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Biocatalysis in Agricultural Biotechnology

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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 Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

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Preface

THE GOLDEN ERA OF BIOLOGY, including the agricultural sciences, is taking place in the 1980s, just as the early part of this century was considered the golden era for organic chemistry and the 1960s–1980s the golden era for electronics. Biology shares the present limelight with high-energy physics and computer sciences. The current focus on biology can be traced to several advances that are now affecting research concertedly. These developments include the solution of the structures of DNA and RNA by Watson and Crick, the remarkable progress in understanding protein structure and biological function, including the catalytic efficiency and specificity of enzymes, and the recognition of genomes as myriad genes and control mechanisms.

Genetic engineering, recombinant DNA, transgenic animals and plants, chimeric macromolecules, gene deletion and addition, and a host of other terms are all "buzzwords" of biology in the 1980s. The nonscientific public is confused, and no wonder! Plant and animal breeding, fermentation-derived foods, processed and formulated foods, digestive aids, protease-containing detergents, rennin puddings, and cornstarch-derived sweeteners, although not really understood by the general public, have won their confidence. Yet we as biologists have not secured the trust and confidence of the public, and our modern buzzwords may be less a source of reassurance than of concern. Perhaps society's wariness stems from the fear that scientists will produce undesirable plants and animals through random, hit-and-miss genetic alterations. With detailed knowledge of the substrate and the catalyst, changes more precise than the surgeon's knife can be accomplished.

This volume emphasizes the application of enzymes, the biological catalysts that can change plants and animals in precise and often remarkably dramatic fashion. In the near future, many of the chemicals that are now used to control pests and diseases will be eliminated, because the genetic composition of plants and animals will have been modified to make them resistant, or because the pests and plague organisms have been modified so that they cannot survive in the same environment with the desired plants and animals. Advances such as nitrogen fixation, tolerance to salinity, drought, and cold, and increased efficiency in fixing sunlight and nutrients into raw material for food uses

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are no longer 20 years in the future. Cultivation of plant and animal cells to produce needed flavors, colors, pharmaceuticals, and other desired products by methods now employed for culturing microbial cells is less than five to ten years away. All of this progress is possible because society, governments, educational institutions, research institutes, and scientists recognized that understanding the structure and function of biological molecules must precede the biotechnology and biology revolution that we are witnessing in the 1980s.

We wish to express our appreciation to all the scientists who worked so diligently to describe their research. Initially these efforts were directed to the participants of the Symposium on Biocatalysis and Biomimetics: Aspects of Enzyme Chemistry for Agriculture. Subsequently, they sought to engage you, the reader, while meeting the exacting standards of the Books Department of the American Chemical Society and the idiosyncrasies of the co-editors. We also appreciate the support and confidence of the Division of Agricultural and Food Chemistry and the Division of Agrochemicals who encouraged us to undertake this project, under the auspices of the Biotechnology Secretariat. We especially appreciate the untiring efforts of Ms. Cheryl Shanks, who set high standards and strict guidelines for us, so that we never lost sight of the opportunities at hand. We trust that you, the reader and final judge, will agree with us that it was all worthwhile.

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Chapter 1

Interdependence of Enzymology and Agricultural Biotechnology

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There is a strong dependence of advances in enzymology on agriculture, based on both historical and present data. Discovery of enzymes and elucidation of many of their properties were by agricultural chemists. The earliest enzymes studied were primarily from agriculturally important animals, plants and microorganisms, with substantial levels of enzymes and ready availability of raw material. The rapidly developing broad field of biochemical engineering/biotechnology, and its tremendous promise for the future, have brought basic and applied scientists together in private venture companies, at universities, and in government and industrial laboratories. Knowledge of enzymes and their utilization for the betterment of humans is literally exploding. This chapter provides a brief history of the contributions of enzymology to agricultural biotechnology, present state of applications of enzymology and a look at some future directions where enzymology can contribute.

Enzymes have been associated with humans and their food since creation. Many thousands of years must have passed before humans began to wonder how the berries they ate and the meat they relished were converted to other substances in the body and to recognize that there must be a connection between food consumption and human growth and mobility. They also observed the processes of fermentation and the ease of making buttermilk, beers, wines and cheeses, depending only upon the microorganisms ubiquitously present in their surroundings. However, it was not until 1833 that Payen and Perzog (1) for the first time specifically recognized that there was some compound present in malt, used for brewing, with the ability to solubilize the starch from the kernel

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of grain leaving behind the insoluble envelopes. They further noted that this substance could be precipitated from a malt extract, and that it was denatured by heat. Payen and Perzog named this substance diastase, because of its separating ability. We now know the enzyme as amylase. To this day, the names of enzymes generally contain the ending -ase. Remarkable contribution on the part of Payen and Perzog!

Soon, there were reports of other discrete activities in plant materials caused by some endogenous compounds. In 1855, Schoenbein described a substance (peroxidase) in plants that caused a solution of gum guaiac to turn from brown to blue in the presence of hydrogen peroxide (2). A year later, Schoenbein identified another compound (polyphenol oxidase) in mushrooms that brought about the aerobic oxidation of certain compounds in the presence of molecular oxygen, thereby causing browning in the mushrooms (3). Berthelot, in 1860 (4) discovered a substance in yeast (invertase) that caused a change in the optical rotation of sucrose solutions. Liebig, in exploring how food is digested, observed that the stomach of vultures contained something (pepsin) that dissolved away meat that had been placed in a perforated cartridge and was swallowed by the bird. Pasteur recognized that fermentation is caused by microorganisms, and that there was something in the microbial cells that was associated with this fermentation (5). Because of the close association between these substances and fermentation, they were named ferments. Until 1879, when Kühne proposed the word *enzyme* (Greek, in yeast) (6), these substances were called *organized ferments* (thought to require intact cells for activity) and *unorganized ferments* (activity in absence of cells; pepsin for example).

Agricultural chemists and agricultural products continued to contribute to the development of enzymology in major ways during the 19th and 20th centuries. These contributions included showing that enzymes were still active when separated from living cells (7), the close stereochemical relationship between an enzyme and its substrate (8), and qualitative methods for describing the action of enzymes (9-12). Pepsin, trypsin and chymotrypsin were purified, particularly from cows and hogs because of the availability of their organs as byproducts (13, 14). The first enzyme to be obtained in crystalline form was from jackbeans, and it was shown to be a protein (15). The relationship between enzyme activity and pH, using invertase, was studied extensively by Sørensen (16). The application of endogenous and exogenous enzymes for quality control and food modification, and for making specialty products, became major activities.

Enzymes are widely used in the food industry to modify properties of raw products in their conversion to foods (17). Most of these applications use rather crude enzyme systems, where a number of reactions occur. In recent years, there has been a tendency to use more specific enzymes in order to bring about selective and limited changes, as will be described below.

Endogenous enzymes in raw food materials also are of major importance during postharvest storage and in storage after processing (18). Enzymes have become very important in deter-

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mining and describing the genetic diversity of plant and animal genomes, in developing new plants, and in the control of pests and pathogens.

The section below will briefly summarize some of these important uses of enzymes in agriculture. Emphasis is on using enzymes in more specific ways, such as uniquely targeting compounds, or small segments of large polymers, for modification.

SOME NEW AND POTENTIAL APPLICATIONS OF ENZYMES IN AGRICULTURAL BIOTECHNOLOGY

Heat Treatment for Preservation. Enzymes have pronounced effects on the color, flavor, aroma, texture and nutritional quality of foods during growth and maturation, during harvest and postharvest storage and storage after processing. The important enzymes vary from one food product to another. In tomatoes, the softening phenomenon in ripening is caused by its pectic enzymes. In peaches, apples, plums, grapes and avocados, the browning reaction is the result of polyphenol oxidase. In green leafy vegetables, lipoxygenase and other enzymes cause flavor and aroma deterioration, but other enzymes may also cause discoloration (loss of green color and/or browning).

Development of the frozen food industry was based on observations that sufficient heat treatment of vegetables and fruits to inactivate peroxidase, followed by freezing and frozen storage, could extend shelf life from a few days or a few weeks to one to two years (19-24). Other enzymes have been used less frequently to monitor the adequacy of heat treatment. These include catalase (25) for English green peas and a few other vegetables, polyphenol oxidase in fruits, polygalacturonase for loss of consistency of tomatoes, potatoes and eggplant; and lipoxygenase and lipase for off-flavor development in soybeans and cereal products, respectively.

Recently, Williams et al. (18) made a strong case for using the specific enzyme responsible for quality loss for monitoring the heat treatment by the food industry. These workers showed that lipoxygenase is the major enzyme responsible for aroma deterioration in English green peas and green beans, while cystine lyase is responsible for aroma deterioration in broccoli and cauliflower (18). The frozen food industry is beginning to adopt these suggestions, because they use less heat to inactivate some of these enzymes compared to peroxidase inactivation; at the same time they realize considerable energy savings. To assist in these changes, faster semiquantitative detection methods are needed for the indicator enzymes.

The consumers' demand for more fresh-like products and for formulated products led to other enzyme-caused problems. Vegetables packeted together with starch-based sauces serve as an example. Relatively heat stable amylases from exogenous microorganisms are often the problem, requiring modified washing conditions and/or longer heat treatment of the raw vegetables. This was a problem in canned apricots a few years ago, where a relatively heat-stable polygalacturonase from the brown rot fungus was the culprit (26).

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Enzymatic Modification of Proteins for Food Use. Proteolytic enzymes are used extensively for modifying proteins in various ways in food products and for waste management (17, 27). These are used in baked and brewed products, cereals, cheese, chocolate/cocoa, egg and egg products, feeds, fish, legumes, meats, milk, protein hydrolysates and wines. But there are many other uses, and potential uses, of enzymes to modify protein. Whitaker (27, 28), and Whitaker and Puigserver (29) have described more than 100 enzymatic modifications of proteins in vivo, challenging scientists to look at the possibilities of modifying the amino acid side chains by proteolytic and other methods. Feeney and Whitaker (30, 31) have emphasized the importance of the enzymes transglutaminase, lipoxygenase, polyphenol oxidase and peroxidase in the crosslinking of proteins. With transglutaminase, chimeric proteins can be made readily by crosslinking two proteins, each with quite different properties (32, 33).

Proteolytic enzymes have long been used to produce protein hydrolysates for use in soups, bouillon, soy sauce, tamari sauce, etc. Recent interest in producing large polypeptides of controlled size having improved solubility and functional properties for use in the food industry has led to investigation of highly specific proteolytic enzymes for that purpose (34, 35).

Specialty Products. Enzymes, because of their high substrate specificity and stereospecificity, are ideal for producing special compounds required by the food and pharmaceutical industries (36). By all measures, the conversion of corn starch, produced by wet milling, to glucose and fructose has been the most successful commercial operation (37). More than ten billion pounds of fructose are produced and used by the food industry each year. The industry uses relatively heat stable α-amylase to partially hydrolyze and solubilize starch, followed by glucoamylase to produce glucose and immobilized glucose isomerase to convert approximately 50% of the glucose to fructose in an equilibrium-controlled process. The following enzymatic hydrolyses are also being used, but with more limited success: conversion of cellulose to feedstock for single cell protein production and other uses, lactose hydrolysis to glucose and galactose (to remove lactose from milk or for sweeteners); xylan hydrolysis to xylose and xylitol; DNA and RNA hydrolysis to mononucleotides as flavor enhancers, and lignin and mannan degradation for waste treatment.

Lipases are now being studied intensively to alter triglyceride fatty acid composition. They are also being evaluated for selective formation of mono- and diglycerides and for producing waxes. The driving force for the research is related both to the continuing abundance, especially in the U.S., of fats and oils, and to the renewed interest in all classes of enzymes that can act on fatty acids and their derivatives. These workers pin their hopes on improved understanding of enzyme stability and mechanism of action, and to finding new enzymes that

have unique selectivity with respect to fatty acid chain length, degree of unsaturation, and alcohol residue (of the triglyceride).

Another large successful commercial application of enzymes is in the amino acid industry. Amino acids for food and feed fortification, nutritional supplements, or as feedstock for downstream products can be made by fermentation processes, from protein hydrolysates or by chemical synthesis. While chemical synthesis is cheaper for a number of amino acids, it often produces a racemic mixture. The racemic mixture is successfully resolved on a commercial scale by acylating the amino acids, then using an aminoacylase to remove the acyl group from the L-amino acid and separating the free L-amino acid from the still acylated-D-amino acid. Ajinamoto and other companies, especially in Japan, make large amounts of amino acids by this process.

The plastein reaction is being used successfully in Japan to produce phenylalanine-free peptide products for patients with phenylketonuria, surfactants for the cosmetic and food industry and antifreeze type compounds that have the potential to prevent "hard freezing" of foods, blood and sperm (38). Aspartame (L-aspartyl-L-phenylalanine methyl ester) can be produced chemically, or more recently by microorganisms, using recombinant DNA technology to produce a large polypeptide of repeating aspartylphenylalanine units, then using a specific protease to hydrolyze the polypeptide to the dipeptide, followed by esterification with methanol. Another application of enzymes is the use of glucosyl transferases and glycosylating enzymes for the production of modified carbohydrates that retain their sweetness but are not metabolized by the human. Stevioside, a natural sweetener from the plant *Stevia rebaudiana*, has been modified by the use of α-glucosidase in order to bring its taste closer to that of sucrose (39, 40).

Enzymes are especially useful in adding and removing residues from compounds, because of their substrate and product stereospecificity. Only the biologically active isomer of a compound is formed by the enzyme because of the strict stereochemistry required for binding of substrate to the enzyme in order to have proper orientation to the catalytic site of the enzyme. Use of more traditional chemical methods leads to mixtures of isomers. Organic chemists have found enzymes to be invaluable in particular steps of synthesis. Enzymes are used quite frequently in the synthesis of pharmaceutical compounds, for example. Use of aminoacylases to permit separation of D- and L-mixtures of amino acids is described above. The stereospecificity of enzymes is also invaluable in analytical uses for sequencing polymers, for configuration determination of monomeric units in polymers (for example carbohydrates), as well as for determining how much of each isomer is present.

Enzymes and Recombinant DNA Technology. Recombinant DNA technology for whatever purpose depends absolutely upon enzymes. Keys to the rapid advancement of biochemical engineering have been: 1) understanding of the primary and secondary structures of DNA and RNA; 2) enzymes with strict specificity that permit the

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relatively easy sequencing of DNA and RNA (with the knowledge of primary structures of proteins encoded); 3) the ability to remove, via hydrolysis, specific nucleotide segments from plasmids (by use of restriction enzymes); and 4) ability to incorporate nucleotide sequences (by use of ligases) from other organisms or by chemical synthesis into the host organism. This relatively easy chemistry, mediated by the specificity of enzymes, permits enhanced production of a valuable protein, enzyme or other product, either identical to that produced naturally by the donor organism, or with selected changes that enhance stability or different functional properties. Examples of this include chymosin, insulin, bovine growth hormone, and proteolytic enzymes for use in detergents.

