process employs specific alkanophilic *Bacillus sp.* at high pH (17). Following reaction, the mixture is concentrated and a small amount of CD is added to seed crystallization of the CDs. CD yields from this process are roughly half that of the solvent processes.

Several variations on these production methods also exist, such as the use of immobilized CGTase (21, 22), continuous ultrafiltration (23) and the use of isoamylase to increase CD yield. Variations of purification methods include the addition of glucoamylase to degrade non-CD starch hydrolysates to simply separation and the use of various synthetic ion exchange resins in chromatographic separations (24-26) and affinity columns (27).

With the tremendous utility and functionality of CDs, these novel biological materials will continue to see dramatic growth in applications over the next decade. Despite recent advances in the chemical synthesis of CDs (11), the predominant industrial means of CD production will remain enzymatic. Basic improvements of the CGTase biocatalyst to increase the efficiency of CD synthesis and decrease costs is therefore a high priority.

Cyclodextrin Glucanotransferases

Amylolytic glucosylases are probably the most widely studied of the carbohydrate enzymes, due to their biological and industrial importance, as well as their ubiquitous distribution. Many different types of amyloglucosylases exist, with a variety of physical, chemical and catalytic properties. Recent reviews (28-30) describe the classification, characterization, action patterns and biochemistry of different enzymes.

Cyclodextrin glucanotransferase (CGTase) [EC 2.4.1.19] is one of the more unusual members of the amylyolytic glucosylase family. Whereas most

glucosylases predominantly catalyze a single reaction (either hydrolytic or synthetic), CGTase possesses both strong hydrolytic and synthetic capabilities, as well as having multiple product specificity. CGTases produce cyclodextrins with 6, 7 and 8 glucosyl residues and a variety of linear maltooligosaccharides, via disproportionation and coupling reactions (31). While this wealth of catalytic capability provides a unique opportunity to study glycosylase mechanisms, it also is a tremendous industrial problem, incurring high costs due to low CD yields and product separation.

Bacterial organisms are the only known sources of CGTases. CGTases from several microbiological species have been isolated, however the predominant genus is *Bacillus*. CGTases are extracellular enzymes of approximately 70,000 molecular weight. Most require calcium and have pH optima in the slightly acidic range, similar to α -amylases. More recently, several alkanophilic CGTases have been discovered with pH optima of 9-10. Temperature optima range from 50°C to 60°C.

Most CGTases catalyze the formation of all 3 types of CDs, with specific equilibrium distributions. *B. macerans* (32), *B. stearothermophilus* (33) and *Klebsiella pneumoniae* (34) are categorized as α -CGTases, producing primarily α -CD. Several alkanophilic *Bacillus* sp. (35,36), *B. obhensis* (37), *B. megaterium* (38), *B. circulans* (39) and *Micrococcus* sp. (40) are classified as predominantly β -CD-producers. The only CGTases classified as γ -CD-producers are *Bacillus subtilis* 313 (41) and alkanophilic *Bacillus* 290-3 (20). Through the use of various precipitants, the product distribution of CDs can be altered (20, 42, 43)

CGTases have been reported from approximately 13 organisms. The genes coding for several CGTases have been isolated and cloned in *E. coli* (See Table II.). The use of genetic expression promoters has been reported to increase cloned CGTase protein expression several thousand-fold (67).

Two references of X-ray crystallographic data for CGTases have been published (47, 48). Only one of these presents a structural model, for *Bacillus circulans* CGTase(48). This structure is divided into five subdomains, labeled A-E (See Figure 2). The N-terminal domain forms a $(\beta\alpha)_8$ TIM barrel structure similar to triose phosphate isomerase (49). This structure is a connected set of alternating, parallel beta sheets and alpha helices organized in a barrel-like structure with the beta sheet on the interior (See Figure 3). Domain B is a region with beta sheet structure following the third β -strand. Domain C consists of a greek key β -sheet structure. Domains D and E are antiparallel β -sheets, with the former having an immunoglobulin topology (50).

Significant primary sequence homology (> 70%) exists among the *Bacillus* sp. CGTases (51). Interestingly, *K. pneumoniae* CGTase only shows about 30% overall homology despite similar molecular size. However, predicted secondary protein structure among CGTases from different origins indicates a high degree of structural homology, despite differences in primary sequence (51).

Interestingly, primary sequence comparisons of CGTases to various α -amylases indicate moderate sequence homology, roughly 15% and 25%. Specific regions of high sequence homology have been identified as important catalytic sites and calcium binding sites for the α -amylases. However, α -amylases primarily only catalyze the hydrolysis of glucans,

Table II. Cloned CGTases

Organism	Reference
<i>Bacillus</i> 290-3	20
<i>Bacillus macerans</i>	32, 33
<i>B. stearothermophilus</i>	33
<i>Klebsiella pneumoniae</i>	34
<i>Bacillus</i> 38-2	35
<i>Bacillus</i> 1011	36
<i>B. subtilis</i> No. 313	41
<i>Bacillus circulans</i>	44
<i>Bacillus</i> 17-1	45
<i>Bacillus</i> 1-1	46

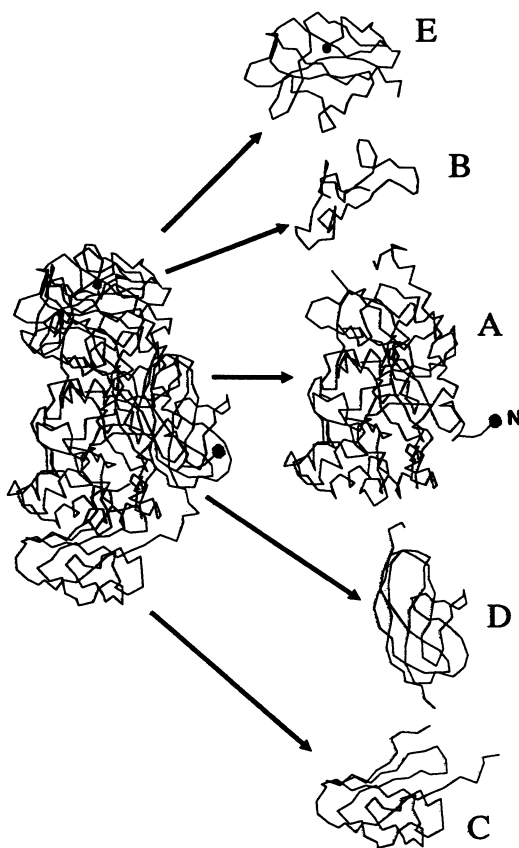


Figure 2. X-ray Crystallographic Structure of *B. circulans* CGTase
(Adapted and reproduced from Ref. 48 with permission. Copyright 1989 J. Mol. Biol., Academic Press)

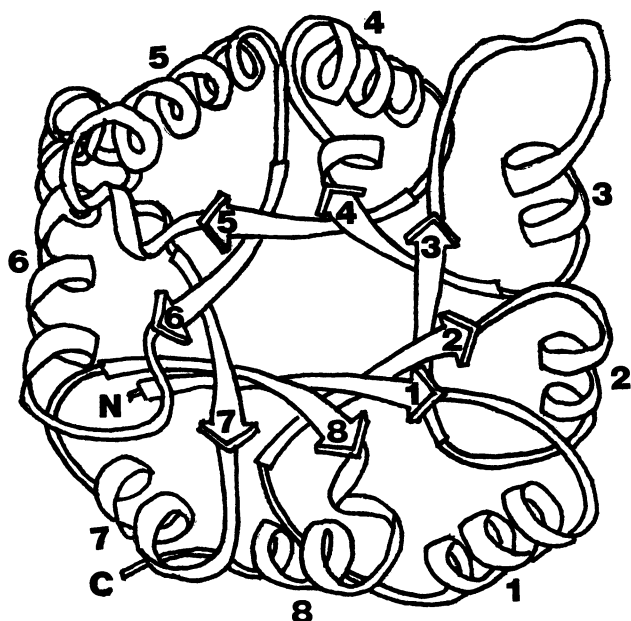


Figure 3. Model of $(\beta\backslash\alpha)_8$ TIM barrel from triose phosphate isomerase. Numbered arrow and twisted ribbon structures are beta sheets and alpha helices, respectively. (Adapted and reproduced from Ref. 66 with permission. Copyright 1984 American Association for the Advancement of Science.)

whereas CGTases also catalyze the cyclization and ligation of oligosaccharides. Despite this apparently limited primary sequence homology, predicted secondary structure similarity is very high (51). This has presented scientists with a unique opportunity to dissect the functional/structural relationships for this family of biocatalysts by direct comparison of structural and functional characteristics of different, naturally-occurring enzymes.

Similarities between CGTase and α -Amylase.

Amylases are ubiquitous in living systems and are important for the utilization of carbohydrates in many industrial processes (brewing, baking, sweeteners, alcohol fuels, etc.). α -Amylases are generally calcium-dependent proteins of approximately 50,000 molecular weight (29). They catalyze the hydrolysis of starch, glycogen and other α -1,4-glucans to form maltooligosaccharides. Despite the functional similarity among α -amylases from various mammalian and bacterial, primary sequence homology among the α -amylases is quite low, on the order of about 5% (52). It is noteworthy that this homology is all concentrated in 3 short regions for all these enzymes. More recently, predicted secondary structures for several amylases based on primary sequence data have been found to be similar (53).

Recently 3-dimensional structures for porcine pancreatic α -amylase (54) and Taka- α -amylase (55) have been published. These structures contain three subdomains, A-C. The amino terminal domain (A) is a TIM barrel structure (49). In between the 3rd β -strand and the 3rd α -helix of the barrel there is a large antiparallel β -sheet structure (domain B). The C domain is a set of 8 antiparallel β -sheets in a greek key topology (56). Significantly, this is the same basic structure as observed for CGTases.

The 3 short regions of high primary sequence homology were all found to be located in either the catalytic site or the calcium binding site. Therefore, it has been hypothesized that these conserved regions are required for the catalytic actions of these glucosylases. Physically, these three regions are located on beta loop turns between corresponding alpha helices and beta sheets of the TIM barrel (54).

Structural analyses from X-ray models and predicted super-secondary models imply that CGTase structure is primarily a super-set of α -amylase structure. The active sites and calcium binding sites of α -amylase are believed to reside in the ($\beta \backslash \alpha$)₈ barrel and β -strand loops of domains A and B. The various β -strand loops of the ($\beta \backslash \alpha$)₈ barrel are also purported to be involved in starch binding. The antiparallel β -sheet (domain C) is hypothesized to possess starch binding capability.

To this amylase structure, CGTase adds 2 other subdomains (D & E), which are antiparallel β -sheets at the C-terminal end of the protein. The structure of domain D is similar to immunoglobulin topology. The functional role of these domains is not known. However, proximity of domain E (an antiparallel β -sheet structure) to the proposed catalytic site, suggests a role in substrate binding and conformation.

Comparison of amylolytic glucosylases

While the literature is rich in scientific information on glucosylases, recent interest has focused on the hypothesis that all these enzymes share a common catalytic mechanism, despite differences in their product specificity (57). Indeed, it has been proposed that all glucosylases share the same basic chemical mechanism (58). The α -amylases have been the focus of much of this attention, as the primary protein sequence (59), tertiary protein structure (54,55) and catalytic mechanism (57) have been recently delineated.

Recently, much attention has been devoted to analyzing the structural and functional relationships of these biocatalysts. Despite limited primary sequence homology among the various enzymes (60-62), predicted secondary structure (51) and limited X-ray crystallographic structures (48, 54, 55) indicate that all these proteins are basically variations of the same structure ((β \backslash α) β TIM barrel, See Figure 3). This suggests similar mechanisms, albeit with differences in product specificity and substrate binding. An X-ray model of xylose isomerase (63) and predicted secondary structure of α -glucosidase (51) also indicate the same (β \backslash α) β barrel structure. It has been speculated that a similar extension might be made to other glucosylases such as amylosucrase and dextransucrase (64).

Genetic Mutations of Amyloglucosylases

Based on primary sequence and X-ray crystallographic structures of amylases and CGTases, it is believed that the overall tertiary structure of amyloglucosylases is conserved. The basic α -amylase TIM barrel construction with β -strand loops between the helices and β -sheets of the (β \backslash α) β barrel structure and large antiparallel β -sheet domain appear to be universal elements of these proteins. The conservation of primary sequence regions involved in catalysis and calcium binding throughout these proteins implies a common mechanism. However, distinct variations in β -strand loop structures are believed to be responsible for differences in product specificity of the different α -amylases (62). The additional domains present in CGTases, along with variations in the β -strand loop structures, are hypothesized to be responsible for the cyclization phenomena of CGTases and the ability to couple glucosyl residues.

Current research is exploring the effects of altering these active sites and binding structures to "engineer" new catalytic specificity and modify biocatalyst behavior. Site-directed mutagenesis on alpha amylase (Vihinen, M.; Ollikka, P.; Niskanen, J.; Meyer, P.; Suominen, I.; Kearp, M.; Holm, L.; Knowled, J.; Mantsala, P., unpublished data) have been used to identify catalytic amino acid residues. Similar work on glucoamylase (Sierks, M.; Svensson, B.; Ford, C.; Reilly, P. Personal communication) has been performed, with current work focusing on changing the catalytic action of this enzyme. Schmid (65) noted that deletion of part of the E subdomain of *K. pneumoniae* CGTase reduced the activity of its CGTase, but did not alter its product specificity. In our laboratory we have recently cloned CGTase genes from several *Bacillus sp.* and expressed native CGTase in *E. coli* and yeast. Using the polymerase chain reaction method for genetic mutation, we are creating site-directed mutants of the β -strand loops in domain B of these CGTases to investigate the role of specific residues in the catalytic

properties of this enzyme. We are also creating regional deletion of subdomains D and E to determine if the resulting protein retains conventional hydrolytic capability. It is our hope that we will eventually be able to unravel the synthetic and hydrolytic structural-functional relationships of CGTases and begin to apply these principles to build more effective glucan biocatalysts.

Conclusions

One of the powerful driving forces behind biotechnology is the promise of tailor-made enzymes and proteins. Such materials would have "engineered" properties, such as enhanced stability, altered substrate specificity and novel catalytic capabilities. While this dream of "manufacturing" new biocatalysts has yet to be realized, significant progress has been achieved towards developing the fundamental structural-functional relationships required to achieve this goal. One promising family of enzymes which are being studied in this regard are the glucosylases, comprised of the amylases, glucosidases, sucrases and glucosyltransferases.

Studies of observed and predicted structure point to the conclusion that despite significant differences (> 70%) in primary sequence, α -glucosylases form a family of biocatalysts with very similar protein tertiary and secondary structure. Small variations in specific areas of protein structure account for very different product specificity, due to changes in substrate binding. The presence of additional domains, in conjunction with some variation in sequence homology, can produce dramatic product configurational changes, as witnessed by CGTase-catalyzed cyclization and multiple product specificity.

By direct comparison of structural and functional characteristics of different, naturally-occurring enzymes and modified biocatalysts, researchers hope to delineate the structural features controlling the hydrolytic and synthetic catalytic activities of this family of glucosylases. This understanding will eventually lead to the ability to "engineer" glucan biocatalyst capability.

These engineered biocatalysts will in turn provide new means to efficiently utilize natural biopolymers to synthesize chemical intermediates, fuels, food products, pharmaceuticals and plastics. New synthetic biocatalysts will allow the creation of a host of new biopolymers possessing new functions and properties for industrial, pharmaceutical and agricultural uses.

Acknowledgments

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Literature Cited

1. Villiers, A. *Compt. Rend. Acad. Sci.* **1891**, *112*, 536.
2. French, D. *Adv. Carbohydr. Chem.* **1957**, *12*, 189.
3. Szejtli, J. In *Cyclodextrin Technology*; Davies, J. E., Ed.; Kluwer Academic Publishers: Dordrecht, 1988; Chapter 3-6.
4. Cramer, F. In *First Proc. Intl. Symp. Cyclodextrins*; Szejtli, J., Ed.; Reidel: Dordrecht, 1982 p 3.
5. Armstrong, D. W. *Anal. Chem.* **1987**, *59*, 84A.

6. Cramer, F. In *Cyclodextrins and their Industrial Uses*; Duchene, D., Ed.; Editions de Sante: Paris, 1987 p 11.
7. Szejtli, J. *Tibtech* **1989**, *7*, 170.
8. Bender, M. L.; Komiyama, M. In *Cyclodextrin Chemistry*; Springer-Verlag: New York, **1978**; p 2.
9. Szejtli, J. In *Cyclodextrin Technology*; Davies, J. E., Ed.; Kluwer Academic Publishers: Dordrecht, 1988; Chapter 6.
10. Krechl, J.; Castulik, P. *Chemical Scripta*; **1989**, *29*, 173.
11. Takahashi, Y.; Ogawa, T. *Carbohydr. Res.*; **1987**, *164*, 277.
12. Mori, M.; Ito, Y.; Ogawa, T. *Tetrahedron Lett.*; **1989**, *30*, 1273.
13. Vaution, C.; Hutin, M.; Glomot, F.; DuChene, D., In *Cyclodextrins and Their Industrial Uses*; DuChene, D., Ed.; Editions de Sante: Paris, 1987; Chapter 8.
14. Special Edition on Cyclodextrins, *Carbohydr. Res.* **1989**, *192*.
15. *Cyclodextrin News*; Szejtli, J.; Pagington, J., Eds.; FDS Publications: P.O. Box 41 Trowbridge, Wiltshire England.
16. *Proc. of the 5th Intl. Symposia on Cyclodextrins*; March 28-30, 1990, Paris, Kluwer Academic Publishers: Dordrecht, 1990.
17. Sicard, P. J.; Saniez, M.-H. In *Cyclodextrins and Their Industrial Uses*; DuChene, D., Ed.; Editions de Sante: Paris, 1987; Chapter 2.
18. Matsutani Kagaku Kogyo Japanese Patent 9224 **1971**.
19. Nihon Shokuhin Kako; Rikagaku Kenkyusho Japanese Patent 92288 **1974**.
20. Schmid, G.; Huber, O.; Eberle, H.-J. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988 p 87.
21. Kato, T.; Horikoshi, K. *Biotech. Bioeng.* **1984**, *26*, 595.
22. Crump, S. P.; Rozzell, J. D. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988; p 47.
23. Hokse, H. U.S. Patent 4477568 **1984**.
24. Hokse, H. J. *Chromatogr.* **1980**, *189*, 98.
25. Rikagaku Kenkyusho, Nihon Shokuhin Kako European Patent Spec. 45464 **1985**.
26. Nihon Shokuhin Kako, Rikagaku Kenkyusho U. S. Patent 4418144 **1983**.
27. Mattsson, P.; Makela, M.; Korpela, T. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988; p 65.
28. Vihinen, M.; Mantsala, P. In *Critical Reviews in Biochemistry and Molecular Biology*; **1989**, *24*, 329.
29. Robyt, J. F. In *Starch: Chemistry and Technology, 2nd edition*; Whistler, R. L.; BeMiller, J.; Paschall, E. F., Eds.; Academic Press: New York, 1984; p. 87.
30. Cote, G.; Tao, B. *Glycoconjugate J* **1990**, *7*, 145-162.
31. French, D.; Levine, M.; Norberg, E.; Nordin, P.; Pazur, J.; Wild, G. J. *Am. Chem. Soc.* **1954**, *76*, 2387.
32. Takano, T.; Fukuda, M.; Moma, M.; Kobayashi, S.; Kainuma, K.; Yamane, K. *J. Bacteriol.* **1986**, *166*, 1118.
33. Sugimoto, T.; Kubota, M.; Sakai, S. UK Patent GB 2169902 **1986**.
34. Binder, F.; Huber, O.; Bock, A. *Gene* **1986**, *47*, 269.
35. Kaneko, T.; Hamamoto, T.; Horikoshi, K. *J. Gen. Microbiol.* **1988**, *134*, 97.
36. Kimura, K.; Takano, T.; Yamane, K. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 149.
37. Yagi, Y.; Iguchi, H. Japan Patent 74124385 **1974**.
38. Okada, S.; Tsuyama, N. U. S. Patent 3812011 **1974**.
39. Nakamura, N.; Horikoshi, K. *Agric. Biol. Chem.* **1976**, *40*, 1785.

40. Yagi, Y.; Kuonon, K.; Inui, T. Eur. Patent 0017242 1983.
41. Kato, T.; Horikoshi, K. *Agric. Biol. Chem.* **1986**, *50*, 2161.
42. Armbruster, F. C. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988; p 33.
43. Seres, G.; Barcza, L. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988; p 81.
44. Nitschke, L. Diplomarbeit, Universitat Freiburg i.Br 1989.
45. Horikoshi, K. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988; p 87.
46. Schmid, G.; Englbrecht, A.; Schmid, D. In *Proc. 4th Intl. Symp. on Cyclodextrins*; Huber, O.; Szejtli, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1988; p 71.
47. Kubota, M.; Mikami, B.; Tsujisaka, Y.; Morita, Y. *J. Biochem.* **1988**, *104*, 12.
48. Hofmann, B.; Bender, H.; Schulz, G. *J. Mol. Biol.* **1989**, *209*, 793.
49. Banner, D. W.; Bloomer, A. C.; Petsko, G. A.; Phillips, D. C.; Pogson, C. I.; Wilson, I. A.; Corron, P. H.; Furth, A. J.; Milman, J. D.; Offord, R. E.; Priddle, J. D.; Waley, S. G. *Nature (London)* **1974**, *255*, 609.
50. Schiffer, M.; Girling, R. L.; Ely, K. R.; Edmundson, A. B. *Biochemistry* **1973**, *12*, 4620.
51. MacGregor, A.; Svensson, B. *Biochem. J.* **1989**, *259*, 145.
52. Nakajima, R.; Imanaka, T.; Aiba, S. *Appl. Microbiol. Biotechnol.* **1986**, *23*, 355.
53. MacGregor, E. A. *Protein Chem.* **1989**, *7*, 399.
54. Buisson, G.; Duee, E.; Haser, R.; Payan, F. *EMBO J.* **1987**, *6*, 3908.
55. Matsuura, Y.; Kusunoki, M.; Harada, W.; Kakudo, M. *J. Biochem.* **1984**, *95*, 697.
56. Richardson, J. S. *Adv. Protein Chem.* **1981**, *34*, 167.
57. Tao, B.; Reilly, P.; Robyt, J. *Biochem. Biophys. Acta* **1989**, *995*, 214.
58. Hehre, E.; Okada, G.; Genghof, D. *Adv. Chem. Ser.* **1973**, *117*, 309.
59. Pasero, L.; Mazzei-Pierron, Y.; Abadie, B.; Chicheportiche, Y.; Marchis-Mouren, G. *Biochim. Biophys. Acta* **1986**, *869*, 147.
60. Sakai, S.; Kubota, M.; Yamamoto, K.; Nakada, T.; Torigoee, K.; Ando, O.; Sugimoto, T.; *Denpun Kagaku* **1987**, *34*, 140.
61. Svensson, B. *FEBS Let.* **1988**, *230*, 72.
62. Svensson, B.; Jespersen, H.; Sierks, M.; MacGregor, A. *Biochem. J.* **1989**, *264*, 309.
63. Carrel, H. L.; Rubin, B. H.; Hurley, T. J.; Glusker, J. P. *J. Biol. Chem.* **1984**, *259*, 3220.
64. Tao, B. Y.; Reilly, P. J.; Robyt, J. F. *Carbohydr. Res.* **1988**, *181*, 163.
65. Schmid, G. *Tibtech* **1989**, *7*, 244.
66. Richardson, J. In *The Protein Folding Problem*; Wetlaufer, D., Ed.; Am. Assn. Adv. Sci. Selected Symp. No. 89: Wash., DC, 1984 Chapt. 1.

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Chapter 29

Starch Liquefaction with a Highly Thermostable Cyclodextrin Glycosyl Transferase from *Thermoanaerobacter* Species

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A novel process is proposed for enzymatically liquefying starch with a highly thermostable cyclodextrin glycosyl transferase (CGTase). Conditions are described for liquefying 35% dry solids starch at pH 4.5 without added calcium, followed by saccharification to 96% dextrose. The CGTase is produced extracellularly by *Thermoanaerobacter* sp. ATCC 53,627, a thermophilic obligate anaerobe. This enzyme has a pH optimum activity observed at pH 3.5. The temperature optimum is 95°C. In the absence of starch and calcium, 95% of the activity remains after incubation at 80°C and pH 5.0 for 40 minutes. This CGTase shows the highest stability reported for any CGTase to date. These remarkable properties enable liquefaction of starch at pH 4.5, resulting in a process that can operate at the saccharifying pH of 4.5, thus eliminating the need for pH adjustment.

The production of high fructose syrup from starch requires three steps called liquefaction, saccharification, and isomerization. Liquefaction is generally accomplished with an alpha-amylase at a pH of approximately 6.0-6.5 followed by saccharification at a pH of approximately 4.5 with an amyloglucosidase. As a result of this pH incompatibility, the starch industry has long sought starch liquefaction enzymes capable of operating at the saccharifying pH of 4.5 in order to eliminate the need for pH adjustment of the starch as it arrives in the plant. The availability of these enzymes would provide significant process advantages with regard to saving costs and reducing undesirable by-products.

The starch industry has adopted standard conditions for liquefaction. These conditions constitute a short-term jet-cooking treatment of a 35-40% dry solids (DS) starch slurry at 105°C for 5 minutes, known as gelatinization or primary liquefaction, followed by a 90-minute hold at 95°C, known as dextrinization or secondary liquefaction (1).

Alpha-amylases (1,4-alpha-D-glucan glucanohydrolase, EC 3.2.1.1) have conventionally been employed in starch liquefaction. Alpha-amylases hydrolyze the alpha-1,4-glucosidic linkages of the starch, producing maltodextrins. The alpha-amylases

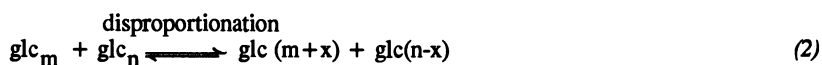
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commercially used today are produced by *Bacillus licheniformis* and *Bacillus stearothermophilus*.

Starch can also be degraded by a group of enzymes known as cyclodextrin glycosyl transferases [CGTase, 1,4-alpha-D-glucan 4-alpha-D-(1,4-alpha-D-glucano)-transferase, E.C. 2.4.1.19]. The CGTase enzymes degrade starch by catalyzing cyclization, coupling, and disproportionation reactions as shown below (2):



x = 6 alpha-cyclodextrin
 x = 7 beta-cyclodextrin
 x = 8 gamma-cyclodextrin



The cyclization reactions produce cyclodextrins (also known as Schardinger dextrans) which are cyclic molecules comprised of six, seven, or eight alpha-D-glucopyranose residues linked by alpha-1,4 bonds (3,4). They are known as alpha-, beta-, or gamma-cyclodextrin depending on the number of glucose residues, 6, 7, or 8, respectively. These cyclized molecules have neither a non-reducing nor reducing end-group.

Since CGTases can degrade starch by catalyzing cyclization and disproportionation reactions, a CGTase characterized by a high thermostability similar to the alpha-amylases employed by the starch industry should be able to solubilize the starch. In general, CGTases have lower pH optima than alpha-amylases. The low pH optima of these enzymes should eliminate the need for pH adjustment prior to saccharification thereby providing a major process improvement.

Known producers of CGTase include *Bacillus mascerans* (7), *Bacillus megaterium* (8), *Bacillus ohbensis* (9), *alkalophilic Bacillus* sp. (10-14), *Bacillus amyloliquefacien* (15), *Bacillus subtilis* (16), *Klebsiella oxytoca* (17), and *Micrococcus* sp. (18). However, none of these CGTase enzymes are sufficiently thermostable for use in industrial starch liquefaction.

Novel CGTases possessing this high thermostability property have now been discovered in a group of thermophilic anaerobic microorganisms belonging to the genus *Thermoanaerobacter*. The ability of one of these enzymes from *Thermoanaerobacter* sp. ATCC 53627 to liquefy starch is reported. The cyclodextrin-producing ability of this enzyme has previously been reported (Starnes, R.L.; Flint, V.M.; Katkocin, D.M. Cyclodextrin Production with a Highly Thermostable Cyclodextrin Glycosyl Transferase from *Thermoanaerobacter* sp. presented at the Fifth International Symposium on Cyclodextrins, Paris, France, March 27-30, 1990).

Materials and Methods

Enzymes. Termamyl, AMG, and Dextrozyme were obtained from Novo Nordisk Bioindustrials, Inc., Danbury, CT. *Bacillus stearothermophilus* alpha-amylase was supplied by Enzyme Bio-Systems Ltd., Englewood Cliffs, NJ.

CGTase Production. Strain ATCC 53627 was cultured for 40 hours at 70 °C in a prereduced liquid medium under argon at pH 7.0 comprised of the following components in grams/liter: Maltrin M-100, 5.0; KH₂PO₄, 2.0; K₂HPO₄, 6.0; NaCl, 1.0; (NH₄)₂SO₄, 2.5; MgSO₄·7H₂O, 0.5; CaCl₂·2H₂O, 0.05; yeast extract, 2.0; Na₂S, 0.5; cysteine-HCl, 0.5; resazurin (redox indicator), 2 ng; and trace metals 5.0 ml. The trace metals solution was comprised of the following components in grams/liter: FeCl₃·6H₂O, 5.40;

ZnSO₄·7H₂O, 1.45; MnCl₂·4H₂O, 1.00; CuSO₄·5H₂O, 0.25; and H₃BO₃, 0.10. The cell-free culture broth was concentrated 200-fold to a volumetric activity of 40 Phadebas units per milliliter.

CGTase Activity Assay. Activity was measured by the Pharmacia Phadebas Amylase Assay at pH 6.0, 60°C in 0.1M sodium acetate (100 ppm Ca⁺) for 15 minutes using *B. stearothermophilus* alpha-amylase as a standard. Alpha-amylase preparations were assayed under the same conditions. One Phadebas unit is defined as the amount of enzyme that will catalyze the hydrolysis of 1.0 micromole of glucosidic linkages of Lintner starch per minute at 60°C, pH 6.0.

Liquefaction - Lab-Scale. 35% DS corn starch slurries were liquefied with the CGTase at a dose of 4.46 Phadebas units/gram DS starch at pHs 4.5-5.5 for 14 minutes at 105°C (primary liquefaction) and for 4 hours at 90 °C (secondary liquefaction) +/- 40 ppm calcium. Final volumes were 5-10 ml. Termamyl and *Bacillus stearothermophilus* alpha-amylase were run as controls at pHs 6.2 and 5.8, respectively in the presence of 40 ppm calcium.

Cloning of CGTase Gene. Chromosomal DNA was isolated from *Thermoanaerobacter* sp. ATCC 53,627 cells, and purified by sucrose gradient velocity sedimentation. A collection of DNA fragments were generated by partial digestion with EcoRI endonuclease, and fragments 7-15 kilobase pairs (kbp) in length were separated by velocity sedimentation in sucrose gradients and purified. These fragments were ligated to the EcoRI site of the *E. coli* plasmid pBR322, and tetracycline-resistant-transformants of strain HB101 were isolated to constitute a gene library. Tetracycline-resistant colonies were plated onto amylopectin-LB plates containing tetracycline (15 micrograms/ml) and incubated overnight at 37°C. CGTase production was detected as clearing zones after exposure to iodine vapor.

Liquefaction - Pilot Plant-Scale. 35% DS corn starch slurries (30 lbs.) were liquefied with the CGTase at a dose of 4.46 or 8.92 Phadebas units/gram DS starch at pH 4.5 for 5 minutes at 105°C (primary) followed by 2 hours at 95 °C or 4 hours at 90 °C (secondary).

Viscosity Determinations. The viscosity determinations were conducted at 60°C with a Haake Rotovisco RV12 viscometer or at 90 °C with a Nametre viscometer.

Saccharification. Saccharifications were performed with amyloglucosidase (AMG) or amyloglucosidase/pullulanase (Dextrozyme) at a dose of 0.18 AG/units/gram DS for 48 hours at 60°C, pH 4.3-4.5. One AG unit is the amount of enzyme which hydrolyzes 1.0 micromole of maltose per minute at 25 °C, pH 4.3.

Dextrose Quantitation. The dextrose yield was determined by BioRad Aminex Carbohydrate HPX-87C HPLC at 85°C eluting with glass distilled water at a flow rate of 0.7 ml/minute. Detection was by refractive index.

Cyclodextrin Action Pattern. The action pattern of alpha-, beta-, or gamma-cyclodextrin was determined by BioRad Aminex Carbohydrate HPX-42A HPLC with two columns in tandem at 85°C eluting with glass distilled water as at a flow rate of 0.6 ml/minute. Detection was by refractive index.

Results and Discussion

Properties of the *Thermoanaerobacter* sp. CGTase. The CGTase is produced extracellularly at 70°C in a prerduced liquid maltrin medium under argon. The maximal activity level after 40 hours of cultivation is 200 Phadebas units per liter culture broth.

The CGTase preparation is comprised of three components with molecular weights of 117,000 (I), 110,000 (II), and 108,000 (III) daltons based on SDS-polyacrylamide gel electrophoresis. The isoelectric points are 4.55 (I), and 4.50 (II and III). No detectable differences are observed between these enzymes with regard to their starch degradation and cyclodextrin production abilities. The pH and temperature optima for activity are 5.0 (60°C) and 95°C (pH 5.0) as shown in Figures 1 and 2, respectively. The CGTase produces an action pattern from 5% Lintner starch when incubated at 60°C, pH 6.0 for 24 hours that is typical of a cyclodextrin-producing enzyme. (Figure 3).

Thermostability of *Thermoanaerobacter* sp. CGTase. The addition of 40ppm Ca^{++} to the CGTase preparation during incubation at high temperatures in the absence or presence of starch substrate provided no enhancement of the thermostability of the enzyme. A comparison of the thermostable CGTase was made to other thermostable enzymes used in starch liquefaction including Termamyl (*Bacillus licheniformis*) and *Bacillus stearothermophilus* alpha-amylase.

The CGTase was observed to retain 100% of its activity when incubated in 5% Lintner starch 0.1M sodium acetate pH 5.0 (50ppm Ca^{++}) for 50 minutes at 95°C while Termamyl and *B. stearothermophilus* alpha-amylases retained 0% and 38% activity, respectively. In the absence of starch substrate and Ca^{++} , the residual CGTase activity is approximately 95% when incubated at 80°C, pH 5.0 for 40 minutes while no activity remains in the case of the alpha-amylases (Figure 4).

The standard CGTase employed in the cyclodextrin industry is produced by *Bacillus mascerans*. This enzyme is reported to be stable only at temperatures around 50°C and loses activity rapidly above 50°C (19). A more thermostable CGTase compared to the *B. mascerans* CGTase is produced by *Bacillus stearothermophilus* with a temperature optimum of 70°C (20). However, the *Thermoanaerobacter* CGTase is far more thermostable with its optimum of 95°C, and is to our knowledge, the most thermostable CGTase.

Liquefying Activity. The liquefying activity of the CGTase was determined under simulated industrial conditions. The CGTase was incubated with 35% DS corn starch +/- 40ppm Ca^{++} at either pH 4.5, 5.0, or 5.5 for 14 minutes at 105°C followed by 4 hours at 90°C. *B. stearothermophilus* alpha-amylase was run for comparison. *B. stearothermophilus* alpha-amylase and Termamyl were also run as controls at pHs 5.8 and 6.2, respectively. The enzyme dose was 4.46 Phadebas units/gram DS starch.

The results demonstrated that the CGTase is able to liquefy corn starch at any pH in the range 4.5-5.5 (Table I). Liquefaction was considered positive if the starch syrup was pourable. The starch was liquefied to a negligible dextrose equivalent (DE); i.e., without the formation of reducing sugars as expected with a CGTase. The presence of calcium was not required. The *B. stearothermophilus* amylase, on the other hand, provided suitable liquefaction only at pH 5.5 and calcium was required, but still not optimal as evidenced by the results obtained at pH 5.8.

The saccharification ability of the liquefied starches was also determined using Dextrozyme at a dose of 0.18 AG/gram DS (48 hours, 60°C). The results (Table I) demonstrated good saccharification of the starches liquefied with the CGTase relative to Termamyl at pH 6.2 and *B. stearothermophilus* alpha-amylase at pH 5.8. While amyloglucosidase is unable to hydrolyze cyclodextrins, the combination of amyloglucosidase and residual CGTase provides a mechanism for degradation as a result of the cyclization/coupling equilibrium. The ability to saccharify the pH 4.5 liquefied starch demonstrates a clear process advantage over current industrial amylases since pH adjustment prior to saccharification is not necessary.

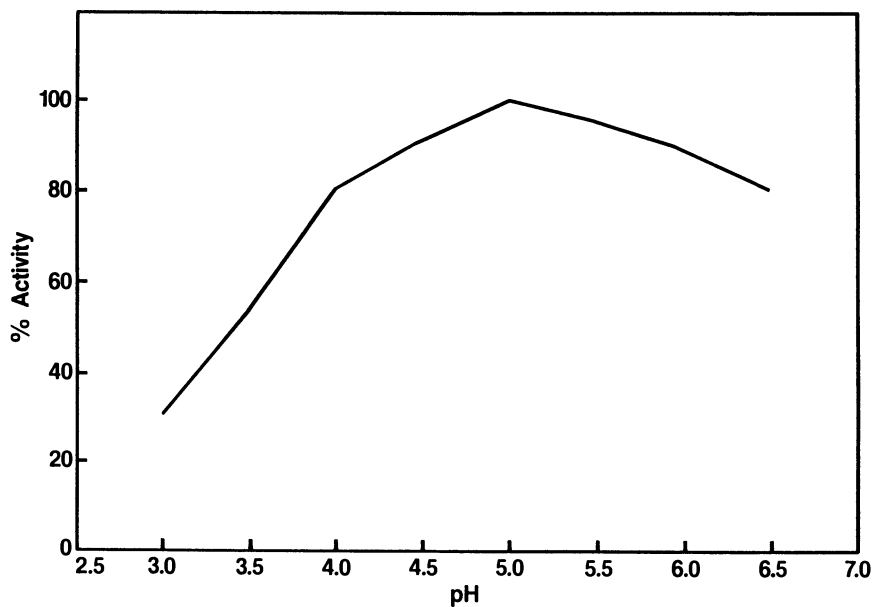


Figure 1. CGTase activity versus pH at 60°C.

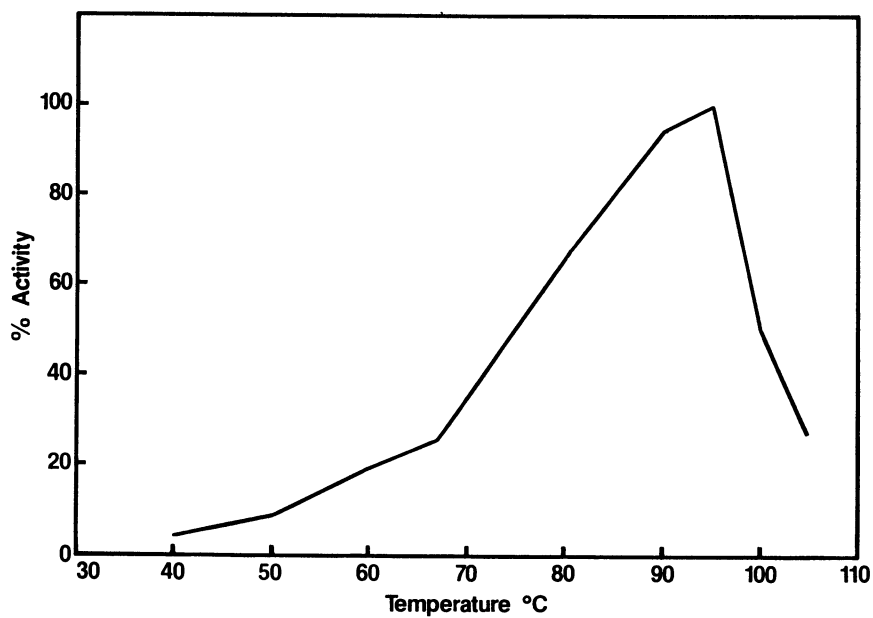


Figure 2. CGTase activity versus temperature at pH 5.0.

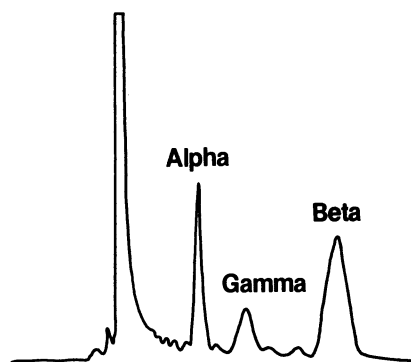


Figure 3. Action pattern from Lintner starch at 60°C, pH 6.0.

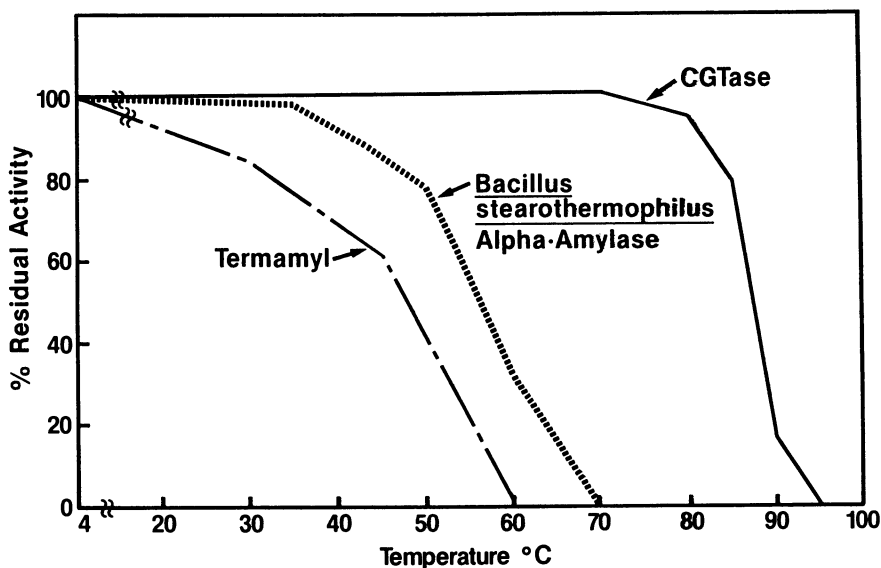


Figure 4. Thermostability of CGTase relative to Termamyl and *Bacillus stearothermophilus* alpha-amylase at 80°C, pH 5.0.

Table I. Liquefying Activity and Dextrose Yields after Saccharification

Enzyme	pH	Ca ⁺⁺	DE	Liquefied	% Dextrose
Amylase*	4.5	+	Not determinable	No	Not determinable
Amylase	4.5	-	Not determinable	No	Not determinable
Amylase	5.0	+	4.78	Yes**	96.0
Amylase	5.0	-	Not determinable	No	Not determinable
Amylase	5.5	+	9.78	Yes	95.8
Amylase	5.5	-	5.58	Yes	95.9
CGTase	4.5	+	0.51	Yes	96.0
CGTase	4.5	-	0.44	Yes	96.0
CGTase	5.0	+	0.73	Yes	95.6
CGTase	5.0	-	0.69	Yes	95.5
CGTase	5.5	+	1.10	Yes	95.7
CGTase	5.5	-	0.83	Yes	95.8
Amylase	5.8	+	13.6	Yes	96.8
Termamyl	6.2	+	14.4	Yes	96.4

* *Bacillus stearothermophilus*

** Rated as liquefied, but very viscous.

Confirmation of the liquefying ability of the CGTase at pH 4.5 in the absence of added Ca⁺⁺ was obtained by measuring the viscosity reduction as a function of enzyme dosage. Termamyl at pH 6.2 and *B. stearothermophilus* alpha-amylase at pH 5.8 were run as controls. All the primary liquefactions were at 105°C, except those with CGTase at 0.446 and 0.223 Phadebas units/gram DS were at 100°C to compensate for the lower enzyme dosage. Secondary liquefactions were all performed at 90°C for 4 hours (Table II).