Recombinant DNA techniques permit both an increase, as well as decrease, in the level of enzymes or other products in an organism. Would increased levels of ribulose bisphosphate carboxylase engineered into a plant increase the amount of carbon dioxide fixed, or would it be better to decrease the oxygenase activity of this enzyme, or change the concentration of hydrogenase? Glyphosate (N-phosphonomethylglycine; trade name Roundup) is an effective general herbicide, effective against plants that contain 5-enolpyruvylshikimate-3-phosphate synthase. This enzyme has now been enhanced in several agriculturally important plants, permitting the use of Roundup to control weeds and grasses in these crops.

Polyphenol oxidase causes up to 50% loss of tropical fruits because they brown when bruised, otherwise damaged or become too ripe. Frozen strawberries become mushy and brown when they are allowed to warm to room temperature following thawing. Yet, they cannot be blanched to inactivate the polygalacturonase, because heating is detrimental to flavor and texture. Lipoxygenase causes off-flavor and off-aroma in soybeans (beany flavor), English green peas, green beans, corn and probably other vegetables (18). Can the levels of these enzymes be reduced, or eliminated entirely, without an adverse effect on the plant and product? Traditional breeding methods have been used to decrease the levels of these enzymes, without apparent effect on productivity and quality. "Lipoxygenase-free" soybeans have been bred, whereby the level of the major lipoxygenase isoenzyme has been greatly reduced.

Bitterness in orange and grapefruit juices is a major economic problem. Orange juice is produced primarily from Valencia-type oranges since there is less associated bitterness. Limonin and naringin are the major causes of bitterness (41). Limonin concentrations as low as 5-6 ppm give an unacceptable level of bitterness. Naringin is about 0.01 as bitter as limonin but is often produced in higher amounts. Bitterness due to limonin can be eliminated by: a) preventing its biosynthesis (preharvest treatment with 1-naphthalene acetic acid); b) removing the rag and pulp from freshly expressed juice as soon as possible to prevent the precursor, limonic acid A-ring (mono)lactone, from being converted to limonin (42, 43) or, c) enzymatic hydrolysis of limonin to non-bitter products by use of immobilized microbial cells containing an NADP-dependent limonin dehydrogenase (44, 45).

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Bitterness, caused by naringin, is also removed by an enzyme, naringinase. Another possibility would be to eliminate one or more of the enzymes in the limonin biosynthetic pathway by using recombinant DNA techniques. Limonin, a triterpenoid, is probably synthesized via the mevalonate pathway, as are the monoterpenoid flavor compounds. It appears that nomilin, a precursor of limonin, is synthesized in the stems and roots of citrus and then the precursor transported to the fruit where it is converted by several enzymes to limonin and other bitter limonoids (46).

Integrated Control of Pests and of Synthetic Pesticides. Much emphasis is placed on reducing the use of synthetic pesticides and in designing pesticides that have a more restricted spectrum of activity with shorter lifetimes in the environment. There is also much emphasis on better management of timing applications and in using biological control methods. This focuses much attention on enzymes of the plants and of the pests. Enzyme and isoenzyme activities, distinguished via separations by polyacrylamide gel electrophoresis and activity stains, are used routinely in determining changes in plants during breeding by traditional and nontraditional methods. Enzyme patterns established by the above techniques are often related to pathogen and insect resistance, thus saving considerable amount of time in analyses. The correlation may be the result of compounds, such as phenolics (47), alkaloids (47), protease inhibitors (48, 49) and α-amylase inhibitors (50; Ho and Whitaker, University of California, Davis, unpublished data) produced by the plant as a defense mechanism against insects and microorganisms.

It is also important to understand how pesticides are metabolized by plants and other organisms. This will aid in determining the residence time of the pesticides in the environment, and could alert us to the possibility of more toxic, and more persistent products that may be formed.

Removal of Unwanted Compounds. The removal of phenylalanine from proteins by the plastein reaction was mentioned above (38). The plastein reaction has also been used to remove the bitter peptides from protein hydrolysates by causing resynthesis of some peptide bonds catalyzed by proteolytic enzymes when the substrate concentration is around 35% (w/w (51). A number of food plants, including cassava and lima beans, contain toxic levels of cyanogenic glycosides (52). Soaking these vegetables overnight permits hydrolysis of the cyanogenic glycosides by specific glycosidases (linamarinase for example when the substrate is linamarin) to glucose, HCN and acetone. The toxic compound, HCN, is then removed by evaporation from the food as it is cooking.

When freshly squeezed orange juice is allowed to stand, the suspended pectic compounds ("cloud") separate and settle from the juice. This phenomenon can be prevented by heating the freshly squeezed juice to inactivate the pectin methylesterase, producing a "cooked flavor." Alternatively, additional polygalacturonase can be added to the juice (53). Cloud precipitation is caused by Ca^{2+} chelating the pectic acid produced by pectin methylesterase.

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The role of polygalacturonase is to hydrolyze the pectic acid to fragments that do not precipitate when they chelate with Ca^{2+}, but are still insoluble to give the cloud appearance of natural juice.

CONCLUSION

As briefly documented above, there has been a close association between the discovery of enzymes, and the development of agricultural biotechnology. Each period seems to bring more exciting advances in use of enzymes. But the role of enzymes in agricultural biotechnology has never been more exciting and important than in the present period. New enzymes, and old enzymes, are being used more extensively to transform microorganisms, plants and animals, to make specialty food products and to carry out difficult stereochemical syntheses of needed biological compounds, with a purity and yield that the organic chemist could only dream of in the past.

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Chapter 2

Design of Enzymatic Catalysts

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Two approaches to catalyst design are presented; these are site-directed mutagenesis of existing proteins and development of antibody-based catalysts with rationally designed immunogens. Specifically, semisynthetic seleno-enzymes (proteases in which the active site nucleophile has been converted chemically into selenocysteine) and monoclonal antibodies with chorismate mutase activity are described. The complementary strategies yield tailored binding sites that couple novel chemical activity with high selectivity. The success achieved augurs well for the development of practical catalysts for general use in medicine and industry.

Enzymes are remarkable catalysts. Few chemical agents can match the rate accelerations or tremendous specificity that enzymes achieve under mild aqueous conditions. Consequently, it is no surprise that such molecules are being used increasingly in medicine and industry to carry out important chemical transformations. The potential usefulness of biocatalysts would be greatly increased, however, if it were possible to design and synthesize them de novo. But how is this to be accomplished? In the following article two viable approaches to catalyst design are illustrated with examples from our laboratory. On the one hand, we are chemically mutating existing protein active sites to develop artificial selenoenzymes with potentially useful properties. On the other, we are employing rationally designed immunogens to generate antibodies that catalyze concerted chemical transformations.

Semisynthetic Enzymes

As it is not yet possible to prepare tailored protein binding pockets from their constituent amino acids, existing, well-characterized protein structures represent attractive starting materials for the design of new enzymes. These can be used as scaffolding on which to mount catalytic groups. For instance, Kaiser and co-workers have

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constructed successful semisynthetic flavoenzymes by incorporating reactive flavin analogs into the active sites of papain (1) and glyceraldehyde-3-phosphate dehydrogenase (2). The resulting hybrid enzymes couple the unique chemistry of the cofactor with the binding specificity of the protein template.

A complementary approach utilizes chemical or recombinant DNA methodologies to alter selected amino acid residues in the protein binding site. We are interested, for instance, in the properties of selenium in biological systems and are currently converting the active site -OH and -SH groups of several serine and cysteine proteases into -SeH groups. Our reasons are threefold. First, selenium can serve as a molecular probe of enzyme mechanism. By comparing the properties of the analogous oxygen, sulfur and selenium variants it may prove possible to sort out steric and electronic factors which are important for catalysis. We also want to take advantage of some of the rich organic chemistry of selenium (3) to develop protein-based catalysts which may have practical utility. Finally, semisynthetic selenoenzymes are likely to be interesting models of naturally occurring selenoproteins, like glutathione peroxidase. The latter enzyme protects mammalian cells against oxidative damage and contains a catalytically essential selenocysteine residue (4).

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Selenosubtilisin. Scheme 1 illustrates our strategy for converting a serine residue in a protein into a selenocysteine. The two step process is analogous to that used by Bender (5) and Koshland (6) 20 years ago to convert a serine protease into a cysteine protease. It involves activation of the side chain alcohol group of a seryl residue by formation of a sulfonyl ester, followed by displacement of the sulfonate with an appropriate selenium nucleophile. We have successfully carried out this sequence on the bacterial protease subtilisin Carlsberg [EC 3.4.21.14] (Wu and Hilvert, unpublished results). Serine 221 in the active site of this enzyme was specifically modified with ^{35}S-labelled phenylmethanesulfonyl fluoride (PMSF). The resulting PMS-subtilisin was treated with a large excess of hydrogen selenide at 40 °C and pH 6.8. After 36 hours, more than 95% of the radioactivity associated with the enzyme was lost. In the absence of hydrogen selenide less than 10% sulfonate was released from the enzyme. Selenosubtilisin was separated from unreacted hydrogen selenide by gel filtration on a Sephadex G-25 column and purified by ion exchange chromatography on CM-50 Sephadex. Greater than 0.9 equivalents of selenium were incorporated per mole of subtilisin as judged by anaerobic titration of the reduced enzyme with 5,5'-dithio-bis(2-nitrobenzoic acid) (7).

Scheme 1. Preparation of Semisynthetic Selenoenzymes

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Serine and cysteine proteases cleave their substrates by a two step mechanism in which the active site nucleophile is transiently acylated. In many cases it is possible to isolate the acyl enzyme intermediate. We have prepared an authentic acyl derivative of selenosubtilisin by treating the reduced protein (-SeH form) with excess cinnamoyl imidazole. Se-Cinnamoyl-selenosubtilisin is relatively stable at pH 5 and can be separated from unreacted reagent by gel filtration. A difference spectrum of the Se-cinnamoylated protein and unmodified subtilisin showed an absorbance maximum at approximately 308 nm for the enzyme-bound chromophore; this value is red-shifted by 18 nm relative to the λ_{max} of the model compound N-carbobenzoxy-Se-cinnamoyl-selenocysteine methyl ester (Wu and Hilvert, unpublished results). For comparison, the absorbance maxima for the analogous cinnamoyl derivatives of native subtilisin and thiosubtilisin are 289 and 310 nm, respectively (5).

Kinetics. The availability of a stable acyl enzyme intermediate offers the possibility of determining directly the deacylation rate for the modified protein (9). Selenoesters undergo non-enzymatic hydrolysis at roughly the same rate as structurally analogous esters and thiolesters, while their aminolysis is significantly faster (9). Consequently, comparison of the partitioning of the cinnamoyl-enzyme species between water and amine for the isologous oxygen, sulfur and selenium cases is of particular interest. Rate constants were determined with the selenoenzyme by following decrease of absorbance at 310 nm in aqueous buffer (pH 9.3) in the presence and absence of glycinamide. Both the hydrolysis and aminolysis reactions were first order in acyl enzyme. The second order rate constants for hydrolysis (k_1) and aminolysis (k_2) are given in Table I, together with data for native Carlsberg subtilisin and thiosubtilisin.

Table I. Second Order Rate Constants for Hydrolysis (k_1) and Aminolysis (k_2) of Cinnamoylated Subtilisins at 25.0 °C and pH 9.3

	Cinnamoyl derivatives of		
	Subtilisin	Thiosubtilisin	Selenosubtilisin
k_1 (M^{-1} s^{-1})	4.2×10^{-3}	2.1×10^{-5}	1.8×10^{-5}
k_2 (M^{-1} s^{-1})	0.13	0.20	0.35
k_2/k_1	30	9,200	21,000

Although S-cinnamoyl-thiosubtilisin and Se-cinnamoyl-selenosubtilisin are cleaved by water at comparable rates, their hydrolysis is slower than that of native O-cinnamoyl-subtililsin by more than two orders of magnitude. The decrease in hydrolytic rate constant may reflect subtle structural changes within the relevant active site geometries: the van der Waals radii of sulfur and selenium are similar (1.85 and 2.0 Å, respectively) but that of oxygen is much smaller

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(1.45 Å) (10). What is particularly striking about the data summarized in Table I, though, is the fact that the ratio of rate constants for aminolysis and hydrolysis of the acyl enzyme species (k_2/k_1) increases substantially as one proceeds from subtilisin to selenosubtilisin. This ratio is less than 30 for the native enzyme, while for selenosubtilisin aminolysis is favored over hydrolysis by a factor of 21,000. Thus, by converting the active site nucleophile of a serine protease into a selenocysteine the selectivity of the enzyme can be altered in a dramatic fashion. The enhanced selectivity of deacylation suggests that semisynthetic selenosubtilisin, like thiosubtilisin (11), may be a useful catalyst for peptide bond formation, for example in fragment condensations. We are currently evaluating this possibility, as well as studying the ability of the modified protein to participate in other hydrolytic and redox processes.

Catalytic Antibodies

Ideally, one would like to be able to generate a unique active site for any chemical transformation. However, the number of structurally well-characterized proteins for construction of semisynthetic enzymes is limited. An alternative approach exploits the mammalian immune system to produce binding pockets that are specifically tailored to the reaction of interest.

The immune system is a prolific source of specific receptor molecules called antibodies (12-14). They are large, dimeric proteins (Mr 160,000), consisting of two heavy and two light chains. Their normal function in the body is to recognize and tightly bind foreign materials, targeting them for eventual elimination. Significantly, immunoglobulins can be elicited against virtually any material, man-made or natural. The dissociation constants for typical antigen-antibody complexes are in the range of 10^{-4} to 10^{-12} M (12-14), and binding apparently involves the same factors that are important for ligand binding to enzymes: hydrophobic interactions, ion pairing and dipolar interactions, and hydrogen bonding (13). Moreover, structural studies reveal that the size and shapes of the binding pockets of enzymes and antibodies are similar (12). Nevertheless, immunoglobulins do not typically catalyze reactions.

Antibodies, according to Pauling (15), leave the molecules they bind chemically unchanged, because their combining sites are complementary to the ground state of the antigen. An enzyme's active site, on the other hand, must be complementary to the ephemeral, high energy transition state of the reaction it catalyzes. In principle, it should be possible to prepare antibodies with catalytic activity by challenging the immune system with compounds that resemble the high energy transition state of selected chemical reactions (16). This notion was recently reduced to practice. Phosphonates and phosphonamidates have been studied for some time as inhibitors of hydrolytic enzymes (17). The tetrahedral phosphorus is apparently an excellent mimic of the transition state geometry expected for ester and amide hydrolysis, and antibodies elicited against aryl phosphonate esters catalyze the cleavage of structurally analogous esters (18,19) and carbonates (20). Ester hydrolysis is unlikely to be the optimal process for uncovering enzyme-like behavior in

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antibodies, however. This is because there presumably exists a low probability for generating an effective constellation of catalytic groups (eg. general acids, general bases and nucleophiles) in the antibody combining site during immunization. Consequently, we have targeted concerted reactions that do not require chemical catalysis. Such reactions, including Claisen rearrangements and Diels-Alder cyclizations, are expected to be especially sensitive to the principal catalytic effects antibodies are likely to impart: induced strain and proximity (21). Moreover, these transformations are of enormous practical and theoretical interest, especially for the synthesis of biologically active molecules (22).

Chorismate Mutase Antibodies. An example of a biologically important Claisen rearrangement is the conversion of (-)-chorismate 1 to prephenate 2, shown in Scheme 2. This reaction occurs at a branch point in the biosynthesis of aromatic amino acids in bacteria, fungi and higher plants (23). The enzyme chorismate mutase [EC 5.4.99.5] catalyzes the rearrangement of chorismate to prephenate by a factor of two million over background (24). Although the mechanism of action of the enzyme is still controversial (25,26), inhibitor studies (27) and elegant stereochemical experiments (28) have implicated a diaxial chair-like geometry for the transition state (Scheme 2). The oxabicyclic compound 3 was designed by Barlett and co-workers (29) to mimic the putative transition state structure. It is currently the best known inhibitor of chorismate mutase, binding 125 times more tightly to the enzyme than does chorismate itself. We have used this material to prepare antibodies with chorismate mutase activity (30).

Scheme 2. Rearrangement of Chorismate to Prephenate

We synthesized 3, and its exo epimer 4, according to published procedures (29). Since small molecules are generally not immunogenic, these compounds were linked individually to a carrier protein, keyhole limpet hemocyanin (KLH), with a glutaric acid linker. The resulting protein conjugates, containing approximately 15 to 20 molecules of 3 or 4 per KLH molecule, were used to immunize mice. Forty-five

different hybridomas which secreted monoclonal IgG antibodies specific for the transition state analog 3 were obtained using standard techniques (31,32). Antibodies were propagated in ascites and purified by affinity chromatography on immobilized Protein A (32) followed by FPLC ion-exchange chromatography on a Mono Q HR 10/10 column (33). The resulting antibodies were nearly homogeneous as judged by SDS PAGE (34) with Coomassie blue staining (Figure 1). Two of the purified monoclonals (1F7 and 27G5) were shown to catalyze the rearrangement of chorismate to prephenate to a significant extent (30, Hilvert et al., unpublished results). An antibody with very high chorismate mutase activity has also been generated independently using immunogen 3 by Bartlett, Schultz and their coworkers (35).

Kinetics. Disappearance of chorismate was monitored spectroscopically. The catalyzed reaction was first order in antibody, and formation of prephenate was verified by a standard assay (36). Furthermore, at high antibody concentrations the rate of chorismate disappearance equaled the rate of prephenate formation. At high substrate concentrations saturation kinetics were observed which suggests that catalysis involves formation of a Michaelis-type complex. The kinetic parameters determined for 1F7 at 14 °C were: k_{cat} = 0.025 min^{-1} and K_m = 22 μM (30). Under these conditions the rate acceleration in the presence of the antibody is roughly 250-fold over background (k_{cat}/k_{uncat}). The fact that the transition state analog 3 is a competitive inhibitor (K_i is ca. 0.6 μM) indicates that rearrangement is occurring in the induced binding pocket of the antibody and that binding interactions contribute to transition state stabilization. The kinetic profile of 27G5 was similar to that of 1F7 (Hilvert et al., unpublished results). Hence, any structural differences between the two immunoglobulins are likely to be distant from the active site.

Values for the enthalpy and entropy of activation for the reaction catalyzed by 1F7 were determined from the temperature dependence of k_{cat}. Apparently, the observed rate acceleration is due entirely to a lowering of the enthalpic barrier (15 kcal/mol versus 21 kcal/mol for the uncatalyzed reaction (24)), consistent with the notion that induced strain might be an important component of catalysis. The entropy of activation for the antibody-promoted reaction (-22 eu) is actually less favorable than for the spontaneous reaction (-13 eu) (23). This fact may reflect the need for some conformational change in the antibody binding pocket during catalysis. However, possible solvent effects make the interpretation of $\Delta S^{\ddagger}$ difficult.

The promoted rearrangement of chorismate is notable as the first example of an antibody-catalyzed carbon-carbon bond forming reaction. The Claisen rearrangement is, moreover, a prototype of a broad and important class of concerted chemical reactions. It now seems all the more likely that this strategy for catalyst design can be extended to related transformations, including additional sigmatropic rearrangements and bimolecular Diels-Alder cycloadditions. Bimolecular reactions are particularly attractive candidates for study, since the entropic advantage to be gained by binding two reactants together in an active site can approach 10^8 M in rate for 1 M standard states (21).

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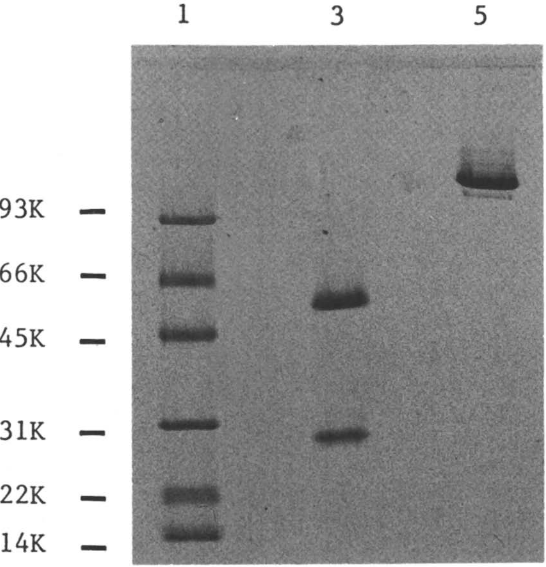

Figure 1. SDS-PAGE (34) of purified monoclonal antibody 1F7. Lane 1, protein relative molecular mass markers; lane 3, reduced 1F7; lane 5, non-reduced 1F7; lanes 2 and 4 are blank. The gel was stained with Coomassie brilliant blue.

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Stereospecificity. Rate accelerations are only one aspect of enzyme catalyzed reactions. More important for practical applications are the exacting regio- and stereoselectivity displayed by biocatalysts. Since antibodies are chiral molecules, they might be expected to exert considerable control over reactions they promote. In fact, an antibody-catalyzed lactonization reaction was recently reported to be stereospecific (19). Not surprisingly, experiments with racemic chorismate establish that the antibodies with chorismate mutase activity also exhibit high enantioselectivity (37).

Under conditions in which all of (-)-chorismate rearranges, only half of the racemic substrate is converted to prephenate by 1F7 (37). The k_{cat} value for (±)-chorismate is the same as that measured for the pure (-)-isomer, but its apparent K_m is twice larger. Because the k_{cat} value determined for the racemate is unchanged relative to the optically pure material, (+)-chorismate can be treated as a competitive inhibitor. From our data, the term $0.5K_m/K_i$ must be much less than 1, indicating that binding of the (+)-isomer to the antibody is at least one or two orders of magnitude weaker than that of (-)-chorismate.

In order to provide a better estimate of the enantioselectivity of the catalyst, we prepared an authentic sample of (+)-chorismate by kinetic resolution of the racemate with 1F7 (37). Circular dichroism spectroscopy confirmed the identity and high optical purity of the recovered, HPLC-purified compound. Initial rate measurements with the individual isomers show that (-)-chorismate is favored over (+)-chorismate by the antibody by a factor of at least 90 to 1 at low substrate concentrations. The slight rate enhancements above background observed for the (+)-isomer may be due to general medium effects rather than interaction with a specific locus on the antibody surface. To test this possibility we are currently examining the ability of the transition state analog 3 to inhibit rearrangement of this optical isomer.

Since the immunizing antigen 3 is racemic, catalytic antibodies specific for rearrangement of both (-)- and (+)-chorismate might have been induced. Assays with racemic chorismate of the 45 monoclonals specific for 3, however, did not reveal any antibodies with reversed substrate preference. We are currently screening a second fusion for which the immunizing antigen was compound 4. More than 40 hybridomas that secrete antibody specific for this compound have been identified (Hilvert, Carpenter and Auditor, unpublished results). Antibodies from this fusion may also catalyze the rearrangement of chorismate to prephenate. Comparison of the specific activities, stereoselectivities and structures of such molecules with the properties of 1F7 and 27G5 may lead to a better overall understanding of the mutase reaction.

Conclusions

Binding interactions are essential for efficient biological catalysis. The construction of enzyme-like molecules consequently requires the design of suitable binding sites for organizing reactants and reducing the energy of the rate limiting transition state. Although it is not yet possible to prepare protein binding pockets from their constituent amino acids, chemical principles can successfully inform

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the redesign of existing structures as well as the manipulation of the immune system to provide tailored active sites. The success of semisynthetic selenosubtilisin and the chorismate mutase antibodies, described above, illustrates the potential of these complementary strategies to exploit binding interactions for highly selective catalysis. Continued study of these systems will allow us to resolve important mechanistic issues and develop comprehensive structure-function relationships. The tandem use of DNA-directed mutagenesis, random mutagenesis and genetic selection may ultimately lead to the development of even more powerful catalytic species. Exploration of this exciting frontier in molecular engineering will bring us closer to our ultimate goal of designing enzymatic catalysts for virtually any chemical reaction.

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Chapter 3

Tailoring Enzyme Systems for Food Processing

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Three illustrations are used to review the various approaches taken by the enzyme industry in tailoring enzyme preparations to meet the production and product quality needs of the food industry. Tailored enzyme preparations have been able to convert the corn syrup industry from an acid-based industry to an enzyme-based industry, to overcome the problems created in the baking industry as grain technology improved and automation was introduced, and to rescue the cheese industry as the supply of bovine rennet decreased and the demand for cheese and cheese flavor increased.

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The use of enzymes by the food industry as a processing tool is increasing rapidly due to the tailoring of enzyme preparations to fit the processing needs of the industry. The potential of using enzymes for converting agricultural products into food products is enormous, and has minimally been tapped as a method of production. To date, the major modern applications coming after the age-old applications in baking, brewing, and cheese have centered on the production of commodity ingredients such as corn syrups, whey upgrading and hydrolyzed proteins. Today food processors are starting to use enzymes as processing tools to improve the attributes of their products. Appearance, texture, flavor, color, stability, and nutrition are attributes being influenced by applying tailored enzyme preparations during the process of converting agricultural products into processed foods. Food processors are also finding several processing advantages in using enzymes, such as increased yields and improved processing parameters like viscosity, filtration rates, and ease of handling (1).

Enzymes offer several advantages to the food processor over physical and chemical methods of processing. Enzymes work under very mild conditions of pH and temperature while accomplishing the same reaction that otherwise would require extreme pH and temperature conditions. Consumers are becoming increasingly aware of artificial and potentially toxic chemicals present in foods. The consumer, as well as the government, is asking for less use of chemical additives to control product attributes. Enzymes are also

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very specific and can select out one specific reaction, such as starch hydrolysis, in a mixture of starch, protein, and fat. Chemical and physical processing methods are not selective and in the starch, protein, fat mixture would hydrolyze all three of the polymers. Thus, by performing specific processing tasks under relatively mild processing conditions, enzymes can offer selected chemical reactions in a heterogeneous mixture, free of undesirable side reactions.

The factors to be considered in tailoring enzymes for the food industry can be divided into five areas: a) sensitivity to processing conditions; b) catalytic specificity or action profile; c) purity; d) source; and e) application economics.

Sensitivity to Processing Conditions. In most food applications, the environment in which an enzyme must work has very narrow limits. The composition of the raw materials and the final product often dictate the pH, salt composition, and inherent enzyme inhibitor level. The temperature ranges allowable are often controlled by the temperatures required to obtain the desired product attributes from temperature-dependent physical and chemical changes in the raw materials such as starch gelatinization and heat induced protein changes. This temperature range restriction is also controlled by temperature above or below which undesirable product attributes develop. It is often desirable to be able to control the activity of an enzyme during processing by altering the conditions of its environment such as pH or temperature.

Catalytic Specificity. The catalytic specificity or action profile of an enzyme is the tailoring factor that offers unlimited potential. Being able to select just the exact bond to cleave in a polymer, or to control within a very narrow range the size distribution in a polymer digest, offers enormous product quality potential.

Purity. The issue of obtaining purity economically is a challenging tailoring factor. Purity, in an industrial application sense, is defined by the absence of side reactions that would cause negative impacts on one or more product attributes. Unlike most ingredients used by the food processor, enzymes are sold by their activity rather than their weight. Therefore, the activity per unit weight of the enzyme product is of prime importance. The enzymes used in industry are rarely crystalline, chemically pure, or even single protein preparations. These impurities may not interfere with enzyme activity but enzyme inpurities can catalyze the formation of side products, present a toxicological hazard, or decrease a desirable attribute of the final food product such as color, taste, or texture. What is being added to the food as part of the enzyme preparation has to be evaluated as to its impact on final product attributes.

In most applications the level of enzyme used is very low and is inactivated during the processing, allowing the enzyme to be regarded as a processing aid. There are many potential food applications for enzymes which require activity levels or processing conditions which would either make the economics unfavorable or would leave undesirable levels of active enzyme in the final

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product. Immobilized enzymes are a way of overcoming these types of problems. Immobilized enzymes are usually completely recoverable from the reaction mixtures allowing them to be reused repeatedly without any contamination of the final product. Immobilized enzymes offer the added advantage of eliminating the need to heat the product to inactivate the enzyme. In many applications heating cannot be done due to the temperature sensitivity of the product. Immobilization also allows the use of higher activity levels compared to using free enzyme, resulting in a shortening of reaction times or reducing the size of the vessel needed to carry out the reaction. The absence of enzyme in the final product is often a major driving force in using immobilized over soluble enzyme.

Source. Enzymes for food applications come from all three kingdoms; plant, animal, and microbial. Traditionally used plant and animal enzymes are the plant proteases such as papain, ficin and bromelain, plant amylases from malt, and animal rennin which is used in cheese manufacture. Microbial cells are the usual and most promising future source of industrial enzymes. Estimates of the number of microorganisms in the world tested as potential sources of enzymes fall around 2% with only about 25 organisms, including a dozen or so fungi, currently used for industrial enzymes.

Application Economics. The bottom line is always the cost vs. benefit aspect of the process. The goal is always to be the least cost producer of the highest quality product. Cost is the most important decision-making aspect in whether to put an enzyme-based process into production once the technical feasibility has been demonstrated. The tailoring of cost effective enzyme preparations often becomes the challenge more than the tailoring of catalytic properties. Besides the direct cost of the enzyme product, enzymes need to be tailored to allow the processing of raw materials without adding long reaction times or costly unit operations such as material separation, cooling, heating, evaporation, or ion exchange, to the normal process flow.

Tailoring Opportunities. There are many methods or approaches available to tailor enzyme products. Early in the history of enzyme companies, methods such as source selection, microbial strain selection, growth conditions, media, purification, and recovery systems, were primarily used to make each enzyme preparation unique. Later, immobilization, encapsulation, and chemical modification of the enzyme molecule itself were added as methods of tailoring enzymes to better fit industrial applications. Today, all of these methods are still being used, and now we have added genetic engineering to our tailoring expertises.

Microbial enzymes offer a much higher potential for being tailored into cost effective highly specific enzyme preparations for use in food processing than do plant and animal enzymes. They offer a great variety of catalytic activities and ranges of environmental adaptability. They have the greatest potential for being produced in an inexpensive, regular and abundant supply. Microbial enzymes in general are more stable than their corresponding plant and animal counterparts having similar activity. Microbial cells also offer an unlimited potential for genetic and environmental manipulation to

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increase yields, to produce altered enzymes, or to introduce new genes to produce totally new enzymes.

Several papers in this symposium use site-directed mutagenesis as a means of tailoring the properties of an enzyme. Today, recombinant DNA techniques are being used in many laboratories to tailor cost-effective enzyme products directed to the food industry. The technologies needed to synthesize a new gene for a new enzyme in the laboratory, attach it to an expression vector, and insert it into a microorganism are available today. It is only a matter of time before it's done.