Flow curves for these liquefactions at an enzyme dose of 4.46 Phadebas units/gram DS starch showing torque reading versus drive rotation speed at 60°C demonstrated a negligible increase in the rate at which torque reading increases as drive rotation speed is increased for liquefactions involving Termamyl and *B. stearothermophilus* alpha-amylase (Figure 5). There is a slight increase in this rate for liquefaction with CGTase. As the CGTase dose is decreased from 4.46 to 0.223 Phadebas units/gram DS starch, there is a corresponding increase in the torque measurements.

Table II. Viscosity Measurements

Enzyme	Dosage Phadebas	Added U/g DS	Primary Ca ⁺⁺	pH	Viscosity Liq. temp.	centipose
CGTase		0.223	-	4.5	100°C	*
CGTase		0.446	-	4.5	100°C	*
CGTase		0.892	-	4.5	105°C	208
CGTase		2.23	-	4.5	105°C	66.9
CGTase		4.46	-	4.5	105°C	42.4
Termamyl		4.46	40 ppm	6.2	105°C	41.6
Ba amylase		4.46	40 ppm	5.8	105°C	34.1

* measurement not possible at this speed

The viscosity achieved by the CGTase at pH 4.5 is very similar to the viscosities produced by Termamyl at pH 6.2 and *B. stearothersophilus* alpha-amylase at pH 5.8 as measured at a drive speed of 32 (Table II). Reducing the CGTase dose to 2.23 Phadebas units still yielded acceptable viscosity. These results establish that the CGTase can liquefy starch under simulated industrial conditions of high starch but at a pH; i.e., 4.5 where conventional alpha-amylases do not function.

Jet-Cooking. Industrially, heating of the starch during primary liquefaction is accomplished by jet-cooking. Consequently, survival of the CGTase during jet-cooking is important in order to be suitable for industrial use. Thermophilic anaerobic bacteria are poor enzyme-producers. It was, therefore, necessary to clone the gene into a suitable expression host in order to produce a sufficient quantity of enzyme for jet cooking.

The CGTase gene was expressed in *E. coli* HB101 using pBR322 as the vector. Restriction mapping of the recombinant plasmid revealed a DNA fragment 12.8 kbp in size had been inserted into the EcoRI site of pBR322. Deletion analysis with the restriction enzyme Bam HI showed the CGTase gene was located on a 6.0 kbp BamHI-BamHI fragment. Characterization of the CGTase enzyme revealed it was indistinguishable from the native CGTase with regard to pH and temperature optima, molecular weight, and thermostability.

The CGTase has a molecular weight roughly 50% larger than other reported CGTases. However, initial sequencing of the gene revealed that the molecular weight is very close to other known CGTases. The larger molecular weight obtained by SDS-PAGE suggests the high thermostability of this enzyme prevents complete reduction-denaturation.

The CGTase was produced by culturing the recombinant *E. coli* strain in Luria-Bertani medium containing tetracycline (15 mg/liter) at 37°C for 24 hours. The CGTase was recovered by lysis of the cells. A 35% DS corn starch slurry (30 lb.) was liquefied with the recombinant CGTase at pH 4.5 without added Ca⁺⁺ at a dosage of 8.92 Phadebas units/gram DS starch by jetting at 105°C for 5 minutes (primary liquefaction) followed by a hold at 95°C for 2 hours or 90°C for 4 hours (secondary liquefaction).

During secondary liquefaction at 95°C or 90°C, a rapid reduction in viscosity was observed. At 90°C, the viscosity reduction was monitored over time with a Nametre viscometer. The results demonstrated there was a rapid reduction in viscosity to 400 centipoise x gm/cm³ by 7 minutes into secondary liquefaction (Figure 6). The action patterns of the liquefied starches following secondary liquefaction demonstrated the characteristic cyclodextrin action pattern at both temperatures. DE values were < 1.0 indicating the absence of reducing end-groups consistent with the mechanism of a CGTase.

The liquefied starch solutions were saccharified with AMG and Dextrozyme at pH 4.5, 60°C for 48 hours (0.18AG/gram DS). The results (Table III) demonstrated a good yield of dextrose in all cases with minor amounts of DP (degree of polymerization) products. The highest yield was achieved with secondary liquefaction at 95°C and saccharification with Dextrozyme. Sediment volumes were less than 1 ml in all cases based on a 100 ml volume. Similar saccharification results were achieved with starch liquefied with the CGTase at a dose of 4.46 Phadebas units/gram DS starch (data not shown).

Initial optimization of the CGTase dose requirements suggests that the dose can be lowered to 3.58 Phadebas units per gram DS without compromising viscosity reduction and dextrose yields upon saccharification. This dosage enables the CGTase to be competitive with Termamyl in enzymatic liquefaction.

The thermostable CGTase produced by *Thermoanaerobacter* sp. ATCC 53,627 is able to liquefy starch at pH 4.5 under standard industrial conditions. It is, therefore, unnecessary to pH adjust the dextrin solution prior to saccharification as is normally done in the industry today. Since there is no need for pH adjustment, significant process advantages are realized. There is a substantial cost improvement with regard to chemicals, ion-exchange media, charcoal, etc. Also, unwanted by-product formation; e.g., maltulose, colored products, base-catalyzed products are reduced. Consequently, these advantages will translate into real savings to the starch industry.

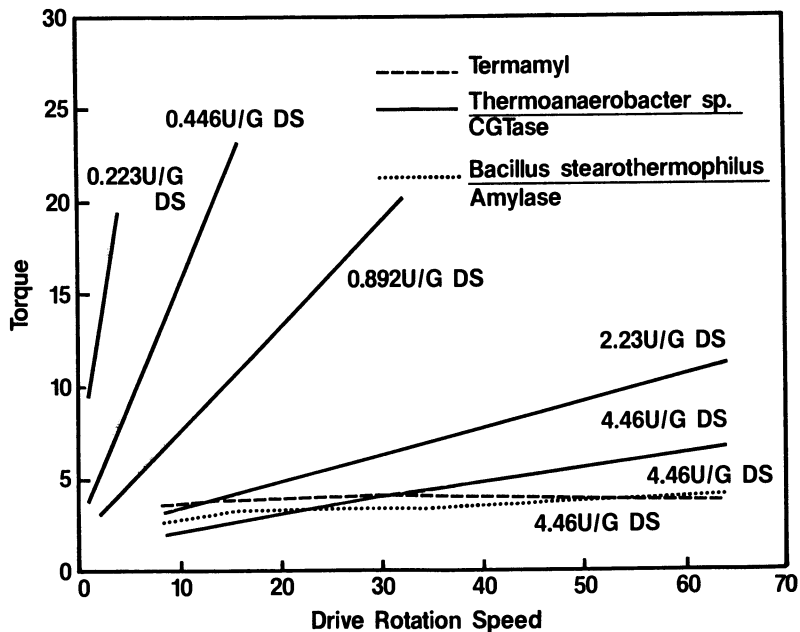


Figure 5. Flow diagrams for CGTase as a function of dose at 60°C.

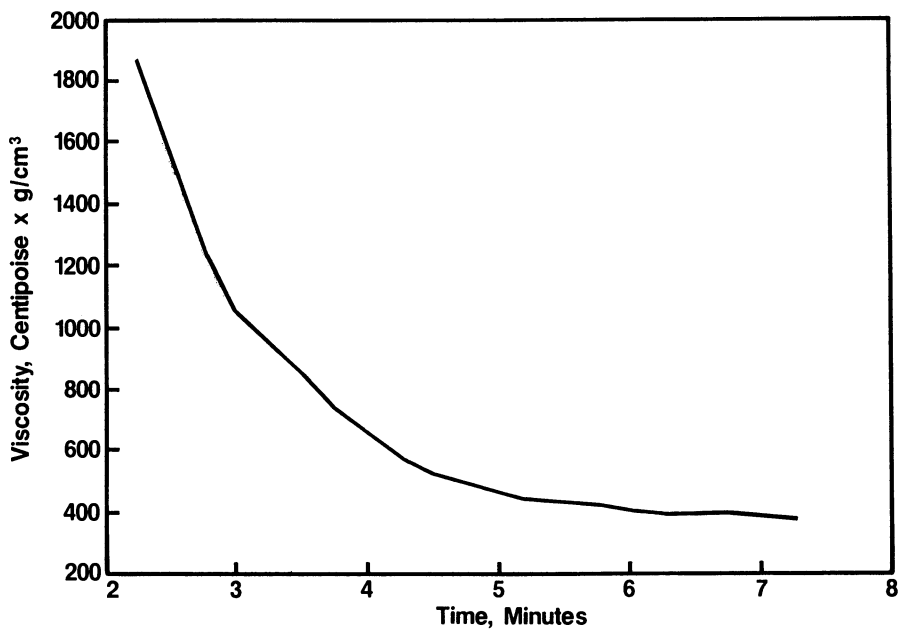


Figure 6. Viscosity reduction during secondary liquefaction at 90°C, pH 4.5.

Table III. Saccharifications From Cyclodextrin Glycosyl Transferase Jetting

Sediment	% Yield				
	Enzyme	Dextrose	DP2	DP3	DP4+
95°C Secondary Liquefaction					
Dextrozyme	95.87	2.44	0.39	1.30	0.5 ml
AMG	95.09	2.27	0.36	2.28	0.5 ml
90°C Secondary Liquefaction					
Dextrozyme	95.37	3.34	0.40	0.89	0.5 ml
AMG	95.36	3.21	0.38	1.05	0.5 ml

Literature Cited

1. Novo Industri A/S, U. S. Patent 3,912,590, 1975.
2. Bender, H. *Adv. in Biotechnol. Processes* 1986, 6, 31.
3. French, D.; Rundle, D.E. *J. Amer. Chem. Soc.* 1942, 64, 1651.
4. Freudenberg, K.; Cramer, F. *Z. Naturforsch* 1948, B3, 464.
5. Tilden, E.B.; Hudson, C.S. *J. Bacteriol.* 1942, 43, 527.
6. Pongsawasdi, P.; Yagisawa, M. *Agric. Biol. Chem.* 1988, 52, 1099.
7. Kitahata, S.; Okada S. *J. Jap. Soc. Starch Sci.* 1982, 29, 7.
8. Kitahata, S.; Okada, S. *Agric. Biol. Chem.* 1974, 12, 2413.
9. Yagi, Y.; Sato, M.; Ishikura, T. *J. Jap. Soc. Starch Sci.* 1986, 33, 144.
10. Nakamura, N.; Horikoshi, K. *Agric. Biol. Chem.* 1976, 40, 1785.
11. Kanedo T.; Hamamoto, T.; Horikoshi, K. *J. Am. Microbiol.* 1988, 134, 97.
12. Kimura, K.; Takano, T.; Yamani, K. *Appl. Microbiol. Biotechnol.* 1987, 26, 149.
13. Schmid, G.; Englbrecht, A.; Schmid, D. *In Proceedings of the Fourth International Symposium on Cyclodextrins*; Huber, O.; Szejtli, J. Eds.; pp 71-76, Kluwer Academic: Munich, Germany, 1988.
14. Schmid, G.; Huber, O.S.; Eberle, H.J. *Proceedings of the Fourth International Symposium on Cyclodextrins*; Huber, O.; Szejtli, J. Eds.; pp 87-92, Kluwer Academic: Munich, Germany, 1988.
15. Yu, E.K.C.; Aoki, H.; Misawa, M. *Appl. Microbiol. Biotechnol.* 1988, 28, 377.
16. Kato, T.; Horikoshi, K. *J. Jap. Soc. Starch Sci.* 1986, 2, 137.
17. Yagi, Y.; Kuono, K.; Inui, T., United States Patent No. 4,317,881, 1982.
18. Binder, F.; Huber, O.; Bock, A. *Gene* 1986, 47, 269.
19. Stayn, A.; Granum, P.E. *Carb. Res.* 1970, 75, 243.
20. Kitahata, S.; Okada, S. *J. Jap. Soc. Starch Sci.* 1982, 29, 13.

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Chapter 30

Reactions of Glucansucrases in the Biomass Conversion of Sucrose

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Many different kinds of glucansucrases are elaborated by different species of *Leuconostoc* and *Streptococcus* to give different kinds of glucans from sucrose. Mechanistic studies have shown that two different dextransucrases and a mutansucrase form covalent glucosyl and glucanosyl intermediates and that the glucosyl moiety is transferred to the reducing-end of the growing glucanosyl chain. The synthesis of the glucan is terminated when the glucan chain is released by acceptor reactions. Acceptor reactions occur when other carbohydrates besides sucrose are added to the digests. When glucan itself is the acceptor, a branch linkage is formed. Over 50 different carbohydrates are known acceptors. The different acceptors react at different rates and efficiencies. The structures of the acceptor products depend on the structure of the acceptor. The amounts of glucan and acceptor product can be altered by the concentration ratio of acceptor to sucrose. Glucansucrases can, thus, be utilized to produce new products (glucans and oligosaccharides of different structures) from the biomass material, sucrose.

Starch, cellulose, and sucrose are abundant, renewable raw materials that can be used in biomass conversions. Glucansucrases are enzymes that convert sucrose into glucans, and on the addition of other carbohydrates to the sucrose digests, the glucose moiety of sucrose is transferred to the carbohydrates to give oligosaccharide acceptor products. Glucansucrases, thus, are important enzymes in the biomass conversion of sucrose into new products, either polysaccharides or oligosaccharides.

Glucansucrases are elaborated by *Lactobacilli* (*Leuconostoc* and *Streptococcus* species) and catalyze the synthesis of glucans from sucrose. In 1954, Jeanes *et al.* (1) surveyed 96 *L. mesenteroides* species that produce glucans from sucrose and found that the glucans

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had a wide variety of physical and chemical properties. Some of the species produce two kinds of glucans that can be separated by differential alcohol precipitation (2).

L. mesenteroides B-512F produces only one glucan, the classical dextran that has 95% α -1 \rightarrow 6 linkages in the main chains with 5% α -1 \rightarrow 3 branch linkages. The branch chains consist of both single glucose residues and long α -1 \rightarrow 6 linked chains with many glucose residues. To date, this is the only commercially produced glucan from sucrose. The resulting dextran is of very high molecular weight ($> 5 \times 10^7$). It serves as the starting material for the epichlorohydrin cross-linked, gel-filtration material, Sephadex. Low molecular weight dextran ($5-10 \times 10^4$) produced by controlled acid hydrolysis and alcohol fractionation is used as a blood-plasma substitute (3) (see Fig. 1A for the structure). A low molecular weight dextran sulfate will serve as a substitute for the anticoagulant polysaccharide, heparin.

Streptococcus mutans produces two glucansucrases, dextransucrase and mutansucrase. Dextransucrase synthesizes a water soluble dextran that is relatively highly branched with 64% α -1 \rightarrow 6 linkages in the main chains and 36% α -1 \rightarrow 3 branch linkages (4). The branch chains consist primarily of single glucose residues attached to every other glucose residue in the main chains (4). There are some long branch chains that also have single glucose units attached by α -1 \rightarrow 3 branch linkages to every other glucose residue in the chain. This type of structure represents a bifurcated, alternating comb polymer and hence differs significantly in structure from B-512F dextran. It is resistant to endo-dextransucrase hydrolysis. Mutansucrase synthesizes a glucan that has $>93\%$ α -1 \rightarrow 3 linkages. This glucan is extremely insoluble (< 0.01 mg/mL). Both of these glucans are synthesized in the oral cavity and go to make-up dental plaque. The two enzymes have been separated (5) and the soluble alternating comb dextran might have some uses as a dextransucrase-resistant glucan, with properties different from those of B-512F dextran.

L. mesenteroides B-742 produces a dextransucrase that synthesizes the most highly branched dextran known. It has 50% α -1 \rightarrow 6 linkages in the main chains and 50% α -1 \rightarrow 3 branch linkages. Its structure is similar to that of *S. mutans* dextran with the branch chains being primarily single glucose residues. It differs from the *S. mutans* dextran in that every glucose residue in the α -1 \rightarrow 6 linked main chains has attached to it an α -1 \rightarrow 3 linked glucose residue. There are also some long branch chains as well. This structure is a bifurcated, regular comb polymer. These types of polymers are called comb polymers because the single glucose branches are "hung" off of the main chains as teeth on a comb. The B-742 structure is considered regular in that every glucose residue in the main chains are branched, whereas the *S. mutans* comb polymer has "gaps" in the teeth as only every other glucose residue in the main chains are branched (see Fig. 1B and 1D for a comparison of the two structures). This dextran is also resistant to endo-dextransucrase hydrolysis. The high degree of branching of these dextrans also make them very water soluble.

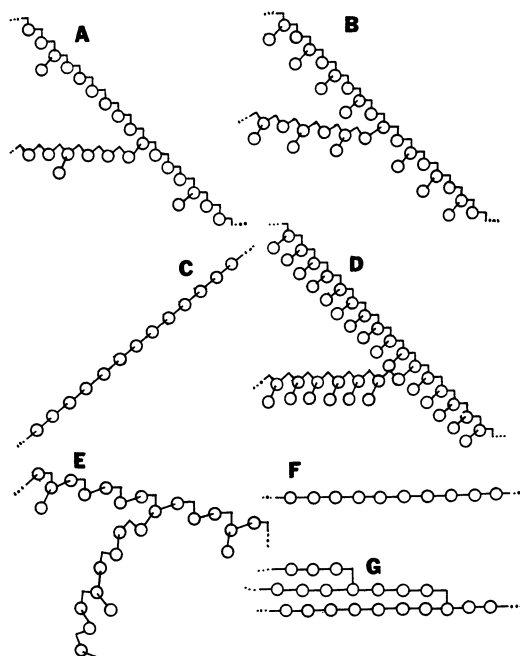


Fig. 1 Structures of some glucans synthesized by glucansucrases

(A) dextran (95% α -1+6 linkages and 5% α -1+3 branch linkages) synthesized by *Leuconostoc mesenteroides* B-512F dextransucrase;

(B) alternating comb dextran (65% α -1+6 linkages and 35% α -1+3 branch linkages) synthesized by *Streptococcus mutans* dextransucrase;




(C) mutan (>93% α -1+3 linkages) synthesized by *S. mutans* mutansucrase;

(D) regular comb dextran (50% α -1+6 linkages and 50% α -1+3 branch linkages) synthesized by *L. mesenteroides* B-742 dextransucrase;

(E) alternan (50% α -1+6 linkages alternating with 40% α -1+3 linkages and 10% α -1+3 branch linkages) synthesized by *L. mesenteroides* B-1355 alternansucrase;

(F) amylose (100% α -1+4 linkages) synthesized by *Neisseria perflava* species amylosucrase;

(G) amylopectin (95% α -1+4 linkages with 5% α -1+6 branch linkages) synthesized by *N. perflava* species amylosucrase.

Where  represents a glucopyranosyl residue attached to another glucose residue by an α -1+6 linkage;  represents a glucopyranosyl residue attached to another glucose residue by an α -1+3 linkage; and  represents a glucopyranosyl residue attached to another glucose residue by an α -1+4 linkage.

L. mesenteroides B-1355 produces an interesting glucan from sucrose that is called alternan (6) because it has alternating α -1 \rightarrow 6 and α -1 \rightarrow 3 linkages in the main chains. It also has a significant number (11%) of α -1 \rightarrow 3 branch linkages. Because of the unusual alternating structure, alternan is also quite resistant to endo-dextranase hydrolysis and has a very different physical appearance from that of B-512F dextran when precipitated by alcohol. B-512F dextran appears as a translucent, gel-like material, and alternan is a dense white, opaque-material (3,6).

We have recently isolated a series of *Neisseria perflava* species from dental plaque that synthesize glucans with different iodine-staining properties from sucrose. Some stain a very dark blue-black, indicating an amylose-like structure with a high content of consecutive α -1 \rightarrow 4 linkages; while others stain purple, indicating an amylopectin structure with α -1 \rightarrow 4 linkages in the main chains and α -1 \rightarrow 6 branch linkages; and still others stain red-brown, indicating a more highly branched, glycogen-like structure. Further work on the enzymes that synthesize these polysaccharides could produce starch or glycogen products from sucrose.

Three of the glucansucrases, *L. mesenteroides* B-512F dextran-sucrase and *S. mutans* dextran-sucrase and mutansucrase have been purified (5,7-9), and their mechanism and specificity of action studied by pulse and chase labeling techniques with ^{14}C -sucrose (5,10-13). It has been shown that these enzymes form covalent glucosyl and glucanosyl intermediates during synthesis and the glucose unit is added to the reducing end of the growing glucan chain (5,10). For *L. mesenteroides* B-512F dextran-sucrase, the proposed mechanism for the synthesis of a sequence of α -1 \rightarrow 6 linkages involves two nucleophiles at the active site that attack sucrose and displace fructose to give two β -glucosyl enzyme intermediates. The C-6-OH of one of the glucosyl residues is oriented to attack C-1 of the other to form an α -1 \rightarrow 6 linkage. The nucleophile that is released attacks another sucrose forming a new enzyme-glucosyl intermediate. The C-6-OH of this new glucosyl intermediate attacks C-1 of the isomaltosyl unit forming isomaltotriosyl enzyme intermediate and in effect is added to the reducing end of the growing dextran chain. The process continues in which the glucosyl and the growing dextranosyl chain are alternately transferred between the two nucleophiles as the dextran chain is elongated (see Fig. 2A). A similar mechanism can be postulated for mutansucrase, but with the glucosyl units being oriented so that C-3-OH attacks C-1 of the growing mutan chain to give α -1 \rightarrow 3 instead of α -1 \rightarrow 6 linkages (see Fig. 2B). For the mechanism of alternansucrase, it can be postulated that the two nucleophiles, X and Y, orient the glucosyl units so that X places the C-6-OH in position to attack C-1 and form an α -1 \rightarrow 6 linkage and Y places the C-3-OH in position to attack C-1 and form an α -1 \rightarrow 3 linkage. In this manner the alternating α -1 \rightarrow 6, α -1 \rightarrow 3 structure of alternan can be synthesized (see Fig. 2C). It should be noted that in D-glucopyranose, the configuration of both the C-6-OH and the C-3-OH are both on the same side of the pyranose ring and the different stereo-orientation of the two hydroxyl groups can be obtained by

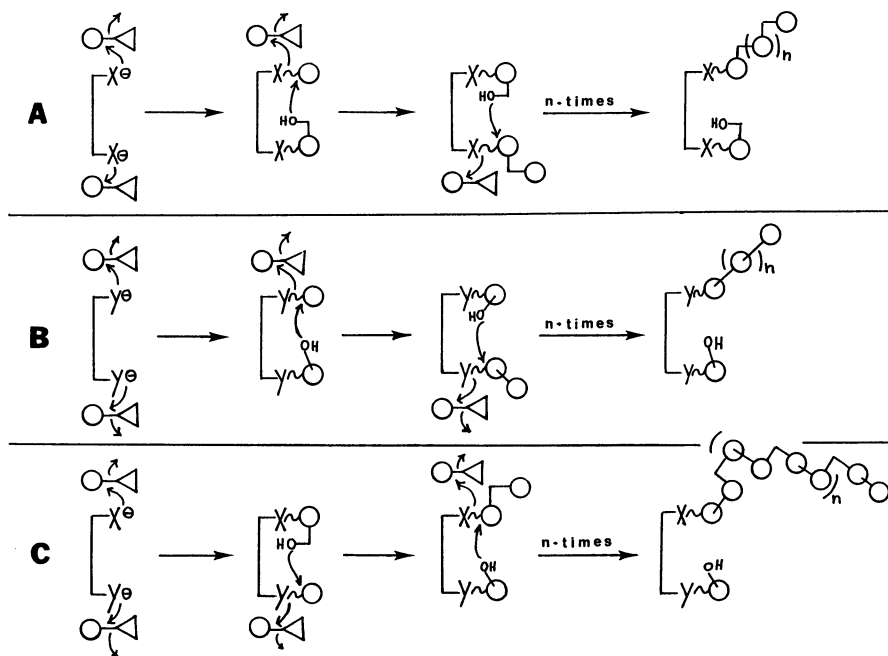







Fig. 2 Mechanisms proposed for the polymerization of glucans by glucansucrases by addition of glucose to the reducing end of the glucan chain.

- (A) Synthesis of dextran by B-512F dextransucrase.
 (B) Synthesis of mutan by mutansucrase.
 (C) Synthesis of alternan by alternansucrase.

Where  represents sucrose with  being glucopyranose and  being fructofuranose;  represents glucopyranose residue attached to glucose residue by an α -1 \rightarrow 6 linkage;  represents glucopyranose residue attached to glucose residue by an α -1 \rightarrow 3 linkage; X^- represents a nucleophile at the active-site that when attached to glucopyranose orients the glucopyranose residue so that its C-6-OH is in position to attack C-1 of the other glucopyranosyl residue to form an α -1 \rightarrow 6 linkage; Y^- also represents a nucleophile at the active-site that when attached to glucopyranose orients the glucopyranose residue so that its C-3-OH is in position to attack C-1 of the other glucopyranosyl residue to form an α -1 \rightarrow 3 linkage.

rotation of the D-glucopyranose residue 180° in the plane of the ring.

Besides synthesis of glucans these enzymes also catalyze reactions called "acceptor reactions". Acceptor reactions occur when other carbohydrates, besides sucrose, are present or are added to the digests. When the glucan being synthesized is itself the acceptor, a branch linkage is formed. This was demonstrated for B-512F dextranucrase using labeled, immobilized enzyme to which a low molecular weight, nonlabeled dextran was added (11). It was shown that the labeled dextran, attached to the enzyme, was transferred to the nonlabeled, added dextran, forming an α -1 \rightarrow 3 branch linkage. Fig. 3A shows the mechanism for the synthesis of branch linkages by the acceptor reaction of a glucan chain.

Many other acceptor reactions occur when low molecular weight carbohydrates are added to the reaction digest. These acceptors react with different efficiencies to give different kinds of products (14). The best known acceptor is maltose, followed by isomaltose, nigerose, α -methyl-D-glucopyranoside, D-glucose, lactose, cellobiose in decreasing order of their efficiencies. Table I gives the relative efficiencies of 17 acceptors. For many of these acceptors, glucose is transferred from the enzyme to the C-6-OH group at the nonreducing-end to form a product that is also an acceptor. This results in a homologous series of acceptor products in which isomaltosyl chains of varying length are attached to C-6 of the non-reducing-end of the acceptor (13).

Other acceptors, such as D-fructopyranose, lactose, melibiose, D-mannopyranose, and D-galactofuranose, only a single product is formed. The resulting products have unusual structures. D-fructopyranose gives the disaccharide, leucrose, in which the glucose is transferred to C-5 (15). Lactose gives a trisaccharide in which the glucose is transferred to C-2 of the reducing glucose residue (16). This is in contrast to the isomer of lactose, cellobiose, which also has the glucose transferred to C-2 of the reducing glucose residue (17), but this transferred glucose residue can act as an acceptor to give a homologous series with isomaltosyl chains attached to C-2 (14). Thus, the inversion of the hydroxyl group at C-4 on the non-reducing-end glucose residue of cellobiose to give lactose affects the binding of lactose so that its acceptor product cannot act as an acceptor to give a homologous series as can cellobiose. This same kind of binding impediment is also observed for melibiose and raffinose both of which contain D-galactose as part of their structure. The cause of this binding specificity awaits further study and interpretation.

D-mannose and D-galactose also give unusual acceptor products both of which are nonreducing sugars (18). D-mannose gives α -D-glucopyranosyl-1 \rightarrow 1- β -D-mannopyranoside, an isomer of α , β -trehalose and D-galactose gives α -D-glucopyranosyl-1 \rightarrow 1- β -D-galactofuranoside, an isomer of sucrose (see Fig. 4 for these structures).

The mechanism for the acceptor reactions involve the binding of the acceptor at the active-site in such a manner that one of their hydroxyl groups is specifically oriented to make a nucleophilic attack onto C-1 of the enzyme linked glucosyl unit or dextranosyl

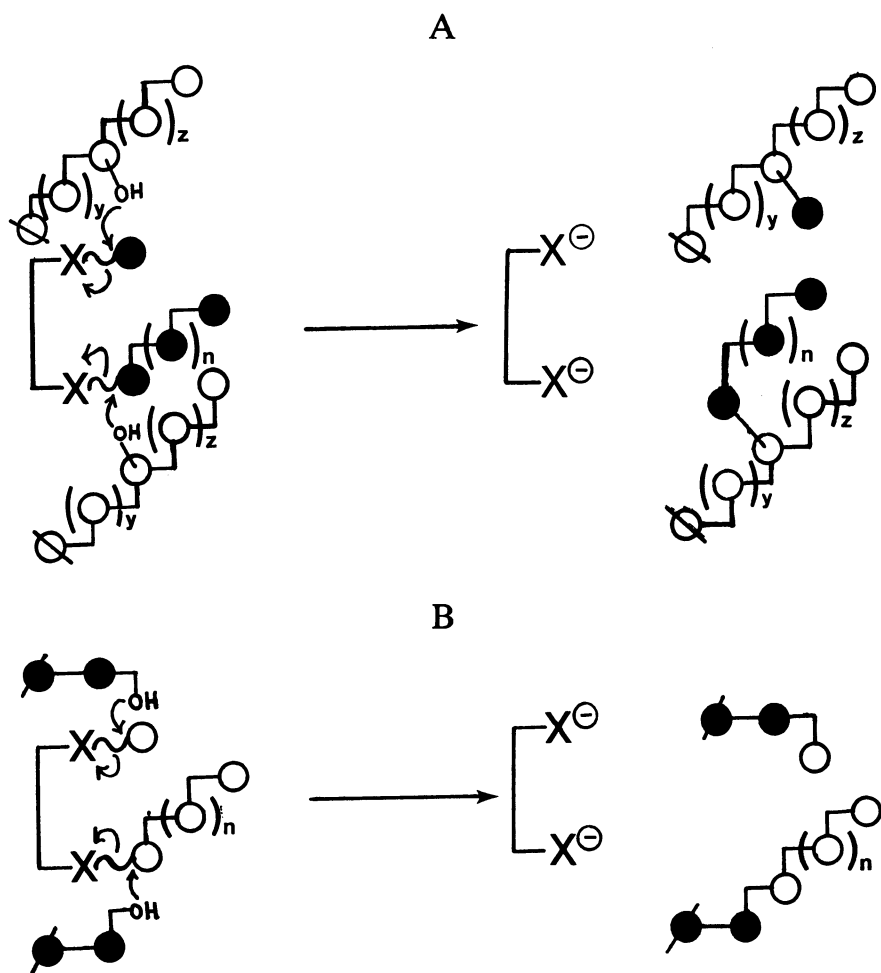


Fig. 3 Mechanisms for the synthesis of branch linkages and for acceptor reactions of B-512F dextranase.

- (A) Synthesis of α -1 \rightarrow 3 branch linkages by acceptor reactions of exogenous dextran.
- (B) Acceptor reactions of maltose

Symbols are the same as those defined for Figures 1 and 2.

**Table I Quantitative Effects of Acceptors in the B-512F
Dextranucrase Reaction**

<i>Acceptor</i>	<i>% Glucose incorporated into acceptors</i>	<i>% Glucose incorporated into dextran</i>	<i>No. of acceptor products</i>	<i>Relative acceptor efficiency^a</i>
Maltose	80.7	19.3	5	100.0
Isomaltose	71.9	28.1	6	88.7
Nigerose	47.4	52.6	6	58.1
α -Me-D-Glc	42.3	57.7	7	51.5
1,5-anhydro- D-glucitol	24.7	75.3	7	29.9
D-glucose	14.2	85.8	7	17.1
Turanose	11.2	88.8	7	13.4
β -Me-D-Glc	10.2	89.8	4	12.3
Lactose	9.0	91.0	1	10.7
Cellobiose	7.5	92.5	4	9.0
D-Fructose	5.3	94.7	1	6.4
Raffinose	3.7	96.3	1	4.4
Melibiose	3.5	96.5	1	4.2
L-sorbose	2.6	97.4	4	3.2
D-Mannose	2.4	97.6	1	2.9
D-Galactose	1.4	98.6	1	1.7
D-Xylose	0.4	99.6	1	0.5

^aRelative efficiency determined by comparing the amount of dextran synthesized in the presence of each acceptor to the amount synthesized in the presence of maltose and assigning 100% efficiency to maltose.

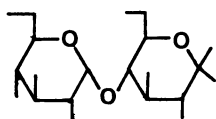
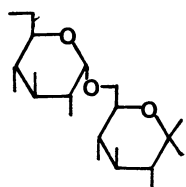
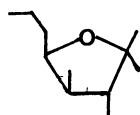
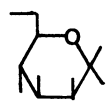
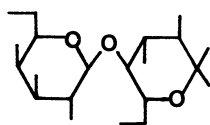
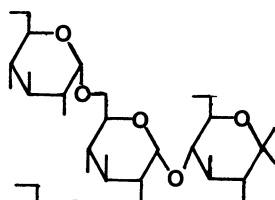
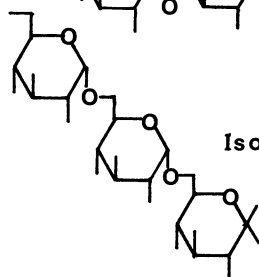
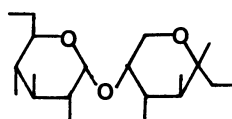
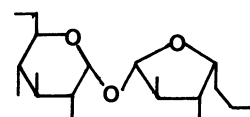
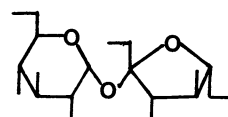
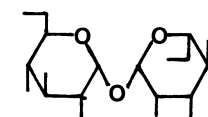
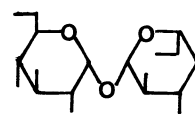
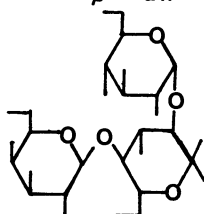
Acceptor**Product****Maltose****Isomaltose****Fructopyranose****Galactofuranose****Mannose****Lactose****Panose****Isomaltotriose****Leucrose****Analogue** **α -Glc-p β -Gal-f****Sucrose** **α -Glc β -Man** **α,β -Trehalose** **α -Glc-(1 \rightarrow 2)-Lactose**

Fig. 4 Structures of some acceptor products of B-512F dextran sucrose.

chain. This results in the release of glucose and/or dextran from the active-site and the formation of an α -glucosidic linkage between the acceptor and glucose or dextran (13). See Fig. 3B for the mechanism of the acceptor reaction.

α -Methyl-D-glucopyranoside is a more efficient acceptor than D-glucose, which in turn is a better acceptor than β -methyl-D-glucopyranoside (14). The acceptor binding specificity for monosaccharides has been studied using α -methyl-D-glucopyranoside analogues, modified at C-2, C-3, and C-4 positions by (a) inversion of the hydroxyl groups and (b) replacement of the hydroxyl group with hydrogen (19). The relative effectiveness of each of the analogues is given in Table II along with the position of attachment of the glucose residue in the acceptor product. D-Glucopyranose is transferred to the C-6-OH of the analogues so modified at C-2 and C-3; it is transferred to C-4-OH of the 4-inverted analogue, and to the C-3-OH of the 4-deoxy-analogue. The relative efficiency of the acceptor reactions with these analogues indicates that the hydroxyl group at C-2 is not as important for acceptor binding as the hydroxyl groups at C-3 and C-4. The hydroxyl group at C-4 is particularly important as it determines the binding orientation of the acceptor pyranose ring.

An interesting result is obtained for C-6 modified analogues, α -methyl-6-deoxy-D-glucopyranoside and α -methyl-6-deoxy-6-fluoro-D-glucopyranoside, neither of which are either acceptors or inhibitors of dextran synthesis. It is concluded that neither are bound at all (19). This observation indicates that the acceptor binding site is distinct from the sucrose binding site as both 6-deoxy- and 6-fluoro-sucrose are potent competitive inhibitors for dextran synthesis (20). If the 6-modified α -methyl-D-glucopyranosides were bound at the sucrose site, it would be expected that the 6-deoxy- and 6-fluoro-analogues of α -methyl-D-glucopyranoside would also be potent inhibitors.

Water also is an acceptor giving approximately 7% hydrolysis of sucrose (14). The first acceptor product for the reaction with maltose is the trisaccharide, panose (6^2 - α -glucopyranosyl maltose) (13,21). The amount of dextran synthesized decreases exponentially as the ratio of maltose to sucrose increases (14) and the number of homologous products also decrease (*Su, D.-1.; Fu, D., and Robyt, J.F., unpublished results*). The use of relatively high concentrations of maltose in the reaction digest has been used as a preparative method for panose (21). Similarly, high concentrations of D-fructose in the reaction digest has been used to prepare leucrose (15).

The structures of the products resulting from the reaction of the higher maltodextrins, G3--G8, have been determined (*Fu, D.; Robyt, J.F., unpublished results*). Each of these maltodextrins gave two products: the first, and major product, had the glucose attached to C-6 of the nonreducing glucose residue and the second, had the glucose attached to C-6 of the reducing glucose residue. The former products were also acceptors to give a homologous series with isomaltosyl chains of various sizes attached to C-6 of the nonreducing glucose residue of the maltodextrins, while the acceptor

Table II Quantitative Effects of α -Methyl-D-glucopyranoside Analogues as Acceptors in the B-512F Dextranase Reaction

Acceptor	% Glucose incorporated into acceptor	% Glucose incorporated into dextran	Position of linkage	No. of Acceptor product	Relative efficiency ^a
α -Me-Glc-p	43.4	55.4	C-6	10	100.0
2-deoxy- α -Me-Glc-p	32.0	67.3	C-6	7	73.7
α -Me-Man-p	18.2	80.4	C-6	6	42.0
3-deoxy- α -Me-Glc-p	16.9	81.7	C-6	8	39.0
α -Me-All-p	6.2	92.5	C-6	5	14.2
α -Me-Gal-p	4.0	94.2	C-4	1	9.2
4-deoxy- α -Me-Glc-p	2.9	95.5	C-3	2	6.6
6-deoxy- α -Me-Glc-p	0.0	100.0	-	-	0.0
6-fluoro- α -Me-Glc-p	0.0	100.0	-	-	0.0
α -Me-Xyl-p	0.0	100.0	-	-	0.0

^aRelative efficiency determined by comparing the amount of dextran synthesized in the presence of each analogue to the amount synthesized in the presence of α -methyl-D-glucopyranoside and assigning 100% efficiency to α -methyl-D-glucopyranoside.

product with glucose attached to C-6 of the reducing glucose residue were very poor acceptors, if they were acceptors at all. The efficiency of the maltodextrins as acceptors decreases as the chain length increases. With the efficiency of maltose being assigned 100%, the efficiency of maltotriose drops to 40%, and maltooctose to only 6% (Fu, D.; Robyt, J.F., unpublished results). Going to larger maltodextrin chains, it would be expected that the efficiency is even lower. Attempts to form acceptor products with starch have been unsuccessful. The maltodextrin acceptors are unusual in that both the nonreducing glucose residue and the reducing glucose residue are sites for glucose transfer from the enzyme. This means that an asymmetric maltodextrin molecule can be bound in the acceptor site at either end and that the binding of the maltodextrin is in such a manner that it is not allowed to "slide" along the binding site and accept the transfer of glucose at any of the interior glucose residues. This is in contrast to dextran chains that can accept glucose and glucan at interior glucose residues to give a branched dextran product (11).

The reaction of maltose as an acceptor with alternansucrase gave panose as the first product. There were two acceptor products formed from panose: (a) the transfer of glucose to C-6 of the nonreducing residue to give 6²- α -isomaltosyl maltose and (b) the transfer of glucose to C-3 of the nonreducing residue to give 6²- α -nigerosyl maltose. The latter product is an unusual structure that has a sequence of glycosidic linkages from the reducing-end of (α -1 \rightarrow 4), (α -1 \rightarrow 6), and (α -1 \rightarrow 3) (22). Isomaltose also gave two products in which (a) glucose was transferred to C-6 of the nonreducing-end to give isomaltotriose and (b) glucose was transferred to C-3 of the nonreducing-end to give 3²- α -glucopyranosyl isomaltose. The specificity of alternansucrase for acceptor reactions, thus, requires first the formation of an α -1 \rightarrow 6 linkage before the formation of an α -1 \rightarrow 3 linkage, but the presence of an α -1 \rightarrow 6 does not preclude the formation of an α -1 \rightarrow 6 linkage.

Recently, it has been reported that a low molecular weight alternan, with lowered viscosity, can be produced by conducting the synthesis of alternan in the presence of D-glucose and endodextranase (23). The latter prevents the formation of dextran by contaminating dextranucrase and the former decreases the molecular size of the alternan by acceptor reactions that terminates alternan polymerization.

B-512F Dextranucrase has also been used in conjunction with endodextranase to produce a product with a high content of isomaltose and D-fructose (24). The D-fructose can be removed by a Ca-cation exchange resin, giving an isomaltose syrup. The isomaltose can then be catalytically reduced to isomaltitol, which is sweet and of low caloric value.

In this paper, it has been shown that glucansucrases with different synthetic specificities can synthesize glucans with different structures and can synthesize oligosaccharides with different structures by acceptor reactions. The mechanisms by which these enzymes carry out the polymerization and by which the acceptor reactions occur has been discussed. The products resulting from the

action of these enzymes are potentially new products that could be produced from the biomass material, sucrose, and could have new uses and applications.

Literature Cited

1. Jeanes, A.; Haynes, W. C.; Wilham, C. A.; Rankin, J. C.; Melvin, E. H.; Austin, M. J.; Cluskey, J. E.; Fisher, B. E.; Tuschya, H. M.; Rist, C. E., *J. Amer. Chem. Soc.* 1954,76,5041-4.
2. Wilham, C. A.; Alexaqnder, B. H.; Jeanes, A., *Arch. Biochem. Biophys.* 1955,59,61-6.
3. Robyt, J. F., "Dextran", in *Encyclopedia of Polymer Science and Engineering*, H. F. Mark, N. M. Bikales, C. G. Overberger, and G. Menges, Eds. 2nd Ed.; John Wiley & Sons, NY 1986, Vol. 4; 752-767.
4. Shimamura, A.; Tsumori, H.; Mukasa, H., *Biochim. Biophys. Acta* 1982,702,72-8.
5. Robyt, J. F.; Martin, P. J., *Carbohydr. Res.* 1983,113,301-15.
6. Cote, G. L.; Robyt, J. F., *Carbohydr. Res.* 1982,101,57-74.
7. Robyt, J. F.; Walseth, T. F., *Carbohydr. Res.* 1979,68,95-111.
8. Miller, A. W.; Eklund, S. H.; Robyt, J. F., *Carbohydr. Res.* 1986,147,119-33.
9. Fu, D.; Robyt, J. F., *Prep. Biochem.* in press 1990.
10. Robyt, J. F.; Kimble, B. K.; Walseth, T. F., *Arch. Biochem. Biophys.* 1974,165,634-40.
11. Robyt, J. F.; Taniguchi, H., *Arch. Biochem. Biophys.* 1976,174,129-34.
12. Robyt, J. F.; Corrigan, A. J., *Arch. Biochem. Biophys.* 1977,183,726-31.
13. Robyt, J. F.; Walseth, T. F., *Carbohydr. Res.* 1978,61,433-45.
14. Robyt, J. F.; Eklund, S. H., *Carbohydr. Res.* 1983,121,279-86.
15. Stodola, F. H.; Sharpe, E. S.; Koepsell, H. J., *J. Amer. Chem. Soc.* 1956,78,2541-8.
16. Bourne, E. J.; Harrington, J.; Weigel, H., *J. Chem. Soc.* 1959,2332-6.
17. Bourne, E. J.; Harrington, J.; Weigel, H., *J. Chem. Soc.* 1961,1088-92.
18. Iriki, Y.; Hehre, E. J., *Arch. Biochem. Biophys.* 1969,134,130-6.
19. Fu, D.; Slodki, M. E.; Robyt, J. F., *Arch. Biochem. Biophys.* 1990,276,460-5.
20. Eklund, S. H.; Robyt, J. F., *Carbohydr. Res.* 1988,177,253-8.
21. Killey, M.; Dimler, R. J.; Cluskey, J. E., *J. Amer. Chem. Soc.* 1955,77,3315-8.
22. Cote, G. L.; Robyt, J. F., *Carbohydr. Res.* 1982,111,127-42.
23. Cote, G. L., *Abst. Papers, 199th Amer. Chem. Soc. Meeting, 1990, No. 49 CARB, Boston, MA.*
24. Paul, F. B.; Monsan, P. F.; Remand, M. M. C.; Pelenc, V. P., *U.S. Patent No. 4,861,381,1989.*

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Chapter 31

Biotechnological Potential and Production of Xylanolytic Systems Free of Cellulases

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Xylanolytic enzymes free of cellulases can be applied in the pulp and paper, textile, and food industries and in basic research. However, most microorganisms grown under natural conditions produce both xylanases and cellulases. Strategies to produce xylanolytic systems free of cellulases are elimination of cellulase activity by separation or inhibition, selection and construction of cellulase-negative strains, and finding conditions for separate production of xylanolytic systems by cellulolytic strains.

The last five years have seen growing interest in microbial xylanolytic systems free of cellulases. The reason is that such systems can be applied in processes where xylan is to be removed from cellulose with cellulose fiber length being preserved. These include mainly the purification of pulp and modification of its paper-making properties. In 1984 Paice and Jurasek (1) suggested the application of xylanases free of cellulases for removal of xylan from chemical pulp in the production of high-quality dissolving pulp. The intention was to replace the ecologically harmful alkaline extraction of hemicellulose with an enzymic treatment, leading to a mixture of fermentable sugars instead of polluting waste liquors. Xylanolytic enzymes attracted further attention after Viikari *et al.* (2,3) announced that xylanases may facilitate chemical extractibility of lignin from crude pulp by their hydrolysis of xylan in lignin-xylan complexes, leading to a significant saving of chemicals used for bleaching. Other important information was that a controlled xylanase treatment of pulp enhances its beatability and binding ability, thus affecting its paper-making properties (4,5). The pulp and paper industry's interest in xylanolytic enzyme systems should increase with growing pressure of governments and "green" initiatives to reduce or replace the ecologically harmful technologies of pulp bleaching.

In addition to the pulp and paper industry, xylanases free of cellulases may be applied in the textile and food industries and in basic research. They can be applied together with pectinases in the processing of plant fiber sources such as flax and hemp. Classical fiber liberation is caused by natural retting *in situ* by microorganisms, leading to the removal of pectin and hemicellulose binding material. Pectinases are believed to play the main role in the process, but xylanases may also be involved. Replacement of slow natural retting, which is strongly dependent on weather conditions, by controlled enzyme retting using defined enzyme mixtures may lead to new fiber lib-

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eration technology. Expected advantages of enzyme retting are high reproducibility, better yield, and higher fiber strength, caused by a higher degree of cellulose polymerization (6).

The application of xylanases in the food industry should not so strictly require the absence of cellulases. Xylanases can be used for modification of bakery products by affecting dough development (7) and by changing rheological properties of cooked rice (8). Xylanases have been used also in the starch and gluten processing industry. Treatment of flour slurries with xylanase decreases viscosity and allows easier separation of small starch granules and gluten (7,9). Together with pectinases, xylan-degrading enzymes can be used to dissolve of precipitates in fruit juices (Pokorny, M., KRKA Pharmaceuticals, Yugoslavia, personal communication, 1989).

Pure xylanases can be useful in basic research. They find application in analytical procedures designed to quantify specific xylan constituents (10) and to study xylan structure. They can be used for preparation of defined xylooligosaccharides, either by hydrolysis of xylan (11-14) or by transglycosylation at high substrate concentrations (15,16). These products may serve as assay substrates and model compounds to study the mechanism of action of xylanolytic enzymes. Finally, xylanolytic enzymes play an important role in complex enzyme preparations used for the release of plant protoplasts.

Strategies for Xylanolytic Systems Free of Cellulases

Most microorganisms grown under natural conditions produce both cellulases and xylanases. Biochemistry, microbiology, and molecular biology offer several approaches to obtain xylanase preparations largely or completely free of cellulases (17).

Elimination of Cellulases from Xylanases. Classical methods of protein fractionation can be used for to separate cellulases and xylanases on a large scale only when they differ considerably in molecular weight or isoelectric point. The *Trichoderma harzianum* enzymes were separated by ultrafiltration because the xylanase was smaller and passed through the membrane into the ultrafiltrate (18). Fractional precipitation with organic solvents is another possibility (1).

Cellulases can also be eliminated from a mixture with xylanases by selective thermal inactivation. Cellulases are more thermolabile than xylanases in the cellulolytic systems of the fungus Y-94 (19), *T. harzianum* (20), and *Thermoascus aurantiacus* (17), but not in the *Trichoderma reesei* system (Biely, P. and Vrsanska, M., Slovak Academy of Sciences, Bratislava, unpublished results). Since cellulase thermal inactivation causes a significant loss of xylanase also, a more convenient way to eliminate cellulase activity is by selective chemical or biological inhibition or inactivation. There appear, however, to be no reports on the existence of natural inhibitors that would be specific for cellulases. Such inhibitors of amylases and pectinases are known to occur in plants (21).

Selective inhibition of cellulases by Hg^{2+} in a mixture of cellulases and xylanases of *Sporotrichum dimorphosporum* used in a laboratory experiments (22) cannot be considered for industrial application for obvious reasons.

Specific active-site directed inactivation of cellulases can be achieved using 4,5-epoxy pentyl β -D-glycosides of celooligosaccharides, the cellobioside being the most efficient in the inactivating the cellulases (23,24). The derivatizing agents appear to esterify the carboxylate groups of acidic amino acids. The specificity of the reaction was documented by the fact that the control compound 1,2-epoxyhexane did not affect the activity of *Schizophyllum commune* endo-(1 \rightarrow 4)- β -glucanase (24).

Non-Cellulolytic Xylanolytic Microorganisms. Despite the fact that xylan never occurs in nature in the absence of cellulose, there are microorganisms that produce only xylanolytic systems. One may speculate that such microorganisms are sym-

biotic with cellulolytic ones. Since cellulase production has been examined in only a minority of xylanolytic microbes, in this section only those xylanolytic strains that are unequivocally cellulase-negative will be mentioned.

Xylanolytic strains that do not produce cellulases, as well as xylanases that do not attack cellulose, can be found among yeasts and yeast-like microorganisms (Table I).

Table I. Xylanolytic Yeasts

<i>Genus or species</i>	<i>Note</i>	<i>Reference</i>
<i>Aureobasidium pullulans</i>	^a EG-positive	25,26
<i>A. pullulans</i> Y-2311-1	Xylanase hyperproducer	26
<i>Candida</i> sp.		27
<i>Candida shehatae</i>		27
<i>Cryptococcus</i>		25-27
<i>Pichia stipitis</i>		27
<i>Trichosporon cutaneum</i>	^a EG-positive	25,28

^aEndo-(1→4)-β-glucanase positive.

However, some of the species give a positive test for endo-(1→4)-β-glucanase activity although they do not grow on cellulose or its soluble derivatives (26-28). It should be noted that the pathogenic yeast *Cryptococcus neoformans* does not belong to the xylanase-positive family of the *Cryptococcus* strains (Biely, P., unpublished results). Unfortunately, xylanase production by yeasts is generally low compared to that of filamentous fungi. Exceptions are color variants of *Aureobasidium pullulans* Y-2311, which hyperproduce extracellular xylanase (26).

Other non-cellulolytic xylanase producers are *Butyrivibrio fibrisolvens* (29) and a thermotolerant *Streptomyces* T₇ strain (30).

Cellulase-Negative Xylanase-Positive Mutants. There are two reports concerning the selection of such mutants from filamentous fungi, one on *Polyporus adustus* (31) and the other on *Trichoderma reesei* (Durand, H. *et al.*, Societé CAYLA, Toulouse, France, unpublished results). An analysis of the eliminated cellulase genes has not been done, so it is not known if the mutants negative in endo-(1→4)-β-glucanase were deficient also in cellobiohydrolases.

The production of cellulase-negative xylanase-positive mutants is much easier from prokaryotic microorganisms that produce less complex enzyme systems and do not have such complicated genetic material. An efficient cellulase-negative xylanase-producing strain of *Streptomyces lividans* was obtained by nitrosoguanidine mutagenesis (32). A double cellulase- and xylanase-negative mutant has also been isolated from the same strain (33). The double mutant was transformed to a xylanase-positive one by returning the xylanase gene from the parent *S. lividans* strain. The newly constructed transformant gave a considerable overproduction of inducible xylanase (34).

Mutants of *Polyporus adustus* (31), *T. viride* (35,36), and *T. reesei* (Durand, H. *et al.*, unpublished results) that produced neither cellulases nor xylanases have been reported. With the *P. adustus* mutant it was shown that the pleiotropic mutation was not due to a deletion of cellulase and xylanase genes, but rather to a deficiency of a single regulatory gene that governs the induction of both cellulase and xylanase (31). A similar interpretation was advanced in the case of a *T. viride* mutant lacking the ability to hydrolyze soluble and crystalline cellulose and xylan (36). A defect in a general ability to secrete proteins could be an alternative to explain the phenotypes. Whether the cellulase- and xylanase-negative mutants of filamentous fungi become candidates

for the introduction of xylanase genes will depend on their ability to harbor and express them. In this context, the efficient host-vector system for genetic modification of *T. reesei* developed by Penttilä *et al.* (37) should be mentioned.

Cloning of Xylanase Genes. Recombinant DNA techniques offer great opportunities for obtaining microbial producers of cellulase-free xylanases. The strategy is simply to clone a xylanase gene in a cellulase-negative host. Cloning techniques also enable the multiplication of the expression of genes already present. The production of xylanase in *Bacillus subtilis* was enhanced several times using a plasmid vector carrying the *Bacillus pumilus* gene (38).

The literature contains numerous examples of cloning xylanase genes with bacterial gene donors and acceptors (Table II). In most cases strains of *Escherichia*

Table II. Cloning of Xylanase Genes

<i>Gene donor</i>	<i>Enzyme localization in the host and other notes</i>	<i>Ref.</i>
Bacilli		
<i>Bacillus pumilus</i>	intracellular	39
<i>B. subtilis</i>	intracellular	40
<i>B. pumilus</i> (a)	intracellular	38
<i>B. subtilis</i>	secreted and periplasmic	41
<i>B. polymyxa</i>	cell-bound	42
<i>B. polymyxa</i>	periplasmic	43
<i>Bacillus</i> C-125 (alkalophil.)	extracellular	44
<i>Bacillus</i> C-125 (alkalophil.)	extracellular (secretory vector)	45
<i>B. circulans</i>	intracellular	46
<i>B. subtilis</i> (b)	intracellular	47
Thermophilic bacteria		
<i>Aeromonas</i>	intracellular	48
<i>Aeromonas</i>	secreted	49
<i>Bacteroides succinogenes</i>	intracellular (high expression)	50
<i>Clostridium acetobutylicum</i>	intracellular (high expression)	51
<i>Clostridium thermocellum</i>	xylanase Z active on 4-methylumbelliferyl β-D-cellobioside	52
<i>Clostridium thermocellum</i>	three xylanase genes	53
<i>Caldocellum saccharolyticum</i>	intracellular	54
Other strains		
<i>Pseudomonas fluorescens</i>	periplasmic, active on 4-methylumbelliferyl β-D-cellobioside	55
<i>Pseudomonas fluorescens</i> (c)	cell-bound	55
<i>Ruminococcus albus</i>	intracellular, non-specific	56
<i>Cryptococcus albidus</i>	intracellular (eukaryotic donor)	57

Recipients of xylanase genes other than standard *E. coli*: (a) *B. subtilis*; (b) "leaky" mutant of *E. coli*; (c) *Pseudomonas putida*.

coli were used as recipients. In xylanase-positive transformants the products of the expressed genes were only rarely secreted out of the cells, and in a majority of cases they remained localized either intracellularly or in the periplasmic space (Table II). The problems with enzyme secretion from bacterial hosts can be overcome by using "leaky"

mutants of *E. coli*, which lyse spontaneously at elevated temperatures, as the hosts (47). Secretion of the expressed gene products can occasionally be achieved by more complicated cloning strategies, which have to be developed for each particular protein. It happens frequently that even when the gene cloned in *E. coli* is provided with the segment coding for the secretory signal peptide, the expressed gene product stays imprisoned in the periplasmic space. However, there are ways to achieve the extracellular production of foreign proteins by *E. coli*. One possibility is the fusion of the foreign proteins with some outer membrane proteins (58) or the use of the export system of colicins and hemolysins (59). These approaches have not yet been applied in cloning of xylanase genes. Hamamoto and Horikoshi (45) achieved the secretion of an alkaline xylanase in *E. coli* by constructing a special secretion vector. Even this vector did not work with some other proteins.

Special attention has been paid by several groups to cloning xylanase genes from thermophilic microorganisms as a source of thermostable xylanases. Cloning of such genes in mesophilic recipients offers a convenient way to purify xylanases simply by a heat denaturation of the more heat-labile proteins of the host (54). Applications are limited to those enzymes that possess thermostability considerably higher than the majority of the host cell proteins.

Selective Production of Xylanases by Cellulolytic Microorganisms. Until recently there was little information on common or separate genetic control of cellulase and xylanase synthesis in microorganisms (60). Studies on this subject were complicated by the fact that numerous microbial cellulases and xylanases are non-specific with respect to cellulose and xylan as substrates. As could be expected from a comparison of both polysaccharide structures, non-specificity is more frequently observed with cellulases, because their substrate binding sites can easily accommodate substrate using an unsubstituted β -(1 \rightarrow 4)-linked chain of D-xylopyranosyl units.

Recent experimental evidence suggests that production of cellulases and xylanases is under separate regulatory control in several filamentous fungi. Selective production of xylanase can be achieved in *Trichoderma* and *Aspergillus* species during growth in media containing xylan as the only carbon source (61-67). On cellulose the strains produce both cellulases and xylanases. The reason for this may be that cellulose is always contaminated by xylan remnants. However, an exception is bacterial cellulose that proved to be a poor growth support for fungi (61). The degree of selectivity of formation of xylanases on xylan in *T. reesei* QM 9414 is dependent on the nitrogen/carbon ratio in the medium (61,62). The mechanisms that govern the formation of extracellular enzymes with respect to the carbon source present in the medium are influenced by the availability of precursors for protein synthesis. A shortage of such precursors causes a more strict separate regulation of the synthesis of cellulases and xylanases by available carbon sources. Therefore, the strategy for producing xylanolytic systems free of cellulases in some fungi might be to grow the cells on xylan not contaminated by cellulose, and under a lower nitrogen/carbon ratio in the medium. However, this strategy cannot be applied to all fungi. For instance, xylanase production in *S. commune* could not be separated from cellulase synthesis (62,68).

Unfortunately, pure xylan is an expensive carbon source for commercial-scale xylanase production. Therefore, several groups have tried to develop xylanase production on cheaper xylan-rich materials. The best candidates for the purpose appear to be water-soluble hemicellulose from steam-treated wood (63,69) and residues of annual plants like wheat bran (70).

Low-Molecular Weight Xylanase Inducers. The response of cellulolytic fungi to low-molecular weight fragments of xylan and cellulose confirmed the separate regulatory control of the formation of xylanases and cellulases. Xylobiose [Xylp- β -(1 \rightarrow 4)-Xylp] was a specific inducer of xylanases in *T. reesei* (61) and *Aspergillus terreus* (67). Sophorose and other glucobioses selectively induced the synthesis of

cellulases (61,67). The non-specific endo-(1→4)- β -glucanase (EG I) of *T. reesei* that attacks both cellulose and xylan is inducible by sophorose and not by xylobiose, supporting the conclusion that the enzyme is a component of the cellulolytic system of the fungus (61).

Xylan fragments induced xylanase also in non-cellulolytic microorganisms like *Streptomyces* sp. (71) and yeasts of the genus *Cryptococcus* and *Trichosporon* (72-74). In these strains xylanase could be efficiently induced also by methyl β -D-xylopyranoside, which is extremely slowly metabolized in the cells (72-74). The glycoside accelerated xylanase production in hyperproducing color variants of *A. pullulans* (75) and in several strains of *Aspergillus* (70). Low cost and easy preparation of methyl β -D-xylopyranoside favors its use for large-scale xylanase production.

Xylanolytic systems of yeasts and fungi can also be induced by positional isomers of xylobiose. Induction with Xylp- β -(1→2)-Xylp is analogous to the sophorose induction of cellulase in filamentous fungi (76). Xylp- β -(1→2)-Xylp and Xylp- β -(1→3)-Xylp induced xylanase in *C. albidus* (77), *Trichosporon cutaneum* (73), *A. pullulans* (78) and *A. terreus* (Hrmova, M. *et al.*, Slovak Academy of Sciences, Bratislava, submitted for publication, 1990). In *C. albidus* the positional isomers behave differently than does xylobiose (77), in that the response of the cells to them was slower but the enzyme yields were higher than in the presence of xylobiose. This indicated that isomeric xylobioses were not direct inducers. In agreement with this idea, both Xylp- β -(1→2)-Xylp and Xylp- β -(1→3)-Xylp were transformed to Xylp- β -(1→4)-Xylp, the natural inducer (79).

The variety of control mechanisms of xylanase synthesis is demonstrated by examples of the synthesis of xylanase induced by xylose (38,74,75). To a list of low-molecular weight xylanase inducers one can add 4-thioxylobiose (80,81) and 4-O- β -D-xylopyranosyl-L-arabinopyranose (73,77).

Future Directions

No doubt the cost of xylanolytic enzymes will be one of the factors determining their application in the pulp and paper industry as well as in other areas. Economically feasible xylanase production can be achieved in paper mills employing xylanase-positive transformants of common industrially used microorganisms that are capable of utilizing inexpensive carbon sources originating there. A substantial improvement in the production of xylanolytic systems can be expected from mutants of non-cellulolytic microorganisms that are resistant to catabolic repression. Such mutants usually exhibit hyperproduction of extracellular enzymes.

More investigation is needed to establish requirements for the properties of xylanolytic enzymes used for upgrading pulp. A question is whether the applied enzyme preparations should be complex xylanolytic systems or just a single endo-(1→4)- β -xylanase. The presence of β -xylosidase may not be required because the primary goal will not be xylose production. Other properties of xylanases may be of interest in connection with their application in the pulp-bleaching sequence. These include their molecular weights, thermostabilities, operational stabilities in alkaline or acidic media, and sizes of their substrate binding sites. Smaller xylanases may penetrate more easily into the pulp structure. Xylanases with smaller binding sites, *i.e.* smaller number of subsites, will be more efficient in the depolymerization of xylans substituted to a higher degree.

Further progress can be expected in the area of selective inhibition and inactivation of cellulases undesirable in xylanase preparations. There is a possibility of finding natural selective cellulase inhibitors or developing highly reactive derivatives of cellobiose and cellodextrins that inactivate cellulolytic enzymes.

Finally, attention should also be devoted to the elaboration of simple and reliable assays of xylanolytic enzymes that provide information on their efficiency in the application process.

Literature Cited

1. Paice, M. G.; Jurasek, L. *J. Wood Chem. Technol.* **1984**, *4*, 187-98.
2. Viikari, L.; Rauna, M.; Kantelinen, A.; Sundquist, J.; Linko, M. In *Proc. 3rd Int. Conf. Biotechnology in the Pulp and Paper Industry*; Eriksson, K.-E.; Ander, P., Eds.; STFI, Stockholm, 1986; pp 67-9.
3. Viikari, L.; Rauna, M.; Kantelinen, A.; Linko, M.; Sundquist, J. In *Proc. 4th Int. Conf. Wood and Pulping Chemistry*, Paris, 1987, Vol. I; pp 151-4.
4. Mora, F.; Comtat, J.; Barnoud, F.; Pla, F.; Noe, P. *J. Wood Sci. Technol.* **1986**, *6*, 147-65.
5. Noe, P.; Chevalier, J.; Mora, F.; Comtat, J. *J. Wood Sci. Technol.* **1986**, *6*, 167-84.
6. Van Sumere, C. F. *Proc. Danish Technical Days in Prague*, 1986; pp. 1-16.
7. McCleary, B. V. *Int. J. Biol. Macromol.* **1986**, *8*, 349-54.
8. Shibuya, N.; Iwasaki, T. *Nippon Shokuhin Kogyo Gakkaishi* **1984**, *31*, 656-60.
9. Wieg, A. J. *Starch/Stärke* **1984**, *36*, 135-40.
10. Puls, J.; Poutanen, K. *Proc. 4th Int. Conf. Wood and Pulping Chemistry*, Paris, 1987, Vol. I; pp. 211-4.
11. Timell, T. G. *Svensk Pappersindn.* **1962**, *65*, 435-47.
12. Kusakabe, I.; Yasui, T.; Kobayashi, T. *Agr. Biol. Chem.* **1975**, *39*, 1355-62.
13. Cavagna, F.; Deger, H.; Puls, J. *Carbohydr. Res.* **1984**, *129*, 1-8.
14. Kusakabe, I.; Ohgushi, S.; Yasui, T.; Kobayashi, T. *Agr. Biol. Chem.* **1983**, *47*, 2713-23.
15. Kratky, Z.; Biely, P.; Vrsanska, M. *Carbohydr. Res.* **1981**, *93*, 300-303.
16. Biely, P.; Vrsanska, M. *Eur. J. Biochem.* **1983**, *129*, 645-51.
17. Senior, D. J.; Mayers, P. R.; Saddler, J. N. In *Plant Cell Wall Polymers: Biogenesis and Biodegradation*; Lewis, N. G.; Paice, M. G., Eds.; Am. Chem. Soc. Symp. Ser. 399, Washington, D. C., 1989; pp 630-40.
18. Tan, L. U. L.; Yu, E. K. C.; Luis-Seize, G. W.; Saddler, J. N. *Biotechnol. Bioeng.* **1987**, *30*, 96-100.
19. Mitsuishi, Y.; Yamanobe, T.; Yagashiwa, M.; Takasaki, T. *Agr. Biol. Chem.* **1987**, *51*, 3207-13.
20. Todorovic, R.; Matavujl, M.; Grujic, S.; Petrovic, J. *Mikrobiologia* **1986**, *23*, 33-8.
21. Albersheim, P.; Anderson, A. J. *Proc. Nat. Acad. Sci. U.S.A.* **1971**, *68*, 1815-9.
22. Barnoud, F.; Comtat, J.; Joseleau, J. P.; Mora, F.; Ruel, K. *Proc. 3rd Int. Conf. Biotechnology in the Pulp and Paper Industry*; Eriksson, K.-E.; Ander, P., Eds.; STFI, Stockholm, 1986; pp 70-2.
23. Legler, G.; Bause, E. *Carbohydr. Res.* **1973**, *28*, 45-52.
24. Clarke, A. J.; Strating, H. *Carbohydr. Res.* **1989**, *188*, 245-50.
25. Biely, P.; Kratky, Z.; Kockova-Kratochvilova, A.; Bauer, S. *Folia Microbiol.* **1978**, *23*, 366-71.
26. Leathers, T. D.; Kurtzman, C. P.; Detroy, R. W. *Biotechnol. Bioeng. Symp.*, **1984**, *14*, 225-40.
27. Lee, H.; Biely, P.; Latta, R. K.; Barbosa, M. F. S.; Schneider, H. *Appl. Environ. Microbiol.* **1986**, *52*, 320-4.
28. Stevens, B. J. H.; Payne, J. *J. Gen. Microbiol.* **1977**, *100*, 381-93.
29. Van Der Toorn, J. J. T. K.; Van Gylswyk, N. O. *J. Gen. Microbiol.* **1985**, *131*, 2601-7.
30. Keskar, S. S.; Srinivasan, M. C.; Deshpande, V. V. *Biochem. J.* **1989**, *261*, 49-55.
31. Eriksson, K.-E.; Goodell, E. W. *Can. J. Microbiol.* **1974**, *20*, 371-8.
32. Mondou, F.; Shareck, F.; Morosoli, F.; Kluepfel, D. *Gene* **1986**, *49*, 323-9.

33. Morosoli, R.; Bertrand, J.-L.; Mondou, F.; Shareck, F.; Kluepfel, D. *Biochem. J.* **1986**, *239*, 587-92.
34. Bertrand, J.-L.; Morosoli, R.; Shareck, F.; Kluepfel, D. *Biotechnol. Bioeng.* **1989**, *33*, 791-4.
35. Mandels, M.; Weber, J.; Parizek, R. *Appl. Microbiol.* **1971**, *21*, 152-4.
36. Nevalainen, K. M. H.; Palva, E. T. *Appl. Environ. Microbiol.* **1978**, *35*, 11-16.
37. Penttilä, M.; Nevalainen, H.; Ratto, R.; Salminen, E.; Knowles, J. *Gene* **1987**, *61*, 155-64.
38. Panbangred, W.; Fukusaki, E.; Epifanio, E. C.; Shinmyo, A.; Okada, H. *Appl. Microbiol. Biotechnol.* **1985**, *22*, 259-64.
39. Panbangred, W.; Kondo, T.; Negoro, S.; Shinmyo, A.; Okada, H. *J. Mol. Gen. Genet.* **1983**, *192*, 334-41.
40. Bernier, R. Jr.; Driguez, H.; Desrochers, M. *Gene* **1983**, *26*, 59-65.
41. Paice, M. G.; Bernier, R., Jr.; Jurasek, L. *Biotechnol. Bioeng.* **1988**, *32*, 235-9.
42. Sandhu, J. S.; Kennedy, J. F. *Enzyme Microb. Technol.* **1984**, *6*, 271-4.
43. Yang, R. C. A.; MacKenzie, C. R.; Bilous, D.; Seligi, V.; Narang, S. A. *Appl. Environ. Microbiol.* **1988**, *54*, 1023-9.
44. Honda, H.; Kudo, T.; Horikoshi, K. *J. Bacteriol.* **1985**, *161*, 784-5.
45. Hamamoto, T.; Horikoshi, K. *Agr. Biol. Chem.* **1987**, *51*, 3133-5.
46. Yang, R. C.; MacKenzie, C. R.; Bilous, D.; Narang, S. A. *Appl. Environ. Microbiol.* **1989**, *55*, 1192-5.
47. Pechan, P.; Barak, I.; Jucovic, M.; Biely, P.; Timko, J. *Biológia (Bratislava)* **1989**, *44*, 1137-45.
48. Kudo, T.; Ohnishi, A.; Horikoshi, K. *J. Gen. Microbiol.* **1985**, *131*, 2825-30.
49. Kato, C.; Kobayashi, T.; Kudo, T.; Horikoshi, K. *FEMS Microbiol. Lett.* **1986**, *36*, 31-4.
50. Sipat, A.; Taylor, K. A.; Lo, R. Y. C.; Forsberg, C. W.; Krell, P. J. *Appl. Environ. Microbiol.* **1987**, *53*, 477-81.
51. Zappe, H.; Jones, D. T.; Woods, D. R. *Appl. Microbiol. Biotechnol.* **1987**, *27*, 57-63.
52. Grepinet, O.; Chebron, M.-C.; Beguin, P. *J. Bacteriol.* **1988**, *170*, 4582-8.
53. MacKenzie, C. R.; Yang, R. C. A.; Patel, G. P.; Bilous, D.; Narang, S. A. *Arch. Microbiol.* **1989**, *152*, 377-81.
54. Patchett, M. L.; Neal, T. L.; Schofield, L. R.; Strange, R. C.; Daniel, R. M.; Morgan, H. W. *Enzyme Microb. Technol.* **1989**, *11*, 113-5.
55. Gilbert, H. J.; Sullivan, D. A.; Jenkins, G.; Kellett, L. E.; Minton, N. P.; Hall, J. *J. Gen. Microbiol.* **1988**, *134*, 3239-47.
56. Romaniac, M. P.; Davidson, K.; White, B. A.; Hazlewood, G. P. *Lett. Appl. Microbiol.* **1989**, *9*, 101-4.
57. Morosoli, R.; Durand, S. *FEMS Microbiol. Lett.* **1988**, *51*, 217-24.
58. Nagahari, K.; Kanaya, S.; Munakata, M.; Aoyagi, K.; Mizushima, S. *EMBO J.* **1985**, *4*, 3589-92.
59. Kobayashi, T.; Kato, C.; Kudo, T.; Horikoshi, K. *J. Bacteriol.* **1986**, *166*, 728-32.
60. Dekker, R. F. H.; Richards, G. N. *Adv. Carbohydr. Chem. Biochem.* **1976**, *32*, 277-352.
61. Hrmova, M.; Biely, P.; Vrsanska, M. *Arch. Microbiol.* **1986**, *144*, 307-11.
62. Biely, P.; MacKenzie, C. R.; Schneider, H. *Can. J. Microbiol.* **1988**, *34*, 767-72.
63. Senior, D. J.; Mayers, P. R.; Saddler, J. N. *Appl. Microbiol. Biotechnol.* **1989**, *32*, 137-42.
64. Royer, J. C.; Nakas, J. P. *Enzyme Microb. Technol.* **1989**, *11*, 405-10.

65. Eriksson, K.-E.; Rzedowski, W. *Arch. Biochem. Biophys.* **1969**, *129*, 683-8.
66. Stewart, J. C.; Lester, A.; Milburn, B.; Parry, J. B. *Biotechnol. Lett.* **1983**, *5*, 543-8.
67. Hrmova, M.; Biely, P.; Vrsanska, M. *Enzyme Microb. Technol.* **1989**, *11*, 610-16.
68. Steiner, W.; Lafferty, R. M.; Gomes, I.; Esterbauer, H. *Biotechnol. Bioeng.* **1987**, *30*, 169-78.
69. Schmidt, O.; Puls, J.; Sinner, M.; Dietrichs, H. H. *Holzforschung* **1979**, *33*, 192-6.
70. Bailey, M.; Poutanen, K. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 5-10.
71. Nakanishi, K.; Yasui, T. *Agr. Biol. Chem.* **1980**, *44*, 2729-30.
72. Biely, P.; Krátky, Z.; Vrsanska, M.; Urmanicova, D. *Eur. J. Biochem.* **1980**, *108*, 323-9.
73. Hrmova, M.; Biely, P.; Vrsanska, M.; Petrakova, E. *Arch. Microbiol.* **1984**, *138*, 371-6.
74. Yasui, T.; Nguyen, B. T.; Nakanishi, K. *J. Ferment. Technol.* **1984**, *62*, 353-9.
75. Leathers, T. D.; Detroy, R. W.; Bothast, R. J. *Biotechnol. Lett.* **1986**, *8*, 867-72.
76. Mandels, M.; Parrish, F. W.; Reese, E. T. *J. Bacteriol.* **1962**, *83*, 400-8.
77. Biely, P.; Petrakova, E. *J. Bacteriol.* **1984**, *160*, 408-12.
78. Pou-Llinas, J.; Driguez, H. *Appl. Microbiol. Biotechnol.* **1987**, *27*, 134-8.
79. Biely, P.; Petrakova, E. *FEBS Lett.* **1984**, *178*, 323-6.
80. Defaye, J.; Driguez, H.; John, M.; Schmidt, J.; Ohleyer, E. *Carbohydr. Res.* **1985**, *139*, 123-32.
81. Biely, P.; Defaye, J. *Abstracts FEMS Symp. Biochemistry and Genetics of Cellulose Degradation*, Paris, 1987; p 98.

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Chapter 32

Catalytic Properties and Partial Amino Acid Sequence of an Actinomycete Endo-(1→4)-β-D-Xylanase from *Chainia* Species

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An endo-(1→4)-β-D-xylanase from a cellulase-free *Chainia* strain was substantially purified and subjected to amino acid sequencing. The first forty N-terminal amino acid residues show high homology with endo-xylanases from *Bacillus pumilus*, *B. subtilis*, *B. circulans*, and *Schizophyllum commune*, less homology with endo-xylanases from *Aureobasidium* sp. and *Pseudomonas fluorescens*, and slight homology, but including a possible catalytic Asp residue, with catalytic domains of endo-xylanases from *Clostridium thermocellum*, *Cryptococcus albidus*, and an alkalophilic *Bacillus* and with a cellobiohydrolase from *Cellulomonas fimi*. The enzyme attacks substrates as small as xylo-tetraose and has xylosyltransferase activity. It is most active at pH 6 and 60°C and most stable between pHs 5 and 7.

Endo-xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), which hydrolyze mainly interior β-(1→4)-D-xylosyl bonds in the plant hemicellulose xylan, have been isolated from many fungi and bacteria and occasionally from plants and invertebrates. Because xylan is one of the most common of all natural materials, and its removal or conversion is often desirable, the endo-xylanase family has high commercial as well as scientific interest.

Many actinomycetes are able to degrade at least a portion of the plant lignocellulosic complex, and this has led to extensive research into the identities and characteristics of the enzymes that are involved in the degradation. Much of this work has recently been reviewed (1,2). In summary, actinomycete endo-xylanases are often produced in multiple forms by the same strain and are often associated with cellulases. They have optimal pHs for activity and stability between pHs 5 and 8, retain activity for substantial periods at 50°C or above, have molecular weights from 20 to 50 kDa, and have widely varying isoelectric points. Different forms give xylo-oligosaccharide mixtures of different average chain length, depending on the number of subsites in their active sites and therefore by the smallest substrate that they can cleave (3). Generally it is to be expected that endo-xylanases will have increasing maximum rates and decreasing Michaelis constants as substrate chain length increases.

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Endo-xylanases can be used industrially in two ways: 1) to remove xylan from paper pulp to give purer cellulose, and 2) to convert xylan into D-xylose or xylo-oligosaccharides that can be further converted into useful materials. In the first case, at least, it is clear that the enzyme preparation should be free of cellulases, either by choosing, or producing by mutation, a strain that makes xylanases but not cellulases, or by separating the latter from the former after they are produced. In nearly all cases the use of a cellulase-free strain is preferable.

We report in this article work on a cellulase-free strain, NCL 82-5-1 (also NCIM 2980 and ATCC 53812), a member of the actinomycete genus *Chainia*. Its microbiological properties and its production of separate D-glucose and D-xylose isomerases have previously been reported (4,5). This strain also elaborates endo-xylanase activity, with the highest levels occurring when it is grown on xylan (6). The endo-xylanase activity was partially purified by ethanol precipitation and appeared, by polyacrylamide gel electrophoresis (PAGE) at pH 4.3, to be from one protein. Activity was highest in a 30-min assay on xylan at pH 5.0 and almost 65°C, and was unchanged after 10 min at pH 6.0 and 60°C. Isoelectric focusing yielded an isoelectric point of 8.0. The activity was adsorbed by CM-cellulose at pH 6.0 and eluted at pH 7.5 (6).

This article describes the further purification of this endo-xylanase and the measurement of its isoelectric point, partial amino acid sequence, activity and stability at various pHs and temperatures, and characteristic products when it hydrolyzes linear xylo-oligosaccharides.

Experimental

Xylanase Assay. Xylanase activity was determined with the ferricyanide assay (7). Xylan suspension of approximately 5% concentration was made from Sigma larchwood xylan, Lot 125C-00582, which was suspended in 0.025M potassium phosphate buffer at pH 6.0. A mixture of 1.95 mL of this and 0.05 mL of enzyme dissolved in the same buffer was incubated at 50°C, and 0.2-mL samples taken at various times were added to 0.8 mL of 2% Na₂CO₃ solution. This mixture was added to 5 mL of ferricyanide solution (2 g Na₂CO₃, 0.5 g KCN, and 0.09 g K₃Fe(CN)₆ in 100 mL water). The mixture was heated to 100°C for 10 min and then cooled to room temperature. Absorbance of the resulting solution in a 10-mm path length cuvette was measured at 420 nm. One unit (IU) is the activity that produces 1 μmol of reducing sugar in 1 min under the conditions of the assay.

Tests of Purity. Isoelectric focusing (IEF) in a 0.5-mm thick horizontal slab gel was performed with LKB pH 7-9 ampholyte (Ampholine 1809-136) (8). Electrophoresis was run at 10°C for 6 h at a constant voltage of 1800 V. Protein was visualized using silver stain (9) or Sigma Coomassie Brilliant Blue G-250.

PAGE was carried out according to the modified method of Reisfield *et al.* (10), using Fuchsin Red as a marker. Gels were run under cathodic conditions and then stained with Coomassie Blue.

Sodium dodecyl sulfate-PAGE (SDS-PAGE) was conducted in a 0.5-mm thick 15% horizontal slab gel (11). Samples were prepared in buffer with dithiothreitol and heated to 100°C for 5 min. The gel was prerun for 3 h at 10°C, pH 8.3, and 150 V, and then samples were run for 3 h at 250 V. Protein was again visualized with silver or Coomassie Blue stain.

Glycoprotein Carbohydrate Analysis. Gels from PAGE were fixed with isopropanol-acetic acid-water (25:10:65 v:v:v), stained with 0.2% thymol in the fixing solution, and washed two or three times with the same fixing solution. This was followed by staining with concentrated H₂SO₄-ethanol (4:1 v/v) at 35°C for 2-3 h (12).

Protein Sequencing. The xylanase protein sequence was obtained by stepwise automated Edman degradation after an initial double-coupling protocol, using an Applied Biosystems model 470A gas-phase sequenator equipped with an on-line Applied Biosystems model 120A PTH-amino acid analyzer (13).

HPLC of Carbohydrates. Samples of carbohydrates after enzymatic hydrolysis were analyzed by two methods. Those from the hydrolysis of xylan, xylopentaose (X_5), xylohexaose (X_6), and xyloheptaose (X_7) were passed through a Bio-Rad HPX-42A strong-acid ion-exchange column (7.8 mm i.d. and 300 mm long), using 85°C water flowing at 0.4 mL/min as eluent. Samples from the hydrolysis of xylooctaose (X_8) and xylononaose (X_9) were separated with a Supelcosil LC-NH2 amino-bonded silica column (4.6 mm i.d. and 250 mm long) eluted with 70% acetonitrile-30% water (v/v) at room temperature and 1.3 mL/min. A Rheodyne 7125 injector, Erma ERC-3510 solvent degasser, Waters M-6000A pump, and Erma ERC-7510 differential refractive index detector were used.

Xylo-oligosaccharides. Samples of X_4 through X_7 of 98% purity or better and of X_8 and X_9 of 90% or better were prepared by acidic hydrolysis of larchwood xylan and preparative chromatography, as previously described (3).

Xylanase Purification

Xylanase was obtained from filtered fermentation broths of *Chainia* NCL-82-5-1 (6). It was precipitated by the addition of three volumes of 0-4°C ethanol, separated from the supernatant by centrifugation, and lyophilized. Four column chromatographic steps, DEAE-cellulose anion exchange, Fractogel TSK DEAE-650S anion exchange, Sephadex G-50-50 gel permeation, and CM-Trisacryl cation exchange, were used for purification.

In the first step, 2.2 g of the ethanol-precipitated solid were dissolved in 55 mL of 0.05M sodium phosphate buffer at pH 7.3, the undissolved residue was removed by centrifugation, and the supernatant was added to a 50-mm i.d., 180-mm long DEAE-cellulose column held at room temperature and eluted with 3.33 mL/min of the same buffer. Since the isoelectric point of the desired xylanase was above the buffer pH, it passed through the column without being retarded, and contaminating protein was removed.

The 600 mL eluate was concentrated at 4°C, the temperature used for all further purification steps, with an Amicon YM-2 membrane to 30 mL and divided into three equal parts. Each was loaded on to a 20-mm i.d., 900-mm-long Fractogel TSK DEAE-650S column and eluted with 4 mL/min of 0.05M sodium phosphate buffer at pH 7.2.

The three pools of xylanase activity contained 224 mL; they were combined, concentrated as before to 12 mL, and split into two portions of 6 mL. Each was added to a 45-mm i.d., 1.3-m-long Sephadex G-50-50 column and eluted with 1 mL/min of the pH 7.2 sodium phosphate buffer. Two peaks of xylanase activity resulted. The first to elute, Xylanase I, represented approximately 2.5% of the original endo-xylanase activity, while Xylanase II represented about 10%.

Attempted purifications of Xylanase I, which has an apparent molecular weight four to six times that of Xylanase II by gel permeation chromatography, by various chromatographic procedures all failed, as more Xylanase II continued to form as the solution underwent dilution (Pestlin, S., Iowa State University, unpublished data). Xylanase I therefore appeared to be cluster of Xylanase II molecules.

Xylanase II was subjected to cation exchange chromatography with a 20-mm i.d., 700-mm long CM-Trisacryl column eluted with 0.8 mL/min of 0.025M sodium acetate buffer at pH 4.5, with a 0-0.2M sodium chloride gradient. Only one protein peak eluted from the column; it contained all the Xylanase II activity. This material appeared

pure by PAGE, SDS-PAGE, and IEF. However, silica-bonded C_8 reverse-phase HPLC (Strickland, W. N., University of Wisconsin Biotechnology Center, personal communication, 1989), matrix-assisted laser desorption mass spectroscopy (Chait, B., Rockefeller University, personal communication, 1989), and automated Edman degradation all indicated the presence of moderate amounts of lower-molecular-weight polypeptides.

In various runs, specific activity went from an average of 2.75 IU/mg protein (based on the Bio-Rad protein assay with bovine serum albumin standard) for the centrifuged crude preparation after alcohol precipitation, to 8 IU/mg after DEAE-cellulose chromatography, to 100 IU/mg after DEAE-Fractogel chromatography, to 1000 IU/mg after Sephadex G-50 chromatography, and to 3200 IU/mg after CM-Trisacryl chromatography.

Xylanase II Characterization

Isoelectric Point. The isoelectric point of Xylanase II, measured by IEF, was found to be pH 8.5.