Three food industries, corn syrup, baking, and dairy, will be used to illustrate how enzyme preparations have been and are being tailored for use in the food industry.

Corn Syrup

The corn syrup industry is a good example of how enzymes took over and improved an industry after it was established as a profitable and important industry. In the beginning, acid was used as the catalyst to hydrolyze starch into syrups in order to impart new and desirable attributes to food products. The process involved treating a 40%-45% solution of refined starch with hydrochloric acid at pH 1.5 and a temperature of 150°C for a few minutes. The acid was not selective and hydrolyzed the protein and fat present in the starch preparation as well as the starch. This led to extensive undesired browning due to Maillard reactions. The syrup was neutralized, filtered, carbon-treated, and ion-exchange treated to become a food product or an ingredient to be used by other food processors in preparing products.

The properties and subsequently the applications of the resulting corn syrup products, were dependent upon the extent of hydrolysis. The extent of hydrolysis in corn syrup is expressed in dextrose equivalents (DE) which is the percentage of glucosidic bonds hydrolyzed with native starch being 0 and pure glucose being 100. The DE of acid-catalyzed corn syrups depend only upon the time of reaction. All acid-catalyzed syrup at any single DE level will always have the same dextrin profile.

Amylases. Acid hydrolyzed corn syrups were a great innovation for the food industry, but both the process and the product had their shortcomings. During the late 1950's, low DE corn syrups were gaining wide acceptance but had two major drawbacks. One was the development of a haze after concentration which limited its usefulness. The other was the high viscosity of the hydrolyzates which made them difficult to process. The use of enzymes as a processing tool to improve the attributes of an existing food product was introduced to the industry. Up to that time enzymes were used only to enhance products that by their complex nature required enzyme reactions such as brewing and cheese production. In 1960, Corn Products Co. obtained a patent for using an alpha amylase to partially hydrolyze the high molecular weight dextrins in low DE acid hydrolyzed syrups without substantially increasing the DE (2). After concentration, the amylase-treated acid hydrolyzed syrups had all of the desirable characteristics of low DE acid syrup but none of the undesirable features. During this same period of

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time, high DE acid hydrolyzed syrups were being used as adjuncts in fermentations but had a major problem of crystallization due to the high level of glucose. In 1962, Anheuser-Busch obtained a patent describing the production of a highly fermentable corn syrup in which 80% to 90% of the total carbohydrate is fermentable and there is no undesirable tendency toward crystallization. Their process involved acid conversion of starch to about DE 20 and then adding malt to increase the maltose level as high as possible which resulted in a syrup of about 55 DE. Next, a microbial amylase was added to further reduce the longer chain dextrins to glucose to give a final DE in the low 70's. This was the beginning of an industry that now offers products prepared by converting starch in a variety of ways: acid, acid-enzyme, enzyme, or dual enzyme.

Today there are two primary types of enzyme produced high maltose syrups available commercially. One has a DE of about 42 and contains about 40% maltose and only about 8% glucose. This product is widely used in jams and confectioneries because it is resistant to color formation, is non-hygroscopic and does not crystallize as readily as glucose syrups. The other commercial high maltose syrup has a DE of about 62 and contains about 35% maltose and about 40% glucose. This syrup is highly fermentable and stable to storage; therefore, it is used widely in brewing and bread making.

The properties of low DE corn syrup and its effect when used in a food system is very dependent upon its dextrin profile. By tailoring the action pattern of the amylase, corn syrups of unique and highly varying dextrin profiles can be produced. Today this is an undeveloped area. Knowledge is currently being generated to understand how enzyme digested starch of controlled dextrin profile can serve as fat mimetics, foam and gel stabilizers and many other food applications previously fulfilled by chemical additives or high levels of fat. The tailoring of the action patterns of amylases will be a major field in the future. The action pattern of an amylase and the resulting composition of its digest are controlled by the binding energies of the active site to the glucose residues in the starch chain. Each amylase has its own unique binding energy profile across its active site. In addition, amylases differ in the number of glucose units that will fit into the active site. These two factors will control the dextrin distribution profile of the resulting syrups. For example, the amylase from *Bacillus licheniformis* produces maltose, maltotriose and maltopentose as its main end products while the amylase from *Bacillus amyloliquifaciens* produces maltohexose as its major end product.

The active site of the amylase from *Bacillus amyloliquifaciens* has been extensively studied to the point that it is known that the active site interacts with ten glucose units along the starch chain with binding of the tenth glucose having a large positive value that hinders binding of substrate (3). The catalytic site of the enzyme is in proximity of the glucosidic bond between glucose units six and seven, resulting in the major product being maltohexose.Many amylases have the ability to cleave off multiple products from each successful enzyme/substrate encounter. This property allows for accumulation of small oligosaccharides in the early phases of amylase hydrolysis. The amylase has the ability to cleave at the end of a large substrate producing small dextrins after the first random attack. Although the number of ruptures per effective

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encounter is basically a kinetic phenomenon, the size of the products released will probably be influenced by monomer-subsite interactions. After the first cleavage, rearrangement of the enzyme-substrate complex will yield a series of complexes whose relative concentration partially depend upon the subsite-monomer interaction energies. The degree of multiple attack has been quantitated for several alpha-amylases (4). The highest value is for porcine pancreatic alpha amylase with six subsequent attacks per effective encounter at its optimal pH while *Aspergillus oryzae* amylase undergoes two subsequent attacks per effective encounter. If porcine pancreatic alpha amylase is studied at pH 10, its subsequent number of attacks drops to 0.7. This repetitive attack feature is one that could be tailored into the action properties of laboratory designed amylases.

In the future, tailored amylases will be able to produce a range of products offering a wide variety of unique properties that can be used to control the attributes of food products.

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Heat Stable Alpha Amylase. One of the key enzymes in today's corn syrup industry is a heat stable alpha amylase. It is now used in the first step in producing all varieties of corn syrups such as low DE, high maltose, high glucose, and high fructose corn syrups. In 1973, enzyme tailoring introduced the corn syrup business to an enzyme preparation that was designed to replace the acid starch converters. The alpha amylase from *Bacillus licheniformis* became available at a cost effective price. The enzyme had to function at 105°C with a half-life long enough to reduce the chain length of the starch molecules to a point that would prevent precipitation when the temperature dropped to 95°C. One reason the acid catalyzed reactions were carried out at 150°C was due to the need to open up the inner structure of the starch granule to make the bonds available for hydrolysis. At lower temperatures, yield was lost due to undigested starch

In the enzyme process using heat stable alpha amylase, the reaction is carried out in a jet cooker where mechanical shear is applied as the temperature is raised to 105°C by injecting pressurized steam. Under these conditions, the enzyme catalyzes enough hydrolysis of the inner core of the starch granule to prevent significant yield losses. If the jet cooking process is run at 95°C, where the enzyme is much more stable, a significant yield loss is obtained. After about five minutes at 105°C the starch slurry is flash cooled to 95°C and held at that temperature while the enzyme increases the DE to about twelve. The temperature of this syrup can now be lowered to 60°C or less and enzyme treated in a variety of ways to produce a wide range of corn syrup products of varying properties and dextrin distributions.

Today, there are several heat stable alpha amylase products on the market, many of which were tailored using mutation and recombinant DNA techniques. A major tailoring target has been improvements of yield during enzyme production in order to be more cost effective in the marketplace. All of the heat stable alpha amylases require calcium for stabilization which must be added to the starch slurry and then removed by ion exchange later in the process. The calcium requirements vary widely. For example, the amylase from *B. licheniformis* requires only 5 ppm calcium while the

amylase from B. amyloliquifaciens requires 150 ppm calcium. A tailoring need for the future would be a very stable non-calcium requiring amylase obtained in high yield during its production.

Amyloglucosidases. Amyloglucosidases or glucoamylases as they are often called, catalyze the stepwise hydrolysis of the alpha 1-4 links in starch releasing beta glucose from the non-reducing end of the chain. Since glucoamylases can only bind at the end of each polymer chain its activity is somewhat controlled by the size of the dextrin or starch molecules in solution. Most glucoamylases will also hydrolyze alpha 1-6 and alpha 1-3 bonds but do so at very slow rates.

The major use of glucoamylases in the corn starch industry today is to digest to as near completion as possible the product resulting from the hydrolysis of starch with heat stable alpha amylase. The starting syrup is usually at about 30% dry solids with a DE of about 12 to 15. Two major things must be done to the syrup as it exits the heat stable amylase step. First, it has to be cooled to 60°C because a heat stable glucoamylase has not yet been tailored. Second, the pH must be adjusted to about pH 4.5. A major need of the industry is a cost effective heat stable glucoamylase that performs at about pH 6.5. This would eliminate the pH adjustment step as well as to allow both the alpha amylase and the glucoamylase reactions to proceed at the same time. Glucoamylases also require calcium ions for stability but in practice the calcium level is adjusted before the alpha amylase step to stabilize it.

The resulting product from glucoamylase treatment, a glucose syrup, usually has a DE of about 97 and a glucose content of 95% to 97% with 3-5% higher saccharides present. The glucose syrup is then used in several ways. It can be used as a syrup or crystallized to give pure solid glucose. Today a very large percentage of it is used as feed stock to produce high fructose corn syrup. In all cases, the higher the glucose level the better and each percent of carbohydrate not converted to glucose equates to millions of pounds of product lost annually for the industry. To push the reaction to as close to completion as possible, glucoamylase is used batchwise for reaction periods of 48 to 92 hours.

At pH 4.5 and 60°C the 30% syrups used for glucoamylase feed stocks are stable microbiologically since microorganisms will not grow under these conditions. In practice, the maximum glucose level can be missed due to the thermodynamics of the system. In a 30% solution of glucose, there is a small level of alpha 1-4, alpha 1-6, and alpha 1-3 disaccharides produced due to the systems thermodynamics. Since glucoamylase can catalyze all three of these reactions, we now have conditions that allow for the formation of alpha 1-6 and alpha 1-3 disaccharides. If the reaction is allowed to continue a glucose syrup reaching a level of 97% glucose would slowly decrease with time. Operating the glucoamylase reaction at a lower dry solids level will greatly reduce the reversion reactions but unacceptable evaporation costs result.

Immobilization is often suggested as a method of tailoring enzymes for more efficient industrial use. In the case of glucoamylase very superior properties would have to be obtained to warrant immobilizing the enzyme due to its relatively low cost in the soluble form. Some of the potential benefits would be logistic

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convenience, lower capital and energy cost, reduction in reaction time from 48 hours to less than one hour ,less color development during processing and lower in-process volume inventories.

Glucoamylase has been successfully immobilized with half-lives of a month while operating under conditions that prevent microbial growth. Immobilized glucoamylase systems have a very high enzyme to substrate ratio. This causes a higher rate of formation of alpha 1,6 and 1,3 disaccharides leading to lower final glucose levels than in batch systems.

A major problem during the 1970's with commercial glucoamylase products was the presence of a contaminating enzyme which formed di- and trisaccharides with alpha 1-6 linkages. Thus, relatively high levels of isomaltose and panose were observed which were much higher than predicted by recombination via glucoamylase reversion. Tailoring of preparations first by purification procedures and later by mutation and media control removed the contaminating transglucosidase from commercial products.

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Debranching Enzymes. One major source of yield loss in the production of high fructose corn syrup is the loss due to incomplete conversion of starch to glucose. When a need exists with a significant dollar value associated with it, someone will develop a cost effective solution if given enough time.

There are enzymes whose primary reaction is to catalyze the cleavage of the alpha 1-6 bonds of the amylopectin in liquefied starch. These enzymes are referred to as debranching enzymes and include pullulanase and isoamylase. Enzyme companies are now tailoring products for the primary purpose of obtaining another percent yield of glucose during glucoamylase conversion of starch. One such product currently available commercially is derived from *Bacillus acidopullulyticus* (5-6). The application conditions of this product are such that it can be used at the same time as glucoamylase and results in higher yields in shorter time.

Another company has taken a different approach to solving the glucose yield problem (7-8). Its scientists identified a new amylolytic enzyme from *Bacillus megaterium*. The enzyme hydrolyzes alpha 1-4 bonds and does not directly hydrolyze alpha 1-6 bonds. The unique property of this enzyme is its ability, via a transfer reaction, to convert the glucoamylase resistant saccharides present in a syrup into forms easily hydrolyzed to glucose by the glucoamylase. This new amylolytic enzyme is produced by *Bacillus megaterium* as a trace contaminant in a complex mixture of proteins which also include a large amount of beta amylase. By genetic engineering the *B. megaterium* gene for this enzyme was transferred into *B. subtilis* (9) resulting in a 10,000-fold increase in yield and a product free of other amylases.

Glucose Isomerase. The biggest success story in the enzyme industry has to be glucose isomerase. The very first report of an enzyme that converts glucose to fructose was in 1957 (10). In less than 20 years the enzyme was studied, tailored into a cost effective immobilized form and put into production to make a commodity corn syrup product. In less than 30 years from its first description, it became the largest commercially used immobilized enzyme and responsible for making one of the world's major sweeteners. Since

glucose isomerase is an intracellular enzyme, stable above 60°C and all of its reactants are small molecules, glucose isomerase is well suited for immobilization. Many approaches have been used to immobilize glucose isomerase using both whole cells and cell free preparations. Commercial products of glucose isomerase are constantly being improved and today the enzyme cost per unit of fructose produced is only a small fraction of what it was during the mid and late 1970's.

In spite of the great commercial success of glucose isomerase, there is still room for improvement. There are two major environmental problems that have been present since the beginning that still elude the researcher. All glucose isomerases require one or more heavy metal ions for activity and stability. To make matters worse, calcium ions inhibit the activity. In practice this means that calcium required for stabilization of the alpha amylase and the glucoamylase must be removed by ion exchange before the magnesium ions, which are needed for glucose isomerase activity, can be added to the syrup. Of course, after isomerization the magnesium ions also have to be removed by ion exchange. Thus in the production of high fructose corn syrup the syrup must be treated with ion exchangers twice. An enzyme not inhibited by calcium or not requiring a metal ion would find a ready market. The other big environmental problem is pH. All currently available glucose isomerase products have to be used at a pH above 7.0 for cost effective operation. Color formation and other alkaline catalyzed reactions increase refining cost and cause a loss of yield. An enzyme capable of prolonged operation at a pH below 7 is needed.

Baking

Enzymes play a very critical role in the baking industry. Traditional methods of baking are based on the presence of enzymes in the flour and in the microorganisms used to ferment the dough. The endogenous alpha amylase hydrolyzes the low level of starch made available by the rupture of starch granules during the milling process to dextrins. These dextrins become the substrate for the flour's endogenous beta amylase which produces maltose. The yeast in the dough then uses its alpha glucosidase to convert the maltose to glucose, which the yeast can now use to produce carbon dioxide via its glycolytic pathway. But this is just the gas formation aspect of baking. Wheat flour also contains low levels of proteases, pentosanases, isoamylases, lipoxygenases, lipases, and esterases in addition to the two types of amylases. All of these enzymes play a role in the quality of the baked goods produced. Therefore, the enzyme content of flour is of major importance in the baking industry.