Protein Sequencing and Structure. The sequence of the first forty amino acid residues from the amino terminus of Xylanase II was determined (Figure 1). The identities of residues 7 to 31, less those at 19 and 22, which were uncertain, were confirmed at the University of Wisconsin Biotechnology Center (Niece, R. L., personal communication, 1988); in addition, an endo-xylanase from a closely related strain has the same sequence through residue 40 (Jeffries, T. W., Forest Products Laboratory, USDA Forest Service, personal communication, 1990). The N-terminal region of Xylanase II was highly homologous (36-46% identical residues) with the analogous regions of endo-xylanases from *Schizophyllum commune* (14), *Bacillus pumilus* (15), *Bacillus subtilis* (16), and *Bacillus circulans* (17), which in this region has a sequence identical to that of the endo-xylanase from *B. subtilis*. It has somewhat less homology (18-20% identical residues) with the N-terminal region of an *Aureobasidium* sp. endo-xylanase (18) and with an interior region of an endo-xylanase from *Pseudomonas fluorescens* (19). Homology with endo-xylanases from the catalytic regions of an alkalophilic *Bacillus* sp. (20), *Clostridium thermocellum* (21), and *Cryptococcus albidus* (22,23), and a cellobiohydrolase from *Cellulomonas fimi* (24) is mainly limited to residues 21-24 of Xylanase II, which contain one highly conserved Asp residue and two almost equally highly conserved Gly residues.

The amino acid sequences in Figure 1 are arranged in order of their relationship to each other, as shown in Figure 2. Also shown there are the percentage of gaps necessary to align different sequences.

Concentrations of *p*-chloromercuribenzoate as low as 0.28mM completely suppressed the activity of Xylanase II, suggesting the presence of a sulfhydryl group in the active site.

Carbohydrate Content. Staining of gels from PAGE yielded a single deep red band, signifying that Xylanase II is a glycoprotein. However, the enzyme was not adsorbed on a Concanavalin A column, suggesting that it does not contain α -D-mannopyranosyl or α -D-glucopyranosyl residues.

Specificity. Under the conditions of the assay, Xylanase II had little or no activity on cellulose, carboxymethylcellulose, Avicel, and starch.

Koch-Light 1887-00 oat-spelt xylan (Lot 90560) was split into soluble and insoluble fractions by dissolving it in 0.05M, pH 6.05 sodium citrate buffer and centrifuging out the undissolved material. The insoluble portion was suspended in the buffer in roughly 1% concentration and was assayed with an equal volume of Xylanase II in

buffer. Activity on insoluble xylan was approximately 25% greater than on soluble xylan at the same concentration.

Essentially pure xylo-oligosaccharides in 0.5% concentration were incubated with Xylanase II at 50°C in 0.05M sodium citrate buffer, pH 5.0 for X₅ through X₇ and pH 6.05 for X₈ and X₉, and samples after varying periods were submitted to HPLC. With X₅, X₂ and X₃ were the major products, with smaller amounts of X₄ and D-xylose produced. Incubation of X₆ gave X₃ as the major product, followed by X₄ and X₂. With prolonged incubation the X₄ was consumed to form X₂ and smaller amounts of D-xylose and X₃. Hydrolysis of X₇ led to the formation of X₄ and X₃, with smaller amounts of X₅, X₆, and X₂, and a very small amount of X₈. The course of hydrolysis of X₈ is shown on Figure 3; the formation of materials larger than the initial substrate is noticeable, as are product distributions unbalanced towards the larger product (more X₆ than X₂, for instance, even after prolonged incubation). This pattern is repeated with the incubation of X₉, where X₃ through X₆ were the major products, but where X₇, X₈, and X₁₀ were produced in higher quantities than X₂.

Preliminary kinetic studies indicate that X₄ hydrolysis was extremely slow, while larger straight-chain substrates were cleaved at rates that increased with increasing chain length. It is possible that branched-chain xylo-oligosaccharides were formed during incubation with Xylanase II; their rates of formation and hydrolysis are as yet unknown.

Effect of Temperature and pH on Activity and Stability. When Xylanase II was subjected to the standard assay at pH 5 but at several different temperatures, the highest activity was found after incubation for 30 min at 60°C. From 21°C to 45°C the energy of activation from a linear plot of ln (activity) vs. T⁻¹ was 41.6 ± 2.1 kJ/mol, where the range is the standard deviation. When the standard assay was conducted at various pHs and 50°C, the highest activity was at pH 6.05. Activities half the maximum were found at pHs 4.4 and 8.0.

Decay of the enzyme in pH 6.05 buffer at 50°C to 70°C was generally greater than first order in remaining activity, in that plots of ln (remaining activity) vs. incubation time yielded curves of decreasing negative slope. At 50°C an almost stable level of 85% of the initial activity was attained after more than 60 min incubation. Half-lives at 60° and 70°C were approximately 25 and 10 min, respectively. When the enzyme was assayed at pH 6.05 after 15 min incubation in the assay buffer at 50°C and various pHs, the highest remaining activities were found in samples incubated between pHs 5 and 7.

Discussion

In several respects, this endo-xylanase is characteristic of others produced by actinomycetes. It attacks the interior β-(1→4)-D-xylosidic links of xylo-oligosaccharides more than those near the terminal residues to preferentially yield products of chain lengths close to half that of the substrate. This is characteristic also of the only endo-xylanase, from *Aspergillus niger*, whose mode of action on linear xylo-oligosaccharides has been thoroughly determined (3). Its rate of attack increases with increasing substrate chain length, as expected. The presence of xylosyltransferase activity in an endo-xylanase is unusual but not unknown (25,26). Its pH optima for activity and stability, while still acidic, are similar to other actinomycete endo-xylanases (1,2) but higher than those normal for fungal endo-xylanases. It is a fairly stable enzyme, as are other endo-xylanases from actinomycetes.

There are a number of noteworthy findings issuing from this work. One is the partial amino acid sequence of Xylanase II, the first one obtained for an actinomycete endo-xylanase. The sequence is highly homologous with the N-terminal sequences of endo-xylanases from both the bacterial genus *Bacillus* and the fungal genus *Schizophyllum*, and somewhat less so with one from the fungal genus *Aureobasidium* and

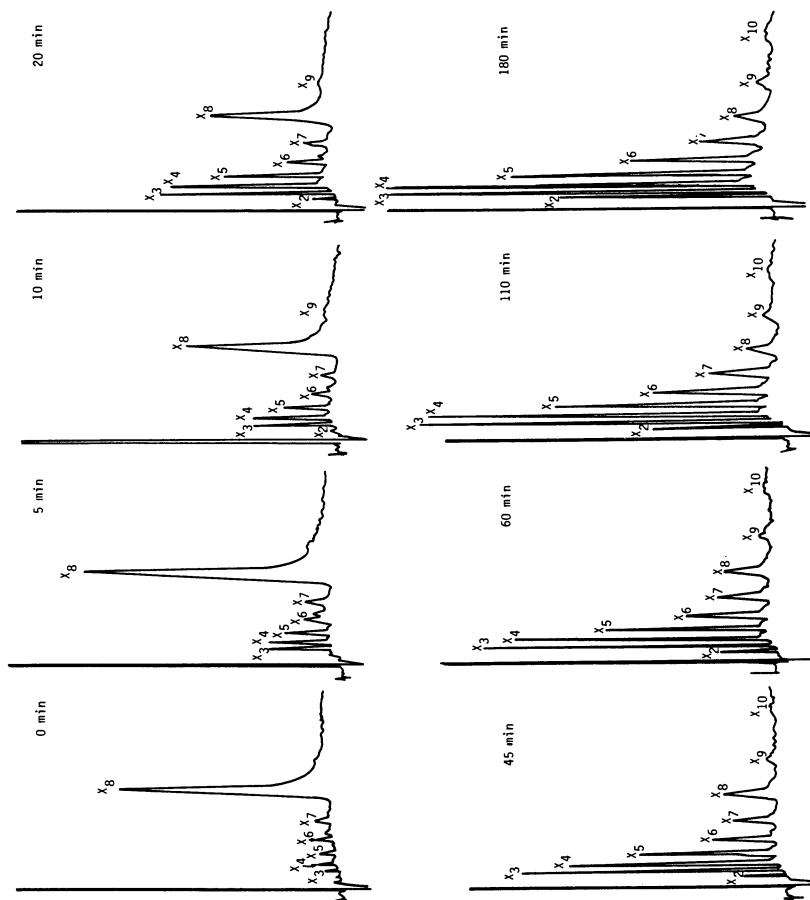


Figure 3. Chromatograms from an amino-bonded silica column of the products of hydrolysis of X₈ by Xylanase II at increasing incubation times.

another from the bacterial genus *Pseudomonas*. It is much more distantly related to endo-xylanases from other bacteria of the genera *Bacillus*, *Clostridium*, and to an endo-xylanase from the yeast genus *Cryptococcus*. Sequences in Figure 1 have been ordered by their relationships to each other. Endo-xylanases from *Aureobasidium* sp., *S. commune*, *Chainia* sp., *B. pumilus*, *B. subtilis*/*B. circulans* form one group, while endo-xylanases from the alkalophilic *Bacillus* sp. and *C. thermocellum*, the cellobiohydrolase from *C. fimi* and, more distantly, the endo-xylanase from *C. albidus* form another. These two classes and the endo-xylanase from *P. fluorescens* are mainly related to each through the almost universal (D-GXG) sequence that starts at residue 21 of the *Chainia* enzyme and is found close to the N-terminus of other closely related enzymes but in the middle of more distantly related enzymes. Homologies between members of the first group, less the *Chainia* endo-xylanase, had been pointed out by Paice *et al.* (16), Leathers (18), and Okada (27), while those between members of the second had been discussed by Grépinet *et al.* (21), Henrissat *et al.* (28), and West *et al.* (29). This appears to be the first publication linking of the two groups through the conserved Asp21 residue in the *Chainia* endo-xylanase, and leads to the supposition that this residue is one of the two acidic residues that are involved in catalysis, a proposal previously put forth by Okada (27) based on the homology between *B. pumilus* and *B. subtilis* endo-xylanases.

Keskar *et al.* (30) have determined that essential Trp and Cys residues are in the active site of an endo-xylanase from a thermotolerant *Streptomyces* sp. Addition of *p*-mercuribenzoate to the *Chainia* endo-xylanase eliminated its activity, supporting the possibility of its having an essential Cys residue also. However, there are no conserved Cys residues in the range covered by Figure 1, and only in the first endo-xylanase group, at position 19 of the *Chainia* enzyme, is there a conserved Trp residue. Therefore, the use of homology to locate these two residues must await the accumulation of more sequences.

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Literature Cited

1. McCarthy, A. J. *FEMS Microbiol. Rev.* **1987**, *46*, 145-63.
2. Ball, A. S.; McCarthy, A. J. *J. Appl. Bacteriol.* **1989**, *66*, 439-44.
3. Meagher, M. M.; Tao, B. Y.; Chow, J. M.; Reilly, P. J. *Carbohydr. Res.* **1988**, *173*, 273-83.
4. Srinivasan, M. C.; Vartak, H. G.; Powar, V. K.; Khire, J. M. *Biotechnol. Lett.* **1983**, *5*, 611-4.
5. Vartak, H. G.; Srinivasan, M. C.; Powar, V. K.; Rele, M. V.; Khire, J. M. *Biotechnol. Lett.* **1984**, *6*, 493-4.
6. Srinivasan, M. C.; Vartak, H. G.; Powar, V. K.; Rele, M. V.; Bastawde, K. B. *Biotechnol. Lett.* **1984**, *6*, 715-8.
7. Hoffman, W. S. *J. Biol. Chem.* **1937**, *120*, 51-55.
8. *LKB Ultramold Brochure*, LKB-Producter AB, Bromma, Sweden, 1983.
9. Heukeshoven, J.; Dernick, R. *Electrophoresis* **1985**, *6*, 103-12.

10. Reisfield, R. A.; Lewis, U. J., and Williams, D. C. *Nature* **1962**, *195*, 281-283.
11. Moore, S.; Stein, W. H. *Meth. Enzymol.* **1963**, *6*, 819-31.
12. Shetlar, M. R.; Masters, Y. F. *Anal. Chem.* **1957**, *29*, 402-5.
13. Hunkapiller, M. W.; Hewick, R. M.; Dreyer, W. J.; Hood, L. E. *Meth. Enzymol.* **1983**, *91*, 399-413.
14. Paice, M. G.; Jurasek, L.; Carpenter, M. R.; Smillie, L. B. *Appl. Environ. Microbiol.* **1978**, *36*, 802-8.
15. Fukusaki, E.; Panbangred, W.; Shinmyo, A.; Okada, H. *FEBS Lett.* **1984**, *171*, 197-201.
16. Paice, M. C.; Bourbonnais, R.; Desrochers, M.; Jurasek, L.; Yaguchi, M. *Arch. Microbiol.* **1986**, *144*, 201-6.
17. Yang, R. C. A.; MacKenzie, C. R.; Narang, S. A. *Nucleic Acids Res.* **1988**, *16*, 7187.
18. Leathers, T. *Biotechnol. Lett.* **1988**, *10*, 775-80.
19. Hall, J.; Hazlewood, G. P.; Huskisson, N. S.; Durrant, A. J.; Gilbert, H. J. *Mol. Microbiol.* **1989**, *3*, 1211-9.
20. Hamamoto, T.; Honda, H.; Kudo, T.; Horikoshi, K. *Agr. Biol. Chem.* **1987**, *51*, 953-5.
21. Grépinet, O.; M.-C. Chebrou; P. Béguin. *J. Bacteriol.* **1988**, *170*, 4582-8.
22. Morosoli, R.; Roy, C.; Yaguchi, M. *Biochim. Biophys. Acta* **1986**, *870*, 473-8.
23. Boucher, F.; Morosoli, R.; Durand, S. *Nucleic Acids Res.* **1988**, *16*, 9874.
24. O'Neill, G.; Goh, S. H.; Warren, R. A. J.; Kilburn, D. G.; Miller, R. C., Jr., *Gene*, **1986**, *44*, 325-30.
25. Kusakabe, I.; Yasui, T.; Kobayashi, T. *Nippon Nôgeikagaku Kaishi* **1977**, *51*, 439-48.
26. Vrsanska, M.; Gorbacheva, I. V.; Kratky, Z.; Biely, P. *Biochim. Biophys. Acta* **1982**, *704*, 325-30.
27. Okada, H. *Abstr., Workshop on Advances in Protein Design*, Gesellschaft für Biotechnologische Forschung, Braunschweig, W. Germany, 1988, p 35.
28. Henrissat, B.; Claeysens, M.; Tomme, P.; Lemesle, L.; Mornon, J.-P. *Gene* **1989**, *81*, 83-95.
29. West, C. A.; Elzanowsky, A.; Yeh, L.-S.; Barker, W. C. *FEMS Lett.* **1989**, *59*, 167-72.
30. Keskar, S. S.; Srinivasan, M. C.; Deshpande, V. V. *Biochem. J.* **1989**, *261*, 49-55.

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Chapter 33

Accessory Enzymes Involved in the Hydrolysis of Xylans

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Xylans are heteropolysaccharides which are depolymerized by β -1,4-D-endoxylanases. Due to the abundance and variety of substituents in native xylans, different accessory enzymes are also needed for the total hydrolysis of xylan. The knowledge of α -glucuronidase, α -arabinosidase, and acetyl xylan- and feruloyl esterases has increased considerably in recent years. In addition to acting in synergism with endoxylanases and β -xylosidase for the complete hydrolysis of xylan, some of these accessory enzymes are also capable of changing the structure of polymeric xylans.

Xylans are the major hemicelluloses of many plant materials, where they often contribute to the rigidity of plant cell walls. Most xylans are heteropolysaccharides with a homopolymeric backbone chain of 1,4-linked β -D-xylopyranose units. The degree and type of substitution of the backbone is dependent on the plant origin of a xylan. In addition to xylose, xylans may contain L-arabinose, D-glucuronic acid or its 4-O-methyl ether, and acetic, p-coumaric, and ferulic acids.

Due to the extreme variety of xylan structures, it is obvious that many kinds of enzymes are needed for their complete hydrolysis in nature. Xylanases (EC 3.2.1.8.) are the polysaccharide hydrolases responsible for the attack of the polymer backbone itself. The total hydrolysis or modification of heteroxylans requires in addition several different exo-glycosidases and esterases. The present knowledge of these enzymes is reviewed in this paper.

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Structure of xylans

Wood xylans are either O-acetyl-4-O-methylglucuronoxylans (in hardwoods) or arabino-4-O-methylglucuronoxylans (in softwoods). The degree of polymerization of hardwood xylans (150-200) is higher than that of softwoods (70-130) (1, 2). Wood xylans have very few branching points, but their degree of substitution is high. 4-O-Methylglucuronic acid and L-arabinofuranoside units are linked to the backbone by α -1,2- and α -1,3-glycosidic bonds, respectively. The average ratio of sugar units in softwood xylans is 8:1.6:1 (xylose:4-O-methylglucuronic acid:arabinose) (2). The average molar ratio of xylose, 4-O-methylglucuronic acid and acetic acid in hardwood xylan is 10:1:7 (3).

Both D-glucuronic acid and/or its 4-O-methyl ether and arabinose are also present in cereal xylans (4). Endospermic arabinoxylans of annual plants, often referred to as pentosans, are because of their branched structures more soluble in water and dilute alkali than xylans of lignocellulosic materials. They also have at least one, or even two, substituents per xylose residue (5).

The presence of feruloyl and p-coumaroyl acids linked via L-arabinose residues has been verified in several studies of xylan (6-8). The amounts of these components, however, are rather small. Every 15th arabinose unit in barley straw arabinoxylan is estimated to be esterified with ferulic acid, and every 31st with p-coumaric acid (7). These hydroxycinnamic acids are bound to C-5 of the arabinose residue (7, 8). It has been suggested that oxidative dimerization of ferulic acid residues crosslinks the arabinoxylan chains and renders them insoluble as a result of the diferuloyl bridges (9).

Hardwood xylans and xylans of annual plants may contain up to 7% O-bound acetyl groups. Seven out of ten xylose residues in native hardwood xylan are acetylated on C-2 and/or C-3 (10). Because of the possible migration of O-acetyl groups between 2- and 3-positions during and after isolation of hemicellulose components, it is difficult to determine their original distribution in nature (11). The ratios reported for 2-, 3-, and 2,3-positions of acetyl groups in birch xylan have been 2:4:1 (3) and 2:2:1 (10) and in bracinga xylan 3:3:1 (12).

The existence of covalent bonds between lignin and hemicellulose, perhaps through xylan substituents in many cases, has been indicated in several studies. Evidence has been shown for the existence of an ether linkage between arabinose and lignin (13) and an ester linkage between glucuronic acid and lignin (14). Feruloyl groups may also crosslink xylan and lignin (15).

β -Xylosidases

Exo-1,4- β -D-xylosidases (EC 3.2.1.37) hydrolyze xylooligosaccharides and xylobiose to xylose by removing successive D-xylose residues from the nonreducing termini. β -Xylosidase is part of most microbial xylanolytic systems, but the highest extracellular production levels have been reported for fungi.

β -Xylosidases are rather large enzymes, with molecular weights exceeding 100 kDa, and are often reported to consist of two or more subunits (16-20). Most purified β -xylosidases show highest activity towards xylobiose and no activity towards xylan. The activity towards xylooligosaccharides generally decreases rapidly with increasing chain length (17, 21). In addition to formation of xylose, many β -xylosidases produce transfer products with higher molecular weights than that of the substrate (17, 22). Some β -xylosidases have also been reported to possess β -glucosidase activity (17, 19). An important characteristic of β -xylosidases is their susceptibility to inhibition by xylose, which may significantly affect the yield under process conditions (17, 20, 23).

β -Xylosidase is the key enzyme for production of monomeric xylose from solubilized xylan fragments, such as those obtained from a steaming process (20). They have been shown to act in synergism with the substituent-cleaving enzymes in the hydrolysis of substituted xylooligosaccharides (24, 25). The β -xylosidase of *Trichoderma reesei* was not able to hydrolyze xylobiose bearing an acetyl substituent at the non-reducing end without the presence of acetyl xylan esterase (Poutanen, K.; Sundberg, M.; Korte, H.; Puls, J. *Appl. Microbiol. Biotechnol.* in press).

α -Arabinosidases

α -L-Arabinofuranosidases (EC 3.2.1.55) hydrolyze non-reducing α -L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans, as reviewed by Kaji (26). The production of α -arabinosidases in microorganisms is often associated with the production of pectinolytic or hemicellulolytic enzymes, e.g. in *Corticium rolfsii* (27), *Sclerotinia fructigena* (28), *T. reesei* (29, 30), and different *Streptomyces species* (31-33). Some reported molecular characteristics of α -arabinosidases are presented in Table I.

Table I. α -Arabinosidases

Microorganism	MW (kDa)	pI	Reference
<i>Aspergillus niger</i>	53 ¹⁾	3.6	34, 35
<i>Trichoderma reesei</i>	53 ²⁾	7.5	30
<i>Streptomyces spp.</i>	92 ²⁾	4.4	31
<i>Streptomyces purpurascens</i>	495 ¹⁾ , 62 ²⁾	3.9	36
<i>Ruminococcus albus</i>	305 ¹⁾ , 75 ²⁾	6.8	37

1) Gel chromatography

2) SDS-PAGE

The purified α -arabinosidase of *Aspergillus niger* (38), as well as that partially purified from a commercial pectinase preparation (39), was able to release L-arabinose from wheat L-arabino-D-xylan. As the reaction proceeded an amorphous precipitate, consisting mainly of D-xylan with only traces of arabinose, was formed. Adrewartha *et al.* (40) prepared a series of arabinoxylans from purified wheat-flour arabinoxylan by partial removal of arabinosyl side branches using an α -L-arabinosidase. They suggested that the solubilizing effect of the arabinosylsubstituents was not a result of increased hydration, but due to their ability to prevent intermolecular aggregation of unsubstituted xylose residues.

Cereal endospermic arabinoxylans especially are known to form viscous solutions and gels. It is obvious that suitable α -arabinosidases could be used to control the degree of substitution and hence the water-binding capacity of these pentosans. In a similar way α -galactosidases have been used in adjusting the degree of α -galactosyl substitution and hence the gelling properties of galactomannans (41, 42).

α -Glucuronidases

α -D-Glucuronidases are required for hydrolysis of the α -1,2-glycosidic linkage between xylose and D-glucuronic acid or its 4-O-methyl ether. The presence of acidic oligosaccharides in xylan hydrolyzates produced by hemicellulolytic enzyme preparations indicates the absence or inadequacy of this enzyme (29, 43). 4-O-Methylglucuronic acid was first detected in the enzymatic hydrolyzates of glucuronoxylan by Sinner *et al.* 1972 (44). The presence of an uronic acid-liberating enzyme was together with β -xylosidase claimed to increase the xylose yield in the enzymatic hydrolysis of hardwood xylan (45). The presence of α -glucuronidase in the hemicellulolytic system of *T. reesei* was demonstrated in 1983 (23).

The production of α -glucuronidase by many fungi and bacteria (Table II) has recently been reported. Only a few α -glucuronidases have been totally or even partially purified and characterized. The α -glucuronidase isolated from a culture filtrate of *Agaricus bisporus* by gel chromatography is a very large protein (450 kDa) (24). The enzyme had a very low isoelectric point and a pH-optimum of about pH 3.3. Of a series of 4-O-methylglucuronosubstituted xylooligosaccharides up to DP 5 tested as substrates, it showed highest activity against 4-O-methylglucuronoxylobiose (52). The α -glucuronidase of *A. bisporus* had no activity towards polymeric xylan.

The α -glucuronidase of *T. reesei* also had an acidic isoelectric point (25). It had a molecular weight of about 70 kDa as estimated by gel chromatography and a pH-optimum at pH 6 with 4-O-methylglucurono-xylobiose as substrate. The very recently characterized α -glucuronidase of the thermophilic fungus *Thermoascus aurantiacus* (51) was a single polypeptide chain with a MW of

Table II. Microbial producers of α -glucuronidases

Microorganism	Reference
<i>Agaricus bisporus</i>	24, 46, 47
<i>Pleurotus ostreatus</i>	46, 47
<i>Trichoderma reesei</i>	25, 46
<i>Trichoderma spp.</i>	47
<i>Schizophyllum commune</i>	48
<i>Aspergillus awamori</i>	46
<i>Aspergillus niger</i>	48
<i>Tyromyces palustris</i>	47
<i>Streptomyces flavogriseus</i>	49
<i>Streptomyces olivochromogenes</i>	50
<i>Streptomyces spp.</i>	33
<i>Thermobacter aurantiacus</i>	51

118 kDa. The enzyme had a pH optimum at pH 4.5 and it hydrolyzed 4-O-methylglucurono-substituted xylooligomers from X₁ to X₇ at rates comparable with that of xylan.

Esterases

In addition to enzymes hydrolyzing the glycosidic linkages in xylans, the requirement of esterases to remove esterified acids from xylans has recently been discovered. Acetyl xylan esterases remove O-acetyl groups from the C-2 and C-3 positions of xylose residues in both xylan and xylooligomers. Feruloyl esterases liberate ferulic acid from arabinoxylans of monocotyledons.

Esterases are classified according to their substrate specificity (53). This classification is not, however, very clear due to the rather wide substrate specificities of many esterases. Acetyl esterases (EC 3.1.1.6), a group of enzymes having highest activity against esters of acetic acid, are widely distributed in nature. They are produced by many animals, plants and microorganisms (54). The occurrence of microbial esterases acting on various synthetic acetyl derivatives of mono- and disaccharides was demonstrated in several studies long ago (55-58), but the role of esterases in the hydrolysis of native acetylated xylans was emphasized only rather recently (59, 60).

Biely *et al.* (59) first reported the presence of acetyl xylan esterases in fungal cellulolytic and hemicellulolytic systems: *Trichoderma reesei*, *Aspergillus niger*, *Schizophyllum commune* and *Aureobasidium pullulans*. As compared with plant and animal esterases, these fungal esterases exhibited high specific activities

towards acetylated glucuronoxylan and were therefore named acetyl xylan esterases. During recent years the production of esterases deacetylating xylan by a number of hemicellulolytic microorganisms has been reported (Table III).

Hitherto, only the acetyl xylan esterases of *T. reesei* have been purified and characterized (67, Sundberg, M.; Poutanen, K., *Biotechnol. Appl. Biochem.*, in press). The enzymes both had neutral isoelectric points, but differed in their native molecular weights as assayed by gel chromatography. Both enzymes showed optimal activity at pH values between 5 and 6. One enzyme released only a little acetic acid from acetylated xylooligomers, but showed high activity towards acetyl xylobiose. The other occurred as multiple isoenzymes and showed high activity towards acetylated xylan fragments and polymeric acetyl-4-O-methylglucuronoxylan (Poutanen, K.; Sundberg, M.; Korte, H.; Puls, J., *Appl. Microbiol. Biotechnol.*, in press). Enzymatic deacetylation of beechwood xylan caused precipitation of the polymer. This was shown to be due to molecular aggregation analogous to the behavior of arabinoxylan after α -arabinosidase treatment.

Feruloyl esterase activity was first detected in culture filtrates of *Streptomyces olivochromogenes* (49), and has thereafter also been reported for some hemicellulolytic fungi (Table III). A partially purified feruloyl esterase from *S. commune* liberated hardly any ferulic acid without the presence of xylanase (65). Very recently a feruloyl esterase was purified from *Aspergillus oryzae* (Tenkanen, M.; Schuseil, J.; Puls, J.; Poutanen, K., *J. Biotechnol.*, in press). The enzyme is an acidic monomeric protein having an isoelectric point of 3.6 and a molecular weight of 30 kDa. It has wide substrate specificity, liberating ferulic, p-coumaric, and acetic acids from steam-extracted wheat straw arabinoxylan.

The late discovery of acetyl xylan and feruloyl esterases has been partly due to the lack of suitable substrates. Xylans are often isolated by alkaline extraction, in which ester groups are saponified. Treatment of plant materials under mildly acidic conditions, as in steaming or aqueous-phase thermomechanical treatment, leaves most of the ester groups intact. These methods, however, partly hydrolyze xylan to shorter fragments (63, 69). Polymeric acetylated xylan can be isolated from delignified materials by dimethyl sulfoxide extraction (70). The choice of substrate is especially important in studies of esterases for deacetylation of xylans. The use of small chromophoric substrates (p-nitrophenyl acetate, α -naphthyl acetate, and methylumbelliferyl acetate) analogously to the assays of disaccharidases may lead to the monitoring of esterases unable to deacetylate xylan (33, 63, 64).

The substituent-cleaving enzymes in the hydrolysis of xylans

It is obvious that some of the accessory enzymes are capable of releasing substituents from polymeric xylan, and consequently they could be used to

Table III. Occurrence of hemicellulolytic esterases

Microorganism	Esterase activity ¹⁾		Reference
	Acetyl xylan esterase	Feruloyl esterase	
<i>Butyrvibrio fibrisolvens</i>	+	nd	61
<i>Streptomyces flavogriseus</i>	+	-	33, 49
<i>Streptomyces olivochromogenes</i>	+, three different	+, three different	29, 33, 49
<i>Streptomyces rubiginosus</i>	+, five different	+	62
<i>Aspergillus awamori</i>	+	nd	29, 63
<i>Aspergillus japonicus</i>	+	nd	63
<i>Aspergillus niger</i>	+	+	48, 59, 63
<i>Aspergillus versicolor</i>	+	nd	63
<i>Aspergillus oryzae</i>	+	+, one purified	64, Tenkanen <i>et al.</i> ²⁾
<i>Fusarium oxysporum</i>	+	nd	29
<i>Schizophyllum commune</i>	+, one	+, two different	48, 59, 65, 66
<i>Trichoderma viride</i>	+, three different	nd	59
<i>Trichoderma reesei</i>	+, two different types purified	-	59, 66, 67, 68, Sundberg and Poutanen ²⁾

1) + = activity detected, - = no activity detected, nd = activity not determined

2) in press, see text for the reference

change the viscosity and solubility of xylans (40, 51, Poutanen, K.; Sundberg, M.; Korte, H.; Puls J., *Appl. Microbiol. Biotechnol.*, in press). On the other hand, some substituent-cleaving enzymes have been reported to prefer short oligosaccharides as substrates (24, 30, 65). In nature, however, all these enzymes usually occur in mixtures with other biomass-degrading enzymes and act synergistically with them to break down plant cell walls.

Synergism between α -arabinosidase, xylanase and β -xylosidase has been demonstrated in the hydrolysis of wheat straw arabinoxylan with purified enzymes of *T. reesei* (71). When only xylanase and β -xylosidase were used in the hydrolysis, the xylose yield was only 66% of that produced by the whole culture filtrate at the same activity levels of these two enzymes, and no arabinose was produced. Addition of α -arabinosidase increased the yields of both xylose and arabinose. Enhanced hydrolytic action of hemicellulolytic or pectinolytic enzymes in the hydrolysis of alfalfa cell wall polymers by addition of *Ruminococcus albus* α -arabinosidase has also been reported (37).

The synergistic action of depolymerizing and side-group cleaving enzymes has most clearly been demonstrated using acetylated xylans as substrates. Due to the high degree of acetylation, xylanases have only limited access to the polymer backbone in the absence of esterases (71). Deacetylation by acetyl xylan esterase prior to the action of xylanases, however, resulted in a lower yield than that obtained by the simultaneous action of xylanase, β -xylosidase and esterase (Poutanen, K.; Sundberg, M.; Korte, H.; Puls, J., *Appl. Microbiol. Biotechnol.*, in press). The sequence of enzyme application not only influenced the extent of hydrolysis but also the nature of the oligomeric end products.

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Literature cited

1. Zinbo, M.; Timell, T. E. *Svensk Papperstidn.* 1965, 68, 647-662.
2. Zinbo, M.; Timell, T.E. *Svensk Papperstidn.* 1967, 70, 695-701.
3. Bouveng, H.O. *Acta Chem. Scand.* 1961, 15, 96-100.
4. Aspinnall, G.O. *Adv. Carbohydr. Chem.* 1959, 14, 429-468.
5. Brillouet, J.M.; Joseleau, J.P. *Carbohydr. Res.* 1987, 159, 109-126.
6. Ahluwalia, B.; Fry, C.S. *J. Cereal Sci.* 1986, 4, 287-295.
7. Mueller-Harvey, I.; Hartley, R.D.; Harris, P.J.; Curzon, E.H. *Carbohydr. Res.* 1986, 148, 71-85.
8. Kooshima, T.; Kato, A.; Azuma, J. In *Proc. Int. Symp. Wood and Pulp Chem.* 1983; Vol. 1, pp 66-77.
9. Harris, P.J.; Hartley, R.D. *Biochem. Syst. Ecol.* 1980, 8, 153-160.

10. Lindberg, B.; Rosell, K.-G.; Svensson, S. *Svensk Papperstidn.* 1973, 76, 30-32.
11. Garegg, P.J. *Acta Chem. Scand.* 1962, 16, 1849-1857.
12. Reicher, F., Correa, J.B.C.; Gorin, P.A.J. *Carbohydr. Res.* 1984, 135, 129-140.
13. Joseleau, J.-P.; Gancet, C. *Svensk Papperstidn.* 1981, 84, 123-127.
14. Das, N.N.; Das, S.C.; Sarkar, A.K.; Mukherjee, A.K. *Carbohydr. Res.* 1984, 129, 197-207.
15. Scalbert, A.; Monties, B.; Lallemand, J.-Y.; Guittet, E.; Rolando, C. *Phytochem.* 1985, 24, 1359-1362.
16. Kersters-Hilderson, H.; Claeysens, M.; Van Doorslaer, E.; Saman, E.; de Bruyne, C.K. *Methods Enzymol.* 1982, 83, 631-639.
17. Rodionova, N.A.; Tavibilov, I.M.; Bezborodov, A.M. *J. Appl. Biochem.* 1983, 5, 300-312.
18. Matsuo, M.; Yasui, T. *Agr. Biol. Chem.* 1984, 48, 1845-1852.
19. Uziie, M.; Matsuo, M.; Yasui, T. *Agr. Biol. Chem.* 1985, 49, 1167-1173.
20. Poutanen, K.; Puls, J. *J. Appl. Microbiol. Biotechnol.* 1988, 28, 425-432.
21. Van Doorslaer, E.; Kersters-Hilderson, H.; de Bruyne, C.K. *Carbohydr. Res.* 1985, 140, 342-346.
22. Conrad, D.; Nöthen, W. *Proc. 3rd Eur. Congr. Biotechnol.* 1984, Vol. 2, pp 325-327.
23. Dekker, R.F.H. *Biotechnol. Bioeng.* 1983, 30, 1127-1146.
24. Puls, J.; Schmidt, O.; Granzow, C. *Enzyme Microb. Technol.* 1987, 9, 83-88.
25. Poutanen, K. Ph.D. Thesis, *VTT Pub.* 47, Technical Research Centre of Finland, 1988.
26. Kaji, A. *Adv. Carbohydr. Chem. Biochem.* 1984, 42, 383-394.
27. Kaji, K.; Yoshihara, O. *Agric. Biol. Chem.* 1970, 34, 1249-1253.
28. Fielding, A.H.; Byrde, R.J.W. *J. Gen. Microbiol.* 1969, 58, 73-84.
29. Poutanen, K.; Rättö, M.; Puls, J.; Viikari, L. *J. Biotechnol.* 1987, 6, 49-60.
30. Poutanen, K. *J. Biotechnol.* 1988, 7, 271-292.
31. Kaji, A.; Sato, M.; Tsutsui, Y. *Agric. Biol. Chem.* 1981, 45, 291-935.
32. Johnson, K.G.; Fontana, J.D.; MacKenzie, C.R. *Methods Enzymol.* 1988, 160A, 551-560.
33. Johnson, K.G.; Harrison, B.A.; Schneider, H.; MacKenzie, C.R.; Fontana, J.D. *Enzyme Microb. Technol.* 1988, 10, 403-409.
34. Kaji, A.; Tayama, K.; Ichimi, T. *Biochim. Biophys. Acta* 1969, 171, 186-188.
35. Tagawa, K.; Kaji, A. *Methods Enzymol.* 1988, 160A, 707-712.
36. Komae, K.; Kaji, K.; Sato, M. *Agric. Biol. Chem.* 1982, 46, 1899-1905.
37. Greve, L.C.; Labawitch, J.M.; Hungate, R.E. *Appl. Environ Microbiol.* 1984, 47, 1135-1140.
38. Tagawa, K.; Kaji, A. *Carbohydr. Res.* 1969, 11, 293-301.
39. Neukom, H.; Providoli, L.; Gremli, H.; Hui, P.A. *Cereal Chem.* 1967, 44, 238-244.
40. Andrewartha, K.A.; Philips, B.R.; Stone, B.A. *Carbohydr. Res.* 1979, 77, 191-204.

41. Overbeeke, N.; Fellingner, A.-J.; Termorshvizen, G.H.M. *Proc. 4th Eur. Congr. Biotechnol.* 1987, Vol. 1, p 509.
42. Fujita, F.; Nakamura, Y. Japanese Patent 61 274 695, 1986; *Chem Abstr.* 107, 76118A.
43. Sinner, M.; Dietrichs, H.H. *Holzforschung* 1976, 30, 50-59.
44. Sinner, M.; Dietrichs, H.H.; Simatupang, M.H. *Holzforschung* 1972, 26, 218-228.
45. Puls, J.; Sinner, M.; Dietrichs, H.H. German Patent 26 43 800, 1976.
46. Puls, J.; Poutanen, K.; Schmidt, O.; Linko, M. In *3rd Int. Congr. Biotechnology in the Pulp and Paper Industry* STFI, Stockholm, 1986, pp 93-95.
47. Ishihara, M.; Shimizu, K. *Mokazai Gakkaishi* 1988, 34, 58-64.
48. Johnson, K.G.; Silva, M.C.; MacKenzie, C.R.; Schneider, H.; Fontana, J.D. *Appl. Biochem. Biotechnol.* 1989, 20/21, 245-258.
49. MacKenzie, C.R.; Bilous, D.; Schneider, H.; Johnson, K.G. *Appl. Environ Microbiol.* 1987, 53, 2835-2839.
50. Fontana, J.D.; Gebara, M.; Blumel, M.; Schneider, H.; MacKenzie, C.R.; Johnson, K.G. *Methods Enzymol.* 1988, 160A, 560-571.
51. Khandke, K.M.; Vithayathil, P.J.; Murthy, S.K. *Arch. Biochem Biophys.* 1989, 274, 511-517.
52. Korte, H.E. Ph.D. Thesis, University of Hamburg, Hamburg, 1990.
53. *Enzyme Nomenclature*, International Union of Biochemistry, Academic Press, 1982.
54. Krisch, K. In *The Enzymes*, Boyer, P.D., Ed.; Academic Press: New York, 1971; pp 43-69.
55. Mandels, M.; Reese, E.T. *J. Bacteriol.* 1960, 79, 816-826.
56. Frohwein, Y.Z.; Zori, U.; Leibowitz, J. *Enzymologia* 1963, 26, 193-200.
57. Fink, A.L.; Hay, G.W. *Can. J. Biochem.* 1969, 47, 353-359.
58. Reuter, G.; Hüttner, R. *Zeitsch. Allg. Microbiol.* 1977, 17, 149-151.
59. Biely, P.; Puls, J.; Schneider, H. *FEBS Lett.* 1985, 186, 80-84.
60. Poutanen, K.; Puls, J.; Linko, M. *Appl. Microbiol. Biotechnol.* 1986, 23, 487-490.
61. Haspell, B.R.; O'Bryan-Shah, P.J. *Appl. Environ. Microbiol.* 1988, 54, 1917-1922.
62. Zimmerman, W. In *Enzyme Systems for Lignocellulose Degradation*, Coughlan, M.P. Ed.; Elsevier: London, 1989; pp 167-181.
63. Khan, A.W.; Lamb, K.A.; Overend, R.P. *Enzyme Microb. Technol.* 1990, 12, 127-131.
64. Puls, J.; Poutanen, K. In *Enzyme Systems for Lignocellulose Degradation*, Coughlan, M.P. Ed.; Elsevier: London, 1989; pp 151-165.
65. MacKenzie, C.R.; Bilous, D. *Appl. Environ. Microbiol.* 1988, 54, 1170-1173.
66. Biely, P.; MacKenzie, C.R.; Schneider, H. *Can. J. Microbiol.* 1988, 32, 767-772.
67. Poutanen, K.; Sundberg, M. *Appl. Microbiol. Biotechnol.* 1988, 28, 425-432.

68. Biely, P.; MacKenzie, C.R.; Puls, J.; Schneider, H. *Bio/Technol.* 1986, 4, 731-733.
69. Puls, J.; Poutanen, K.; Körner, H.-U.; Viikari, L. *Appl. Microbiol. Biotechnol.* 1985, 22, 416-423.
70. Häggglund, E.; Lindberg, B.; McPherson, J. *Acta Chem. Scand* 1956, 10, 1160-1164.
71. Poutanen, K.; Puls, J. In *Biogenesis and Biodegradation of Plant Cell Wall Polymers*, Lewis, G.; Paice, M., Eds.; American Chemical Society: Washington, D.C., 1989; pp 630-640.

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Chapter 34

Comparison of Endolytic Hydrolases That Depolymerize 1,4- β -D-Mannan, 1,5- α -L-Arabinan, and 1,4- β -D-Galactan

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Hydrolysis of mannan-type polysaccharides by β -mannanase is dependent on substitution on and within the main-chain as well as the source of the β -mannanase employed. Characterisation of reaction products can be used to define the sub-site binding requirements of the enzymes as well as the fine-structures of the polysaccharides. Action of *endo*-arabinanase and *endo*-galactanase on arabinans and arabinogalactans is described. Specific assays for *endo*-arabinanase and arabinan (in fruit-juice concentrates) are reported.

Beta-mannanases. Enzymes active on 1,4- β -D-mannans include *endo*- β -mannanase, *exo*- β -mannanase and β -mannosidase. Two types of *exo*- β -mannanase have been purified and characterised. For both enzymes, the preferred substrate has a degree of polymerisation (DP) of 5 or more. However, one of these enzymes (from guar seed(1) releases mannose, while the second type (from *Aeromonas hydrophila*(2) sequentially releases β -D-mannobiose from the non-reducing end of the manno-oligosaccharide. Several β -mannosidases have been purified and characterised (3,4). The enzyme from *Helix pomatia* (4) hydrolysed β -D-mannobiose at the same rate as higher DP manno-oligosaccharides, but had no detectable action on mannobitol. This enzyme cleaves terminal (non-reducing end) D-mannosyl residues from D-gluco-D-manno-oligosaccharides, and with D-galacto-D-manno-oligosaccharides, can cleave the glycosidic linkage of D-mannosyl residues attached to D-galactose substituted D-mannosyl residues, but cannot bypass substituted residues. Consequently, this enzyme has found considerable use in the characterisation of such oligosaccharides.

The most characterised D-mannan degrading enzymes are the *endo*- β -mannanases (5). These enzymes act on a range of '1,4- β -D-mannan-type' polysaccharides including D-mannan, glucomannans (acetylated and deacetylated), galactomannans and galacto-glucomannans. The extent and

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products of hydrolysis are a consequence of both the source of the particular β -mannanase employed, and the sugar composition and physical state of the substrate employed (e.g. the galactose content of galactomannans).

In the following section of this paper, some aspects of the author's work on the characterisation and utilisation of β -mannanases will be described.

Assay and Purification of β -Mannanases. β -Mannanase can be specifically measured in crude enzyme mixtures with a dye-labelled carob galactomannan substrate (6). This substrate has a galactose content high enough (23%) to impart solubility to the substrate, but sufficiently low as not to interfere with the action of β -mannanases on the D-mannan backbone. The soluble dye-labelled substrate is incubated with enzyme preparation under controlled conditions and the reaction is terminated and unreacted dyed polysaccharide precipitated by ethanol addition.

β -Mannanases are generally prepared by conventional chromatographic procedures, which can lead to high yields of enzyme, but in some cases only a poor state of purity. Small quantities of highly purified β -mannanases have been prepared by substrate affinity chromatography of partially purified enzymes on a column of glucomannan immobilised on aminohexane Sepharose 4B (6). The action patterns of enzymes described in this paper were determined using enzymes purified by this latter procedure.

Action of β -Mannanases on Oligosaccharides and Polysaccharides.

(a) **Galactomannans.** The extent of hydrolysis of galactomannans by a particular β -mannanase is dependent on the extent to which the 1,4- β -D-mannan backbone is substituted by D-galactosyl residues as well as the pattern of distribution of these residues. Thus, the degrees of hydrolysis of lucerne (48% Gal), guar (38% Gal), tara (28% Gal) and carob (24% Gal) galactomannans are 1, 5, 20 and 22% respectively (5). However, *Leucaena leucocephala* galactomannan has a galactose/mannose ratio the same as that for guar, yet it is hydrolysed by *A. niger* β -mannanase to an extent of 10% (i.e. twice that for guar). This result, and that obtained with several other galactomannans, shows that the pattern of distribution of the galactosyl residues is also important in determining the extent of β -mannanase action. As a corollary to this, β -mannanase can be used to define the fine-structure of distribution of D-galactosyl residues along the D-mannan backbone (7).

β -Mannanase enzymes from different sources vary in their ability to cleave a given galactomannan, thus the extent of hydrolysis of hot-water-soluble carob galactomannan (18% galactose) by *Aspergillus niger* and guar seed β -mannanases are 26 and 20% respectively. Other β -mannanases studied gave values within this range (5).

The difference in action patterns between these β -mannanases is shown clearly by fractionating and characterising the reaction products (Table I) (8). These products are a consequence of the ability of the enzyme to cleave in the vicinity of D-mannosyl residues substituted by D-galactose, as well as the length of the 1,4- β -D-manno-oligosaccharide chain required by the enzyme for binding. The favoured conformation of the (1-4)- β -D-linked mannan chain is a flat

ribbon with a two-fold axis, which places neighbouring hydroxymethyl groups on opposite sides of the ribbon (9,10), so that, in galactomannan, galactosyl substituents separated by zero or an even number of mannosyl residues lie on opposite sides of the chain and those separated by an odd number, on the same side. Then the enzyme binds to one edge of the ribbon, as in Figure 1. With slight modifications, this schematic representation can be used to define the binding requirements of each of the β -mannanases studied in our laboratory.

For *A. niger* β -mannanase, cleavage at the point marked is only possible if sugar residues B and D are not substituted by D-galactose. For optimal rate of hydrolysis, a 1,4- β -D-manno-oligosaccharide chain length of 4 is required. With guar-seed β -mannanase a chain length of 5 residues is required with disposition around the active site as shown in Figure 1. Also, this enzyme, unlike the *A. niger* β -mannanase, is unable to cleave the glycosidic linkage of a D-galactosyl substituted D-mannosyl residue. Thus guar-seed β -mannanase is unable to cleave at the point marked (Figure 1) if sugar residues B, C or D are substituted by D-galactose and this is reflected by the structures of the oligosaccharides produced (Table I).

(b) **Glucomannans.** Action of β -mannanases on glucomannans is dependent on the glucose/mannose ratio in the glucomannan as well as the degree of acetylation and the physical state of the polysaccharide (i.e. solution or gelatinous suspension) (5). Salep glucomannan (Glc:Man = 20:80) is hydrolysed more extensively than konjac glucomannan (Glc:Man = 40:60). On treatment of salep glucomannan with sodium hydroxide followed by neutralisation (to effect deacetylation), the polymer remains in solution and is more extensively hydrolysed by β -mannanase than is the native polymer. Deacetylation of konjac glucomannan yields a gelatinous precipitate on neutralisation. The acetyl groups impart solubility to the glucomannans; removal of these leads to a self-association of the polysaccharides and consequent precipitation. Konjac glucomannan with a higher glucose content is more 'cellulose-like' and thus precipitates much more rapidly than deacetylated salep glucomannan. The nature of the oligosaccharides released on hydrolysis of glucomannans by pure β -mannanase is consistent with the substrate binding pattern shown in Figure 1. At sugar residues B and D, the enzyme apparently binds through the hydroxymethyl groups of the sugar, and since this is the same in glucose and mannose, substitution of a D-mannosyl residue by a D-glucosyl residue does not prevent cleavage at the point marked. However, for sugar residues C and E, binding apparently occurs through the other edge (i.e. through HO-2 and/or HO-3), and replacement of mannose by glucose would be expected to affect binding of the β -mannanase to the substrate, and thus cleavage. In fact, substitution of a D-mannosyl by a D-glucosyl residue at either of these points prevents cleavage. Thus, none of the oligosaccharides produced have a D-glucosyl group at the reducing end. The disposition of D-glucosyl residues within the oligosaccharides produced is also affected by this binding requirement. Thus, the only glucose containing di- or tri-saccharide permitted

Table I. Oligosaccharides produced on hydrolysis of carob galactomannan by β -mannanases

<i>A. niger</i> β -Mannanase	Guar-seed β -Mannanase
$M-M$ $M-M-M$ $M-M$ $M-M-M$ $M-M-M-M-M$ $M-M-M-M-M$ $M-M-M-M-M$ $M-M-M-M-M-M$	$M-M$ $M-M-M$ $M-M-M-M$ $M-M-M$ $M-M-M-M$ $M-M-M-M$ $M-M-M-M-M$ $M-M-M-M-M$ $M-M-M-M-M-M$

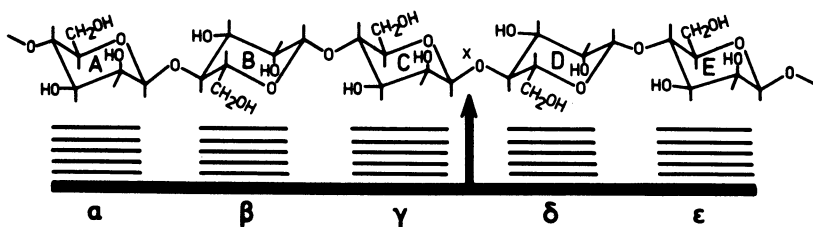


Figure 1. Schematic representation of subsite binding between β -mannanase and the (1 \rightarrow 4)- β -D-mannan chain. (Reproduced with permission from reference 5. Copyright 1983 Elsevier Science Publishers).

(and in fact found) has the **D**-glucosyl residue located at the non-reducing end. The only glucose containing di-, tri- and tetra-saccharides produced are β -Glc-(1 \rightarrow 4)-Man, Glc-Man-Man, Glc-Man-Man-Man, Man-Man-Glc-Man and Glc-Man-Glc-Man.

(c) **Galactoglucomannans.** There have been limited reports on the action of β -mannanases on galactoglucomannans. In our laboratory we have studied the action of β -mannanases on galactoglucomannans which occur as seed-endosperm reserves in *Cercis siliquaestrum* and *Bauhinia* sp. Oligosaccharides produced on hydrolysis of *Cercis* galactoglucomannan by *A. niger* β -mannanase were fractionated by chromatography on Bio-Gel P-2 and characterised using highly purified enzymes (α -galactosidase, β -mannosidase and β -glucosidase). A mixed trisaccharide containing the three monosaccharides was shown to be 1,4 β -D-Glc-(1,6- α -D-Gal)-D-Man. This structure is consistent with the defined sub-site binding requirements of *A. niger* β -mannanase.

Preparation of 1,4- β -D-Manno-oligosaccharides. Manno-oligosaccharides can be prepared by acid or enzymic hydrolysis of mannan (i.e. ivory nut mannan) (12). With acid, the yield of oligosaccharides is low compared to what is recovered when β -mannanases are employed. However, even with the use of β -mannanase, it is difficult to obtain reasonable yields of manno-oligomers of DP>4. This problem can be resolved by hydrolysing galactomannan with β -mannanase, where the degree of hydrolysis can be pre-determined by the particular galactomannan and β -mannanase employed. Following heat inactivation of β -mannanase, the mixture is treated with α -galactosidase to convert the galacto-manno-oligosaccharides to galactose and β -D-manno-oligosaccharides. With such a format, we have successfully produced mixtures containing high levels of manno-oligosaccharides of DP 3-8 (11).

Fine Structure, Gelling Interaction of Galactomannans. Knowing the sub-site binding requirements of a particular β -mannanase, and determining the amounts and structures of the oligosaccharides produced on hydrolysis of a given galactomannan, it is possible to gain information on the fine-structure of distribution of galactosyl residues along the **D**-mannan backbone (7), and in turn, the potential gelling interaction properties of the polysaccharide (13).

This has been achieved by developing computer programs which simulate the sub-site binding properties of the β -mannanase as well as the synthesis of galactomannan (7). In the simulated galactomannan, the mannan backbone is 1,000 units long, and in the 'growing' mannan chain, the probability of a given **D**-mannosyl residue being substituted by **D**-galactose is dependent on the nature of substitution of the previous two residues (nearest neighbour/second nearest neighbour interactions i.e. a second-order Markov process). Thus, four probabilities are involved, P_{00} in which the previous two residues are unsubstituted), P_{01} , P_{10} and P_{11} . The simulated polysaccharide was subjected to cleavage by the simulated β -mannanase and the probabilities were optimised, in turn, through a minimization of the sum of squared differences between the supplied experimental data and the corresponding computed values. These probability values give information on the overall galactose distribution and the

lengths of the unsubstituted regions of the D-mannan backbone, and thus give a direct insight into the gelling interaction potential of galactomannan samples. Gelling interaction of galactomannans with xanthan, K-carrageenan and agarose is dependent on the proportion and lengths of these unsubstituted regions.

Future research. From an industrial standpoint, the major requirements of β -mannanases are thermostability and the ability to hydrolyse crystalline and/or amorphous mannan to low DP oligosaccharides. Enzymes with such properties could find application in such processes as coffee extraction and in enzymic treatment of wood pulp.

Arabinanases

Enzymes active on α -L-arabinan include *endo*-1,5- α -L-arabinanase (*endo*-arabinanase EC 3.2.1.99) (14-16), α -L-arabinofuranosidase (EC 3.2.1.55) (16-19) and a novel *exo*-arabinanase (20) which releases trisaccharide units from highly branched beet arabinan, but has no action on linear 1,5- α -L-arabinan.

Arabinofuranosidases readily hydrolyse 1,3(2)- α -L-arabinofuranosyl residues from sugar-beet arabinan yielding an essentially linear 1,5- α -L-arabinan, which precipitates from solution (21,22). This linear arabinan is hydrolysed by *A. niger* arabinofuranosidase at less than 10% the rate of the original arabinan, suggesting that 1,3- α -L-arabinosyl residues are cleaved more rapidly than 1,5- α -L-linked arabinofuranose. However, an enzyme from *Scopolia japonica* (18) was shown to cleave both linkage types at similar rates. Arabinofuranosidases cleave arabinofuranosyl residues from arabinoxylans at about 10% the rate for beet arabinan. Gum arabic is quite resistant to hydrolysis. Two types of α -L-arabinofuranosidases were reported by Rombouts *et al.* (16) ; one (type B) was similar to that reported by Kaji and Saheki (14), but a second enzyme (type A) acted on 1,5- α -L-arabinofuranosyl oligosaccharides and *p*-nitrophenyl α -L-arabinofuranose but not on beet arabinan.

endo-Arabinanases cleave the 1,5- α -L-arabinofuranosyl backbone of arabinan releasing oligosaccharide fragments. The extent of hydrolysis (14,23) and rate of hydrolysis (15) is affected by the degree of substitution of the arabinan backbone by 1,3(2) α -L-arabinofuranosyl residues. It has been shown (23) that partial debranching of sugar beet arabinan by α -L-arabinofuranosidase increases its susceptibility to degradation by *endo*-arabinanase, but this effect has not been carefully quantitated.

Interest in arabinans has increased significantly in recent years due to their identification as a major cause of haze in apple and pear juice concentrates. These problems can be relieved by using pectic enzyme mixtures containing adequate levels of *endo*-arabinanase to degrade 1,5- α -L-arabinan produced from branched arabinan either by the action of α -L-arabinofuranosidase or a combination of heat and low pH conditions (23).

Our work in this area has two major aims; namely (1) to develop a specific assay for *endo*-arabinanase in pectinase preparations and (2), to develop a specific and reliable assay for arabinan in fruit juice and juice

concentrates. To achieve these goals it has been necessary to purify and characterise α -L-arabinofuranosidase and *endo*-arabinanase and to produce a range of enzymically and chemically modified arabinan materials.

Measurement of *endo*-Arabinanase. The substrate usually employed for *endo*-arabinanase measurement is 1,5- α -L-arabinan recovered from hazy fruit-juice concentrate, and activity is measured by Hplc procedures in which the disappearance of arabinan is monitored. This assay is limited by the restricted availability of the substrate and the time required for the analyses. An alternative substrate (25) is 1,5- α -L-arabinan prepared by α -L-arabinofuranosidase treatment of purified sugar-beet arabinan (termed Debranched Arabinan). Such material was prepared in this laboratory by treatment of beet arabinan (1 litre, 2% w/v) with 1,000 Units of arabinofuranosidase at pH 4 and 40°C for 2 hours. Complete debranching was confirmed by proton resonance NMR (disappearance of resonance peaks at 5.075 and 5.125 (acetone standard) and the recovered polysaccharide had an arabinose: galactose:galacturonic acid composition of 89.5:4.7:5.8 (smaller quantities of rhamnose were also present). This polysaccharide at 2 mg/ml was hydrolysed by a purified *endo*-arabinanase from *A. niger* (sp. activity 8 U/mg) at a rate similar to that for an arabinan purified from pear-juice concentrate. However, the rate of hydrolysis of this substrate by α -L-arabinofuranosidase was less than 0.2% the rate of hydrolysis by *endo*-arabinanase. At this rate difference, and knowing the range of activity values for *endo*-arabinanase and arabino-furanosidase found in commercial pectinase preparations, this substrate can be used directly for measurement of *endo*-arabinanase in crude pectinase preparations using a reducing sugar assay (e.g. Nelson/Somogyi (24)).

The Debranched Arabinan substrate precipitates from solution on storage at 4°C overnight. This problem was alleviated by slight carboxymethylation of the polymer to increase solubility. A carboxymethyl degree of substitution of 0.1 greatly improved solubility properties while giving only a 10% decrease in the rate of hydrolysis by *endo*-arabinanase. Increasing the degree of carboxy-methylation to 0.2 halved the initial rate of hydrolysis of the substrate by *endo*-arabinanase.

endo-Arabinanase from *A. niger* has a high affinity for CM-Debranched Arabinan, with a K_m of 0.37 mg/ml. Thus, a final substrate concentration in assay mixtures of 2 mg/ml was routinely employed. The background reducing sugar level in the substrate was reduced by borohydride reduction. Increase in reducing sugar levels on hydrolysis by *endo*-arabinanase were measured using the Nelson/Somogyi (24) reducing sugar method as this gave the same molar colour response with arabinose, 1,5 α -L-arabinobiose and 1,5- α -L-arabinotriose.

In using this substrate to screen activity in a range of commercial pectinase preparations, it was found that for all preparations, except two, the same ratio of activity on this substrate and linear arabinan (from pear juice) was obtained as was obtained for pure *endo*-arabinanase. One of these preparations was Novo Ultra SP. This preparation hydrolysed CM-Debranched Arabinan at 1.5 times the rate of pear-juice linear arabinan. This additional

activity may be due to rhamnogalacturonanase activity which has recently been reported to be present in Novo Ultra SP (and none of the other preparations examined) (26)

It was thus obvious that CM-Debranched Arabinan did not have the required purity to allow specific measurement of *endo*-arabinanase and that further purification of the Debranched Arabinan was necessary. This was effected by chromatography (at 70°C) through Q-Sepharose CL-6B at pH 8.0. This treatment separated a highly purified Linear Arabinan (unbound) from a charged arabinan material. The Linear Arabinan contained arabinose:galactose:galacturonic acid in the ratio of 97.4:0.5:2.1. On carboxymethylation to an extent of 0.05, the substrate was hydrolysed by pure *endo*-arabinanase at about 110% the rate for pear-juice linear arabinan. Furthermore, the relative rates of hydrolysis of the two substrates by *endo*-arabinanase in a range of commercial pectinase preparations (including Novo Ultra SP) were the same. This substrate was not hydrolysed by *endo*-galactanase or *endo*-polygalacturonanase and the rate of hydrolysis by arabinofuranosidase was less than 0.2% of that for *endo*-arabinanase. Hydrolysis of the substrate by *endo*-arabinanase containing a 20-fold higher level of arabinofuranosidase was only 6% higher than the rate of *endo*-arabinanase devoid of arabinofuranosidase. CM-Linear Arabinan is thus a useful substrate for the specific measurement of *endo*-arabinanase in crude pectinase preparations. In situations where the enzyme preparation contains high levels of reducing sugars, a dye-labelled arabinan substrate (Red Debranched Arabinan) can be employed.

Kinetic Properties of *A. niger* Arabinofuranosidase and *endo*-Arabinanase.

Information on the linkage requirements (for arabinofuranosidase) and the substrate binding requirements of *endo*-arabinanase was obtained by studying the kinetics of hydrolysis of 1,5- α -L-arabino-oligosaccharides (compared to beet arabinan) and by characterising the structures of the branched arabino-oligosaccharides produced by *endo*-arabinanase hydrolysis of partially debranched beet arabinan. With arabinofuranosidase, the rates of hydrolysis of 1,5- α -L-arabinotriitol, -tetraitol, -pentaitol, and -hexaitol were essentially the same. Arabinobiiitol was resistant to hydrolysis. Determined V_{max} and K_m values for this enzyme on beet arabinan (1,3- α -L-arabinosyl linkage hydrolysis) and 1,5- α -L-arabinotriitol are shown in Table II. It is apparent that, contrary to expectation, the 1,5- linkage is hydrolysed more rapidly than the 1,3-. The slow rate of hydrolysis of linear 1,5- α -L-arabinan by arabinofuranosidase is due, in fact, to a low affinity of the enzyme for the arabinan containing the 1,5-linkage. From Hplc studies on these substrates (and consistent with literature reports), the length of these 1,5- α -L-arabinan chains is 50-100 residues. Thus, at a substrate concentration of 2 mg/ml (CM-Linear Arabinan), the concentration of terminal arabinosyl residues is less than 0.2 mM. This explains the slow rate of hydrolysis of this substrate by α -L-arabinofuranosidase.

On incubation of *endo*-arabinanase with reduced arabino-oligosaccharides, the rate of hydrolysis increases up to the hexasaccharide indicating that the enzyme binds over 5-6 arabinosyl residues (11). Further information on these requirements has been gained by characterising the

structures of oligosaccharides produced on *endo*-arabinanase hydrolysis of partially debranched beet arabinan. The smallest branched oligosaccharide was a tetrasaccharide with an arabinosyl residue linked α -L-1,3- to the central residue of 1,5- α -L-arabinotriose i.e. 3²- α -L-arabinofuranosyl 1,5- α -L-arabinotriose (shown by C¹³-NMR), and this was the only branched tetrasaccharide. This oligosaccharide was resistant to cleavage by *endo*-arabinanase at levels which gave rapid hydrolysis of 1,5- α -L-arabinotriose. It would thus appear that for hydrolysis of branched 1,5- α -L-arabinan by *endo*-arabinanase, there is a requirement for at least one unsubstituted α -L-arabinofuranosyl residue on each side of the substituted residue.

Measurement of Arabinan. Excess quantities of arabinan in fruit juice can lead (after prolonged storage) to hazy fruit-juice concentrates. This problem can be resolved by ensuring that the pectinase preparation employed contains adequate levels of *endo*-arabinanase to hydrolyse the amounts of debranched arabinan likely to be present. A limiting factor is that currently, there are no methods available for the measurement of arabinan in fruit juice or juice concentrates.

Table II. Kinetic constants for *A. niger* α -L-Arabinofuranosidase on Arabinotriitol and Sugar-Beet Arabinan

Substrate	Linkage type hydrolysed	K _m	V _{max} U/mg
1,5- α -L-Arabinotriitol	1,5- α -L-	14.3 mM	82.4
Sugar-beet Arabinan	1,3(2)- α -L-	0.18 mg/ml*	40.3

*This is approximately equal to 0.46 mM terminal arabinosyl residues.

This problem has now been resolved by the development of a method which employs *endo*-arabinanase and arabinofuranosidase to hydrolyse arabinan to arabinose, with specific measurement of arabinose with galactose dehydrogenase. Levels of free arabinose and galactose in juice are measured on samples not treated with arabinofuranosidase/*endo*-arabinanase mixtures. The arabinan degrading enzymes are devoid of galactose releasing activities. Experiments to determine the required incubation conditions for 'quantitative' measurement of arabinan in fruit juice concentrate (pear juice) are summarised in Figure 2. Direct analysis of juice gives a measure of total arabinofuranosyl oligosaccharides of DP \geq 2. Higher DP material can be selectively measured by dialysing the juice before analysis.

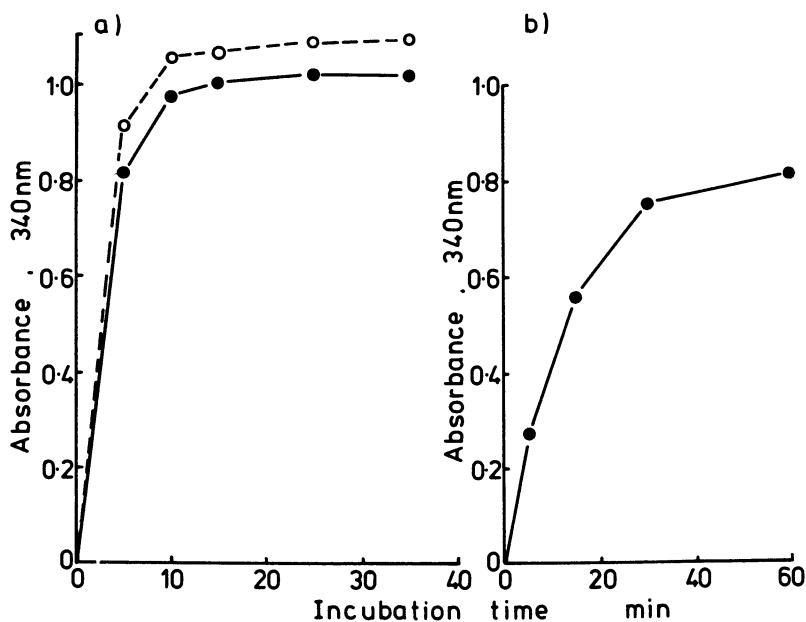


Figure 2. Enzymic measurement of α -L-arabinan in fruit-juice concentrates. (a) Effect of time of incubation of sample with arabinofuranosidase and *endo*-arabinanase. Diluted pear juice concentrate (1:10; 0.1 ml) or sugar beet arabinan solution (0.1 ml) was incubated with an aliquot (0.1 ml) of arabinofuranosidase (5 units) plus *endo*-arabinanase (0.2 units) at 35°C and pH 4.0. Aliquots were analysed for arabinose. (b) Arabinose determination using the NAD-Galactose Dehydrogenase method. Arabinose solution (0.2 ml, 50 μ grams) was incubated with Tris-HCl buffer (2.5 ml, pH 8.6), NAD (0.1 ml, 10 mg/ml) and galactose dehydrogenase (20 μ l, 140 milliunits) at 35°C. Absorbance (340 nm) was measured after various time intervals.

galactanase (approx. 10% of maximum) a range of 1,4- β -D-galactooligosaccharides up to a DP of at least 10 are produced. These oligomers were fractionated on Bio-Gel P-2 and then treated with an excess of *endo*-galactanase. In all cases, the oligomers were hydrolysed almost exclusively to galactobiose and galactose, consistent with previous findings (28) on the structure of the galactan fragments in soybean arabinogalactan.

endo-1,4- β -D-Galactanase is usually assayed with an arabinogalactan type material from soybean or potato with allowance made for the action of other enzymes on these substrates. It would seem likely that a more specific substrate could be prepared by treatment of soybean arabinogalactan with a cocktail of enzymes including *endo*-polygalacturonanase, *endo*-arabinanase and arabinofuranosidase to remove polysaccharide material susceptible to hydrolysis by these enzymes. Such work is currently underway in our laboratories.

Literature Cited

1. McCleary, B.V. *Carbohydr. Res.* **1982**, *101*, 75-92.
2. Araki, T.; Kitamikado, M. *J. Biochem.* (Tokyo) **1982**, *91*, 1181.
3. Boukuelet, S.; Spik, G.; Montreuil, J. *Biochim. Biophys. Acta* **1978**, *522*, 521-530.
4. McCleary, B.V. *Carbohydr. Res.* **1983**, *111*, 297-310.
5. McCleary, B.V.; Matheson, N.K. *Carbohydr. Res.* **1983**, *119*, 191-219.
6. McCleary, B.V. In *Methods in Enzymology*; Wood, W.A.; Kellogg, S.T., Ed.; Academic:New York, NY, 1989; Vol. 160, pages 74-86; 596-610.
7. McCleary, B.V.; Clark, A.H.; Dea, I.C.M.; Rees, D.A. *Carbohydr. Res.* **1985**, *139*, 237-260.
8. McCleary, B.V.; Nurthen, E.; Taravel, F.R.; Joseleau, J.-P. *Carbohydr. Res.* **1983**, *118*, 91-109.
9. Sundararajan, P.R. and Rao, V.S.M. *Biopolymers*, **1970**, *9*, 1239-1247.
10. Bittiger, H. and Marchessault, R.H. *Carbohydr. Res.* **1971**, *18*, 469-470.
11. McCleary, B.V. *Unpublished data*.
12. Kusakabe, I.; Takahashi, R. In *Methods in Enzymology*; Wood, W.A.; Kellogg, S.T. Ed.; Academic:New York, NY, 1989; Vol 160, p.518-523.
13. Dea, I.C.M.; Clark, A.H.; McCleary, B.V. *Carbohydr. Res.* **1986**, *147*, 275-294.
14. Kaji, A.; Saheki, T. *Biochim. Biophys. Acta* **1975**, *410*, 354-360.
15. Weinstein, L.; Albersheim, P. *Plant Physiol.* **1979**, *63*, 425-432.
16. Rombouts, F.M.; Voragen, A.G.J.; Searle-van Leeuwen, M.F.; Geraeds, C.C.J.M.; Schols, H.A.; Pilnik, W. *Carbohydr. Polym.* **1988**, *9*, 25-47.
17. Kaji, A.; Tagawa, K. *Biochim. Biophys. Acta* **1970**, *207*, 456-464.
18. Tanaka, M.; Abe, A.; Uchida, T. *Biochim. Biophys. Acta* **1981**, *658*, 377-386.
19. Brillouet, J.-M.; Moulin, J.-C.; Agosin, E. *Carbohydr. Res.* **1985**, *144*, 113-126.
20. Kaji, A.; Shimokawa, K., *Agric. Biol. Chem.*, **1984**, *48*, 67-72.
21. Gremlı, H.; Neukom, H. *Carbohydr. Res.* **1968**, *8*, 110-112.
22. Tagawa, K.; Kaji, A. *Carbohydr. Res.* **1969**, *11*, 293-301.

23. Voragen, A.G.J.; Rombouts, F.M.; Searle-van Leeuwen, M.F.; Schols, H.A.; Pilnik, W. *Food Hydrocolloids*, **1987**, *1*, 423-437.
24. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19-23.
25. McCleary, B.V. In *Gums and Stabilisers for the Food Industry* Phillips, G.O.; Wedlock, D.J.; Williams, P.A.; Eds., Elsevier:London, 1990; Vol. 5, 291-298.
26. Voragen, A.G.J. *Int. Food Ing.* **1990**, *1*, 18-31.
27. Hashimoto, Y. *Nippon Nogei Kagaku Kaishi*, **1971**, *45*, 147-150.
28. Labavitch, J.M.; Freeman, L.F.; Albersheim, P. *J. Biol. Chem.* **1976**, *251*, 5904-5910.
29. Emi, S.; Yamamoto, T. *Agric. Biol. Chem.* **1972**, *36*, 1945-1954.

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Chapter 35

Microbial Strategies for the Depolymerization of Plant and Algal Polyuronates

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The maceration of plant cell walls requires the depolymerization of the polygalacturonate (pectate) component of rhamnogalacturonan. Analogous processing of the matrix and wall components of the brown algae requires the depolymerization of alginate. Most pectate and all alginate degrading bacteria secrete either pectate or alginate lyases which catalyze trans-eliminase reactions responsible for glycosidic bond cleavage and the depolymerization process. The pectate lyases secreted by the phytopathogen, *Erwinia chrysanthemi*, include a battery of four or more enzymes with pI values ranging from 4.2 to 9.6. These enzymes display unique mechanisms in the depolymerization of polygalacturonate, ranging from predominantly endolytic to exolytic, which collectively contribute to the maceration process associated with the pathogenic response. In contrast, bacteria associated with a stable environment, e.g. *Lachnospira multiparus* in the bovine rumen, *Clostridium populeti* from a poplar based methane digester, or bacteria associated with healthy tissues of the brown algae, *Sargassum*, secrete acidic pectate or alginate lyases with exolytic/endolytic depolymerization mechanisms. Through the secretion of different polyuronate lyases, these different bacteria should provide an useful genetic resource for the directed depolymerization and solubilization of plant tissue.

Structural Properties of Pectate and Alginate

The plant polyuronates, pectate and alginate, are perhaps most well known and conceptually defined for their use in a number of industrial applications. Their ability to form nontoxic gels and thickening agents has led to their extensive application in the food industry. Much of our understanding of their chemical properties comes from investigations of the factors which influence their ability to form stable gels, particularly through the formation of complexes with divalent cations (1,2).

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The pectates are generally part of a more complex structure known as the rhamnogalacturonan (RG), and two main types designated as rhamnogalacturonan I and rhamnogalacturonan II have been isolated from plant cell walls and extensively characterized (3,4). Common to the RG fractions is the polygalacturonate in which uronic acid groups, either free or as methyl esters, are linked $\alpha(1-4)$ in chains up to 25 residues in length and are attached via (1-2) linkages to L-rhamnose residues. These rhamnose residues may be linked to D-galacturonic acid residues (containing either free or esterified carboxyl groups) via $\alpha(1-4)$ linkages which may be extended in chains of variable lengths and then to another L-rhamnose residue. Galactan or arabinogalactan polymers may be attached to the 4 position of the L-rhamnose residues, with these then connecting to the xylan or hemicellulose components of the cell wall (5). An important aspect of the RG structure is the presence of linear galacturonic acid residues, up to 25 in number, which are capable of forming double stranded complexes between two RG systems via calcium ion bridges. Other sequences contain methyl esters of galacturonic acid residues, preventing these sequences from complexing with the calcium ions and thus preventing cross-bridging to other RG systems at these locations (6). It would therefore appear that RG structure provides the rhamnose residues as punctuation points to separate sequences of free from esterified galacturonic acid residues, as well as providing attachment points to the hemicellulose and cellulose systems via the arabinogalactan chains. A schematic depicting this punctuation potential as well as the proposed cross-bridging of RG systems via calcium ions is presented in Figure 1. In considering this proposed structure, the polygalacturonate depolymerases must select a limited region of the RG as substrate prior to the action of esterases on the other sections of the RG.

The role of alginate in the structure of the cell walls of the brown algae (Phaeophyta), especially as it is viewed in relation to cellulose and other polymers, is not well understood. While it may constitute as much as 50% of the dry weight of many of the marine species of Phaeophyta, and be present in quantities much greater than is pectate in the tissues of higher plants, much of the alginate appears as nearly uniform material comprising a matrix which separates the algal cells (7,8). The alginate occurs as an unbranched polymer of $\beta(1-4)$ D-mannuronan and $\alpha(1-4)$ L-guluronan sequences interspersed with alternating D-mannuronate and L-guluronate residues. A critical aspect of the functional structure of alginate is the catenated structure of the adjacent $\alpha(1-4)$ L-guluronate residues versus the ribbon structure of the adjacent $\beta(1-4)$ D-mannuronate residues. The catenated structures of the $\alpha(1-4)$ L-guluronate residues are similar to those found for the $\alpha(1-4)$ linked D-galacturonate residues of pectate, and 18 or more in sequence provide the potential for cooperative calcium ion cross-bridging that is responsible for the structural properties of the alginate as an insoluble matrix material of the algal tissues (9,10). In contrast to the pectate, the development of sequences which complex with calcium is regulated by the conversion of $\beta(1-4)$ linked D-mannuronate residues to $\alpha(1-4)$ linked L-guluronate residues, a process that may be catalyzed by an epimerase acting

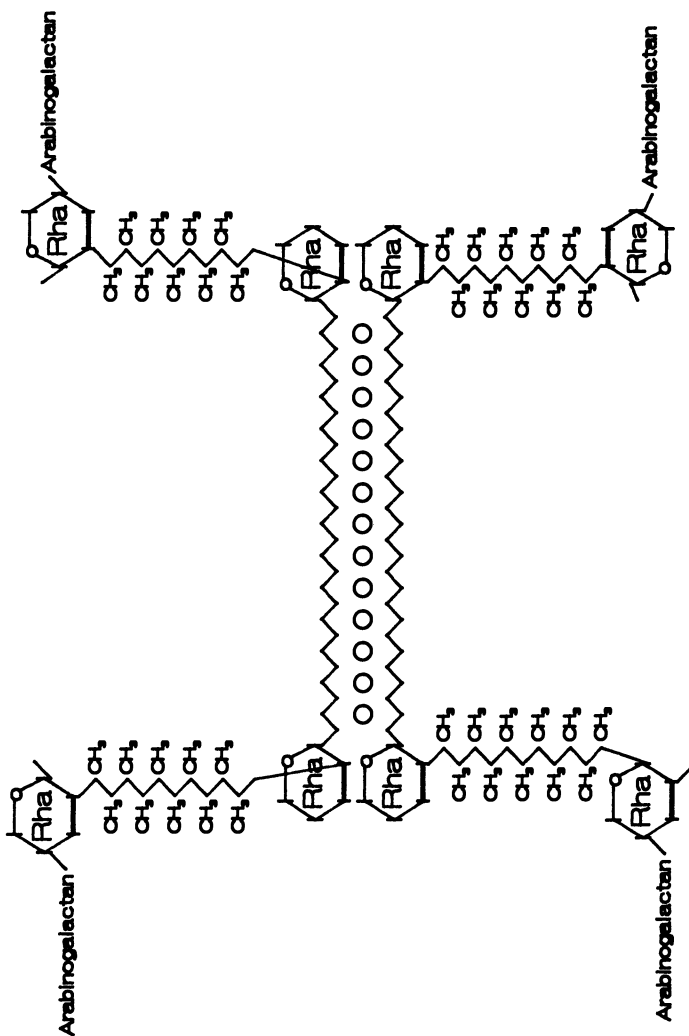


Figure 1. Schematic representation of pectin structure indicating stabilization of catenated polygalacturonic acid chains through Ca^{2+} cross-bridging. Non-bridging sequences of $\alpha(1-4)$ linked β -galacturonic acid methyl ester extend from L-rhamnose via (1-4) linkage to another rhamnose via $\alpha(1-2)$ linkages. Arabinogalactan side chains are linked to rhamnose residues and couple the RG structure to hemicellulose.

on the assembled polymer (11,12). A diagram depicting this process is described in Figure 2. It appears that the structures of both pectate and alginate that are functionally shared are the abilities to complex with calcium ion and form a structure containing two or more polymer chains held together by the divalent calcium ion bridge. The pectate then differs from the alginate in that it is regulated with respect to ion mediated cross-bridging by esterification of $\alpha(1-4)$ linked D-galacturonate residues, while the ionic cross-bridging of alginate is regulated by the epimerase catalyzed conversion of $\beta(1-4)$ linked D-mannuronate to $\alpha(1-4)$ linked L-gulonate. The unesterified homopolymeric sequences of mannuronan and guluronan chains provide a substrate for the enzymes catalyzing the depolymerization of alginate, with specific enzymes attacking each type of polymer.

Pathways for the Depolymerization of Pectate and Alginate

The complex structure of pectate in the RG fraction of the plant cell wall provides several options for its depolymerization. The presence or absence of methyl esters of individual or groups of galacturonate residues alters the nature of substrate, and has resulted in the evolution of enzymes that recognize these differences. As noted in several reviews (13,14,15), the different types of enzymes involved in the depolymerization of RG may involve either lyases or hydrolases, each with unique mechanisms of glycosidic bond cleavage. Different lyases may show specificity for methyl esterified polygalacturonate (pectin) while others are specific for unesterified polygalacturonate (pectate). In addition, pectin esterases may participate in the overall depolymerization process by providing substrate for pectate specific enzymes. A scheme depicting the different options for the depolymerization of pectin and pectate is shown in Figure 3.

The trans-elimination reaction mediated by the lyases is analogous to a base catalyzed beta-elimination reaction, and therefore occurs with pH optima near and above neutrality. The hydrolase catalyzed reaction is analogous to an acid catalyzed glycosidic bond cleavage, and occurs with acidic pH optima. The comparison of these two types of reactions in the cleavage of glycosiduronic linkages of pectate is given in Figure 4.

Both pectin and pectate specific lyases have been characterized. All of the hydrolases which have been studied as purified enzymes are specific for the unesterified pectate. With several notable exceptions, the degradation of pectate by bacteria occurs via secreted lyases in neutral media, while the degradation of pectate by fungi occurs via secreted hydrolases in acidic media (14). The most striking example of pectate depolymerization in nature occurs during the phytopathogenic response to infection by *Erwinia* species, particularly with pathovars of *E. carotovora* and *E. chrysanthemi*, and in both cases the maceration process is primarily effected by secreted pectate lyases (16). Another well documented plant maceration process occurs during ruminant digestion, and the ruminant anaerobe, *Lachnospira multiparus* (17), has been implicated as an active pectate degrading bacterium which secretes pectate lyase. These examples have provided the basis for detailed analysis of individual enzymes. In the case of

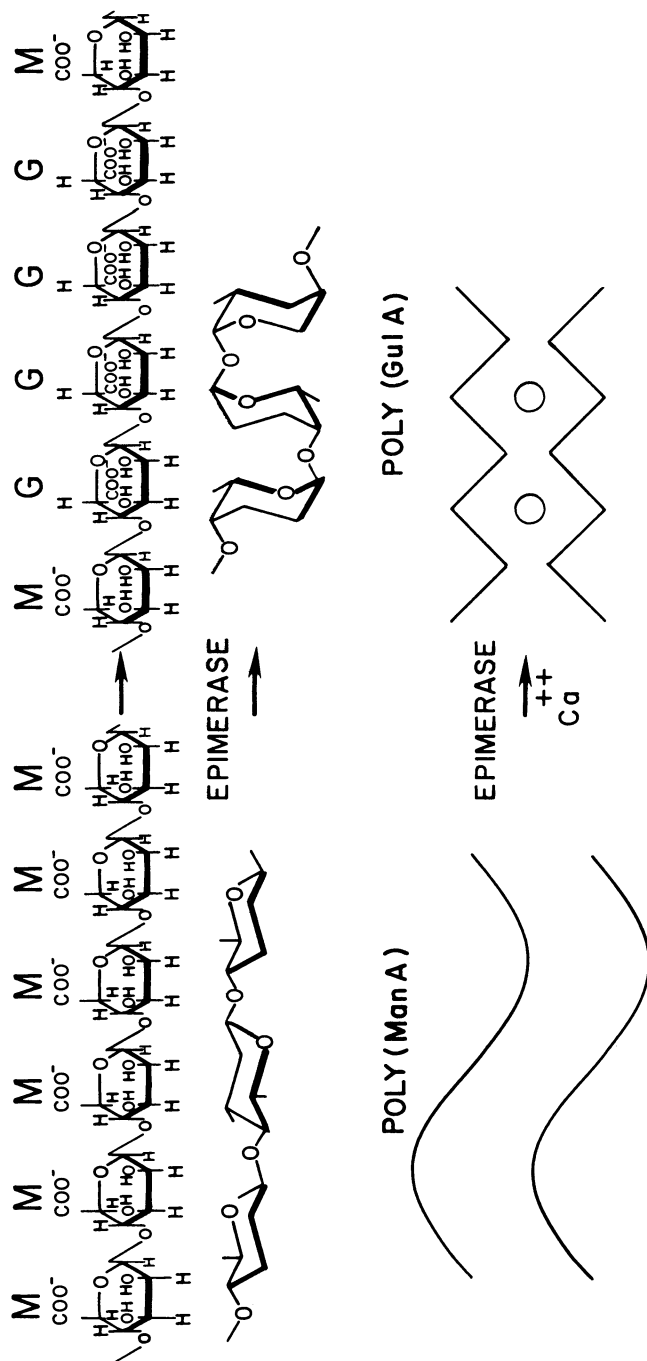


Figure 2. Structural properties of alginate are shown, with the linear array of Haworth structures given at the top, the conformational structure given next, and the effect of calcium on the formation of complexes between two polymeric strands of alginate given at the bottom. The epimerase catalyzed conversion of $\beta(1-4)$ linked D-mannuronate to $\alpha(1-4)$ linked L-guluronate residues of poly(ManA) to the catenated structure of poly(GulA) and the formation of the "eggbox" structure upon the complexing of two polymer strands with Ca^{2+} . (Reproduced with permission from reference 7. Copyright 1988 Elsevier.)

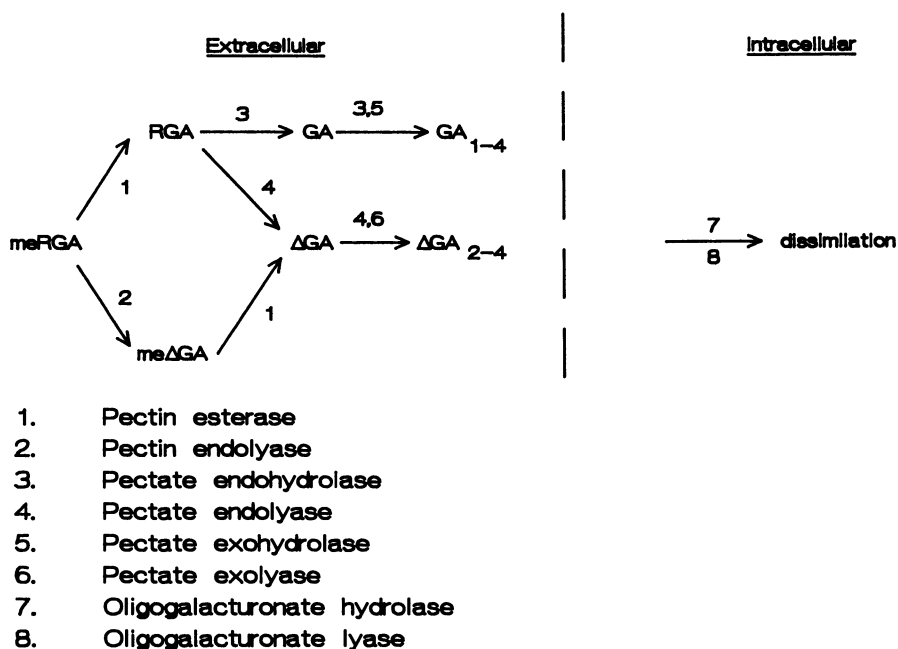


Figure 3. Pathways of pectin depolymerization by extracellular and intracellular enzymes associated with pectin degrading organisms. Methylated rhamnogalacturonan (meRGA) may be deesterified to yield demethylated rhamnogalacturonan (RGA) or may undergo eliminative cleavage via endolyase activity to form Δ -4,5-unsaturated methylated rhamnogalacturonan (me Δ RGA). Demethylation of me Δ RGA and subsequent depolymerization of saturated (GA) and unsaturated (Δ GA) polygalacturonic acid by hydrolyses and lyases in the figure yields oligomeric limit products (degree of polymerization indicated by subscripts) which can be assimilated by the organism and subsequently acted upon by intracellular enzymes.