Flour Amylases. Beta amylase is produced in wheat during the ripening stage and is stored in the aleurone layer until needed during germination and therefore is present in ungerminated grain. Plant alpha amylase, in contrast, is not present in ungerminated grain to any significant extent but is synthesized *de novo* upon germination (11).

Prior to 1920, the growing and harvesting of wheat were poorly controlled. Field sprouting was routine and produced varying levels

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of alpha amylase in the resulting flour. With the introduction of mechanical harvesting of wheat in the 1920's, field sprouting became a rare occurrence. The level of alpha amylase in flour, produced from wheat that has been carefully and expertly grown, harvested, and milled, will be too low to produce acceptable bread by the traditional system of dough preparation.

The history of the use of exogenous enzymes in the food industry has nearly paralleled the history of the development of cereal technology. The benefits of adding malt diastase (amylase) to bread to increase volume and quality were known in the 1800's. The practice of adding malt to flour became an early art in the milling and baking industry. If the alpha amylase level was low, poor fermentations were obtained. If the alpha amylase level was high, the resulting bread had a sticky, moist, inelastic crumb structure. With proper alpha amylase supplementation of flour, dough viscosity is reduced and the rate of fermentation increased resulting in a product of improved volume and texture.

As bread making became more and more automated, replacing the bakers decisions on critical processing end points with preset processing parameters, the amylase level in the flour became a critical factor in the industry. Responsibility for the amylase level fell to the miller to supply flour that would perform to the baker's standards. The miller originally added the alpha amylase in the form of malt. But in 1884, Takamine discovered that the fungus <u>Aspergillus oryzae</u> could be grown on wheat bran by solid state fermentation to produce a high level of alpha amylase. This enzyme would prove to be an ideal amylase for supplementing flour. Today it is the major method used by millers to standardize flour amylase activity. Malt supplemented flour is darker than fungal amylase supplemented flour. This darker color adversely affects the color of the bread. Malt also contains a relatively high level of proteolytic activity which can result in a reduction of gluten quality in low protein flours. <u>A. oryzae</u> amylase products contain a negligible protease content. Another major difference between malt alpha amylase and fungal alpha amylase is in their thermostability. Malt amylase is more stable and if too much is added can lead to the formation of sticky bread. The fungal amylase is inactivated at about 60°C before appreciable amounts of starch are gelatinized, thus making the control of the reaction easier. <u>A. oryzae</u> amylase can be added to low alpha amylase flour with relative safety to obtain an increased loaf volume and less firm crumb with little danger of over treatment.

<u>Flour Proteases</u>. In the baking process, the quality and quantity of gluten protein determines the rheological characteristics of the dough. Wheat flour contains 7% to 15% protein with about 80% being gluten. Ungerminated flour contains low levels of protease which play a minor role in bread making. Since the 1920's, proteolytic enzymes have been used as additives in baking to improve dough handling properties. At that time, the main source was malt and it was often difficult to control the ratio of alpha amylase and protease in malt products. The art of tailoring malt products for the baking industry focused primarily on the time and the condition used for malting. The use of proteases in baking increased significantly in the 1950's after the introduction of fungal

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proteases from A. oryzae. Today the supplementation of flour made from hard, high protein wheat is routinely practiced in order to improve dough handling properties, to control mixing times, and to increase loaf volume. Without protease addition, high gluten doughs produce small loaves with blistered or cracked crusts.

The level of protease activity in a flour is particularly important today due to the use of automated baking operations that require strict control of mixing time. The controlled use of proteolytic enzymes can reduce the dough mixing time by 30% without adverse effect on dough properties and on bread quality. Excess proteolytic activity will cause slack, sticky doughs and poor quality bread. Today the baking industry uses protease products tailored for their use from fungal proteases. Fungal proteases are less stable than malt proteases and are inactivated at a lower temperature. This feature allows for relatively good tolerance to overusage. Fungal protease products contain both endopeptidase and exopeptidase activities. The endopeptidases affect the viscoelastic properties of gluten. The exopeptidases produce free amino acids which improve both bread flavor and crust color through increased Maillard reactions during baking.

Bread Staling. Staling in the baking industry is a problem begging to be solved and the enzyme industry is starting to tailor products directed toward solving the problem. Staling of bakery products is a general term used to cover a large number of changes that occur during the normal storage of bakery products. For the consumer, the staleness of a bakery product is the sum of several perceptive judgements about the changes in several product attributes. The major attributes used by a consumer to judge staleness are loss of perceived moisture and the firming or texture deterioration of the crumb. Up to very recently the dogma regarding the mechanism of staling has focused on the retrogradation of starch as the primary cause. The change in the crystalline structure of starch over time does play a role in the staling processes. Current theories on staling are focusing more on the level of free water in the product with starch retrogradation being one of the major mechanisms through which water becomes bound during staling. It may not be the presence of retrograded starch that causes the firmness but the reduction of free water content below a critical level.

Soon after the practice of supplementing flour with alpha amylase started, observations were made that the higher the amylase level, the longer the shelf life. But, as discussed earlier, excess alpha amylase activity can lead to a gummy bread texture, difficult to slice and unacceptable to the consumer. In 1953, a comprehensive study (12) was reported in which fungal, cereal and bacterial alpha amylases were investigated at equivalent levels for their ability to reduce firmness development. All three amylase products retarded firmness development and their effectiveness to do so was positively correlated to their heat stabilities. The bacterial amylase, having the highest heat stability, produced the biggest effect while the fungal amylase showed the least having been inactivated before the starch was gelatinized. Theories were proposed that the amylase produced dextrins that interfered with the crystallization of starch. However, in 1959, based on X-ray studies of bread (13), it was found that bacterial alpha amylase treatment increased rather

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than decreased starch crystallization. In 1980, another X-ray study (14) looked at the starch crystallization pattern in control bread and breads that had been treated with alpha amylases from malt, fungal and bacterial sources. The basic findings agreed with the 1959 study in that starch crystallinity and bread firming were not synonymous and that crystallization increased upon amylase treatment. The news in 1980 was that the type of crystals found in amylase treated bread were different than the type of crystals found in the control bread. Previous to this 1980 study, it was known that starches found in nature are crystalline and that they exist in two forms, A and B. The A form contains less than 7% water in its crystalline structure while the B form contains over 25% water in its structure (15). In the study, the control bread formed B type crystals while the amylase treated bread formed A type crystals.

Due to the potential of producing gummy bread due to excess alpha amylase activity, the baking industry never adopted for regular use any of the commercially available amylase products for the primary purpose of freshness extension.

Recently the enzyme industry set out to tailor products for the baking industry that would decrease the rate of firming in baked goods without the risk of producing a gummy texture. During the last two years, at least four enzyme companies have developed special products and are promoting them for this application. The two major challenges were to develop an enzyme product that would a) allow bread to retain its moisture as free water for as long as possible and b) would have a built-in application tolerance level that would allow wide variation in use level without causing production or product problems.

The approach one company is employing is to use a debranching enzyme along with a low level of bacterial alpha amylase (16). They had developed a debranching enzyme from the bacterium Bacillus acidopullulyticus for use in the corn syrup industry. By blending their debranching enzyme with an alpha amylase, an enzyme preparation was obtained with the ability to greatly reduce the formation of B starch crystals while maintaining a "forgiveness factor" before product or production problems are experienced.

A second company also focused in on changing the structure of the starch. Their approach is to prevent B type starch crystallization by rearranging the branched portions of the starch chain via a transfer reaction. They developed a debranching system for the corn syrup business by transferring a unique amylase gene from Bacillus megaterium into Bacillus subtilis. This unique amylase has the ability to transfer branched dextrin to glucose. In their anti-staling enzyme preparation they have a blend of the B. megaterium amylase and a glucoamylase. Their literature claims that their new product will improve moulding properties, crust color, height, flavor, and rate of staling.

Another component of flour, the pentosans, have a very high capacity to bind water. Pentosans are present in wheat to about 4-5% and in patent wheat flour to about 2.5-3%. Pentosans contribute to about one quarter of the adsorption of dough water which is disproportionately more than the starch or protein fractions. Pentosans can absorb about 6.5 times their weight of water. A significant anti-staling effect has been claimed by the addition of pentosanase to bread (17-18).

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An enzyme company has now introduced into the market an enzyme preparation produced by Trichoderma reesei that contains a mixture of cellulase, beta-glucanase and pentosanase. The claim is that this new product is tailor made for the baking industry to extend the softness life of bread.

A fourth enzyme company has added cellulase, hemicellulase and pentosanase to fungal alpha amylase as their entry into the anti-staling market. They claim that the added enzymes work synergistically with the amylase to produce a product with reduced tendency to become firmer with time.

Dairy

The dairy industry is very heavily dependent on the enzyme industry. Without enzymes there would be no cheese industry. The principal enzymes used in dairy technology are proteases, lipases/esterases, and lactases. Cheese production is the largest consumer of proteases and has been the target industry for tailored enzyme preparations for many years. Besides tailoring protease preparations, the enzyme industry has developed many lipase/esterase preparations specifically for the ripening and flavor development of cheese products. The whole concept of rapid ripened cheeses and cheese flavoring products is based on the use of tailored enzyme preparations. The flavor levels of enzyme modified cheese (EMC) pastes and powders are 5-100 times greater than cheeses aged by conventional procedures. The potential uses of lactase to overcome nutritional and product attribute problems have led to the tailoring of many lactase products, several in an immobilized state.

Milk Coagulation. The first step in cheese manufacture is the coagulation of milk. Traditionally, this coagulation step is catalyzed by the enzyme rennet. Rennet is a saline extract of the 4th stomach of calves, usually slaughtered before they are 30 days old. The principal protease in rennet is rennin. In an attempt to avoid confusion with the hormone peptide renin, the International Enzyme Nomenclature Committee has assigned the name chymosin to the protease in calf rennet. During the growth of calves, chymosin is replaced by pepsin, the acid protease of the mature stomach. Chymosin initiates the clotting of milk by selectively cleaving a single phenylalanine-methionine bond in kappa-casein. Cleavage of this one bond causes the loss of a peptide from the milk's micelles. This peptide is the part of the native micelle that maintains a net negative charge on the micelle's surface causing the micelles to repel one another. Once this peptide is lost the micellar surface charges (zeta potential) are reduced to about half. The micelles can now associate to form a network structure of protein. On electron microscopy examination the individual micelles can still be clearly distinguished indicating that chymosin does not disrupt the micelle structure. Chymosin is highly specific in its action. By splitting a single peptide bond in kappa casein, which is only about 10% of the total casein in milk and even less than that of the total protein, chymosin can produce dramatic changes in the physical properties of milk. Chymosin is an excellent example of the marked effect enzymes can have on the functional properties of food.

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Unfortunately, cheese consumption is constantly increasing and veal consumption is constantly decreasing. For several years now, there has been a major shortage in chymosin. Chymosin is an acid protease. More specifically it is an aspartate protease. It is so named because it belongs to a class of acid proteases that have two aspartic acid residues involved in the active site. The task for enzyme manufacturers is to develop microbial acid protease preparations that come as close as possible to the catalytic and stability properties of chymosin. Nearly all acid proteases will catalyze the rapid hydrolysis of the key phenylalanine-methionine bond in kappa casein. The problem is that other bonds are cleaved and the relative rates of their hydrolysis is high compared to the relative rate for chymosin. Successful milk coagulants have been produced from microbial sources for use in cheesemaking. The major organisms used to produce microbial rennet products are Mucor pusillus, Mucor meihei, and Endothia parasitica with Mucor pusillus being the most widely used. Mucor pusillus was the first microbial rennet identified that successfully produced a good quality cheese. In the 20 years since their first discovery, microbial rennets have been tailored to improve their functionality in cheese making to more closely match the functionality of chymosin. Microbial rennets in general have the disadvantage of being too stable. The organisms have been mutated and the enzymes chemically modified to increase their specificity and to decrease their stability. In the process of tailoring thermally destabilized microbial rennets, an unexpected benefit was obtained as an increase in the specificity of the microbial enzyme making its performance virtually indistinguishable from calf rennet (20).

Of course, the ultimate tailoring task still needed to be done. Using recombinant DNA techniques, the bovine chymosin gene needed to be expressed in a microorganism. Commercial production of chymosin using recombinant DNA techniques became the target of a number of biotechnology firms. At first the gene was inserted into bacteria and yeast cells mainly because the state of the art in expression vectors was best understood in these organisms. The recombinant DNA procedures worked great but the chymosin that was formed was in an inactive form and required a costly renaturation process to obtain an active enzyme.

The problem was one that is common to a number of complex proteins whose conformation is determined in part by formation of one or more disulfide bonds. When these proteins are produced in a microorganism, the proper disulfide bonds often do not form and the protein assumes an inactive conformation. One way around this problem is to produce the protein in an organism that will secrete it in its active form. Microbial rennets, currently produced and used in cheese production, are aspartyl proteases like chymosin. Therefore, if the bovine gene for chymosin could be transferred to and expressed in the same type of organism that currently produces an aspartyl protease, then the probability of success is much higher. Since all microbial rennets are produced by filamentous fungi, investigators turned to filamentous fungi (19) in order to successfully obtain expression and secretion of bovine chymosin.

Today, active bovine chymosin has been successfully produced from several filamentous fungi and they are indistinguishable from the natural enzyme obtained from calves' stomachs.

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Cheese Ripening. Rennet plays a major role in the texture and flavor development of cheese during the ripening process. Besides the rapid cleavage of the key phenylalanine-methionine bond to coagulate milk, chymosin has been shown to hydrolyze at least 22 other bonds in the casein molecules. The favored amino acids at the point of cleavage are leucine, isoleucine and phenylalanine. Chymosin produces cheese free of bitter flavor and of excellent texture. The use of other acid proteases such as pepsin and many of the microbial rennets in the coagulation step often leads to bitter flavor and a soft texture after ripening.

In traditional cheese making, while milk is being coagulated using a rennet preparation, starter cultures of microorganisms are added which are carefully selected for the type of cheese being produced. Most varieties of cheese undergo some degree of aging which improves flavor and texture. Certain free amino acids appear within a few hours after the curd is harvested indicating the presence of exopeptidases. In mozzarella cheese desirable changes in flavor and texture are evident within two weeks.

Both proteolysis and lipolysis are involved in the cheese ripening process. The rate and extent of their interactions are influenced by the rennet preparation used, characteristics of the starter culture, pH, moisture range, salting practices, temperature, and the activity of adventitious microorganisms present in or on the cheese.

Rapid Ripened Cheese. Hard cheeses require prolonged ripening times thus the cheese manufacturer must accept certain time lags between the initial conversion of milk and the realization of the value of the resulting cheese. The time lag is dependent upon the cheese variety. For example, mature cheddar requires up to one year to achieve full maturity. The maturation period ties up large amounts of capital and is a costly process. The capacity of a cheese production facility is often limited by the space available for maturing the cheese. Being able to accelerate the aging process would be a major cost efficiency improvement.

Since many varieties of cheese are matured by the action of indigenous enzymes rather than by their viable microflora, aging of these varieties has the potential of being accelerated by artificially increasing the concentration of those enzymes that have a definite role in the ripening process. The tailoring of enzyme preparations to accelerate the ripening process, while giving excellent flavor and texture properties, is a major challenge being tackled by the enzyme industry today.

Proteases for Accelerating Cheese Ripening. Proteolysis plays such a major role in developing the flavor and texture of hard cheese that the addition of exogenous microbial proteases has been attempted using every commercially available protease preparation. By very carefully choosing the protease and its level, very strong flavored cheese can be produced in a short time. However, it is very difficult to produce the proper balance of flavor and to avoid bitter defects in real applications. To varying degrees, all protease treatments will increase the softness of the final product, thus adding another defect the cheese manufacturer has to minimize

in the rapid ripened cheese products. The limit of the magnitude by which cheese flavors can be increased with protease treatment is limited by the onset of defects.

When faced with a problem that carries a big bottom line profit potential, technologies will be developed to solve that problem. A new approach now being used to tailor enzyme preparations for the cheese industry is a spin-off of the medical/pharmaceutical industries technology. Liposome technology is the art of producing multilayered lipid particles ranging in size from less than one micron to several microns with non-lipid materials trapped between layers. In one current liposome enzyme preparation being tested now in cheese making, proteases are encapsulated in a system of phospholipid bilayer membranes (21).

This liposome enzyme system is a combination of an endopeptidase and an exopeptidase. The enzymes are slowly released after the cheese is put into the aging chamber. Using these liposomes for cheddar cheese production showed that the liposomes were evenly distributed and 90% of the added enzyme was retained in the cheese curd. The resulting cheese ripened in half the normal time with excellent flavor and textural properties.

The tailoring of protease preparations to rapidly produce hard cheeses in a cost effective manner and with good flavor and textural properties, is progressing rapidly. In the next few years several new and innovatively tailored enzyme preparations will be available for accelerating the production of hard cheeses.

Lipases for Accelerating Cheese Ripening. Acceleration of lipolysis by the addition of either animal or microbial lipases has been successfully applied to the relatively strong flavored cheeses. Lipases derived from the pregastric gland of kid, calf and lamb are currently being added to accelerate ripening in Italian type cheeses where the characteristic rancid flavor of low molecular weight free fatty acids, for example butyric acid, is desirable. However, when these pregastric lipases are used to accelerate mild flavored cheese such as cheddar, too much of the low molecular weight fatty acids are produced and a rancid flavor develops. When the animal pancreas lipases are used in a high concentration, excessive amounts of lauric acid are produced giving the cheese a soapy taste. Also, a major disadvantage of using any of these lipase preparations tailored from animal glands is the protease that is always present. Although the protease is present in small amounts, cheese softening occurs and at times bitter off-flavors are produced. To date, the use of microbial lipase has also been primarily in the very strong flavored cheeses, like blue cheese where rancid and soapy flavor notes are less noticeable.

When there is a need, someone will solve it. A patent was issued in 1987 (22) describing a new and novel lipase produced by a mutant strain of Aspergillus that has an accelerating effect on cheese flavor development without lypolytic enzyme associated rancidity. The patent claims that this new lipase will be useful as a ripening accelerator in the production of mild flavored cheeses such as cheddar.

Enzyme Modified Cheese. If the texture issue is removed from the desirable attributes of cheese production and the only attribute

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desired is flavor, then a whole new area becomes available for tailored enzyme preparations. The importance of free fatty acids to the characteristic flavor of various types of cheese and to dairy products in general, becomes very apparent as technologists evaluate and study the chemistry of food flavors. The mechanisms and chemistry by which relatively tasteless cheese curds become distinctively flavored cheeses have been under research scrutiny for many decades.

Lipolysis is defined as the enzyme-catalyzed hydrolytic cleavage of triglycerides resulting in the release of free fatty acids. But lipases differ greatly in their catalytic specificity and are responsible for many of the differences among cheese flavors. The microbial lipases, produced by the starter culture microorganisms, are often the deciding factor on the type of cheese being produced. The flavor of the Italian cheeses - romano, parmesan, and provolone -are entirely dependent on free fatty acids for their distinctive flavor. The catalytic specificity of various lipases differ in four major areas: fatty acid chain length; position of the fatty acid in the glyceride structure; degree of esterfication in the glyceride (tri-, di-, or mono-); and the physical state of the glyceride. Lipolytic enzymes responsible for various cheese flavors demonstrate preferential release of specific fatty acids from one position in the butterfat when the butterfat is in the correct physical form. Enzyme preparations are now being tailored by preculturing the appropriate organism under conditions that allow the accumulation of the desired enzymes free in solution. Enzyme products are now available that are capable of producing specific cheese flavors in slurries of butterfat or in slurries of cheese curd. For example, a romano cheese flavor was recently reported (23) that was produced from a very simple butterfat emulsion. An emulsion of water washed butter and Tween 80 in phosphate buffer was treated with a crude enzyme mixture isolated from *Candida rugosa*.

Today, cheese flavored paste and powders, containing from 5 to 100 times the flavor level of native cheese, are available in the marketplace for most cheese varieties. Nearly all of these products are made by using added enzymes with most of them using a blend of enzymes and microorganisms. Selected cheese flavors are produced in a liquid or slurry form in a relatively short time compared to making the true cheese and at a flavor level many fold above the maximum flavor obtainable in the true cheese.

The synergistic use of enzymes and microorganisms to produce natural flavors for foods is an industry of its own just now being born. The tailoring of enzyme products with and without microorganisms, will be a major market area for the enzyme industry in the future.

Lactase. Lactose, present in milk, is a disaccharide sugar composed of glucose and galactose. Lactose is a major problem to the dairy industry. It is non-sweet, very low in solubility, cannot be absorbed directly from the intestine of humans and is metabolized very poorly by microorganisms. In contrast, its components (glucose and galactose) have a sweetening power of about 0.8 relative to sucrose, are 3 to 4 times more soluble than lactose, are easily absorbed from the intestine and the glucose is highly fermentable by microorganisms.

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Lactase is an enzyme that selectively hydrolyzes lactose into glucose and galactose thus having the potential for the tailoring of enzyme preparations for wide use in the dairy industry. Lactase treatment provides a method of preventing lactose crystallization in frozen milk and whey based products as well as in liquid concentrated milk products. Humans intolerant of lactose can prevent suffering from milk induced abdominal pain and other gastrointestinal distresses by using lactase-treated milk. Today the largest market for lactase is by individuals suffering from lactose intolerance.

Several years ago the enzyme industry looked at the cheese industry and saw lactase as a big opportunity. Besides the lactose crystallization problem in frozen and concentrated milk products, the enzyme industry saw another potentially big profit application. Over half of the cheese whey produced in making cheese is diverted to waste treatment or waste disposal systems rather than being processed for food and feed use. Upgrading cheese whey became the hot development item for the enzyme industry.

The first problem faced by the enzyme industry was to prepare preparations having the same catalytic properties and yet be able to work at extremely different pH values. The pH of milk is 6.4 to 6.8 while the pH of cheese whey is closer to 4. Adjusting the pH of either feed stream is not a feasible answer to the problem. Enzyme preparations were developed from dairy yeast that are active around pH 6.7 for use in milk systems and other enzyme preparations were developed from fungal organisms active around pH 4.0 for use in cheese whey.

The use of soluble preparations of acid lactase was never developed into a cost effective process for upgrading whey. The low level of lactose in the whey created a large expense in concentrating the products. This plus the high cost of food approved lactase made the production of sugar from whey uneconomical. The next tailoring step, in an attempt to upgrade whey, was to immobilize the fungal lactase and use the hydrolyzed product as feedstock to grow baker's yeast. Several companies spent years and a lot of money developing immobilized lactase products. The one system that was put into commercial production in the United States was tailored by immobilizing fungal lactase onto microporous beads of glass-like carrier. After several attempts to make the operation a profitable one, the facility was closed.

Today, the lactase market is primarily for solving the lactose intolerance problem in human diets. The tailoring of enzyme preparations continues and lactase may still be developed into a big profit item.

Tailoring Enzymes for Future Food Applications

The big tailoring projects in the future will be in designing enzymes using gene synthesis, site-directed mutagenesis and other genetic engineering technologies. The enzyme's responses to its environment will be the primary targets. Responses to temperature, pH, ions, and chemicals will be adjusted to best tailor an enzyme for its intended use. To illustrate the chemical basis for potential changes, the tailoring of an enzyme's response to temperature will be used.

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Enzymes respond to temperature in two ways. First the enzyme's stability is affected by temperature. As the temperature is increased, inactivation of the enzyme due to thermal denaturation of the protein increases resulting in a decrease in the concentration of active enzyme. The second effect of temperature is on the reaction rate directly.

For chemical reactions, the Arrhenius equation describes the relationship between the rate constant of the reaction and the absolute temperature. This equation, which is empirical and has no theoretical basis, was developed in 1889 by Arrhenius to define the activation energy of a reaction. The role of enzymes in chemical reactions is to lower the activation energy. In fact, the activation energy of enzyme-catalyzed reactions appear to be more characteristic of the enzyme than of the substrates involved in the reaction. For most enzyme-catalyzed reactions, the activation energies are between 6,000 and 15,000 cal/mol (24).

Since the rate of reaction is dependent upon the activation energy, the genetic engineering task is to design into the enzyme a temperature sensitive conformation change. This conformation change needs to cause a major change in the activation energy at a desired temperature. This could be to either turn on or turn off the activity depending upon the application. For example, in cheese making it may be desirable to turn off the activity as the temperature rises, while in bread baking it may be desirable to have enzyme activity only at high temperatures. The switch can work in either direction. The other temperature-related property of enzymes needing attention is thermal inactivation. Tailoring enzymes for the cheese industry has already produced less stable proteases. Tailoring alpha amylase preparations for the corn syrup industry has produced amylases with long enough half-lives at 105°C to allow their use in production at that temperature. The current processing trend in the food industry is toward shorter processing times. One of the major processing methods in the food industry today is referred to as high temperature and short time (HTST) processing. Products made under these conditions often have physical properties very similar to products made by much longer processing methods. However, most of the time the flavor development is not acceptable. Enzymes capable of working at high temperatures, even for short time periods, are needed by the food industry. Microwave cooking is able to cook food rapidly producing the physical changes desired but often falls short in flavor development. Enzyme preparations are needed to solve the flavor generation and textural problems associated with microwave cooking and reheating.

Conclusion

The past success of tailoring enzyme preparations for application in the food industry can be expressed by summarizing a few major applications of enzymes in the industry today. Almost all clear fruit juices are enzyme treated. A high proportion of grape juice, grape must and grape wine is enzyme treated. All production of glucose from starch is done using the dual enzyme process. Corn syrups are produced by either an acid-enzyme process or by a dual enzyme process. All isomerizations for the production of high fructose syrups are done enzymatically. Most bread is processed

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with enzyme additives. Egg whites are generally desugared enzymatically prior to drying. Uses of commercial enzymes in the dairy and brewing industries are growing rapidly.

The ability of enzyme manufacturers to design enzyme preparations and processes for specific food applications is limited only by the ability of the food processor to identify and communicate specific application needs to the enzyme manufacturers.

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Chapter 4

Characteristics of Some Enzymes Used in Genetic Engineering

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Fundamental knowledge of the structure, function and mechanism of DNA-modifying enzymes has been important not only in understanding how these enzymes perform a myriad of chemical reactions *in vivo* but also for the development of the field of recombinant DNA technology. The functions of the major groups of enzymes in deoxyribonucleic acid synthesis, hydrolysis and modification are reviewed, as well as some structural and mechanistic aspects of the restriction endonucleases, ligases and polymerases.

Two important commercial applications of enzymes are the use of carbohydrases in making sweeteners from cornstarch and the restriction endonucleases/ligases/polymerases in recombinant DNA technology and in nucleic acid sequence studies. Both technologies developed rapidly as a result of substantial prior basic knowledge of the enzymes. Understanding the key roles of DNA and RNA in microbes, plants and animals developed rapidly after enunciation of the Watson-Crick model, including the multitude of enzymes involved in their biosynthesis, degradation and modification (Table I).

Several enzymes are involved in nucleic acid synthesis, especially when one considers the varied nature of enzymes in each group. For example, with the polymerases, there are separate enzymes important in biosynthesis of DNA and RNA, some with specificity for size of the chain length (gap) to be completed. The enzymes have different structural properties depending on whether they are from microorganisms, plants or animals. There are multiple forms within a single cell or organism. They vary from a single polypeptide enzyme of 40,000 daltons [mammalian β-polymerase (1)], to a seven-subunit complex of about 500,000 daltons [*E. coli* DNA polymerase III (2)].

Unlike the polymerases, which add nucleotides sequentially to the 3′-OH terminus of single strands of pre-existing polynucleotides, the ligases catalyze formation of phosphodiester bonds

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Table I. Some Enzymes that Catalyze Reactions of Nucleic Acids

Enzyme	Reaction Catalyzed
Nucleic Acid Synthesis	
Polymerases	Catalyze addition of nucleoside triphosphates to 3′-OH terminals of single strands of preexisting polynucleotides (primers) with release of pyrophosphate.
Ligases	Catalyze formation of phosphodiester linkages between DNA chain segments.
RecA protein	Essential for homologous genetic recombination, DNA repair and other SOS functions as a result of DNA damage.
Reverse transcriptases	Catalyze transcription of RNA nucleotide sequence into complementary DNA (cDNA).
Nucleotidyl transferases	Catalyze attachment of mononucleotide triphosphates to the 3′-OH of the initiator polymer, with release of pyrophosphate.
Priming enzymes	Provide a free 3′-OH at terminal end of initiator strand to serve as acceptor for transfer of the first nucleotide and formation of first phosphodiester bond in DNA synthesis.
Poly(A) adding enzymes	Attach AMP residues, from ATP, to the 3′, 5′ phosphodiester bond.
Capping enzyme	Attaches 7-methylguanosine to the penultimate nucleoside of RNA via a 5′, 5′-triphosphate bridge.
Qβ replicase	Replication of viral RNA.
Nucleic Acid Hydrolysis	
Nucleases	Catalyze hydrolysis of the phosphodiester bonds of polynucleotides.
Restriction endonucleases	Catalyze hydrolysis of the phosphodiester bonds of polydeoxyribonucleotides. Strict specificity for double stranded DNA and extended nucleotide sequences.

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Table I. Continued.

Enzyme	Reaction Catalyzed
Nucleic Acid Modification	
Kinases	Catalyze the transfer of the α-phosphate of a nucleoside 5′-triphosphate to the 5′-OH terminus of a deoxyribonucleic acid or ribonucleic acid molecule.
Topoisomerases	Catalyze changes in the topological structure of DNA molecules by breakage and resynthesis of the same phosphodiester bond in DNA strands.
Gyrase	Type II topoisomerase that catalyzes conversion of relaxed DNA to a superhelical form; requires ATP.
Unwinding enzymes	Catalyze separation of complementary strands of DNA; require ATP.
Methylases	Catalyzes transfer of methyl groups from donor S-adenosyl-L-methionine (AdoMet) to specific bases of acceptor DNA molecules.
Insertases	Catalyze the reinsertion of a missing base into the appropriate apurinic or apyrimidinic AP sites of DNA.
Glycosylases	Catalyze hydrolysis of nucleoside bases-glycosidic bonds, resulting in production of an apurinic/apyrimidinic (AP) site in DNA and a free nucleoside base.
Alkaline phosphatase	Catalyzes hydrolysis of monophosphate ester groups from compounds, including from the 3′-and 5′-terminal ends of nucleic acids and nucleotides.

between segments within double-stranded DNA chains. By use of restriction endonucleases, segments of double-stranded DNA can then be excised from a donor and the excised segments can then be covalently inserted into the host DNA by using ligases.