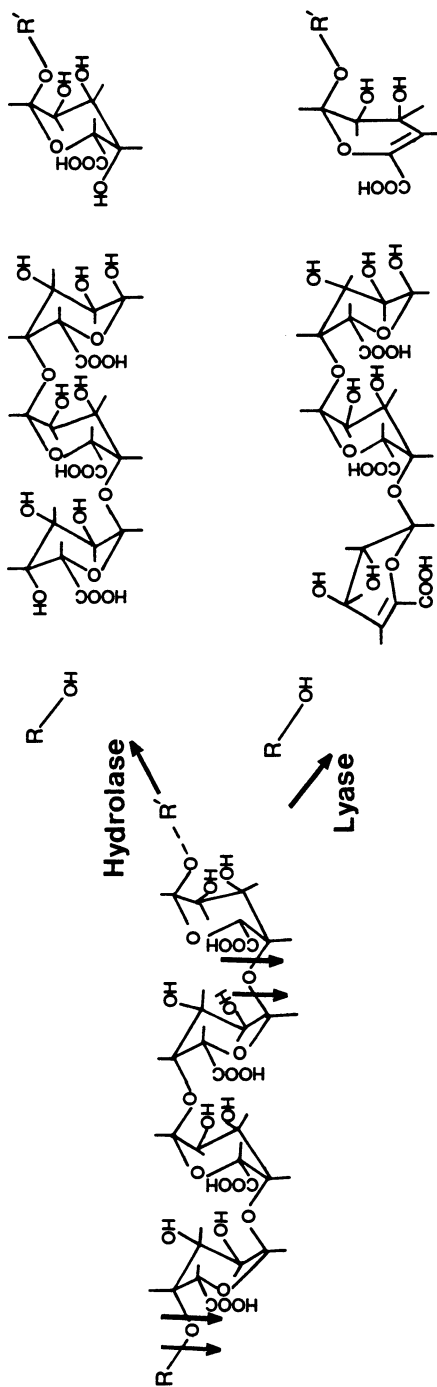


Figure 4. Comparison of hydrolase and lyase mechanisms from the depolymerization of polygalacturonic acid. Polygalacturonic acid sequences (structure at left) may be depolymerized via hydrolytic cleavage, (indicated by arrows to the left of o-linkages) to yield saturated oligomeric products indicated by the structures at upper right. Alternatively, eliminative depolymerization (indicated by arrows to the right of o-linkages) yields Δ -4,5-unsaturated oligomers indicated by the structures at lower right.

Erwinia chrysanthemi, interest in the maceration process has led to the cloning and sequencing of four separate genes coding for different secreted pectate lyases (18,19). The production of these enzymes, either by the parent organism, or as recombinant enzymes in *E. coli*, has now led to their detailed characterization with respect to depolymerization mechanism.

Aside from a single report of an alginate hydrolase, the depolymerization of alginate is mediated through the action of alginate lyases. These enzymes, which have been identified in bacteria, fungi, and molluscs, are specific for the sequences of D-mannuronate or L-guluronate residues within the alginate, and examples of both types of enzymes with endolytic and exolytic mechanisms have been documented (20). While many bacteria secrete a mixture of alginases which degrade both D-mannuronan and L-guluronan polymers, others secrete single enzymes that attack only one type of polymer (21,22,23). Regardless of substrate specificity, all of the alginate lyases which have been purified and characterized are single subunit enzymes with molecular weights close to 30 kD.

Kinetic Analysis of the Lyase Catalyzed Depolymerization Process

The introduction of the unsaturated bond between carbon atoms 4 and 5 in the newly generated nonreducing terminus of product provides a convenient marker with which to follow the lyase mediated depolymerization process (24). The ability to resolve individual oligouronates by reverse-phase ion-pair HPLC and detect and quantify individual oligouronates by their absorptive properties has led to methods for the kinetic analysis of the depolymerization process catalyzed by different alginate and pectate lyases (25,26,27). The appearance of individual products generated by the action of a mixture of pectate lyases secreted by *Erwinia chrysanthemi* UF1 is shown in the HPLC profiles in Figure 5. The generation of an oligomeric series, with increasing resolution obtained with increasing size, is presumably the result of the greater hydrophobic partitioning resulting from a greater number of carboxyl groups pairing with the tetrabutyl ammonium ions in the solvent. A plot of the molecular weight versus the logarithm of the retention time provides an almost linear (slightly curvilinear with increasing size) relationship which allows identification of individual oligouronates relative to reference oligosaccharides. The presence of unsaturated oligogalacturonates ranging in degree of polymerization (DP) from 2 (retention time of 5.36 min) to 8 (retention time of 26.66 min) with decreasing quantities from trimer to octomer, suggests that a combination of exolytic and endolytic activities are contributing to the overall depolymerization process.

When unpurified enzyme preparations from *Erwinia chrysanthemi* UF1 and *Lachnospira multiparus* are compared with respect to kinetics of formation of individual unsaturated oligogalacturonates formed, a clear difference may be observed (Figure 6). The *E. chrysanthemi* preparation generates large oligomers with DP values from 7 to 11 which are further depolymerized with time, finally yielding dimer, trimer, and tetramer as the predominant limit products. The *L. multiparus* preparation produces a heptamer as the largest detectable product, with the production of dimer, trimer, and tetramer as limit products. In this case

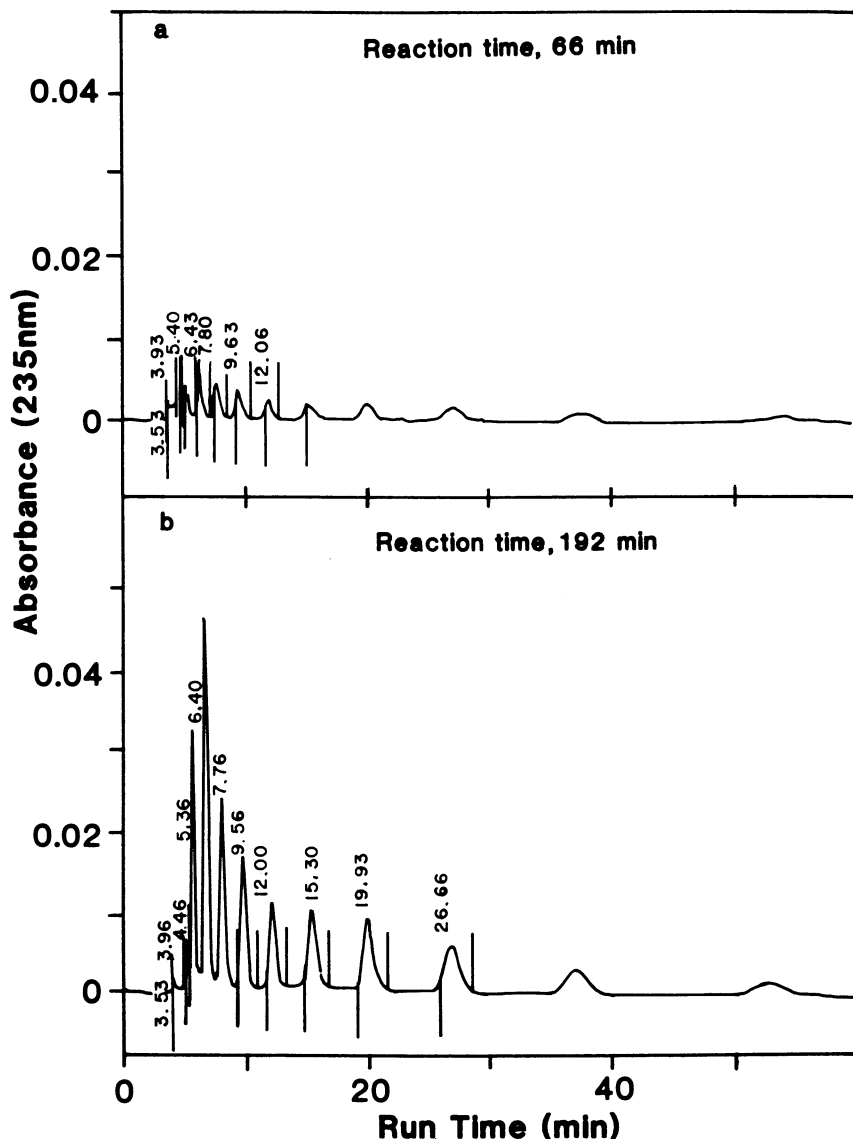


Figure 5. HPLC profiles of products from reactions catalyzed by secreted pectate lyase activities from *Erwinia chrysanthemi* after 66 min and 192 min. Reaction mixtures contained 4 units of enzyme activity per ml and 0.1% PGA in 0.05 M Tris-HCl, pH 8.5, 0.2 mM CaCl₂. Injections of 0.05 ml were made from reaction mixtures with a WISP automatic sample injector and eluted at 2.0 ml/min with a run time of 60 min. Compounds eluting with retention times of 5.36, 6.40, 7.76, and 9.56 min corresponded to unsaturated oligogalacturonate reference standards with DP values of 2, 3, 4, and 5, respectively.

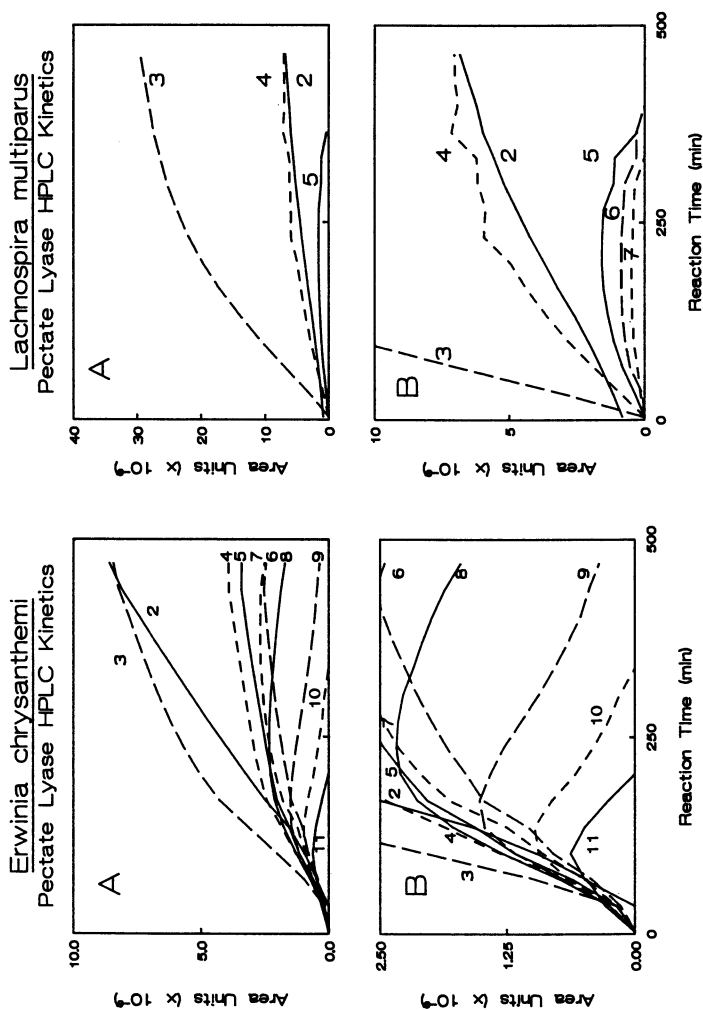


Figure 6. HPLC kinetics of polygalacturonic acid depolymerization by extracellular pectate lyases from crude supernatants of *Erwinia chrysanthemi* and *Lachnospira multiparus* cultures. A panels are full scale representations of products found over the reaction time sequence. B panels have expanded ordinates to better demonstrate the kinetics of minor products. Area unit refers to integration from HPLC tracings of product absorbance at 235 nm. Numbers in the panels refer to the degree of polymerization for individual products. Conditions for enzyme assay and product detection were the same as described for Figure 5.

the appearance of dimer appears to be at the expense of pentamer. The phytopathogen, *E. chrysanthemi*, secretes a complement of pectate lyase activities with a rapid random endolytic attack on the polygalacturonate. The ruminant bacterium, *L. multiparus*, secretes a complement of pectate lyase which generates smaller oligomers and appears to exhibit an exolytic/endolytic attack.

When the secreted pectate lyase preparation from *E. chrysanthemi* UF1 was subjected to isoelectric focusing and analyzed by pectate gel overlay, at least four different pectate lyases were resolved, with pI values ranging from 4.2 to 9.6. These were fractionated and obtained in preparative quantities by chromatofocusing. When kinetically compared for the depolymerization of polygalacturonate by the HPLC method, each of these could be distinguished as providing a unique kinetic profile (Figure 7). Fractions A, C, and D, which include both basic and acidic enzymes, show endolytic depolymerization profiles which may be distinguished with respect to the rates of dimer versus trimer formation, as well as the larger oligomers that persist as apparent limit products. Fraction B, which contained a single protein with a molecular weight of 40 kD, showed a depolymerization profile that was clearly unique. On the basis of the very rapid production of trimer, the formation of dimer at the expense of pentamer, and the transient appearance of oligomers larger than pentamer, this activity might best be classified as having an exolytic or a combination, exolytic/endolytic, mechanism. The structural genes coding for four different pectate lyases secreted by *Erwinia chrysanthemi* EC 16 have been cloned and expressed in *E. coli* by N T. Keen and his colleagues (18,19). Plasmids carrying these genes were provided and expressed in *E. coli* to provide enzymes as individual gene products. When kinetically analyzed by HPLC, once again different depolymerization profiles were observed which were, however, comparable to those for the enzymes secreted by the UF strain (Table I and II). It is clear that the *E. chrysanthemi* secretes a battery of pectate lyases with different depolymerization mechanisms, which may in part contribute to its virulence as a phytopathogen.

The pectate lyase activities secreted by *Clostridium populeti*, isolated from a poplar based methane digester (28), were compared with those secreted by *E. chrysanthemi* and *L. multiparus*. Isoelectric focusing followed by overlay and activity analysis revealed the presence of one or two activity bands with pI values from 4.5 to 4.7 for both the *C. populeti* and the *L. multiparus*. The kinetic depolymerization profiles of *C. populeti* also revealed an exolytic/endolytic mechanism, typified by the high ratio of trimer and/or dimer to hexamer in the early stages of the reaction (Table I).

Alginate lyases secreted by marine bacteria associated with *Sargassum* species have been purified and examined with respect to depolymerization mechanisms (21,22). A D-mannuronan specific enzyme secreted by fermentative Alg-A isolate has a pI of 4.2 to 5.0 and displays a depolymerization mechanism nearly identical to that designated as the exolytic/endolytic mechanism ascribed to one of the pectate lyases secreted by both *E. chrysanthemi* strains (27). The structural gene coding for this enzyme has recently been cloned and expressed in *E. coli* (29), and the enzyme produced in *E. coli* generates products typical of this proposed

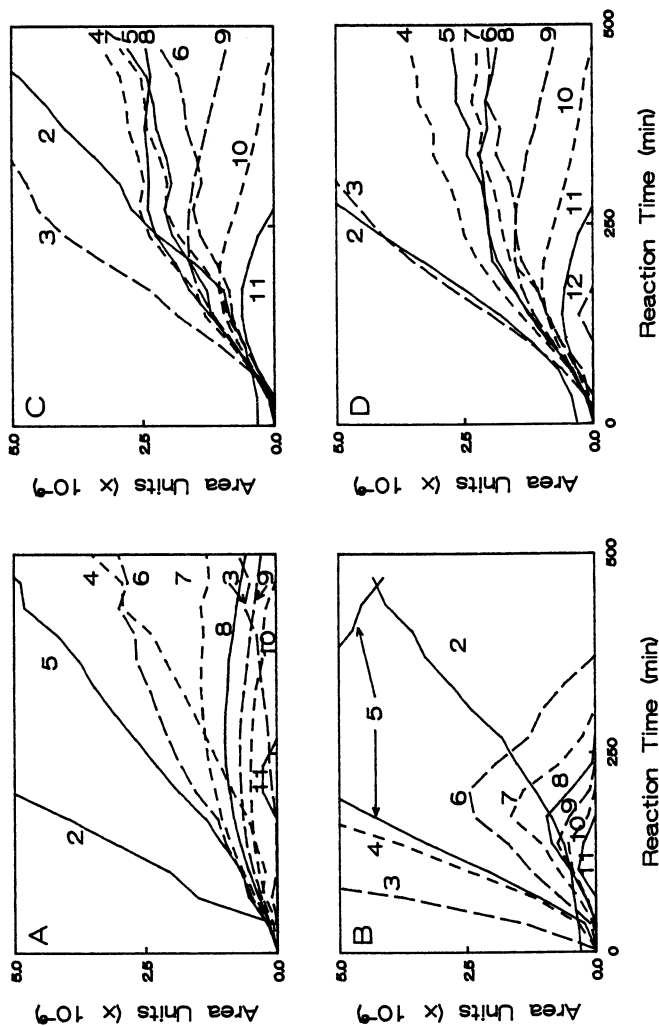


Figure 7. HPLC kinetic profiles of reaction products of pectate lyases obtained from chromatofocused fractions of *Erwinia chrysanthemi* culture supernatants. Chromatofocused enzymes, eluted at pH 8.6 (A), pH 8.3 (B), pH 6.0 (C), and < pH 6.0 (D), were assayed under conditions similar to those described in Figure 5.

Table I. Products formed by Pectate Lyases Secreted by *E. chrysanthemi*, *L. multiparus*, and *C. populeti*, or by Alginate Lyases Secreted by the Alg A Marine Bacterium

Enzyme ^a	Initial product ^b	Ratio ^c , 236 min		Limit products ^d					
		2/6	3/6	2	3	4	5	6	7
<i>E. ch.</i> , medium	3	2.2	3.1	43	31	13	8.0	3.8	1.8
<i>E. ch.</i> , CF-A	2	3.4	0.1	63	5.4	17	14	0.6	<0.1
<i>E. ch.</i> , CF-B	3	0.8	12.9	13	65	17	4	1.1	0.1
<i>E. ch.</i> , CF-C	3	1.6	2.3	45	26	13	8.4	4.7	2.5
<i>E. ch.</i> , CF-C	2/3	3.5	2.9	62	27	9.5	1.4	0.1	<0.1
<i>L.m.</i> , medium	3	5.0	26	30.4	31.7	24.8	11.3	<0.6	<0.6
<i>C.p.</i> , medium	2/3	>450	>500	47.3	52.6	<0.1	<0.1	<0.1	<0.1
Alg A/ <i>E. coli</i>	3	2.2	19.8	10.2	86.6	<0.1	3.2	<0.1	<0.1

^a For all but the recombinant Alg A, extracellular enzymes were obtained from medium concentrated by ultrafiltration. For the *E. chrysanthemi* enzymes, a mixture precipitated between 60 and 90% saturated ammonium sulfate was fractionated into individual activities by chromatofocusing. Enzymes from *Lachnospira multiparus* (strain D15d) and *Clostridium populeti* cultures were analyzed directly in concentrated and dialyzed medium. The Alg A enzyme was expressed in *E. coli* HB101 transformed with plasmid pAL-A3 (Brown, et al., University of Florida, unpublished) from the periplasmic fraction.

^b Initial product refers to that which appeared in greatest quantity during the first 236 min; 2 = dimer and 3 = trimer.

^c Ratios of dimer to hexamer and trimer to hexamer were determined after 236 min.

^d Limit products were determined after 24 hours of incubation, except for *Clostridium populeti*, which was measured after 10 h.

Table II. Comparative Properties and Mechanisms of Polyuronide Eliminases Secreted by Bacteria

Organism	Substrate	pI	MW(kD)	Mech.	Limit (DP)	
<i>E. chrysanthemi</i> ^a	UF CF-D	poly(GalA)	4.0-5.3	ND	endo	2>3>4
	UF CF-C	"	4.9	43	endo	2>3>4>5>6>7
	UF CF-B	"	7.5-8.1	40	exo/endo	3>4>2
	UF CV-A	"	8.6	ND	endo	2>>5=4>3
<i>E. chrysanthemi</i> ^b	EC16PLa	"	4.6	45	endo	2>3>4>5>6>7
	EC16PLb	"	8.8	38	exo/endo	3>4=2
	EC16PLc	"	9.0	39	exo/endo	3>4=2
	EC16PLE	"	9.8	38	endo	2>>4>5>3
<i>L. multiparus</i> ^c	"	4.5-4.7	29	exo/endo	3>4>2	
<i>C. populeti</i> ^d	"	4.5-4.7	190	exo/endo	3>4>2	
<i>B. circulans</i> ^e	poly(ManA)	ND	40	endo	ND	
<i>K. aerogenes</i> ^f	poly(GulA)	8.9	28	endo	2=3>4	
Alg A ^g	poly(ManA)	4.2-5.0	29	exo/endo	3>4>2	
Alg G ^h	poly(GulA)	4.2-5.0	38	exo	3>>2	

^a *Erwinia chrysanthemi* UF was isolated from *Anthurium* species and identified by T. Shubert, Division of Plant Industries, University of Florida. Enzymes were purified by chromatofocusing.

^b *Erwinia chrysanthemi* EC16 (ATCC 1162) originally from *Chrysanthemum x morifolium*, provided purified enzymes, the genes for which were cloned and expressed in *E. coli* HB101. (18,19).

^c *Lachnospira multiparus* D15d was a bovine rumen isolate obtained from R. Hespell, USDA laboratories, Peoria, IL. Enzymes were evaluated in concentrated/dialyzed medium.

^d *Clostridium populeti* was isolated from a wood (poplar) based methane digester by Sleet and Mah (32). Enzymes were evaluated in concentrated/dialyzed medium.

^e *Bacillus circulans* was isolated from soil and characterized by Hansen et al. (31) as producing a poly(ManA) specific enzyme.

^f *Klebsiella aerogenes* type 25 was obtained as a human pathogen and characterized by Boyd and Turvey (30) as producing a poly(GulA) specific enzyme.

^g Alg A was an alginate secreting bacterium isolated from the brown alga, *Sargassum fluitans*, and tentatively assigned to the genus *Photobacterium* (33). Pure enzyme was evaluated on poly(ManA) as substrate (21) and as a recombinant enzyme expressed in *E. coli* HB101 (Brown, B.J., Preston, J.F., Ingram, L.O.; University of Florida, unpublished).

^h Alg G isolated along with Alg A from *Sargassum fluitans* and tentatively assigned to the genus *Photobacterium* (33). Pure enzyme was evaluated on poly(GulA) (Brown, B.J., Preston, J.F., University of Florida, unpublished).

mechanism (Table I). A L-gulonon specific enzyme purified from a fermentative Alg-G isolate has a pI of 4.2 to 5.0 and a predominantly exolytic depolymerization mechanism (22). The acidic properties of the enzymes secreted by the marine bacteria associated with healthy *Sargassum* tissues, as well as their exolytic mechanisms serve to distinguish them from the basic endolytic L-gulonon specific lyase produced by *Klebsiella pneumoniae* (30) and the endolytic D-mannuronon specific lyase secreted by *Bacillus circulans* (31). The properties of these and other alginate lyases are compared with the different pectate lyases discussed above in Table II.

Conclusions

Plant pathogens, e.g. *Erwinia chrysanthemi*, which cause the maceration of plant tissues in conjunction with the phytopathogenic response, secrete a battery of acidic and basic pectate lyases which exhibit a range of endolytic or exolytic/endolytic mechanisms in catalyzing the depolymerization of polygalacturonic acid. It will now be of interest to determine if different depolymerization patterns occur with different rhamnogalacturonon preparations, and if the complement of pectate lyases contributes to pathovar specificity via preference for a specific rhamnogalacturonon.

Saprophytic anaerobic bacteria, e.g. the ruminant bacterium *Lachnospira multiparus* and the methane digester isolate, *Clostridium populeti*, secrete only acidic pectate lyases that exhibit an exolytic/endolytic depolymerization toward polygalacturonate. It will be important to determine if this acidic property contributes to the adsorption of these enzymes to the secreting bacterium, perhaps contributing to the efficiency with which the bacteria are able to assimilate the products generated by these enzymes.

Saprophytic alginase secreting bacteria isolated from healthy Sargassum tissues secrete acidic D-mannuronon or L-gulonon specific lyases that exhibit exolytic/endolytic or exolytic mechanisms in the depolymerization of alginate. Again, localization studies may illuminate their potential function for the efficient utilization of the products they generate.

The availability of pectate and alginate lyases exhibiting different substrate specificities and depolymerization mechanisms should allow the selective modification of these polyuronates for their applications as viscosity modifiers and gelling agents. These enzymes may also be useful as reagents for the isolation of protoplasts from plants and algae for genetic manipulation through tissue culture techniques. Some of these enzymes, as well as the organisms producing them, may be suitable for assisting in the degradation of plant and algal biomass for production of alcohol and methane.

It is apparent that both the pectate lyase and the alginate lyase secreting bacteria represent a rich genetic resource with which to determine the relationships of protein structure, substrate specificity, and depolymerization mechanism. With the recent advances in the cloning and expression in *E. coli* of the structural genes coding for the pectate lyases secreted by *E. chrysanthemi*, as well as structural genes coding for L-gulonon and D-mannuronon specific lyases,

definitive depolymerization mechanisms for specific gene products may now be assigned. The complete sequence analysis of the *E. chrysanthemi* pectate lyase genes provides information to identify domains and search for motifs relevant to mechanism. Sequence analysis of genes coding for alginate lyases should establish domains that are required for a particular depolymerization mechanism versus those that are involved in substrate specificity. We may find ourselves approaching an understanding of structure/function relationships for the polyuronate lyases that has been reached for the cellulases and other polysaccharide degrading enzymes so well described by the contributors to this symposium.

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Literature Cited

1. Whitaker, J.R. *Enzyme Microb. Technol.* **1984**, *6*, 341-349.
2. Gacesa, P. *Carbohydrate Polymers* **1988**, *8*, 161-182.
3. Darvill, A.G.; McNeil, M.; Albersheim, P. *Plant Physiol.* **1978**, *64*, 418-422.
4. York, W.S.; Darvill, A.G.; McNeil, M.; Stevenson, T.T.; Albersheim, P. *Methods in Enzymol.* **1985**, *118*, 3-54.
5. Valent, B.S.; Albersheim, P. *Plant Physiol.* **1974**, *54*, 105-108.
6. Jarvis, M.C. *Plant, Cell and Environment.* **1984**, *7*, 153-164.
7. Preston, J.F.; Romeo, T.; Gibor, A.; Polne-Fuller, M. In *Methane from Biomass. A Systems Approach*; Frank, J.R.; Smith, W.H. Ed.; Elsevier: New York, 1988; pp. 211-234.
8. Vreeland, V.; Laetsch, W.M. *Planta*, **1989**, *177*, 423-434.
9. Rees, D.A.; Morris, E.R.; Thom, D.; Madden, J.K. In *The Polysaccharides*; Aspinall, G.O. Ed.; Academic Press: New York, 1982, Vol. 1; pp. 194-290.
10. Kohn, R. *Pure Appl. Chem.* **1975**, *42*, 371-397.
11. Madgwick, J.A.; Haug, A.; Larsen, B. *Acta Chem. Scand.* **1973**, *27*, 3593-3594.
12. Larsen, B.; Skjak-Braek, G.; Painter, J. *Carbohydrate Res.* **1986**, *146*, 342-345.

13. Rombouts, F.M.; Pilnik, W. In *Economic Microbiology, Microbial Enzymes and Bioconversions*, Rose, A.H. Ed.; Academic Press: New York 1980, Vol. 5; pp 227-283.
14. Fogarty, W.M.; Kelly, C.T. In *Micobial Enzymes and Biotechnology*; Fogarty, W.M. Ed.; Applied Science Publishers: New York, 1983, Vol 4; pp 131-181.
15. Rexova-Benkova, L.; Markovic, O. *Adv. Carbohydrate Chem. Biochem.* 1976, 33, 323-385.
16. Collmer, A.; Keen, N.T. *Ann. Rev. Phytopathol.* 1986, 24, 383-409.
17. Hespell, R.B.; Bryant, M.P. In *The Prokaryotes*; Starr, M.P.; Stolp, H.; Truper, H.G.; Balows, A.; Schlegel, H.G. Ed.; Springer-Verlag: New York 1981; pp 1479-1494.
18. Keen, N.T.; Dahlbeck, D.; Staskawicz, B.; Belser, W. *J. Bacteriol.* 1984, 159, 825-831.
19. Tamaki, J.J.; Gold, S.; Robeson, M.; Manulis, S.; Keen, N.T. *J. Bacteriol.* 1988, 170, 3468-3478.
20. Romeo, T.; Bromley, J.C.; Preston, J.F. In *Biomass Energy Development*; Smith, W.H. Ed. Plenum: New York, 1986; pp 303-320.
21. Romeo, T.; Preston, J.F. *Biochemistry.* 1986, 25, 8385-8391.
22. Brown, B.J.; Preston, J.F. Abstracts Annual Meeting, American Society for Microbiology. 1987.
23. Preston, J.F.; Romeo, T.; Bromley, J.C. *J. Indust. Microbiol.* 1986, 1, 235-244.
24. Preiss, J.; Ashwell, G. *J. Biol. Chem.* 1962, 237, 309-316.
25. Voragen, A.G.J.; Schols, H.A.; DeVries, J.A.; Pilnik, W. *J. Chromatog.* 1982, 244, 327-336.
26. Romeo, T.; Preston, J.F. *Carbohydrate Res.* 1986, 153, 181-193.
27. Preston, J.F.; Rice, J.D. Abstracts Annual Meeting, American Society for Microbiology. 1989.
28. Patel, G.B.; Agnew, B.J. *Arch. Microbiol.* 1988, 150, 267-271.
29. Brown, B.J.; Preston, J.F.; Ingram, L.O. Abstracts of the 199th National Meeting, American Chemical Society. 1990.
30. Boyd, J.B.; Turvey, J.R. *Carbohydrate Res.* 1977, 57, 163-171.
31. Hansen, J.B.; Doubet, R.S.; Ram, J. *Appl. Environ. Microbiol.* 1984, 47, 704-709.
32. Sleat, R.; Mah, R.A., *Int. J. Syst. Bacteriol.* 1985, 35, 160-163.
33. Preston, J.F.; Romeo, T.; Bromley, J.C.; Robinson, R.W.; Aldrich, H.C., *Devel. Indust. Microbiol.* 1985, 26, 727-740.

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Chapter 36

Synergism between 1,3- β -Glucanases in Yeast Cell Wall Zymolysis

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Synergistic effects between four 1,3- β -glucanases GI, GII, GIV, and GVIII, of the *Streptomyces* sp. 1228 lytic system were investigated in both yeast cell wall lysis and yeast cell wall solubilization of glucan. The maximum synergistic effect in *Candida utilis* lysis was observed with GI and GII glucanases. In *Schizosaccharomyces pombe* lysis, GI and GVIII had maximum effect, while in *Saccharomyces carlsbergensis*, the greatest effect was achieved by GI and GIV enzymes. Glucanase GI had the strongest synergistic effect with the other three glucanases. The greatest amount of degradation of the yeast cell wall polysaccharide occurred during GI action after the previous treatment of the yeast with glucanase GII or GIV. The amount of reducing sugars released from *Candida utilis* cells by pairs of glucanases, irrespective of sequence of action, was the same.

Enzymatic lysis of whole yeast cells, which is primarily caused by 1,3- β -glucanases and other enzymes from lytic systems, is becoming increasingly attractive. Applications include upgrading of waste yeasts for poultry feed; preparation of functional carbohydrates from cell walls; extraction of intracellular substances (especially those produced by recombinant DNA technology); treatment of fungal diseases; and as an essential tool for cell fusion, transformation, and the genetic engineering of yeast (1, 2).

Many microorganisms produce enzymes that lyse the cell wall of yeast. The most extensive work has been done with the lytic system from *Arthrobacter* sp., *Cytophaga* sp., *Oerskovia*, *Bacillus circulans*, *Rhizopus*, *Trichoderma*, *Penicillium*, *Pellicularia* sp., *Rhizoctonia*, and *Streptomyces* sp. (3-9).

The lytic enzyme systems, active against yeast cell walls, usually contain 1,3- β -glucanases, proteases, mannanases, chitinases, and 1,6- β -glucanases. The proportion of those enzyme activities, their action pattern, synergism, and dependence on inhibitors constitute the activity profile

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of the lytic system. The effectiveness of yeast cell wall lysis depends upon the degree of hydrolysis of 1,3- β -glucans, the components that are responsible for the shape and rigidity of the yeast cell. Hence, the β -glucanases are the most important enzymes of the lytic system. They have different substrate and product specificities. These enzymes may be classified as exo- β -glucanase that hydrolyze the polysaccharides at the chain ends and release glucose. In addition, endo- β -glucanases may be further divided into two groups depending on whether they produce larger or smaller oligosaccharides (10).

So far the role of particular enzymes from the lytic system in yeast cell wall lysis has not been explicitly explained. Tanaka and Phaff (11) were among the first to demonstrate that 1,3- β -glucanases from *Bacillus circulans* are able to degrade yeast cell walls without participation of other enzymes. McLellan et al. (12) thought that phosphomannanase played a leading role at least at the beginning of yeast cell wall lysis by removing the external layer of mannoproteins and revealing the glucan layer to the action of glucanases. The mannoproteins can be also removed by 1,6- β -glucanase from *Cytophaga* (13), or 1,3- β -glucanases from Zymolase (14), as well as thiocompounds, or lytic protease (15).

It now appears that removal of the mannoprotein layer is not necessary for lysis of yeast cell wall by glucanases. It seems to be sufficient to increase the porosity or "loosen" the mannoprotein layer by addition of agents that disrupt disulphide or thioester bonds in the mannoprotein. Similar effects can be achieved by increasing the solution ionic strength or by adding compounds that react with the hydrophobic regions of mannan (16). Such changes in the structure of cell wall surface layers more readily enable β -glucanases to access the glucan layer, which permits depolymerization and subsequent lysis of the yeast cell wall.

One of the most intriguing phenomena in microbial cell wall hydrolysis by lytic enzymes is the synergistic action shown between individual components of the yeast lytic system. Less is known about the cooperation between different lytic glucanases themselves in the degradation of the rigid β -glucan layer in yeast cell walls. In this paper, we report the synergism exhibited between four 1,3- β -glucanases, isolated from a lytic enzyme system produced extracellularly by *Streptomyces* sp. 1228, in the degradation of the cell walls of *Candida utilis*, *Schizosaccharomyces pombe*, and *Saccharomyces carlsbergensis*. In order to determine the degree of cooperation between the various glucanases in both cell wall lysis and glucan solubilization, a synergistic coefficient was used. This was defined as the ratio between the observed activity of the combined glucanase mixtures and the sum of the observed individual activities.

Materials and Methods

Enzyme Preparation. The four 1,3- β -glucanases, GI, GII, GIV, and GVIII were purified from lytic enzyme system produced by *Streptomyces* sp. 1228 using methods previously described (17).

Yeast Strains. *Candida utilis*, *Saccharomyces carlsbergensis*, and *Schizosaccharomyces pombe* were obtained from the Institute of Microbiology and Fermentation Technology, Technical University of Lodz. The strains were stored at 4 °C. After incubation at 30 °C for 3–4 days on malt agar, the yeasts were cultivated in 500-mL flasks with 50 mL of YPD medium (yeast extract, 1%; peptone, 2%; glucose, 2%) at 30 °C on a rotor shaker until the optical density of the culture reached $A_{500} = 2$. Growth was stopped by adding NaN_3 to a final concentration of 10 mM. Cells were harvested by centrifugation at 3000 *g* for 5 min and washed three times with distilled water. A yeast suspension ($A_{500} = 1.5$) was prepared in 0.02 M phosphate buffer, pH 6.5, and used within four days.

Yeast Zymolysis. To 2 mL of yeast suspension containing 5 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, 0.4 mL of enzyme solution containing 50 units of activity of each glucanase was added in various combinations. The total volume was made up to 4 mL with 0.02 M phosphate buffer, pH 6.5. The A_{500} of the reaction mixture was read at zero time and after 30 and 60 min of incubation at 50 °C with gentle shaking. The decrease in A_{500} in the same volume of yeast suspension, but without addition of enzymes, was taken as the blank. The degree of yeast lysis was expressed as a percent of decrease in the optical density. To determine the amount of total sugars liberated during yeast zymolysis, 30- and 60-min incubation mixtures were boiled for 5 min and centrifuged at 3000 *g* for 10 min. The total amount of sugar in the supernatant was determined by the method of Dubois et al. (18).

Synergism of 1,3- β -Glucanases. The cooperation between glucanases in yeast cell wall lysis was expressed by a synergistic coefficient SCL, defined as the ratio between the degree of lysis obtained by the action of combined glucanases and the sum of the degree of lysis caused by individual glucanases (equation 1)

$$\text{SCL} = \frac{L_G}{L_{GI} + L_{GII} + L_{GIV} + L_{GVIII}} \quad (1)$$

where L_G is the degree of lysis by the combination of glucanases and L_{GI} , L_{GII} , L_{GIV} , and L_{GVIII} are the degrees of lysis by the individual glucanases GI, GII, GIV, and GVIII, respectively.

The cooperation between glucanases in cell wall glucan solubilization was expressed by a synergistic coefficient SCS, defined as the ratio between the amount of total sugars liberated by the action of combined glucanases and the sum of sugars liberated by individual glucanases, as shown in equation 2. In equation 2, C_G is the amount of sugars released

by the combination of glucanases and C_{GI} , C_{GII} , C_{GIV} , and C_{GVIII} are the amounts of sugars released by the individual glucanases GI, GII, GIV, and GVIII, respectively.

$$SCS = \frac{C_G}{C_{GI} + C_{GII} + C_{GIV} + C_{GVIII}} \quad (2)$$

The experiment based on the two-level factorial design as described by Box and Wilson (19) was carried out in order to check the influence of the individual glucanases (g_n) of *Streptomyces* sp. 1228 lytic enzymes system on the degree of yeast cell lysis (y).

To a *Candida utilis* suspension, prepared as described above, individual 1,3- β -glucanases were added at either a lower level of 41 units/ml or an upper level of 102.5 units/ml to the reaction mixtures according to the following planning experiment matrix (equation 3). Each row of the matrix represents an elementary experiment of which result y_k is contained in the result's matrix Y.

k	i →				
	$z_1 = g_1$	$z_2 = g_2$	$z_3 = g_4$	$z_4 = g_8$	
1	-1	-1	-1	-1	Y = $\begin{bmatrix} y_1 \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ \cdot \\ y_n \end{bmatrix}$ (3)
2	+1	-1	-1	-1	
3	-1	+1	-1	-1	
4	+1	+1	-1	-1	
5	-1	-1	+1	-1	
6	+1	-1	+1	-1	
7	-1	+1	+1	-1	
8	+1	+1	+1	-1	
9	-1	-1	-1	+1	
10	+1	-1	-1	+1	
11	-1	+1	-1	+1	
12	+1	+1	-1	+1	
13	-1	-1	+1	+1	
14	+1	-1	+1	+1	
15	-1	+1	+1	+1	
16	+1	+1	+1	+1	

The experimental result was expressed in terms of the regression equation shown in equation 4. The linear effect of individual glucanases on yeast lysis is expressed by b_1 , b_2 , b_3 , and b_4 , whereas coefficients $b_{1,2}$,

$b_{1,3}$, $b_{1,4}$, $b_{2,3}$, $b_{3,4}$, \dots , $b_{1,2,3,4}$ are associated with the synergism of appropriate glucanase mixtures.

$$y = b_0 + b_1g_1 + b_2g_2 + b_3g_3 + b_4g_4 + \dots \quad (4)$$

$$+ b_{1,2}g_1g_2 + \dots + b_{n-1,n}g_{n-1}g_n + b_{1,2,3}g_1g_2g_3 + \dots$$

$$+ b_{n-2,n-1,n}g_{n-2}g_{n-1}g_n \dots$$

where

$$b_0 = \frac{\sum_{k=1}^n Y_k}{n}, \quad b_1 = \frac{\sum_{k=1}^n Y_k Z_{ik}}{n}, \quad \text{and} \quad b_{1,2} = \frac{\sum_{k=1}^n Y_k Z_{1k} Z_{2k}}{n} \dots$$

Results

Synergism between the 1,3- β -glucanases from the lytic system of *Streptomyces* sp. 1228 in yeast zymolysis depends on the composition of the enzyme mixture and also on the yeast species used for hydrolysis (Tables I to III). Positive cooperation (SCL > 1 and SCS > 1) was observed for almost all combination of glucanases which included the GI enzyme. SCL of GI and GII enzymes was the highest in the lysis of *C. utilis* (Table I). There was no synergism (SCL = 0.97) between these enzymes in the zymolysis of *S. carlsbergensis* (Table II), and the lysis of *S. pombe* (SCL = 1.18) was rather low (Table III). This pair of glucanases (GI and GII) cooperate more effectively in the solubilization of *C. utilis* cell wall polysaccharides (SCS = 1.37) than in the liberation of sugars from both *S. carlsbergensis* (SCS = 1.07) and *S. pombe* (SCS = 1.18) (Tables I–III).

GI and GVIII glucanases show high synergism (SCL = 1.51) in the lysis of *S. pombe* (Table III) but low synergism in the lysis of *C. utilis* (SCL = 1.02) and *S. carlsbergensis* (SCL = 1.06) (Tables I and II), although high synergism (SCS = 1.55) between these enzymes in the solubilization of *C. utilis* polysaccharide was also observed (Table I). This suggests that cooperation in the process of yeast cell wall polysaccharide solubilization may not coincide with the cooperation in the lysis.

Such phenomena were also observed for the combination of glucanases GI, GII, and GIV during the zymolysis of *C. utilis*, where SCL = 0.87 but SCS = 1.66, and for GI, GIV, and GVIII during the treatment of the same yeast, where SCL = 0.99 and SCS = 1.15. The combination of four glucanases GI, GII, GIV, and GVIII has higher synergism in the solubilization of polysaccharide of examined yeast cells than in their lysis. The opposite phenomena were observed for some other mixtures of glucanases. The cooperation between glucanases GI and GIV was higher in the lysis of *C. utilis* (SCL = 1.46) than in solubilization of the *C. utilis* polysaccharide (SCS = 1.35). A similar effect was noticed for GI and GII in the

Table I. Synergism between Glucanases in *C. utilis* lysis (SCL) and Solubilization of Cell Wall Glucans (SCS)

Combination of Enzymes	SCL		SCS	
	30 min	60 min	30 min	60 min
GI, GII	1.60	1.02	1.37	1.21
GI, GIV	1.46	1.25	1.35	1.31
GI, GVIII	1.02	1.05	1.55	1.07
GII, GIV	0.92	0.56	0.77	0.84
GII, GVIII	0.61	0.70	0.71	0.73
GIV, GVIII	0.69	0.75	0.92	0.90
GI, GII, GIV	0.87	0.84	1.66	1.09
GI, GII, GVIII	1.03	0.87	1.29	1.10
GI, GIV, GVIII	0.99	0.85	1.15	0.92
GII, GIV, GVIII	0.79	0.60	0.90	0.97
GI, GII, GIV, GVIII	1.16	0.80	1.71	1.09

Table II. Synergism between Glucanases in *S. carlsbergensis* lysis (SCL) and Solubilization of Cell Wall Glucans (SCS)

Combination of Enzymes	SCL		SCS	
	30 min	60 min	30 min	60 min
GI, GII	0.97	0.95	1.07	0.99
GI, GIV	1.10	1.13	1.18	1.11
GI, GVIII	1.06	1.04	1.04	0.86
GII, GIV	0.89	0.73	0.71	0.56
GII, GVIII	0.94	0.77	0.79	0.74
GIV, GVIII	0.90	0.82	0.89	0.81
GI, GII, GIV	0.99	0.97	1.32	1.06
GI, GII, GVIII	1.01	1.03	1.20	0.97
GI, GIV, GVIII	1.09	0.99	1.07	0.91
GII, GIV, GVIII	0.68	0.52	0.85	0.90
GI, GII, GIV, GVIII	1.14	0.90	1.30	1.01

treatment of *C. utilis* (Table I) and for GI and GVIII in the lysis of *S. pombe* (Table III). This can be explained by the fact that the lytic effect can be obtained by bursting of the cell wall at the site of the intensive action of some glucanases, without profound solubilization of cell wall glucan.

Synergism between the enzymes was shown to decrease with time during zymolysis. This may occur because the substrate for a particular glucanase disappeared (but as the substrates were in excess in this case it is unlikely), or the products released during hydrolysis inhibited one or a number of the glucanases.

The synergistic coefficients, SCL and SCS, show clearly the participation of each glucanases in yeast cell wall degradation. These measurements, however, do not take into account the overall dynamics of the zymolysis process, e.g., access to the substrates, their continual changes in concentration, and inhibition of one enzyme with the products of another one. To overcome this problem, the zymolysis of *Candida utilis* was carried out according to the full two-level factorial experiment, and the lytic glucanase action was described by the resulting regression equation:

$$\begin{aligned}
 y = & 56.3 + 9.75g_1 + 0.65g_2 + 0.43g_4 + 1.74g_8 & (5) \\
 & + 0.6g_1g_2 + 1.03g_1g_4 + 0.4g_1g_8 - 0.5g_2g_4 \\
 & - 1.14g_2g_8 + 0.2g_1g_2g_4 + 0.27g_1g_2g_8 - 0.29g_1g_4g_8 \\
 & - 0.24g_2g_4g_8 + 0.91g_1g_2g_4g_8
 \end{aligned}$$

According to the above regression coefficients, which indicate the role of individual glucanases in yeast lysis, the GI enzyme had the greatest influence on the degree of lysis ($b_1 = 9.75$). Synergism ($b_n > 0$) exists between the glucanase pairs GI and GII, GI and GVIII, and GI and GIV (for which the synergism is strongest), with regression coefficients of 0.6, 0.4, and 1.03, respectively. There was no cooperation between the combinations of glucanases GII and GIV; GII and GVIII; GI, GIV, and GVIII; and GII, GIV, and GVIII, for which the regression coefficients are -0.5 , -1.14 , -0.29 , and -0.24 , respectively. The above result clearly confirms the presence of glucanase cooperation first obtained using the synergistic coefficients (Table I).

To check for the possibility of modification of yeast glucan by the action of one glucanase that makes the substrate more available to the action of another glucanase, the zymolysis of *C. utilis* was carried out as described in Materials and Methods, with the exception that, after 30 min of incubation, the same amount of the same or another glucanase was added, and then zymolysis was continued for an additional 30 min. The degree of lysis and the amount of liberated sugars were determined. From the data presented in Table IV, the greatest sugar release occurred when the hydrolysis of yeast with glucanase GI, GII, or GIV was followed by glucanase GI. The pretreatment of yeast with glucanase GVIII did not increase yeast glucan solubilization by enzyme GII or GIV. Independent of the enzyme, which initialized the lytic process, a considerable degree of

Table III. Synergism between Glucanases in *S. pombe* Lysis (SCL) and Solubilization of Cell Wall Glucans (SCS)

Combination of Enzymes	SCL		SCS	
	30 min	60 min	30 min	60 min
GI, GII	1.18	0.98	1.18	0.96
GI, GIV	1.17	1.03	1.41	1.20
GI, GVIII	1.51	1.43	1.31	1.27
GII, GIV	0.88	0.74	0.66	0.62
GII, GVIII	0.84	0.59	0.60	0.53
GIV, GVIII	0.82	0.71	0.83	0.72
GI, GII, GIV	0.99	0.99	0.88	0.85
GI, GII, GVIII	0.99	0.85	1.31	1.04
GI, GIV, GVIII	0.93	0.89	1.11	1.12
GII, GIV, GVIII	0.78	0.72	0.70	0.69
GI, GII, GIV, GVIII	1.03	0.93	1.24	0.91

Table IV. Effect of Sequential Addition of Glucanases on the Lysis and Solubilization of *C. utilis* Cell Wall Glucan

Glucanase Fractions	Total soluble Sugars ($\mu\text{g/mL}$)	Increase of Sugars ($\mu\text{g/mL}$)	Degree of Lysis (%)
GI	145.0	—	20.5
GI + GI	250.0	105.0	40.5
GI + GII	180.0	35.0	23.0
GI + GIV	200.0	55.0	25.0
GI + GVIII	175.0	30.0	22.0
GII	57.5	—	7.5
GII + GII	82.5	25.0	9.7
GII + GI	199.0	132.5	30.0
GII + GIV	75.0	17.5	7.0
GII + GVIII	90.0	32.5	8.0
GIV	62.5	—	7.0
GIV + GIV	77.5	15.0	7.7
GIV + GI	205.0	142.5	34.0
GIV + GII	67.5	5.0	6.5
GIV + GVIII	70.0	7.5	6.7
GVIII	75.0	—	9.0
GVIII + GVIII	95.0	20.0	11.1
GVIII + GI	185.0	110.0	32.5
GVIII + GII	80.0	5.0	10.0
GVIII + GIV	72.5	-2.5	11.1

lysis could be achieved by the addition of GI, and it is higher than the degree of lysis obtained by addition of glucanase GII, GIV, or GVIII to the process initialized by glucanase GI.

Discussion

It has been shown that all four glucanases isolated from *Streptomyces* sp. 1228 culture filtrate can act synergistically in the zymolysis of yeast cell walls. The synergistic effect depends on the composition of the glucanase mixture and on the yeast species used for lysis. In enzyme mixtures without glucanase GI, no synergism was observed, and the degree of yeast lysis was 2- to 3-fold lower than that obtained with a mixture of all glucanases (9). The leading role of 1,3- β -glucanase GI in the zymolysis of yeast might be explained by its reported properties (17, 20). GI is the least specific glucanase of the four; 1,4- β -, 1,3- β -, and 1,6- β -glucosidic bonds are hydrolyzed by this enzyme. Also, of the four glucanases, the GI enzyme is most active against yeast glucan. Due to the low molecular weight (Mr 19000) of GI, the enzyme can penetrate deeply into the glucan layer of the cell wall.

Surprisingly, there was a lack of synergism between both GI and GVIII enzymes in the lysis of both *Candida utilis* and *Saccharomyces carlsbergensis*, although the activities do not impede each other. Probably the molecular weight of the GI products may be too high for the GVIII enzyme, which prefers a low-molecular-weight substrate. The high synergism seen between these enzymes in *Schizosaccharomyces pombe* lysis may be the result of a different structure of glucan present in that yeast strain and its access for that enzyme mixture. The synergism between GI and GII glucanases was strongly dependent on the yeast species. High synergism was obtained for the action of GI and GII glucanases in the lysis of *Candida utilis*, lower synergism in the lysis of *Schizosaccharomyces pombe*, and no synergism in the lysis of *Saccharomyces carlsbergensis*.

Evidence presented earlier (9) suggests that the main reason for the decrease of synergism between the glucanases after prolonged yeast hydrolysis, seems to be inhibition of the enzymes by the products released from zymolysis. The more effective GI glucanase was inhibited by 50% by glycoprotein fraction F5 isolated from yeast with *Streptomyces* sp. 1228 lytic system. Glucanase GIV was inhibited by 45% by this glycoprotein fraction, and glycoprotein fraction F2 inhibited GVIII by 39%. Glucanases GIV and GVIII are inhibited by a disaccharide that is released in the time course of yeast zymolysis by the mixture of glucanases.

The results presented above, however, convincingly document the synergism of individual glucanases in cell wall lysis but do not explain the dynamics of the overall process. In addition, the cooperation between different ratios of glucanases has not been explored. Lysis carried out according to the full two-level planning experiment entirely confirms the role of individual glucanases in *C. utilis* lysis. There are other reactions occurring during the lysis of yeast cell wall, such as splitting of disulphide or

thioester bonds, changes in hydrophobic regions, or electrostatic repulsions (16). These reactions could differently affect the access of suitable substrates for individual enzymes from the lytic enzyme system. Hence synergism between glucanases is not only the result of their hydrolytic capability (specificity, mechanism of action, types of end product, inhibition, and activation) but also depends upon the capability of the enzymes to be adsorbed onto insoluble substrates like yeast cell walls and the competition between the individual enzymes to occupy the available adsorption sites.

Yeast populations subjected to lysis are not homogeneous, and most physical and chemical properties, such as cell volume and mass contents of protein, DNA, reserve carbohydrate, and other components, can vary severalfold. Hunter and Asenjo (21) developed a population balance model of enzymatic lysis of microbial cells in order to evaluate the extent to which such distribution will affect reactor performance and lytic process behavior. Such a model also considering the above mentioned properties of the enzymes is likely to be useful for the design and optimization of yeast cell lysis both in batch and continuous processes. Synergism between individual glucanases of the lytic system is of great importance for yeast cell wall zymolysis. Cooperation between lytic proteases and lytic glucanases may also be important. Furthermore, the lytic process can be achieved by the action of one low-molecular-weight 1,3- β -glucanase (GI) with broad specificity, but the solubilization of yeast cell walls is much more effective when synergism is present between different glucanases.

Literature Cited

1. Kabayashi, R. K.; Miwa, T.; Yamamoto, S.; Nagasaki, S. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *15*, 14.
2. Asenjo, J. A.; Andrews, B. A.; Hunter, J. B.; Lecorre, S. *Process Biochem.* **1985**, *10*, 158.
3. Doi, K.; Doi, A. *J. Bacteriol.* **1986**, *168*, 1276.
4. Hunter, J. B.; Asenjo, J. A. *Biotech. Bioeng.* **1987**, *30*, 471.
5. Tanaka, H.; Kobayashi, K.; Hakura, K.; Kamimiya, S. In *Current Developments in Yeast Research*, Advances in Biotechnology; Stewart, G. G.; Russel, I., Eds.; Pergamon Press: Canada, **1980**; p.503.
6. Yamamoto, S.; Shiraishi, T.; Nagasaki, S. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 1802.
7. Elad, Y.; Chet, I.; Henis, Y. *Can. J. Microbiol.* **1982**, *28*, 719.
8. Yamamoto, R.; Arai, M.; Murao, S. *Agric. Biol. Chem.* **1978**, *42*, 1593.
9. Bielecki, S. *Sci. Bull. Lodz Tech. Univer.* **1989**, *544*, 84.
10. Mori, H.; Yomamoto S.; Nagasaki, S. *Agr. Biol. Chem.* **1977**, *41*, 611.

11. Tanaka, H.; Phaff, H. J. *J. Bacteriol.* **1965**, *89*, 1570.
12. McLellan, W. L.; McDaniel, L. E.; Lampen, J. O. *J. Bacteriol.* **1970**, *102*, 261.
13. Bacon, J. S. P.; Gordon, A. N.; Jones, D.; Taylor, I. F.; Webley, D. M. *Biochem. J.* **1970**, *120*, 67.
14. Shibata, N.; Mizugami, K.; Takano, K.; Suzuki, S. *J. Bacteriol.* **1983**, *156*, 552.
15. Hunter, J. B.; Asenjo, J. A. *Ann. N.Y. Acad. Sci.* **1987**, *506*, 649.
16. Zlotnik, H.; Fernandez, M. P.; Bowers, B.; Cabib, E. *J. Bacteriol.* **1984**, *159*, 1018.
17. Bielecki, S.; Wnuk, M.; Szczesna, M.; Bobowicz-Lassocinska, T.; Antczak, T.; Galas, E. *Biotech. Lett.* **1989**, *11*, 281.
18. Dubois, M.; Giles, K. A.; Hamilton, M. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350.
19. Box, G. E. P.; Wilson, K. B. *J. Roy. Stat. Soc. B.* **1951**, *13*, 1.
20. Bielecki, S.; Antczak, T.; Wnuk, M.; Bobowicz-Lassocinska, T.; Galas, E. *Biotech. Lett.* **1989**, *11*, 287.
21. Hunter, J. B.; Asenjo, J. A. *Biotech. Bioeng.* **1990**, *35*, 31.

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Chapter 37

Chitinases

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Chitin is a major bioresource, with an estimated annual production and standing crop of 10 to 100 billion tons. Major sources are wastes from shellfish and krill processing and fungal fermentations. There is great scope in chemical and biochemical processing of chitin to make a wide range of derivatives of value. Chitinases are widely produced in nature, by some bacteria and vertebrates, and by most fungi, plants and invertebrates. These chitinases show a range of properties, notably with respect to substrate specificity and products. Most common are endo-chitinase activities, giving a range of oligomeric products. Transglycosylation is also an important activity in special conditions. Other enzymes with potential for processing chitin are deacetylases to produce chitosan, and chitosanases producing a range of oligomers.

Nature, Occurrence and Abundance of Chitin

Chitin, the (1-4)- β -linked homopolymer of *N*-acetyl-D-glucosamine is an immense renewable resource in the biosphere. Gooday (1) estimates both its annual production and standing crop to be of the order of 10 to 100 billion tons. Annual production from Antarctic krill, *Euphausia superba*, alone, is estimated to be 5.8 - 8.4 million tons.

Chitin is used in nature as a structural polysaccharide, notably in the exoskeleton of most invertebrates and in the cell walls of most fungi. Its most common form is as α -chitin, where the unit cell is of two *N,N*-diacetylchitobiose units of two chains in an antiparallel arrangement (2). The final structure has extensive intermolecular hydrogen-bonding, with the exclusion of water, leading to great stability. A less common form is β -chitin, in which the unit cell is of one *N,N*-diacetylchitobiose unit, giving a final structure of parallel poly-*N*-acetylglucosamine chains, extensively hydrogen bonded in sheets, but without the intersheet hydrogen bonds of α -chitin (3). A further form, gamma-chitin, has a mixture of parallel and antiparallel chains, but has not been investigated in details (4).

Apart from the β -chain flotation spines of diatoms (5), chitin always occurs in nature in states other than the ideal crystalline homopolymers suggested above. It shows one or more of the following features: a variable degree of crystallinity; varying amounts of deacetylation; cross-linking with other molecules. Thus in growing fungal hyphae, the nascent chitin chains at the apices only slowly become crystallized as microfibrils (6). In both fungi and insects there is evidence for a

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controlled deacetylation of chitin, eventually to give chitosan, the (1-4)- β -linked homopolymer of D-glucosamine (7). Covalent cross-links are formed in fungal walls between chitin and β -glucans (8) and in invertebrates between chitin and specific structural proteins (9). A range of other post-polymerisation covalent modifications are possible. Thus the chitin of the test of a tunicate has been reported to be extensively 6-O sulphated (10). The precise physical and chemical state of the chitin clearly has a major bearing on its suitability as a substrate for an enzyme, and must be borne in mind during the discussions to follow.

Although the glycosidic chain of chitin is similar to the backbone of peptidoglycan in bacteria, and both polymers are biosynthesized via UDP-*N*-acetylglucosamine, chitin is not produced by bacteria. Two possible exceptions are in spore coats of streptomycete spores and the stalks of some prosthete bacteria. The occurrence of chitin in eukaryotic organisms has been reviewed elsewhere (1,11,12) and will be briefly summarised here. In protozoa it is formed in the cyst walls of amoebae, flagellates and ciliates. In algae β -chitin forms the flotation spines of centric diatoms. In fungi α -chitin is the characteristic component of nearly all cell walls. In invertebrates α -chitin is a major component of exoskeletons and egg shells, while β -chitin is in the peritrophic membranes lining the guts, and in a range of other internal structures, such as squid pen. Plants, vertebrates and a few groups of invertebrates, such as platyhelminths, ctenophores, nemertean and echinoderms do not produce chitin.

Thus chitin is abundant in the sea, in diatom blooms and in the zooplankton, most notably in the shoals of krill; and on the land, in invertebrates and in fungi in the soil. Potential industrial sources are wastes from shrimps and crabs, krill, squid, clams and oysters, and fungal fermentations (13). The krill fishery alone produces 3000 tons per year, currently going to waste.

Uses of Chitin, Chitosan and their Oligomers

Actual and potential uses of chitin and its derivatives have been the impetus for several conferences and reviews (14-21). Hirano (21) provides figures for Japan in 1986, when 1270 tons of chitin were produced. Most of this was deacetylated with hot alkali to give chitosan, of which 500 tons was used as a flocculant and 200 tons for formulation in cosmetics and animal and human foodstuffs. A small proportion of the chitin, 40 tons, was used as such for a wide variety of uses, and 60 tons was depolymerised to sugars and oligosaccharides, again with a wide variety of uses. Specialist uses for chitin include non-woven artificial skin for burns, biodegradable sutures, and as the vibrating panel in audio speakers (imitating the wing of a cricket). Uses for chitosan are very diverse (22), with specialist applications including enzyme and cell immobilization, wound healing, drug delivery and seed treatments. Oligomers of chitin and chitosan have been reported to have powerful biological activities both in animals and plants. In animals these include immunopotentiating, antitumour and antimetastatic effects (23, 24); in plants they include potent elicitor action, activating defense genes (25,26).

Nature and Activity of Chitinases

The simple definition of chitinase activity, EC 3.2.1.14, "hydrolysis of *N*-acetyl-D-glucosaminide (1-4)- β -linkages in chitin and chitodextrins", belies the complexity and diversity of this group of enzymes. When chitinolytic organisms are investigated in detail, they are found to produce a range of chitinase activities. Usually these can be separated readily by chromatography or electrophoresis. However, it is more difficult to define their precise activities, chiefly because of the uncertain nature of the available assays for chitinases, with non-linear time courses

and non-physiological pH optima being common. With care, however, a classification into exo- and endo-chitinases can be made (27, 29). These activities may be defined as follows:

Exo-chitinases catalyse processive release of diacetylchitobiose units from the non-reducing ends of chitin chains;

Endo-chitinases randomly catalyse hydrolysis of *N*-acetylglucosaminide (1-4)- β -linkages of chitin chains.

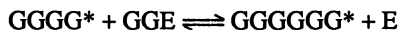
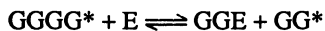
Related enzymes are *N*-acetylglucosaminidases, EC 3.2.1.30, some of which can act to hydrolyse the terminal, non-reducing *N*-acetylglucosamine residue of chitin; and lysozymes, EC 3.2.1.17, some of which can act slowly as endohydrolases.

As stated earlier, the nature of the substrate clearly has a major influence on its susceptibility to chitinase attack. Nascent chitin (e.g. as newly formed in fungal hyphal apices) and "colloidal chitin" (chitin dissolved in strong mineral acid and then precipitated) are most readily attacked. Highly ordered ("crystalline") chitin and chitin that has been subjected to "purification" by treatment with acids and oxidising agents are most resistant to attack. Bade *et al.* (28) describe methods of preparing fibrous chitins from invertebrates, by replacing the proteins stabilizing their structures by sulphate ester groups. The resultant chitins, retaining their native fine structure, then provide good model substrates for chitinases. Several bacterial, plant and fungal chitinases have now been cloned (27, 30-32), and so detailed studies of the mechanisms of action of different chitinases on different substrates are becoming possible for the first time. Detailed methods for the preparation of a range of chitinases have recently been published (33).

The diversity of chitinases is also evident in their different responses to the antibiotic allosamidin. This is a specific inhibitor of invertebrate, vertebrate, fungal and bacterial chitinases, but does not inhibit the plant chitinase from yam (34-37). Of the invertebrate chitinases reported, activity from a nematode was exquisitely sensitive, with that from an insect a thousand-fold less so; a chitinase from turbot fish plasma was a thousand-fold more sensitive than one from the fish stomach.

Transglycosylation Activities of Chitinases

Glycosylase activity, as well as hydrolysing glycosidic linkages, can also result in formation of new glycosidic linkages, by transglycosylation (37-39). This reaction has been investigated for hen egg-white lysozyme, where the rate constant of transglycosylation is much larger than that for hydrolysis (40-42). Transglycosylase activity has been reported for a chitinase from *Nocardia orientalis* (43, 44). It is favoured by a high substrate concentration and a lowered water activity, i.e. in increasing concentrations of ammonium sulphate. Thus Usui *et al.* (43), observing accumulating hexamer while incubating tetramer with the enzyme, propose the scheme:



(E = chitinase, G = *N*-acetylglucosamine, G* = reducing end group).

Thus in this example the tetramer serves as both the donor and the acceptor. Usui *et al.* (43) propose this reaction for the synthesis of hexa-*N*-acetylchitohexaose, an oligosaccharide with reported antitumour activity (24). They also observed formation of heptamer by incubating the pentamer with the *Nocardia* enzyme, but no chain elongation was observed with the hexamer as initial substrate. Similar activities with the formation of chitin oligosaccharides have also been observed for

hen egg-white lysozyme, in this case being favoured by high substrate concentration and lowered water activity in water-methanol solution (45). It is possible that in some situations *in vivo* the transglycosylation activities of "chitinases" may be their important roles, rather than their lytic activities.

Occurrence and Roles of Chitinases

Bacteria. Chitinase activities of bacteria have been reviewed by Gooday (1, 4, 6). Most notable among the Gram-negative bacteria are species of *Pseudomonas*, *Serratia*, *Vibrio*, *Photobacterium*, *Aeromonas*, *Chromobacterium*, and the gliding bacteria, *Cytophaga*, *Lysobacter* and *Chitinophaga*. Amongst the Gram-positive bacteria chitinase production is widespread in the Actinomycetes, e.g. species of *Streptomyces*, in *Arthrobacter* and *Nocardia*, and in the sporing genera *Bacillus* and *Clostridium*. These activities clearly have a nutritional role, as many of these bacteria can grow on chitin as sole carbon and nitrogen source. In some cases there is also a pathogenic role, for example with *Serratia* species when they are acting as insect pathogens. The sugar beet root maggot, however, appears to have a symbiotic relationship with *Serratia* bacteria, utilising their chitinases to emerge from its pupa (47). A strain of *Serratia marcescens* was the most active producer of chitinases of many bacteria and fungi tested by Monreal and Reese (48). Bacterial chitinases that have been cloned include those from *S. marcescens* (30) and from *Streptomyces* species (27). A strain of *Vibrio anguillarum* has recently been reported to accumulate diacetylchitobiose when cultivated in a medium containing colloidal chitin, giving a 40% conversion of chitin to its biose, presumably by the continued action of an exochitinase (49). Further study of the diversity of bacterial chitinases is clearly worthwhile.

Fungi. Nearly all fungi, certainly all of those containing chitin in their cell walls, produce a range of chitinases. The occurrence and roles of these have been discussed by Gooday (46) and Gooday *et al.* (50). Their roles can be nutritional, as in many soil saprophytes, such as *Aspergillus* and *Trichoderma* species, and as in pathogens of other fungi or invertebrates, such as *Beauveria*; or autolytic, as in *Coprinus* ink-caps and *Lycoperdon* puff-balls, and in ageing cultures; or morphogenetic, during branching, cell separation, anastomosis, and perhaps during apical hyphal growth and spore germination. They may have more subtle roles in the modelling and cross-linking of the chitin in the fungal wall to give it its characteristic shape and physical properties.

Invertebrates. As for fungi, nearly all invertebrates, and certainly all of those containing chitin, also produce a range of chitinases (11). Again these may be nutritional, occurring in guts and saliva; or morphogenetic, concerned with molting and egg-hatching.

Plants. A range of chitinases are found in plants, and are major components of the pathogenesis-related proteins, characteristically being induced when the plant is challenged in some way, such as attack by a fungus or virus, or treatment with an oligosaccharide elicitor or ethylene, or wounding (1, 31). The plant chitinases are antifungal, so this response can be seen as a first line defense against fungal attack. Seeds and latex also have chitinase activities. The insectivorous plants use chitinases to help digest their chitinous prey.

Vertebrates. Many vertebrates, notably insectivores and others eating prey with chitinous exoskeletons, produce digestive chitinases in their guts (1). In the case of fish this has been investigated in detail, and the chitinases are used as food-

processing enzymes, enabling other digestive enzymes, notably proteases, to get to the meat within the chitinous shells. Some vertebrates also have distinct chitinase activities in body tissues, such as blood plasma (51). These may have a defensive role, as for lysozyme. They may however not have chitin as their natural substrate, but instead may have unsuspected roles in processing glycoproteins or mucopolysaccharides.

Activity and Occurrence of Chitosanases

The chitosanases are a group of enzymes discovered by Monaghan *et al.* (52). They are produced by a limited range of bacteria and fungi, and act on chitosan in an endohydrolytic manner (46, 53). The major natural occurrence of chitosan is as the chief component of the cell walls of Zygomycete fungi, such as *Mucor*, *Rhizopus* and *Absidia* (12). Cultures of these fungi accumulate autolytic chitosanases as they age (54).

Activity and Occurrence of Chitin Deacetylase

Chitin deacetylase, EC 3.5.1.41, hydrolyses the *N*-acetamido groups of *N*-acetylglucosamine residues in chitin, releasing acetate, and eventually giving chitosan. The enzyme is best characterised from *Mucor rouxii*, where it acts on newly synthesized chitin as it is being incorporated into the growing cell walls (7). It is also reported from invertebrates, where it seems to be associated with loosening of chitinous tissues to allow their expansion (55). Hillman *et al.* (56) detected considerable activities of chitin deacetylase in estuarine sediments, associated with an accumulation of chitosan.

Uses of Chitinases, Chitosanases and Chitin Deacetylases

The use of the transglycosylase activities of chitinases to produce defined oligosaccharides has already been discussed (42-45). These procedures use oligosaccharides as substrates, but obviously carefully prepared chitin itself can be used as substrate for the lytic activity of chitinases to produce a range of oligomers. Likewise, controlled hydrolysis of chitosan to yield D-glucosamine oligosaccharides by the use of chitosanase from a *Bacillus* species has been reported by Izume and Ohtakura (57).

Chitinases have found extensive use in the preparation of protoplasts from fungi, a technique of increasing importance in biotechnology (58, 59). Chitosanases are required to make protoplasts from species of Mucorales (54).

As chitinases are a natural part of the plant's defense repertoire, there has been much interest in boosting plants' resistance to fungal attack by getting them to express cloned chitinases. This has now been achieved successfully in several plants (60).

The use of chitinases in the bioconversion of shellfish wastes to single-cell protein has been suggested (61), with concepts drawn from work on the use of cellulases in the bioconversion of cellulose. There is clearly much potential in this area.

Endogenous chitinases are already fortuitously used in food processing; in the de-shelling of shrimps, which is accomplished after a period of storage during which time the dead shrimp's enzymes loosen the chitinous attachments between meat and shell.

As was made clear earlier, chitosan and its derivatives are of more value than chitin. Currently the chitin is deacetylated by a harsh alkali treatment. If they

became available, the use of chitin deacetylases for this process would be an attractive alternative, which likely would give a new range of potential products.

Currently there are increasing numbers of bacterial, fungal and plant chitinases being sequenced and cloned. This information will give us new insights into the comparative biochemistry of these enzymes, and undoubtedly will aid in the development of a wide range of novel applications for them.

Literature Cited

1. Gooday, G.W. *Adv. Microb. Ecol.* **1990**, *11*, 387-430.
2. Minke, R.; Blackwell, J. *J. Mol. Biol.* **1978**, *120*, 167-81.
3. Gardner, K.H.; Blackwell, J. *Biopolymers.* **1975**, *14*, 1581-95.
4. Ruddall, K.M.; Kenchington, W. *Biol. Rev.* **1973**, *48*, 597-636.
5. Lindsay, G.J.H.; Gooday, G.W. *Carbohydr. Polymers* **1985**, *5*, 131-40.
6. Vermeulen, C.A.; Wessels, J.G.H. *Protoplasma* **1984**, *120*, 123-30.
7. Davis, L.L.; Bartnicki-Garcia, S. *J. Gen. Microbiol.* **1984**, *130*, 2095-102.
8. Wessels, J.G.H. *Acta Bot. Neerl.* **1988**, *37*, 3-16.
9. Giraud-Guille, M.M.; Bouligand, Y. In *Chitin in Nature and Technology*; Muzzarelli, R.; Jeuniaux, C.; Gooday, G.W., Eds.; Plenum: New York, **1986**; pp. 29-35.
10. Seno, N.; Matsumoto, I.; Doi, A.; Anno, K. In *Chitin and Chitosan*; Hirano, S.; Tokura, S. Eds.; Japanese Society of Chitin and Chitosan: Tottori, **1982**, pp. 159-164.
11. Jeuniaux, C. *Chitine et Chitinolyse*; Masson: Paris, **1963**.
12. Gooday, G.W. *Prog. Indust. Microbiol.* **1983**, *18*, 85-127.
13. Allan, G.G.; Fox, J.R.; Kong, N. In *Proceedings of the First International Conference on Chitin/Chitosan*; Muzzarelli, R.A.A.; Pariser, E.R., Eds.; MIT Sea Grant Report MITSG 78-7, Massachusetts Institute of Technology: Cambridge, MA, **1978**, pp. 64-78.
14. Muzzarelli, R.A.A. *Chitin*; Pergamon, Oxford, **1977**.
15. Muzzarelli, R.A.A.; Pariser, E.R. *Proceedings of the First International Conference on Chitin/Chitosan*; MIT Sea Grant Report MITSG, **1978**.
16. Hirano, S.; Tokura, S. *Chitin and Chitosan*; Japanese Society of Chitin and Chitosan: Tottori, **1982**.
17. Zikakis, J.P. *Chitin, Chitosan and Related Enzymes*; Academic: Orlando, **1984**.
18. Muzzarelli, R.A.A.; Jeuniaux, C.; Gooday, G.W. *Chitin in Nature and Technology*; Plenum: New York, **1986**.
19. Skjaek-Braek, G.; Anthonsen, T.; Sandford, P.; *Chitin and Chitosan*; Elsevier: London, **1989**.
20. Deshpande, M.V. *J. Sci. Indust. Res.* **1986**, *45*, 273-81.
21. Hirano, S. In *Chitin and Chitosan*; Skjaek-Braek, G.; Anthonsen, T.; Sandford, P., Eds.; Elsevier: London, **1989**; pp. 37-43.
22. Sandford, P.A. In *Chitin and Chitosan*; Skjaek-Braek, G.; Anthonsen, T.; Sandford, P., Eds.; Elsevier: London, **1989**; pp. 51-69.
23. Suzuki, S.; Suzuki, K.; Tokoro, A.; Okawa, Y.; Suzuki, M. In *Chitin in Nature and Technology*; Muzzarelli, R.; Jeuniaux, C.; Gooday, G.W., Eds.; Plenum: New York, **1986**; pp. 485-92.
24. Suzuki, S.; Matsumoto, T.; Isukada, K.; Aizawa, K.; Suzuki, M. In *Chitin and Chitosan*; Skjaek-Braek, G.; Anthonsen, T.; Sandford, P., Eds.; Elsevier: London, **1989**.
25. Hadwiger, L.A.; Kendra, D.F.; Fristensky, B.W.; Wagoner, W. In *Chitin in Nature and Technology*; Muzzarelli, R.; Jeuniaux, C.; Gooday, G.W., Eds.; Plenum: New York, **1986**; pp. 209-14.

26. Roby, D.; Gabelle, A.; Toppan, A. *Biochem. Biophys. Res. Comm.* **1987**, *143*, 885-92.
27. Robbins, P.W.; Albright, C.; Benfield, B. *J. Biol. Chem.* **1988**, *263*, 443-7.
28. Bade, M.C.; Stinson, A.; Moneam, N.A. *Connective Tiss. Res.* **1988**, *17*, 137-51.
29. Cabib, E. *Adv. Enzymol.* **1986**, *59*, 59-101.
30. Fuchs, R.; McPherson, S.; Druhos, D. *Appl. Environ. Microbiol.* **1988**, *51*, 564-9.
31. Broglie, K.; Guymer, J.; Broglie, R. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 6820-4.
32. Kuranda, M.J.; Robbins, P.W. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 2585-9.
33. Wood, W.A.; Kellogg, S.T. *Methods in Enzymology Vol.161, Biomass*; Academic: New York, **1988**.
34. Sakuda, S.; Isogai, A.; Matsumoto, S.; Suzuki, A. *J. Antibiot.* **1987**, *40*, 296-300.
35. Somers, P.J.B.; Yoo, R.C.; Doolin, L.R.; McGowan, M.J.; Fukuda, D.S., Myndase, J.S. *J. Antibiot.* **1987**, *40*, 1751-6.
36. Gooday, G.W.; Brydon, L.J.; Chappell, L.H. *Mol. Biochem. Parasitol.* **1988**, *29*, 223-5.
37. Gooday, G.W. In *Biochemistry of Cell Walls and Membranes in Fungi*; Kuhn, P.J.; Trinci, A.P.J.; Jung, M.J.; Goosey, M.W.; Copping, L.G., Eds.; Springer-Verlag: Berlin, **1990**; pp. 61-79.
38. Bacon, J.S.D. In *Microbial Polysaccharides and Polysaccharases*; Berkeley, R.C.W.; Gooday, G.W.; Ellwood, D.C., Eds.: Academic: London, **1979**; pp. 269-84.
39. Nilsson, K.G.I. *Trends Biotech.* **1988**, *6*, 256-64.
40. Tadu, H.; Kakitani, T. *Bull. Chem Soc. Japan* **1973**, *46*, 1226-32.
41. Chipman, D.M. *Biochemistry* **1971**, *10*, 1714-22.
42. Masaki, A.; Fukamizo, T.; Ohtakara, A.; Torikata, T.; Hayashi, K.; Imoto, T. *J. Biochem.* **1981**, *90*, 527-33.
43. Usui, T.; Hayashi, Y.; Nanjo, F.; Ishido, Y. *Biochim. Biophys. Acta* **1984**, *923*, 302-9.
44. Nanjo, F.; Sakai, K.; Ishikawa, M.; Isobe, K.; Usui, T. *Agric. Biol. Chem.* **1989**, *53*, 2189-95.
45. Usui, T.; Hayashi, Y.; Nanjo, F.; Ishido, Y. *Biochim. Biophys. Acta* **1988**, *953*, 179-84.
46. Gooday, G.W. In *Microbial Polysaccharides and Polysaccharases*; Berkeley, R.C.W.; Gooday, G.W.; Ellwood, D.C., Eds.: Academic: London, **1979**; pp. 437-60.
47. Iverson, K.L.; Bromel, M.C.; Anderson, A.W.; Freeman, T.P. *Appl. Environ. Microbiol.* **1984**, *47*, 22-7.
48. Monreal, J.; Reese, E.T. *Can. J. Microbiol.* **1969**, *15*, 689-96.
49. Takiguchi, Y.; Shimahara, K. *Letts. Appl. Microbiol.* **1988**, *6*, 129-131.
50. Gooday, G.W.; Humphreys, A.M.; McIntosh, W.H. In *Chitin in Nature and Technology*; Muzzarelli, R.; Jeuniaux, C.; Gooday, G.W., Eds.; Plenum: New York, **1986**; pp. 83-92.
51. Manson, F.D.C.; Gooday, G.W.; Fletcher, T.C. In *Chitin and Chitosan*; Skjaek-Braek, G.; Anthonsen, T.; Sandford, P., Eds.; Elsevier: London, **1989**; pp. 243-53.
52. Monaghan, R.L.; Eveleigh, D.E.; Tewari, R.P.; Reese, E.T. *Nature New Biology* **1973**, *245*, 78-80.
53. Davis, B.; Eveleigh, D.E. In *Chitin, Chitosan and Related Enzymes*; Zikakis, J.P., Ed.; Academic: Orlando, **1984**. pp. 161-79.

54. Reyes, F.; Lahoz, R.; Martinez, M.J.; Alfonso, C. *Mycopathologia* **1985**, *89*, 181-7.
55. Aruchami, M.; Sundara Rajulu, G.; Gowrie, N. In *Chitin in Nature and Technology*; Muzzarelli, R.; Jeuniaux, C.; Gooday, G.W., Eds.; Plenum: New York, **1986**; pp. 263-5.
56. Hillman, K.; Gooday, G.W.; Prosser, J.I. *Est. Coast Shelf Sci.* **1989**, *29*, 601-12.
57. Izume, M.; Ohtakara, A. *Agri. Biol. Chem.* **1987**, *51*, 1189-91.
58. Peberdy, J.F. *Annu. Rev. Microbiol.* **1979**, *33*, 21-39.
59. Yabuki, M.; Kasai, Y.; Ando, A.; Fujii, T. *Exptl. Mycol.* **1984**, *8*, 386-90.
60. Dunsmuir, P.; Suslow, T. In *Cell Culture and Somatic Genetics of Plants*, Vol. 6; Schell, J.; Vasil, I.K., Eds.; Academic: San Diego, **1989**; pp. 215-27.
61. Carroad, P.A.; Tom, R.A. *J. Food Sci.* **1978**, *43*, 1158-61.

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Chapter 38

Xylose–Glucose Isomerases Structure, Homology, and Function

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Interest in the bacterial enzyme xylose/glucose isomerase has been driven by its use in the isomerization of glucose to fructose to produce high-fructose corn syrups, and in the isomerization of xylose to xylulose for the conversion of the more fermentable xylulose to ethanol. In this work, a brief historical perspective is presented, followed by a summary of the current understanding of the enzyme's major features. Also, a useful compilation of available xylose isomerase DNA sequences is presented with annotation of some of the major areas identified as being of functional significance. The extent of homology between the xylose isomerases is discussed with reference to differences in their function.

Xylose isomerases (EC 5.3.1.5), often referred to as glucose isomerase, have been studied extensively, in large part because of their use in the conversion of glucose to fructose for high-fructose corn syrup (HFCS). The world market for HFCS is expected to reach a total of 7.9 million metric tons in 1990 which, at a cost of \$0.20/LB, would amount to \$3.2 billion (1), and sales of xylose isomerase is expected to be about \$15 million (T. Wallace, International Biosynthetics, personal communication). Research on xylose isomerase has produced DNA sequences of the gene from a number of bacterial strains, including the detailed structure of the xylose operon (2-7). In addition, x-ray crystallographic studies (8), kinetic measurements (9), and the use of inhibitors (10,11) have led to descriptions of the location of the active site and mechanistic models of its activity.

The enzyme is also being studied for use in converting of biomass to ethanol for fuel usage. Prospects for the conversion of cellulytic biomass to ethanol for fuel or as a fuel additive have improved within the last decade because of the development of methods for the fermentation of xylose, which can comprise as much as 50% of the fermentable sugars in these feedstocks. One of these methods uses xylose isomerase to convert xylose, which is difficult to ferment by ethanol-tolerant yeasts, to the fermentable sugar xylulose (12,13).

Initial Discoveries. Xylose isomerase activity was initially found in 1953 in extracts of *Lactobacillus pentosus* (14), followed by similar activities in extracts of *Pseudomonas hydrophila* and *Pasteurella pestis* in the mid-1950s (15-17). An enzyme activity that was found to convert glucose to fructose was discovered in 1957 (18). This activity, found in sonicated extracts from *Pseudomonas hydrophila*, was enhanced in the presence of

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arsenate and had a substrate specificity that was 100 fold higher on xylose than glucose. The requirement for arsenate and low affinity toward glucose makes the enzyme unsuitable for HFCS production, but the result showed that enzyme-mediated conversion of glucose to fructose was feasible.

Other arsenate-requiring enzymes, isolated from an *Aerobacter* sp. and *Escherichia freundii*, were found to isomerize glucose to fructose (19,20), but, at least in some cases, the enzymes required arsenate only when glucose or fructose, and not when glucose-6-phosphate or fructose-6-phosphate were used as substrates. Purification of the arsenate-dependent activity from *Escherichia intermedia* allowed the conclusion that an arsenate-glucose complex was formed during the reaction that allowed the enzyme, now identified as phospho-glucose isomerase (EC 5.3.1.9), to isomerize the sugar (21,22). True xylose isomerase activity was distinguished by constant glucose-to-xylose conversion ratios through purification and in the presence of inhibitors.

A true xylose isomerase, that did not require arsenate for its activity, was found in strains of *Lactobacillus*, especially *L. brevis* (23). In this study, it was found that the activity of isomerization of glucose and xylose were essentially equal, and that ribose was isomerized to ribulose at a reduced rate (24). The xylose isomerases from this strain, like those from other species, requires divalent cations for activity. In this case, Mn^{++} and Co^{++} were found to be required for activity. In other studies, Co^{++} has been found to increase xylose isomerase stability (25,26,27).

Since these early discoveries, xylose isomerases have been isolated from many bacterial species, and these enzymes have been intensely investigated, especially those of the genera *Streptomyces*, *Lactobacillus*, and *Bacillus*. The characteristics of substrate specificity (xylose \geq glucose > ribose), divalent metal cation activation (Mg^{++} , Mn^{++} or Co^{++}), and activity at alkaline pH are properties that most of the enzymes share to a certain extent, but significant variations exist. Some of these enzymes have been immobilized and patented for commercial use. There are many good reviews in the literature that describe the enzymatic characteristics of the xylose isomerases (9,28,29).

Xylose isomerases with higher thermostability were found in the strains of *Streptomyces* and related *Actinoplanaceae* (which includes the genera *Ampullariella* and *Actinoplanes*). High thermo-tolerance is desirable for production of HFCS because at equilibrium, as the temperature of the enzyme reaction is increased, the ketose/aldose ratio increases proportionately (30). In addition, reactors running at higher temperatures have less risk of microbial contamination, allowing for less frequent and less costly enzyme replacement.

Another factor contributing to enzyme cost is the need for xylose, an expensive substrate, to induce the biosynthesis of the enzyme. This consideration has led to the discovery of strains having constitutive production of the enzyme (31,32) and work that significantly increased enzyme titres through recombinant DNA procedures have been successful (33,34).

Mechanism of Isomerization. The overall reaction for xylose isomerase in the isomerization of aldose to ketose sugars is essentially the intramolecular transfer of a hydrogen from C2 to C1 of the sugar, accompanied by loss of a proton from the C2-hydroxyl and proton addition to the carbonyl group at C1. Although there is some controversy over the detailed mechanism of activity, it is generally agreed that there are two cation-binding sites (35), and that the primary substrate of the forward reaction is an α -D-pyranose (8). The first step of the reaction is the enzyme-mediated opening of the ring structure, followed by the hydrogen transfers. Studies using specific chemical modification of amino acids identified the presence of a single histidine residue that is essential for enzyme activity (10).