Commercially available restriction endonucleases of well-defined specificity and purity (Table II) have made it possible for recombinant DNA technology to be applied by biologists and chemists with little knowledge of the basic protein chemistry and enzymology that undergird this field. In fact, much of the basic knowledge of these endonucleases was developed in the 1960-1981 period, making the modern era of molecular biotechnology possible.

In the remainder of this paper, I shall restrict discussion to the restriction endonucleases, the ligases and the polymerases as being the most important in genetic engineering.

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THE RESTRICTION ENDONUCLEASES

Type II restriction endonucleases recognize specific base sequences in double-stranded DNA, and cleave both strands of the duplex. More than 600 restriction endonucleases have been reported in the literature. Some 121 restriction endonucleases are listed in Current Protocols in Molecular Biology as commercially available (3). Table II, adapted from the 1988 Sigma Chemical Company Catalog, lists 35 of the endonucleases available from that source. Endonucleases are found in many bacteria where their function is to hydrolyze foreign DNA introduced by phage infection, conjugation or transformation. The first restriction endonucleases were discovered and isolated in 1968 (4,5).

Restriction endonucleases are invaluable in recombinant DNA applications and in sequencing DNA. As shown in Figure 1, specific restriction endonucleases hydrolyze the phosphodiester linkages at precise locations in DNA. In a plasmid, such as pBR322, a portion of the chain can be excised by use of two restriction endonucleases, such as Bsm I and Pvu II in Figure 1, removing a fragment containing 714 base pairs. A fragment of similar size, with the right base sequence at the ends, can be excised from a porcine DNA molecule (for example, the region encoding porcine insulin) with restriction endonucleases and, using DNA ligase to covalently attach the fragment into the vector (here pBR322), the gene for insulin can be inserted into the host DNA. Proper insertion of the modified plasmid into a bacterium (*E. coli* for example) permits expression of porcine insulin by the *E. coli* (Fig. 2).

SPECIFICITY. Restriction endonucleases are highly specific in hydrolyzing doubled-stranded DNA. Not only do they recognize a specific sequence of four to six base pairs (up to 11 base pairs in case of Bgl I; see Table II) but the base pairs must be *palindromic* in the two strands of DNA. The cleavage sites possess twofold rotational symmetry as shown in Scheme I for restriction endonuclease Aat II from *Acetobacter aceti* (Table II; A, T, G, C indicate deoxyribonucleotides in this paper). In this example, the T-C phosphodiester bond in each strand two residues distal to the symmetry axis is cleaved by restriction endonuclease Aat II.

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Table II. Some Commercially Available Restriction Endonucleases[a]

Enzyme	Specificity	No. cleavage sites in λDNA
Aat II	5´-GACGT/C-3´	10
Alu I	5´-AG/CT-3´	>50
Bal I	5´-TGG/CCA-3´	18
Bam H-I	5´-G/GATCC-3´	5
Ban II	5´-GPuGCPy/C-3´	7
Ban III	5´-AT/CGAT-3´	15
Bcl I	5´-T/GATCA-3´	5[b]
Bgl I	5´-GCCNNNN/NGGC-3´	12[b]
Bgl II	5´-A/GATCT-3´	6
Bst EII	5´-G/GTNACC-3´	8[b]
Cla I	5´-AT/CGAT-3´	15
Eco RI	5´-G/AATTC-3´	5
Fsp I	5´-TGC/GCA-3´	15
Hae II	5´-PuGCGC/Py-3´	>30
Hae III	5´-GG/CC-3´	>50
Hha I	5´-GCG/C-3´	>50
Hinc II	5´-GTPy/PuAC-3´	34
Hind II	5´-GTPy/PuAC-3´	34
Hind III	5´-A/AGCTT-3´	6
Hinf I	5´-G/ANTC-3´	>50
Hpa I	5´-GTT/AAC-3´	6[b]
Hpa II	5´-C/CGG-5	>50
Kpn I	5´-GGTAC/C-3´	2
Mbo I	5´-/GATC-3´	8[c]
Mbo II	5´-GAAGA$(N)_8$/-3´	>50
Pst I	5´-CTGCA/G-3´	18
Sac I	5´-GAGCT/C-3´	1[d]
Sal I	5´-G/TCGAC-3´	2
Sau 3A I	5´-N/GATC-3´	50
Sin I	5´-G/G(A,T)CC-3´	17
Sma I	5´-CCC/GGG-3´	3
Sph I	5´-GCATG/C-3´	6
Taq I	5´-T/CGA-3´	>50
Xba I	5´-T/CTAGA-3´	4
Xho I	5´-C/TCGAG-3´	1

[a]Sigma Chemical Co. Catalog Feb. 1988; T, A, C, G specify deoxyribonucleotide residues; (N, nucleotide; Pu, purine; Py, pyrimidine; and /, point of cleavage.

[b]Using Adenovirus 2 DNA.

[c]Using SV40 DNA.

[d]Eco RI fragments of λDNA.

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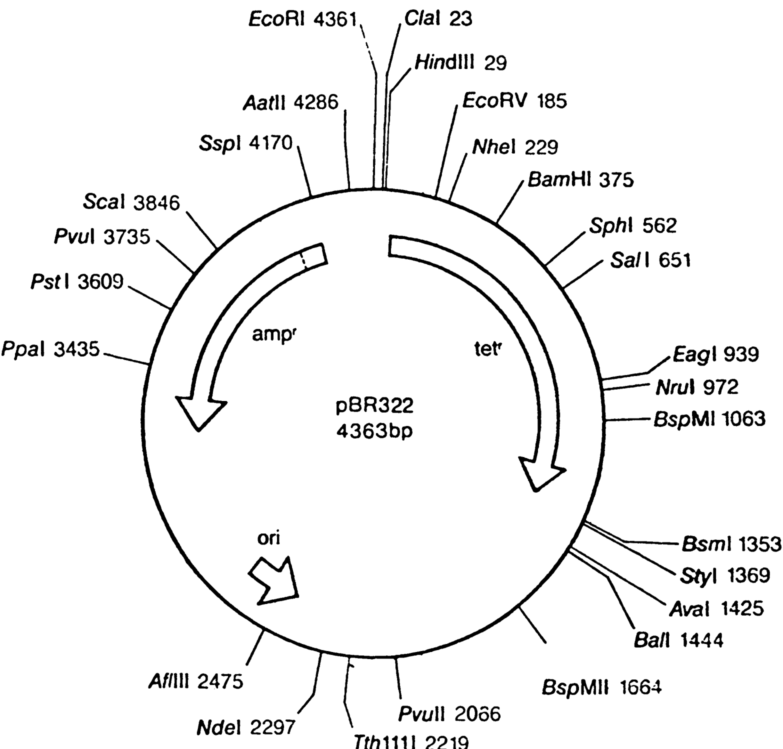

Figure 1. Some restriction endonucleases hydrolysis sites on *Escherichia coli* plasmid pBR322.

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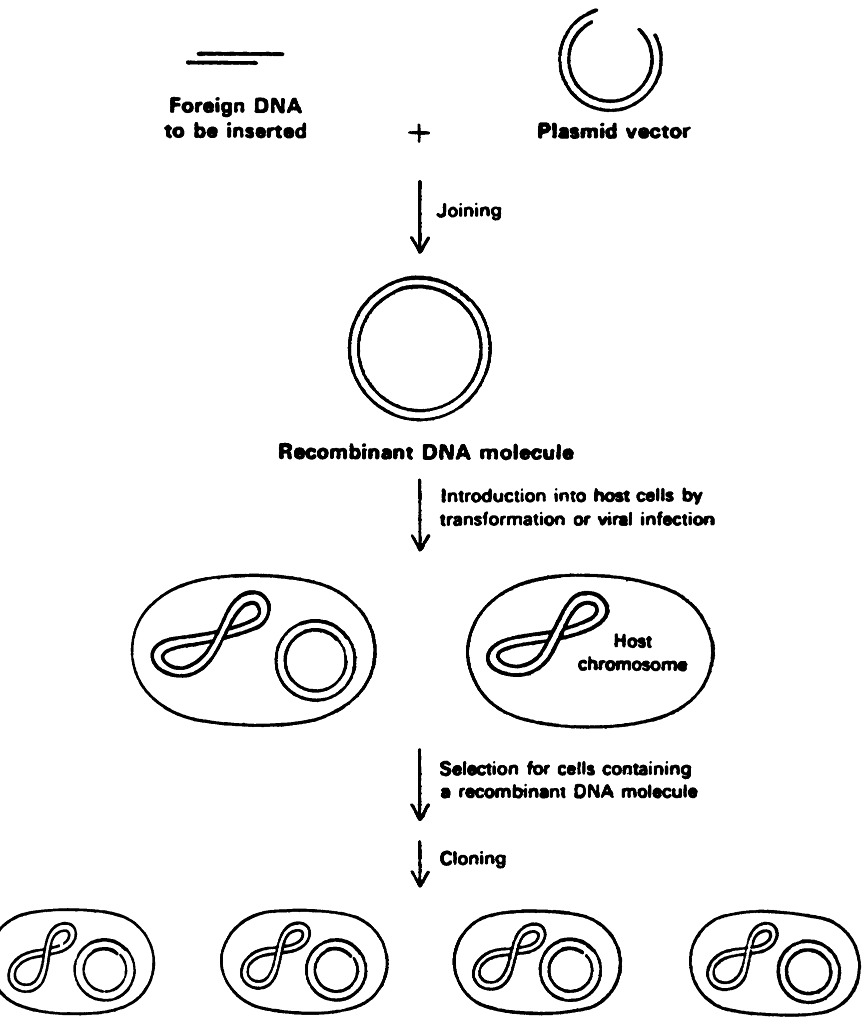
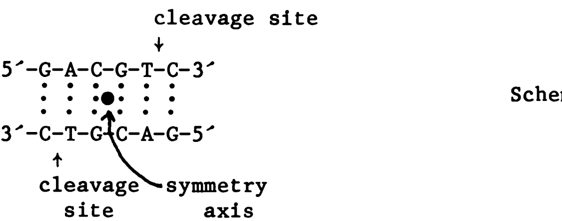


Figure 2. Schematic of insertion of foreign DNA fragment into plasmid vector and cloning of DNA molecules in host cells.



Scheme I

The specificities of some of the restriction endonucleases listed in Table II are included in Figure 3. The cleavage sites may be staggered or even. The high specificity of restriction endonucleases permits precise and reproducible cleavage of phosphodiester bonds in DNA substrates. They are indispensable tools for sequencing very long strands of DNA, isolating genes, analyzing chromosome structures and creating new DNA molecules that can be cloned (recombinant DNA technology). Obviously the enzymes cleave variable numbers of bonds, depending on a given DNA's sequence. For example, as shown in Table II, restriction endonucleases Xho I, Sal I, Hind III and Sau 3A I hydrolyze 1, 2, 6 and 50 sites in λDNA, respectively.

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ECO RI ENDONUCLEASE. Eco RI endonuclease is one of the better studied endonucleases. It is a small protein of 276 amino acid residues (31,065 daltons) of known sequence (6,7). The active enzyme contains two subunits (8,9) and hydrolyzes the phosphodiester bond between the deoxyguanylic and deoxyadenylic acid residues of duplex 5′-GAATTC-3′. On hydrolysis, there is an inversion of configuration at the reactive phosphorus (10) indicating there is an odd number of chemical steps in the overall reaction. This could mean that the endonuclease does not form a covalent intermediate with the DNA. Hydrolysis of DNA requires Mg^{2+}, but binding of duplex 5′-GAATTC-3′ occurs in the absence of Mg^{2+} with a dissociation constant of 10^{-11} M (11,12). Eco RI endonuclease does not cleave duplex 5′-GAATTC-3′ when the central adenine moiety of either one or both chains is methylated (13). DNA of the host organism is protected from hydrolysis by its own restriction endonucleases by such specific methylation, which is catalyzed by a specific methylase that recognizes the same nucleotide sequence as the restriction endonuclease.

Crystalline enzyme-substrate complex. Recently, Eco RI endonuclease has been co-crystallized with the dodeca- and tridecanucleotides 5′-CGCGAATTCGCG-3′ and 5′-TCGCGAATTCGCG-3′ (Eco RI endonuclease recognition sites underlined) (13) and the crystal structure of the complex solved at 3 Å resolution. The enzyme consists of two globular subunits forming a crevice on one side of the molecule. Each subunit forms a single domain consisting of a five-stranded β sheet surrounded on both sides by α-helices. One subunit of the enzyme is shown in Fig. 4, depicting the crevice at the upper center part.

The complex is formed by binding of DNA into the crevice (Fig. 4) with the major groove of the polynucleotide in contact

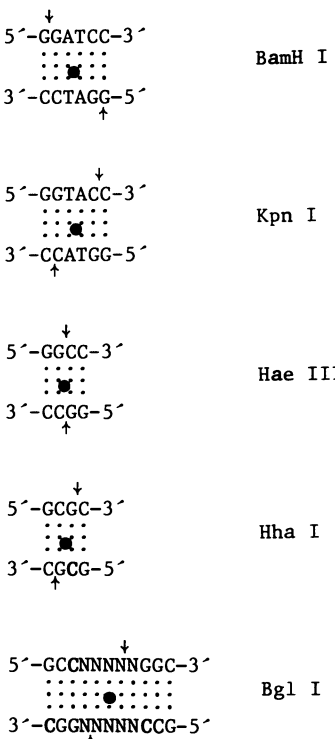

Figure 3. Recognition and cleavage (arrows) sites of double stranded nucleotide sequences by some restriction endonucleases. The closed circle indicates axis of twofold rotational symmetry of the two strands.

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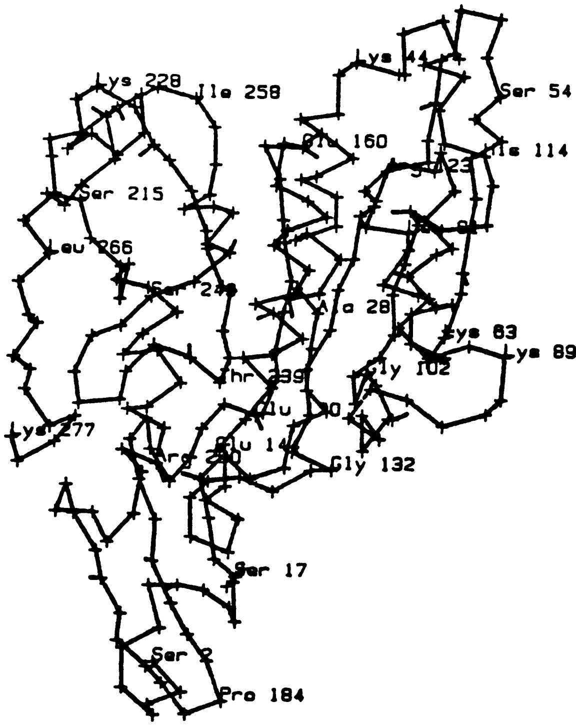

Figure 4. α-Carbon backbone of one polypeptide subunit of Eco RI endonuclease. (Reproduced with permission from Ref. 13. Copyright 1986 American Association for the Advancement of Science.)