Two models for the mechanism of the isomerization have been proposed. The first model was originally proposed because of similarities between enzymes involved in the activation of C-H bonds next to a carbonyl function (Fig. 1a). Stereochemical considerations were used to suggest that the ring structure of the sugar is opened to

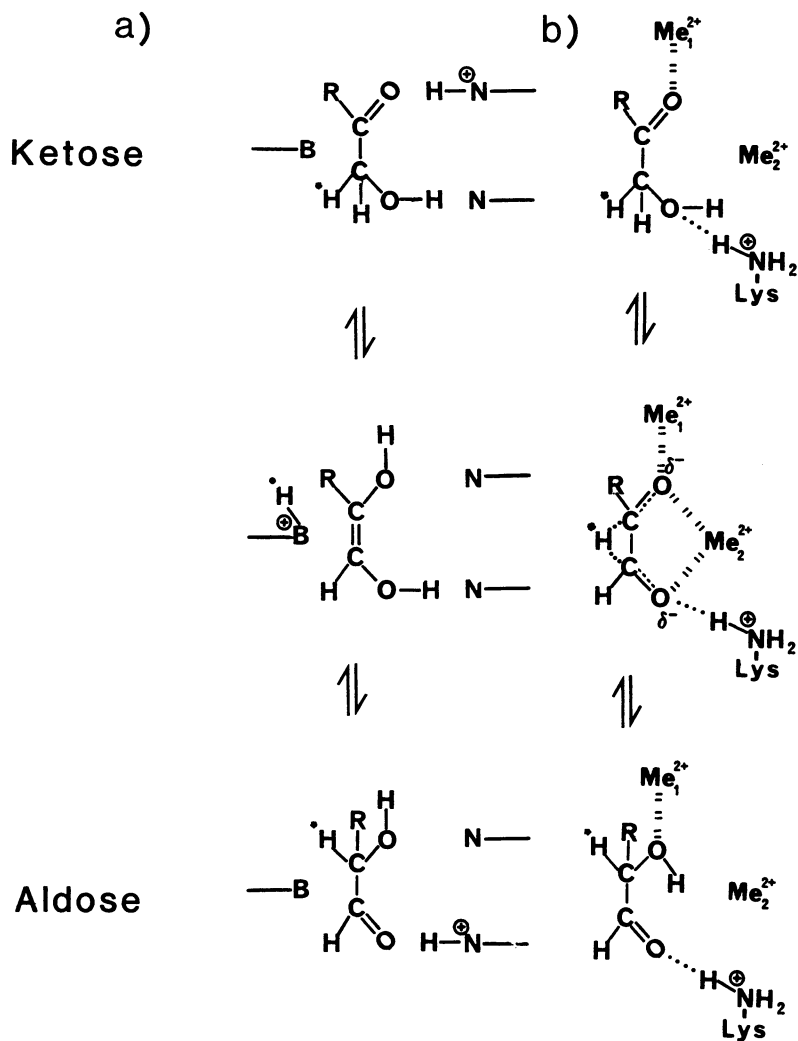


Figure 1. a. *cis*-enediol mediated isomerization (36,37); b. hydride shift mechanism (8).

form a *cis*-enediol intermediate, and kinetic and chemical evidence obtained from studies on triose phosphate isomerases was used to support this view (36).

This mechanism has been supported by x-ray studies on crystallized xylose isomerase that suggest that the "active-site histidine" (presumed to be His 53, or 54 in some sequences) is located correctly so as to properly remove a proton from either C1 or C2 of the substrate, and that two threonine residues (Thr90 and Thr91, or 89,90 in some sequences) are positioned to provide polar oxygens to be either the acceptor or the donor proton in the *cis*-enediol configuration (37).

The second model, referred to as a "hydride shift," predicts the shift of hydrogen atoms without the *cis*-enediol intermediate (38) (Fig. 1b). In the first place, it had been determined that the exchange of hydrogen (^3H) between substrate and solvent was five orders of magnitude lower for xylose isomerase than with other isomerase enzymes known to have enol intermediates. The ratio of exchange of transfer to solvent for xylose isomerase was estimated to be 10^{-4} , indicating virtually no exchange of hydrogen to solvent during transfer.

X-ray crystallographic evidence is also used to support this model (8). These studies suggest that initial opening of the aldose ring is mediated by His53 and Asp56 is followed by interaction with the cations in sites MI and MII. The positive charge of the cations, and the interaction of Lys182 with O1, allows for a transition state in which the proton on O2 is lost, leaving the remaining proton situated between O1 and O2. In this transition state the hydrogen on C2 is able to move between C1 and C2 without an enediol intermediate.

These models have been based on isomerases isolated from *Streptomyces* spp. and closely related bacteria. It is unfortunate that more distant strains (see below) have not been studied in such a rigorous manner.

Categories of Xylose Isomerase. Xylose isomerases from various bacterial sources have been categorized in terms of arsenate requirements, cation requirements, pH activity range, or other features that relate to the conditions under which the enzyme functions. Many of the genes that code for xylose isomerase have been sequenced and translated to the unambiguous amino acid sequences. Homologies between the proteins have been investigated by computer analysis of the amino acid sequences. With these tools, the single letter amino acid code of the enzymes were aligned and the homologous regions between proteins were indicated (6,38). Careful study of these sequences showed that there are significant homologies between all the proteins, but these charts are difficult to read and to convert into the original DNA sequences of the genes.

In this report, five published DNA sequences of the gene are compiled. Regions of homology greater than four amino acids in length were mapped onto the DNA sequences. This procedure was found to emphasize the differences as well as the similarities between sequences. From these comparisons, two different categories of enzyme can be identified with respect to characteristic size and homologous regions. The DNA sequences are also annotated with the locations of some of the amino acids that have been identified as being of functional significance.

Materials and Methods

The xylose isomerase DNA sequences from *Bacillus subtilis* (5), *Escherichia coli* (2,3), *Streptomyces violaceoniger* (4), and an *Ampullariella* sp., (6) were computer down-loaded from GenBank (Mountain View, CA). The sequence from *Actinoplanes missouriensis* (7) was typed into the computer twice. Discrepancies between the sequences were then compared to the original to produce a verified copy. Analysis of these sequences were performed using software developed by D. Mount and B. Conrad at the University of Arizona (Department of Molecular and Cellular Biology, Biosciences West, Tucson AZ).

All sequences were edited to contain only the gene-coding regions before analysis. The DNA sequences were each translated by computer into amino acid sequences and homologies of 4 amino acids or more were then determined. These homologies were then mapped onto the DNA sequence. This stringent method of matching requires a minimum size of 12bp and exact correspondence of amino acid residues. Annotation of functional sites in the sequences of *S. violaceoniger*, *Ampullariella* sp., or *A. missouriensis* are those used by Collyer et al. (8) or Carrell et al. (37). Correspondence of these sites to sites found in sequences obtained from *B. subtilis* and *E. coli* were aided by the amino acid comparisons published by Saari et al. (6).

Results and Discussion

Xylose Isomerase Sequences. The coding regions of five sequenced xylose isomerase genes, and their computer generated translation products, are shown in Figs. 2 and 3. The sequences are aligned from the translation initiation site (ATG) and end with the first non-sense terminator site in the open reading frame. Homologous sites that represent amino acid segments of four or more matching residues are mapped onto the sequences as indicated in the legend. Two of these homologous sequences, WGGREG (Trp-Gly-Gly-Arg-Glu-Gly) and IEPKP (Ile-Glu-Pro-Lys-Pro) are the only matches that are present in all of the xylose isomerase sequences (Table 1); their locations are also indicated in the margin of Fig. 2. The former regions have been discussed as regions that contribute substantially to the structure of the active site of the enzyme, whereas the later contains a Lysine (Lys182) that is important in the mechanism for transfer of the hydrogen atom ("hydride shift") between C1 and C2 of the substrate (8). Other annotations on the sequence show the locations of the amino acids thought to open the aldose ring (His53 and Asp56), and residues (Thr 90, Thr91) that are thought to hold a water molecule in place for the *cis*-enediol model of activity (Table 2) (37). The locations of amino acids that are thought to constitute the two cation binding sites of the enzyme, as determined by x-ray crystallographic studies (8), are listed in Table 3 and also marked on Fig. 2.

Gene-coding Regions of *E. coli* and *B. subtilis*. The xylose isomerase gene-coding sequences of *B. subtilis* and *E. coli* are exactly the same length, 1323bp, 147bp to 162bp longer than the other strains. The first homology match on these sequences is a match at b34-e874; the rest of the homologies follow at even spacings through the two sequences. At the beginning of the sequences there is an initial discrepancy in coding of 21bp caused by seven amino acid residues present in the N-terminus of the *B. subtilis* sequence that do not occur in the *E. coli* sequence. At the site designated as His53, the discrepancy has become 18bp and this six amino acid difference is maintained through the rest of the sequences. Thus, there is a good correspondence between these two sequences despite the fact that they are taxonomically dissimilar. However, there are some differences in the *E. coli* sequence that seem unique: the presence of four cysteine residues (other strains contain none or one), and the fact that it functions as a dimer instead of the usual tetrameric configuration (39). For expediency, these genes and the enzymes they code for will be called "Type 1."

Gene Coding Regions of *S. violaceoniger*, an *Ampullariella* sp. and *Actinoplanes missouriensis*. The xylose isomerase gene coding sequences of these genera ("Type 2") show a significantly increased homology in primary structure. The *Ampullariella* sp. and *A. missouriensis* are more closely related taxonomically than the *Streptomyces* spp., but they are all in the same taxonomic order, the *Actinomycetales* (40). The homology match for these strains shows how closely they are related. This can be demonstrated by counting the number of amino acids that are identical between the species in groups

b = *Bacillus subtilis* 1323bp, (5)
 e = *Escherichia coli* 1323bp, (2),(3)
 s = *Streptomyces violaceoniger* 1170bp, (4)
 a = *Ampullariella* sp. 1185bp, (6)
 m = *Actinoplanes missouriensis* 1182bp, (7)

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1 b ATGGCTCAAT CTCATTCTAG TTCAGTTAAC TATTTTGGAA GCGTAACAA-b34->e874
1 e ATGCAAGCCT ATTTTGACCA GCTCGATCGC GTTCGTTATG AAGGCTCAAA
1 s ATGAGCTTCC AGCCACCCC CSAGSACAAG TTCACCTTCG GTCTGTGGAC
1 a ATGTGCTGCC AGGCCACACC CGATSACAAG TTCTCCTTCG GTCTCTGGAC
1 m GTGTCTGTCC AGGCCACACG CSAAGSACAAG TTCTCCTTCG GTCTCTGGAC

51 b AGTGGTTTTT GAAGGGAAAG CTTCCACTAA TCCTTTAGCA TTTAAATATT
51 e ATCCTCAAAC CCGTTAGCAT TCCGCTACTA CAATCCGCAC GAACTGGTGT
51 s CGTCGGCTGG CAGGGAAGGG ACCCGTTCGG CSAGCCACC CGCCCTGGCC
51 a CGTCGGCTGG CAGGCGCGTG ACGCGTTCGG TSAGCCACC CGTCCGGTCC
51 m CGTTGGATGG CAGGCTCGTG ACGCGTTCGG TSAGCCACC CGTACGGCAC

101 b ATAATCCTCA AGAAGTAATC GCGGAAAAA CGATGAAAGA GCATTTGGCA
101 e TGGTAAGCG TATGGAAGAG CACCTGGCTT TTGGCGCTG CTACTGGCAC
101 s TGACCCGGT CSAGACCGTG CAGCGCTGG CCGAGCTGGG CGCCTACGGG
101 a TGACCCGGT CSAGCCCGTG CACAAGCTGG CCGAGATCGG CGCCTACGGC
101 m TGACCCGGT CSAGCCCGTG CACAAGCTGG CTGAGATCGG CGCCTACGGC

151 b TTTTCTATTG CCTATTGGCA TACATTTACT GCTGATGGCA CAGACGTTTT
151 e ACCTTC TGCT GGAACGGGGG GGATATGTTT GGTGTGGGGG CGTTAATCG
151 s GTGACCTTCC ACGACGACGA CTGTATCCCC TTGCGGTCTG CCGACACCGA
151 a GTGACCTTCC ACGACGACGA CTGTGTCCCG TTGCGGCGCG ACGCCGGGAC
151 m ATCACCTTCC ACGACGACGA CTGTGTCCCG TTGCGGCTCG ACGCCAGAC
His53, Asp56
sam=160,169

201 b TGGAGCAGCT ACAATGCAAA GACCATGGGA TCACTATAAA GGCATGGATC
201 e TCCGTGGCAG CAGCCTGGTG AGGCAC TGGC GTTGGCGAAG CGTAAAGCAG
201 s GCGCGAGTCG CACATCAAGC GGTTCGCCA GGCCTGGAC GCCACTGGCA
201 a CCGCGACGGC ATCGTCGCGG GGTTCGCCA GGCGCTCGAC GAGACC6GCC
201 m CCGCGACGGC ATCATCGCGG GCTTCAAGAA GGCGCTCGAC GAGACC6GCC

251 b TAGCTAGGGC AAGAGTAGAA GCAGCATTG AGATGTTTGA AAACTAGAT
251 e ATGTGCGATT TGAGTTTTTC CACAAGTTAC ATGTGCCATT TTATTGCTTC
251 s TGACGGTGCC GATGGCCACC ACGAACCTCT TCACCCACCC CGTCTTCAAG
251 a TGATCGTCCC GATGGTACC ACGAACCTCT TCACCCACCC GGTGTTCAAG
251 m TGATCGTCCC GATGGTACC ACGAACCTCT TCACCCACCC GGTGTTCAAG
Thr90, 91
sam=268, 271

301 b GCACCATTTT TTGCTTTTCA TGATCGAGAT ATTGCACCAG AAGGAAGTAC
301 e CACGATGTGG ATGTTTCCCC TGAGGGCGCG TCGTTAAAAG AGTACATCAA
301 s GACGGCGGT TCAGCGCAA CGACCCGCAC GTGCGCCGT ACGCGCTGCG
301 a GACGGCGGT TCACGACAA CGACCCGACG GTCCGGCGGT ATGCGATCCG
301 m GACGGCGGT TCACGACAA CGACCGTTCC GTGCGCGCT ACGCGATCCG
His53=b319
His53=e301
Asp56=b328
Asp56=e310

351 b GTTAAAAGAG ACAATCAAA ATTTAGATAT TATCGTGGG ATGATTAAGG
351 e TAATTTTGGC CAAATGTTG ATGTCTGGC AGGCAAGCAA GAAGAGAGCG
351 s CAAGACGATC CGCAACATCG ACCTGGCGG CSAGCTGGG GCCAAGACCT
351 a CAAGGTGCTG CGCCAGATGG ACCTGGCGG CSAGCTGGG GCCAAGACCT
351 m CAAGGTGCTG CGCCAGATGG ACCTGGCGG CSAGCTGGG GCCAAGACCT

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Figure 2. Gene-coding regions of xylose isomerases isolated from 5 bacterial strains. Homologous regions (x) are mapped from the computer-generated amino acid sequences; minimum = 4aa: e x sam = **ATCG**; b x sam = **ATCG**; sam x sam or b x e = **ATCG**

401 b ATTACATGAG AGATAGCAAC **GTAAAGTTAT** TATGSAATAC TGCAAACATG-Thr90=b439
401 e GC**GTGAAGCT** **GCTGTGGGA** ACGGCCAACT GCTTTACAAA **CCCTCGCTAC**-Thr90=e421
401 s AC**GTCCGCTC** **GGGGCCGGT** **GAGGGCCCG** AGTCCGGTGG **CGCCAAGGAC**-WGGREG=s409
401 a **TGGTCTCTC** **GGGGCCGGT** **GAGGGCCCG** AGTACGACT **GGCCAAGGAC**-VLWGGREG=a403
401 m **TCCTCTCTC** **GGGGCCGGT** **GAGGGCCCG** AGTACGACT **GGCCAAGGAC**-VLWGGREG=m403

451 b **TTTACGAACC** **CCCCTTTCGT** CCATGGAGCC GCGACTTCTT GTAATGCAGA
451 e GGC**CGGGT** **CGCGACGAA** **CCCAATCTC** **CAAGTATTCA** GCTGGCGCGC
451 s **GTGCGGACG** **CCCTCGACC** CATGAAGGAG GCGTTCGACC TCCTGGGCGA
451 a **GTCGGCCCG** **CCCTCGACC** CTACC**CGGAG** **GCCCTCAACC** **TGCTCGCGCA**
451 m **GTCAGCGCG** **CCCTCGACC** CTACC**CGGAG** **GCGCTCAACC** **TGCTCGCGCA**

501 b TGTGTTTGG TATGCTGCAG CACAAGTAAA AAAAGGGTTA GAAACAGCAA
501 e AACGCAAGT GTTACAGCGA TGGAAAGCAAC CCATAAAATTG GCGGGT**GAAA**
501 s GTACGTACCC GCCCAGGGCT ACGAC**TCCG** **CTTCGCATC** **GAGCCGAGC**-MI-Glu180
501 a **GTACTCCGAG** **GACCAGGGCT** ACGGC**TGCT** **CTTCGCATC** **GAGCCGAGC** sam=541
501 m **ATACTCCGAG** **GACCAGGGT** ACGGC**TGCT** **CTTCGCATC** **GAGCCGAGC**-Lys183=sam547
IEPKT=sam538
-VLWGGREG=b556

551 b **AAGAGCTTGG** **CGCCGAGAAC** TATGTATTTT **GGGGCCCGC** **TGAAGGATAC**-WGGREG=e562
551 e **ACTATCTCC** **GTGGCCCG** **GCTGAAGGTT** **ACGAAACGCT** **GTTAAATACC**
551 s **GAAACGAGCC** **CCGCGGCGAC** ATCCTCTCTG **CCACCCTCGG** **CCACGCGCTC**
551 a **GAAACGAGCC** **CCGCGGCGAC** ATCCTCTCTG **CGACCCTCGG** **CCACGCGCAT**
551 m **GAAACGAGCC** **CCGCGGCGAC** ATCCTCTCTG **CGACCCTCGG** **CCACGCGCAT**

601 b **GAAACATTGT** **TAAATACCGA** TTTAAATTTT GAGCTTGATA ATTTGGCGAG
601 e **GACTTGCCTC** **AGGAGCTGGA** ACAACTGGGC CGCTTTATGC AGATGGTGGT
601 s GCCTTCATCG AGCGCTTGA **CGCCCGGAG** **CTGTACGGCG** **TCAACCCGGA**-MI-MII-Glu216
601 a **GCCTTCTGTC** **AGGAGCTGGA** **CGCCCGGAG** **CTGTTCGGCA** **TCAACCGGGA** sam=649
601 m **GCCTTCTGTC** **AGGAGCTGGA** **CGCTCCGAG** **CTTTCGGCA** **TCAACCGGGA**

651 b ATTTATGCAT ATGGCAGTAG ATTATGCGAA GGAATCGAG TATACAGGGC-MI-Glu180
651 e TGAGCATAAA CATAAAATCG GTTCCAGGG CACGTTGCTT **ATCGAATCCG**-b=712, e=694
651 s GGT**CGGCCAC** **GAGCAGATGT** **CCGGCTGAA** **CTTCCGCAC** **GGCATCGCCG**-MII-His219
651 a **GACCGGCCAC** **GAGCAGATGT** **CGAACCTGAA** **CTTACCACAG** **GGCATCGCCC** sam=658
651 m **GACCGGCCAC** **GAGCAGATGT** **CGAACCTCAA** **CTTACCACAG** **GGCATCGCCC**-IEPKP=e691

701 b **AGTTTTTGAT** **TGAACCAAAA** CCAAAGAGC CGACCACCCA **TCAATATGAT**-IEPKP=b709
701 e **ATCGCAAGA** **ACCGACAAA** **CATCAATATG** **ATACGATG** **CGCGACGGTC**-Lys182
701 s **AGGCCCTGTG** **GGCGGGCAAG** CTCTTCCACA **TCGACCTCAA** **CGCCAGTCC** b=718, e=700
701 a **AGCGCTGTG** **GCACAAGAAG** CTGTTCCACA **TCGACCTGAA** **CGCCAGCAC**-MI-Asp244
701 m **AGCGCTGTG** **GCACAAGAAG** CTGTTCCACA **TCGACCTGAA** **CGGTACGAC** sam=733

751 b **ACAGATCGAG** **CAACAACCAT** TGCCTTTTGG **AAGCAATATG** GCTTAGACAA
751 e TATGGCTTCC **TGAACAGTT** TGGTCTGGAA AAAGAGATTA AACTGAACAT
751 s GGCATCAAGT **ACGACAGGA** **CCTGCGTTC** GGCGCCGGCG ACCTGCGGGC-MII-
751 a **GGCCGAAGT** **TCGACAGGA** **CCTGCTTTC** **GGTCACGGT** **ACCTGCTCAA**-Asp254, 256
751 m **GGCCGAAGT** **TCGACAGGA** **CCTGCTTTC** **GGCCACGGT** **ACCTGCTCAA** sam=763, 769

801 b TCATTTTAAA TAAATCTAG **AAGCCAATCA** **TGCCACATTA** **GCCGGGATG**-MII-His219
801 e **TGAAGCTAAC** **CACGACCGC** **TGGCAGTCA** **CTCTTCCAT** **CATGAAATG** b=829
801 s **GGCGTCTGG** **CTGGTCGACC** **TCCTGGAGAG** **CGCCGGTTAC** **GAGGGCCCGC** e=811
801 a **CGCGTCTCC** **CTGGTCGACC** **TCCTGGAGAA** **CGGGCCCGAC** **GGCGCCCGG**-MI-MII-Glu216
801 m **CGCGTCTCG** **CTGGTCGACC** **TCCTGGAGAA** **CGGTCCGGAC** **GGCGCCCGG** b=820, e=802

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Figure 2. Gene-coding regions of xylose isomerases isolated from 5 bacterial strains. Homologous regions (x) are mapped from the computer-generated amino acid sequences; minimum of 4aa: e x sam = **ATCG**; b x sam = **ATCG**; sam x sam or b x e = **ATCG**

851 b CATTGAACA TGAATTACGC ATGGCAAGAG TACATGGTCT TCTTGGATCT-MI-Asp244
851 e CCACCGCCAT TGCCTTGGC CTGTTGGTT CTGTGACGC CAACCGTGGC b=904, e=886
851 s **GGCACTTCA** CTTCAGCCG CCGCGGACC **AGGACTTCA** CCGCGTGTG-MI-Asp292
851 a CCTACGACG CCGCGGAC TTCGACTACA **AGCCCTCGC** CACCGAGGAC s=889
851 m **CGTACGACG** ACCCGCTAC TTC**ACT**TACA **AGCCGTCCG** TACCGAGGAC am=874

901 b GTTGATGCGA ACCAGGGTCA TCCTCTTTA **GGCTGGGACA** CCGATGAATT-MII-Asp254
901 e GATGCGCAAC **TGGCTGGGA** CACCGAC CAG TTCCCGAACA GTGTGGAAGA-MII-Asp256
901 s GCCTCGGCCG AGGGCTGCAT GCGCAACTAC CTGATCTCA **AGGAGCGCG** b=937,943
901 a **TTCGACGGC** TCTGGGAGT **GGCCAAGGAC** AACATCCGGA TGTACCTGT e=919,925
901 m TACGACGGC TCTGGGAGT **GGCAAGGCC** AACATCCGGA TGTACCTGT

951 b TCCCACAGAT TTATATTCTA CGACATTAGC **AATGTACGAA** ATCCTGCAAA
951 e GAATGGCGCTG GTGATGTATG **AAATTCTCAA** AGC**AGCGG** TCA**ACC**CGG
951 s **GGCGCCCTT** **CGCGCCGAC** **CGGAGTCA** GGAGGCCCTG CCGCGCCGCG
951 a **GCTCAAGGAG** **CGGGCCAAGG** **CGTTCCGGC** **CGACCGGAG** **GTG**CAGGCGG
951 m **GCTCAAGGAG** **CGGGCCAAGG** **CGTTCCGGC** **CGACCGGAG** **GTG**CAGGCGG

1001 b ATGGCGGCT TGGAAAGCGT **GGCTTAACT** **TGACGCGAA** **GGTCAGAAGA**-MI-Asp292
1001 e **GTGATTTGA** **CTTGATGCC** **AAAGTACGTC** **GTC**AAAGTAC TGATAAATAT b=1033
1001 s GTCYGGACCA GCTGGCCAG CCGACCGCG CGGACGGCT GGAGGCCCTG e=1015
1001 a CGCTGGCCGA GTCGAAGGTC GACGAGTGC GGACCCGAC GCTGAACCCG
1001 m **CGCTGGCGC** CAGCAAGGTC GCGGAGTGA AGACCCGAC CCTGAACCCG

1051 b TCTTCTTTG AGCCTGATGA **TTT**AGTATAT GCCCATATTG CAGGGATGGA
1051 e GATCTGTTTT ACGGTCATAT CCGCGCGATG GATACGATGG CACTGGCGCT
1051 s **CTCGCCGAC** **GCACCGCGT** **CGAGGACTT** **GACGTGGAGG** **CGGCCGCCG**
1051 a GCGGAGACCT ACGCCGACCT **GCTGGCCGAC** **CGTAGCGCT** **TGAGGACT**
1051 m GCGGAGGGAT ACGCCGAGCT **GCTCGCCGAC** **CGCAGCGCT** **TGAGGACT**

1101 b TGCATTTGCA AGAGGATTGA AAGTAGCCCA CAAATTAATC GAAGATCGTG
1101 e GAAAATTGCA GCGCGCATGA TTGAAGATGG CGAGCTGGAT AAACGCATCG
1101 s GCGCGGGCA TGGCCGTTG AACGCCTCGA CCAGCTGGCG AAG**CAACC**
1101 a **CGCGCGGAC** CCGGTGCGG CCAAGGGCTA CGGCTTGCCT AAGCTCAACC
1101 m **CGCGCGGAC** GCCGTGGCG CCAAGGGCTT CGGCTTGCCT AAGCTGAACC

1151 b TGTTTGAAGA TGTGATTCAA CATCGTTATC GCAGTTTTAC TGAAGGAATT
1151 e CGCAGCGTTA TTCCGGCTGG AATAGCGAAT TGGCCAGCA AATCTGAAA
1151 s **TGCTGGGCG** **CGCGGCTGA**
1151 a AGCTGGCGAT **CGACCACCTG** **CTCGGAGCG** **GCTGA**
1151 m AGCTCGGAT **CGACACCTG** **CTCGGAGCCC** **GC**

1201 b GGTCTTGAAT TTACAGAAGG AAGAGCTAAT TTCCATACTC TTGAGCAATA
1201 e GGCCAAATGT CACTGGCAGA TTTAGCCAAA TATGCTCAGG AACATCATT

1251 b TGCGTAAT AATAAAACAA TAAAAATGA **ATCTGGAGA** **CAGGAGCAT**
1251 e GTCTCCGGTG CATCAGAGT **GTCGCCAGA** ACAACTGGAA AATCTGTTAA

1301 b TAAAACCTAT ATTGAACCAA TAA
1301 e ACCATTATCT GTTCGACAAA TAA

Figure 2. Gene-coding regions of xylose isomerases isolated from 5 bacterial strains. Homologous regions (x) are mapped from the computer-generated amino acid sequences; minimum = 4aa: e x sam = **ATCG**; b x sam = **ATCG**; sam x sam or b x e = **ATCG**

Bacillus subtilis Length: 440

1	MAQSHSSSVN	YFGSVNKVVF	EGKASTNPLA	FKYYNPQVEI	GGKTMKEHLR
51	FSIAYWHTFT	ADGTDVFGAA	TMQRPDWYHK	GMDLARARVE	AAFEMFEKLD
101	APFFAFHRDR	IAPEGSTLKE	TNQNLDIIVG	MIKDYMRSN	VKLLWNTANM
151	FTNPRFVHGA	ATSCNADVFA	YAAAQVKKGL	ETAKELGAEN	YVFWGGREGY
201	ETLLNTDLKF	ELDNLARFMH	MAVDYAKEIE	YTGQFLIEPK	PKEPTTHQYD
251	TDAATTIAFL	KQYGLDNHFK	LNLEANHATL	AGHTFEHELK	MARVHGLLGS
301	VDANQGHPLL	GWDTDEFPTD	LYSTTLAMYE	ILQNGGLGSG	GLNFDKAVRR
351	SSFEPDDLVI	AHIAGMDAFA	RGLKVAHKLI	EDRVFEDVIQ	HRYRSFTEGI
401	GLEITEGRAN	FHTLEQYALN	NKTIKNESGR	QERLKPILNQ	

Escherichia coli, Length: 440

1	MQAYFDQLDR	VRYESGKSSN	PLAFRHYNPD	ELVLGKRMEE	HLRFAACYWH
51	TFCWNGADMV	GVGAFNRPWQ	QPGEALALAK	RKADVAFEFF	HKLHVPFYCF
101	HDVDVSPEGA	SLKEYINNFA	QWVDVLGKQ	EESGVKLLWG	TANCFNPNRY
151	GAGAATNPDV	EVFSAATQV	VTAMEATHKL	GGENYVLWGG	REGYETLLNT
201	DLRQERQLG	RFMQMVVEHK	HKIGFQGTLL	IEPKPQEPK	HQYDYDAATV
251	YGFLLKQFGL	KEIKLNIEN	HATLAGHSFH	HEIATAIALG	LFGSVDANRG
301	DAQLGWDTDQ	FPNSVEENAL	VMYEILKAGG	FTTGGNLFDA	KVRRQSTDKY
351	DLFYGHIGAM	DTMALALKIA	ARMIEDGELD	KRIAQRYSGW	NSELGQQILK
401	GQMSLADLAK	YAQEHHLSPV	HQSGRQEQL	NLVNHYLFDK	

Ampullariella sp., Length: 394

1	MSLQATPDDK	FSFGLWTVGW	QARDAFGDAT	RPVLDPIEAV	HKLAEIGAYG
51	VTFHDDDLVP	FGADAATRDG	IVAGFSKALD	ETGLIVPMVT	TNLFTHPVFK
101	DGGFTSNDRS	VRRYAIRKVL	RQMDLGAELG	AKTLVLWGR	EGAEYDSAKD
151	VGAALDRYRE	ALNLLAQYSE	DQGYGLRFAI	EPKPNEPRGD	ILLPTAGHAI
201	AFVQELERPE	LFGINRETGH	EQMSNLNFTQ	GIAQALWHKK	LFHIDLNGQH
251	GPKFDQDLVF	GHGDLNNAFS	LDLLENGPD	GGPAYDGPRH	FDYKPSRTED
301	FDGVWESAKD	NIRMYLLLKE	RAKAFRADPE	VQAAALAESKV	DELRTPTLNP
351	GETYADLLAD	RSAFEDYDAD	AVGAKGYGFV	KLNLQLAIDHL	LGAR

Actinoplanes missouriensis, Length: 394

1	VSVQATREDK	FSFGLWTVGW	QARDAFGDAT	RTALDPVEAV	HKLAEIGAYG
51	ITFHDDDLVP	FGSDAQTRDG	IIAGFKKALD	ETGLIVPMVT	TNLFTHPVFK
101	DGGFTSNDRS	VRRYAIRKVL	RQMDLGAELG	AKTLVLWGR	EGAEYDSAKD
151	VSAALDRYRE	ALNLLAQYSE	DRGYGLRFAI	EPKPNEPRGD	ILLPTAGHAI
201	AFVQELERPE	LFGINPETGH	EQMSNLNFTQ	GIAQALWHKK	LFHIDLNGQH
251	GPKFDQDLVF	GHGHLNNAFS	LDLLENGPD	GAPAYDGPRH	FDYKPSRTED
301	YDGVWESAKA	NIRMYLLLKE	RAKAFRADPE	VQEAALASKV	AELKTPTLNP
351	GEGYAEALLAD	RSAFEDYDAD	AVGAKGYGFV	KLNLQLAIEHL	LGAR

Streptomyces violaceoniger, Length: 389

1	MSFQPTPEDK	FTFGLWTVGW	QGRDPFGDAT	RPALDPVETV	QRLAELGAYG
51	VTFHDDDLIP	FGSSDTERES	HIKRFQRALD	ATGMTVPMAT	TNLFTHPVFK
101	DGGFTANDRO	VRRYALRKTI	RNIDLAAELG	AKTYVAVGGR	EGAESGGAKD
151	VRDALDRMKE	AFDILLGEYVT	AQGYDLRFAI	EPKPNEPRGD	ILLPTVGHAL
201	AFIERLERPE	LYGVNPEVGH	EQMAGLNFPH	GIAQALWAGK	LFHIDLNGQS
251	GIKYDQDLRF	GAGDLRAAFW	LDLLESAGY	EGPRHFDKPK	PRTEFDGQVW
301	ASAEGCMRNY	LILKERAAAF	RADPEVQEAL	RAARLDQLAQ	PTAADGLEAL
351	LADRTAFEDF	DVEAAAAAAA	WPFERLDQLA	MDHLLGARG	

Figure 3. Computer-generated amino acid sequences

Table 1.
CROSS-HOMOLOGIES

#	SEQUENCE	e-s	e-a	e-m	b-s	b-a	b-m
1	LRFA	124 526	-	124 526	-	-	-
2	VEAA	-	-	-	265 1084	-	-
3	EALA	-	-	220 997	-	-	-
4	DPEV	475 967	475 982	475 982	-	-	-
5	ELGA	-	-	553 382	553 382	553 382	-
6	VLWGGREG	-	556 403	556 403	-	556 403	556 403
7	WGGREG	562 409	-	-	562 409	-	-
8	IEPKP	691 538	691 538	691 538	709 538	709 538	709 538
9	DYDA	-	730 1096	730 1096	-	-	-
10	DAAT	-	-	-	-	754 190	754 190
11	DDLV	-	-	-	-	1066 166	1066 166
12	GGFT	985 304	985 304	985 304	-	-	-
13	GLNF	1003 673	-	-	1021 673	-	-

* Single letter sequence identifications (a,b,e,m,s) are described at the top of Figure 2.

Table 2.
LOCATIONS OF SIGNIFICANT RESIDUES

SITE	LOCATION	FUNCTION	Ref.
His53	sam=160 b=319 e=301	Aldose ring opening, active site	(8)
Asp56	sam=169 b=328 e=310	Aldose ring opening, active site	(8)
Thr90	sam=268 b=439 e=421	Base catalyzed cis-enediol formation by H ₂ O interaction	(37)
Thr91	sam=271 (b, e=A1a)	Interacts with Thr90	(37)
Lys182	sam=547 b=718 e=700	Provides R-NH ₃ ⁺ at active site for hydride shift, conserved in amino acid sequence IEPKP	(8)

* Single letter sequence identifications (a,b,e,m,s) are described at the top of Figure 2.

Table 3.
LOCATIONS OF MAJOR METAL BINDING RESIDUES

SITE	LOCATION	FUNCTION
Asp244	sam=733 b=904, e=886	Binds at MI (Mg ⁺⁺)
Asp292	s=889, am=874	Binds at MI
Glu180	sam=541 b=712, e=811	Binds at MI
His219 (Co ⁺⁺), histidine?	sam=658 b=829, e=811	Binds at MII " e s s e n t i a l "
Asp254	sam=763 b=937, e=919	Binds at MII
Asp256	sam=769 b=943, e=925	Binds at MII
Glu216	sam=649 b=820, e=802	Links MI and MII

* Single letter sequence identifications (a,b,e,m,s) are described at the top of Figure 2.

Table 4.

MATCHING AMINO ACIDS

PAIRING OF STRAINS	NUMBER OF MATCHES	TOTAL AA MATCHED	RELATIVE HOMOLOGY
<i>B. subtilis</i> / <i>E. coli</i>	19	111	25.2 %
<i>B. subtilis</i> / <i>S. violaceoniger</i>	7	21	5.4 %
<i>E. coli</i> / <i>S. violaceoniger</i>	6	27	6.9 %
<i>S. violaceoniger</i> / <i>Ampullariella</i> sp.	24	176	45.2 %
<i>S. violaceoniger</i> / <i>A. missouriensis</i>	29	194	49.9 %
<i>A. missouriensis</i> / <i>Ampullariella</i> sp.	18	348	88.3 %

of four or more (Table 4). A relative percentage of homology was calculated by dividing the total number of amino acids that matched by the total number of amino acids in the smallest sequence in the pair ($\times 100$). These numbers show there is significant divergence between these strains and the previously discussed Type 1 strains. The differences in amino acid homology are also reflected in the size of these genes and the locations of functional residues.

Effect of Protein Primary Structure on Enzyme Activity. The primary structures between the two groups identified in the preceding paragraphs have been shown to have some relation to the types of reactions they catalyze. The Type 1 enzymes are in general ones that have lower temperature optima and stability and that can function at lower pH levels than the Type 2 enzymes (9). This makes them more suitable for fermentation processes using yeast because they have limited tolerance for high pH or temperatures. Type 1 enzymes were found to be the best for simultaneous fermentation and isomerization of xylose to ethanol (13). During these investigations, it was found that the rate at which the purified *E. coli* isomerized glucose to fructose was 50 to 100 times slower than the rate at which it isomerized xylose to xylulose. On the other hand, the Type 2 enzymes are more efficient at glucose isomerization, and have the other features that suit the production of HFCS.

Conclusion

The comparison of five DNA sequences that code for xylose isomerase activity, annotated with the sites amino acid homology, and with some of the amino acid residues known to occupy the active site of the enzyme should be useful in both understanding the differences between the genes and a reference for potential use for site-directed mutagenesis. Comparisons between the xylose isomerase sequences show that there are significant differences in the gene-coding sequences and primary structures of these enzymes. As expected, enzymes isolated from more distantly related strains have more differences. Changes in DNA structure were found in many cases to be conservative, i.e. changes that do not change the amino acid structure, or changes that replace an amino acid with one with similar chemical properties. However, there are large differences in structure between the two types of isomerases described in this report. These changes significantly vary the location of sites on the protein that have been implicated as being important in the activity of the enzyme, and in some cases significant changes occur within these sites. However, models derived from x-ray crystallographic studies have been obtained only on data from enzymes of one type (Type 2). Clearly, more study on more divergent strains is necessary to establish common features as well as differences in structure that may relate to enzyme function.

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Literature Cited

1. Vuilleumier, S. *Sugar y Azucar* 1988, 88, 14-27.
2. Schellenberg, G. D.; Sarthy, A.; Larson, A. E.; Backer, M. P.; Crabb, J. W.; Lindstrom, M.; Hall, B. D.; Furlong, C. E. *J. Biol. Chem.* 1983, 256, 6826-6832.
3. Lawlis, V. B.; Dennis, M. S.; Chen, E. Y.; Smith, D. H.; and Henner, D. J. *Appl. and Environ. Microbiol.* 1984, 47, 15-21.

4. Drocourt, D.; Bejar, S.; Calmels, T.; Reynes, J. P.; Tiraby, G. *Nuc. Acid Res.*, 1988, 16, 9337.
5. Wilhelm, M.; Hollenberg, C. P., *Nuc. Acid Res.* 1985, 13, 5717-5722.
6. Saari, G. C.; Kumar, A. A.; Kawasaki, G. H.; Insley, M. Y.; O'Hara, P. J. J. *Bacteriol.* 1987, 169, 612-618.
7. Amore, R.; Hollenberg, C. P. *Nuc. Acid Res.* 1989, 17, 7515.
8. Collyer, C. A.; Henrick, K.; Blow, D. M. *J. Mol. Biol.* 1990, 212, 211-235.
9. Chen, W.-P. *Process Biochem.* 1980, 15, 36-41.
10. Vangrysterperre, W.; Ampe, C.; Kersters-Hilderson, H. *Biochem. J.* 1989, 263, 195-199.
11. Vangrysterperre, W.; Kersters-Hilderson, H.; Callens, M. *Biochem. J.* 1989, 260, 163-169.
12. Jeffries, T. W. In *Yeast: Biochemistry and Biocatalysis*; Verachtert, H.; De Mot, R., Eds.; Marcel Dekker: New York, NY, 1990; pp 349-394.
13. Lastick, S. M.; Tucker, M. Y.; Beyette, J. R.; Noll, G. R.; Grohmann, K. *Appl. Microbiol. Biotechnol.* 1989, 30, 574-579.
14. Mitsuhashi, S.; Lampen, J. O. *J. Biol. Chem.* 1953, 204, 1011-1018.
15. Hochster, R. M.; Watson, R. W.; *Arch. Biochem. and Biophys.* 1954, 48, 120-129.
16. Slein, M. W. *J. Am. Chem. Soc.* 1955, 77, 1663-1667.
17. Green M.; Cohen, S. S.; *J. Biol. Chem.* 1956, 219, 557-568.
18. Marshall, R. O.; Kooi, E. R. *Science* 1957, 125, 648-649.
19. Tsumura, N.; Sato, T. *Bull. Agr. Chem. Soc. Japan* 1960, 24, 326-327.
20. Natake, M.; Yoshimura, S. *Agr. Biol. Chem.* 1963, 27, 342-348.
21. Natake, M. *Agr. Biol. Chem.* 1966, 30, 887-895.
22. Natake, M. *Agr. Biol. Chem.* 1968, 32, 303-313.
23. Yamanaka, K. *Agr. Biol. Chem.* 1963, 27, 265-270.
24. Yamanaka, K.; *Biochim. Biophys. Acta* 1968, 151, 670-680.
25. Kasumi, T.; Hayashi, K.; Tsumura, N.; *Agric. Biol. Chem.* 1982, 46, 21-30.
26. Kasumi, T.; Hayashi, K.; Tsumura, N.; *Agric. Biol. Chem.* 1982, 46, 31-39.
27. Ananichev, A. V.; Ulezlo, V.; Egorov, A. V.; Bezborodov, A. M.; *Biochem. (Engl. Trans.)* 1983, 48, 101-108.
28. Bucke, C. In *Topics in Enzyme and Fermentation Biotechnology*; Wiseman, A., Ed.; John Wiley and Sons: New York, NY, Vol. 1, 1977; pp 147-171.
29. Barker, S. A.; Petch, G. S. In *Enzymes and Immobilized Cells in Biotechnology*; Laskin, A., Ed.; The Benjamin/Cummings Publishing: Menlo Park, CA, 1985; p. 93.
30. Tewari, Y. B.; Steckler, D. K.; Goldberg, R. N. *Biophys. Chem.* 1985, 22, 181-185.
31. Sanchez, S.; Quinto, C. M. *Appl. Microbiol.* 1975, 30, 750-754.
32. Meers, J. L., *Biotech. Lett.* 1981, 3, 131-136.
33. Stevis, P. E.; Ho, N. W. Y. *Enzyme Microb. Technol.* 1985, 7, 592-596.
34. Lastick, S. M.; Tucker, M. Y.; Mackedonski, V.; Grohmann, K. *Biotech. Lett.* 1986, 8, 1-6.
35. Callens, M.; Tomme, P.; Kersters-Hilderson, H.; Cornelis, R.; Vangrysterperre, W.; De Bruyne, C.K. *Biochem. J.* 1988, 250, 285-290.
36. Rose, I. A. *Phil Trans. R. Soc. Lond.* 1981, 293, 131-143.
37. Carrell, H. L.; Glusker, J. P.; Burger, V.; *Proc. Nat. Acad. Sci. USA* 1989, 86, 4440-4444.
38. Farber, G. K.; Glasfeld, A.; Tiraby, G. *Biochem.* 1989, 28, 7289-7297.
39. Tucker, M. Y.; Tucker, M. P.; Himmel, M. E.; Grohmann, K.; Lastick, S. M. *Biotech. Lett.* 1988, 10, 79-84.
40. *Bergey's Manual of Determinative Bacteriology*; Buchanan, R. E.; Gibbons, N. E., Eds.; Williams and Wilkins: Baltimore, MD, 1975; p. 290, 529, 710, 716, 748.

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