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with the enzyme and the minor groove exposed to the solvent. The complex has twofold symmetry as shown in Figure 5. The N-terminal end of each subunit of the enzyme wraps around the double stranded polynucleotide, like arms. More details of the complex are given in Figure 6, showing the backbone schematic drawing of one subunit of the dimeric endonuclease and both strands of the polynucleotide.

The endonuclease-polynucleotide complex is stabilized by 12 hydrogen bonds that determine the substrate specificity of the enzyme (Fig. 7). As shown in Figure 7, six hydrogen bonds between the amino acid side chains of Glu144, Arg145 and Arg200 of the enzyme with the purine and pyrimidine bases of the hexanucleotide specificity region of the substrate are contributed by each subunit. This explains the basis of the twofold rotation symmetry illustrated in Figure 3. In solution, these interactions are more likely ion pairs (9).

When Mg^{2+} is infused into the complex, there is an isomerization of the complex to give the active conformation. McClarin et al. (13) suggested that "the isomerization plays the important functional role of enhancing the specificity of Eco RI endonucleases by allosteric activation." Halford and Johnson (14), in an elegant series of single turnover experiments, have shown also that a conformational change in the enzyme, after DNA is bound, is required before it can cleave DNA. Depending on whether such a conformational change occurs in one or both of the enzyme subunits, one or both strands of DNA are cleaved at the scissile bonds during the lifetime of the enzyme-DNA complex. Under conditions used in steady state experiments, the bonds in both strands are cleaved.

Catalysis by enzyme. The specific events resulting in catalysis of hydrolysis of the phosphodiester bonds are not determinable directly from the X-ray crystallographic structure. Determinable are the physical shape changes (13). When double-stranded polynucleotides (including DNA) with the correct sequence of base pairs bind into the active site of the enzyme, distortions of the double strands occur. These distortions are concentrated into separate features that are localized disruptions of the double helical symmetry. Unwinding of the double helix results in widening of the major groove of the polynucleotide. The phosphate-phosphate distances of the backbone of the polynucleotide are increased by approximately 3.5 Å, while there is no change in the distance between the purine and pyrimidine bases of the two strands. These physical changes result in neokinks in the chains. When Mg^{2+} is added to the complex, conformational changes (isomerization) occur resulting in activation of the enzyme and cleavage of the scissile bonds. Hydrolysis of the phosphodiester bond of the nucleotide occurs at the 3′-phosphate position (Fig. 8). Most likely the mechanism is a general acid-general base catalyzed reaction, such as is known to occur with ribonuclease A where His12 serves as a general base and His119 serves as the general acid (Fig. 9). No covalent intermediate would be formed, which is consistent with data on the mechanism of Eco RI endonuclease (10).

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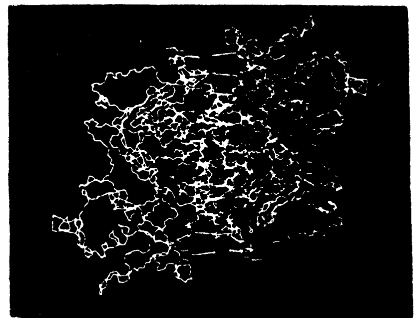

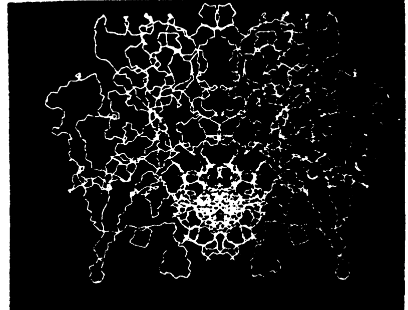

Figure 5. α-Carbon structure of Eco RI endonuclease-DNA complex. The left part shows a front view with double-stranded DNA (heavier white lines running vertically through center) setting in the crevice formed by the two subunits of the enzyme. The right part is a projection from the "top" with axis 90° from that on the left. (The double-stranded DNA is the circular structure at bottom center.) (Reproduced with permission from Ref. 13. Copyright 1986 American Association for the Advancement of Science.)

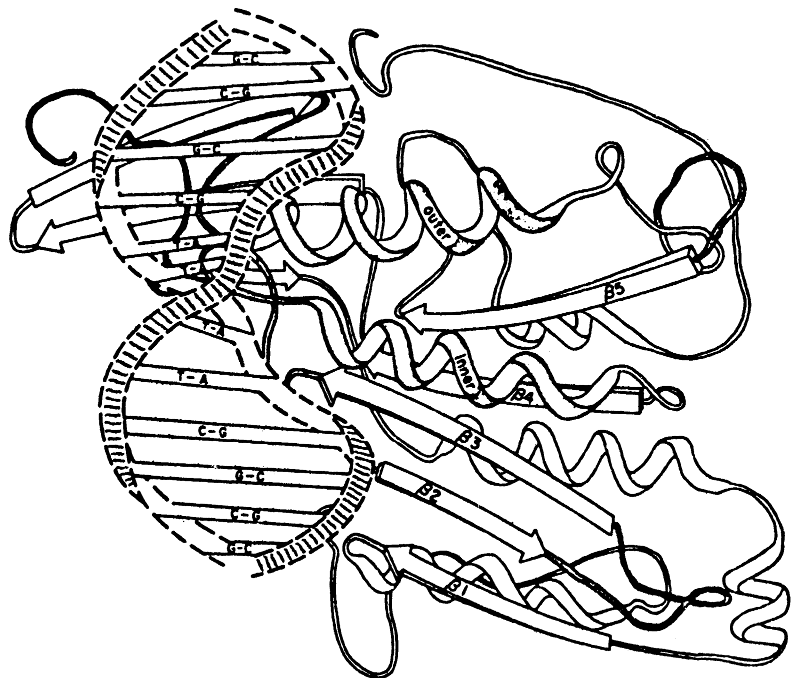

Figure 6. Schematic drawing of the α-carbon backbone of one subunit of Eco RI endonuclease and both strands of DNA in the complex. (Reproduced with permission from Ref. 13. Copyright 1986 American Association for the Advancement of Science.)

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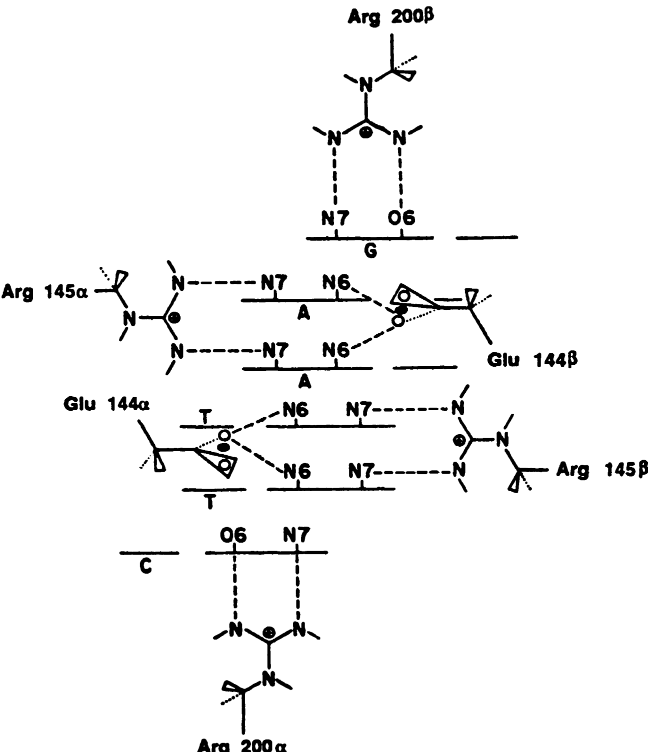

Figure 7. Schematic representation of the hydrogen bond interaction of the deoxynucleotide sequence C-T-T-A-A-G with Arg145, Arg200 and Glu144 residues of the α and β subunits of Eco RI endonuclease. (Reproduced with permission from Ref. 13. Copyright 1986 American Association for the Advancement of Science.)

Figure 8. Hydrolysis of the 3′-O-P bond in a nucleotide by a restriction endonuclease.

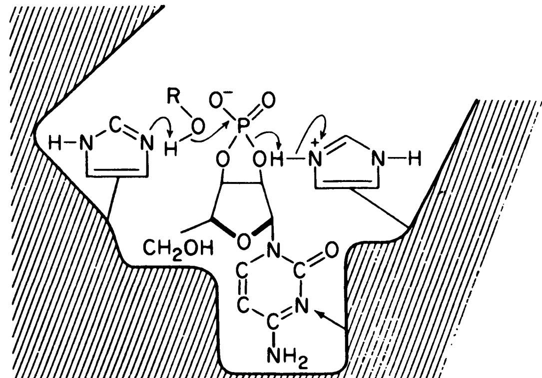

Figure 9. Proposed transition state in the ribonuclease A-catalyzed hydrolysis of cytidine 2′-3′-phosphate. Two histidine residues of the enzyme participate in catalysis.

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McLaughlin et al. (15) have recently examined effects of changes in the functional group pattern of a series of polynucleotides for binding and catalysis by Eco RI. They reported that only the exocyclic amino group of the outer adenine residue of the substrate is necessary for efficient catalysis although both are involved in complex formation (13). They found that the endonuclease could discriminate between the inner and outer A-T base pairs. Furthermore, addition of an amino group to the minor groove of the nucleotide at the outer A-T base pair did not prevent binding to the enzyme active site but the scissile bond was not cleaved.

DNA LIGASES

DNA ligases catalyze the joining of two DNA chain segments or the closure of a single DNA chain, by condensation of the 5′-phosphoryl group with the adjacent 3′-hydroxyl group, coupled with hydrolysis of the pyrophosphate group of NAD^+ (*E. coli* enzyme) or ATP (T4 phage or eukaryotic enzymes).

E. coli DNA ligase is a single-chain protein of 74,000 daltons, thought to be somewhat elongated in shape (16). The T4 DNA ligase is also a single-chain protein of approximately 68,000 daltons, also elongated in shape (17). Bacteriophage T7 DNA ligase is 41,133 daltons (18), while mammalian cells contain at least two DNA ligases of about 200,000 and 85,000 daltons (19).

The mechanism of DNA ligase has been extensively studied as summarized by Lehman (20) and Engler and Richardson (21). The overall reaction catalyzed by *E. coli* DNA ligase consists of three steps as shown in Figure 10. In the first step, the ε-amino group of a lysyl residue of the ligase, in a nucleophilic attack on the adenylyl phosphorus of NAD^+, is phosphorylated to give the adenylated ligase with elimination of nicotinamide mononucleotide (NMN). In the second step, the adenylyl group is transferred from the enzyme to the 5′-phosphoryl terminus of the nicked DNA to form a pyrophosphate bond. In the third step the 3′-OH group, in a nucleophilic attack on the 5′-phosphoryl group, forms a phosphodiester bond, with elimination of AMP, thereby joining the two ends of the nicked chain. There are two covalent intermediates formed in the reaction, both by nucleophilic attack mechanisms. T4 DNA ligase catalyzes a similar condensation reaction except that in the first step there is a nucleophilic attack by the ε-amino group of a lysyl residue of the enzyme on the adenylyl phosphorus of ATP, with elimination of pyrophosphate (PP_i). T4 DNA ligase ligates blunt ends of DNA where both strands have been nicked (22). Presumably, it does this by duplicating the reaction (Fig. 10) for both strands. Both ligases condense strands with cohesive (sticky) ends.

THE POLYMERASES

Both DNA and RNA polymerases have been isolated and studied. I shall concentrate on DNA polymerase and specifically DNA polymerase I from *E. coli* in this paper.

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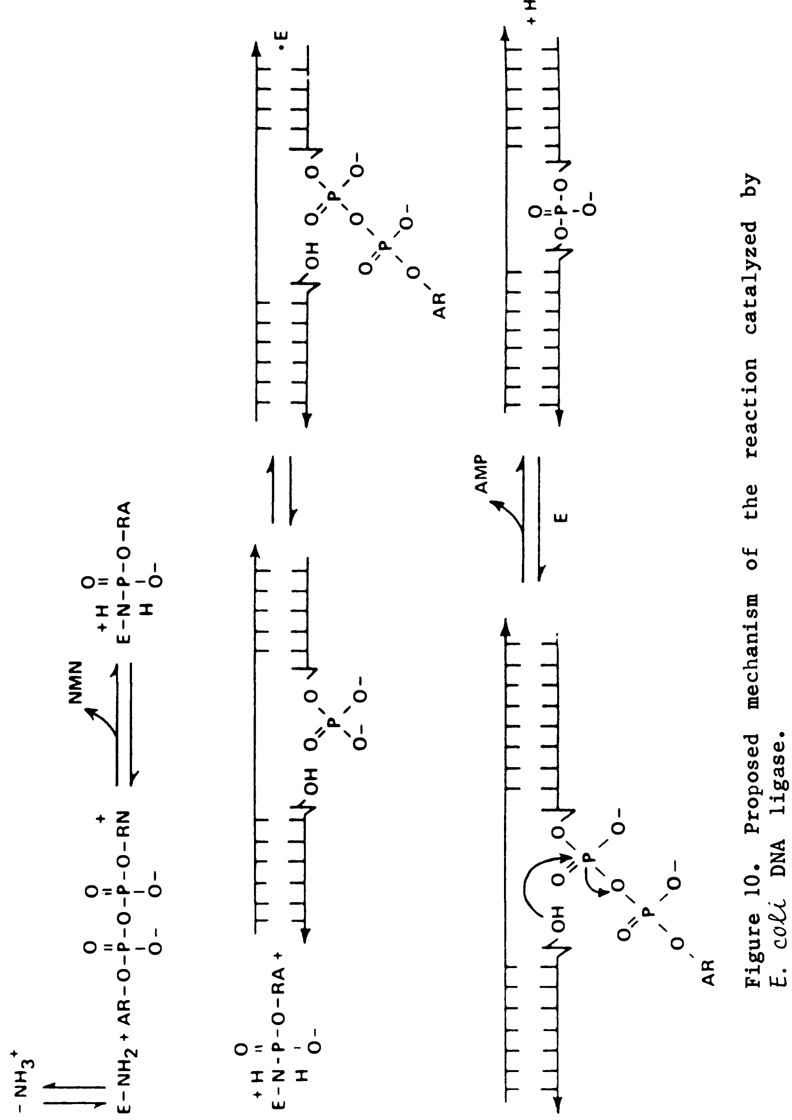

Figure 10. Proposed mechanism of the reaction catalyzed by *E. coli* DNA ligase.

DNA polymerase I, obtained from wild-type *E. coli* in which there are about 400 molecules per bacterium, or by molecular cloning of the *polA* gene in a lambda transducing bacterium, is a single-chain protein of 103,000 daltons. The protein is approximately spherical, folded into three domains (Fig. 11), and contains a substantial amount of α-helical structure (Fig. 12)(23). The enzyme contains one sulfhydryl group which can be modified without loss of activity. The active center of the enzyme contains three distinct but closely juxtaposed binding sites for the DNA template, the nucleotide primer and the deoxynucleoside triphosphates (24). The four deoxynucleoside triphosphates appear to be accommodated equally well in the binding site. There is also a Mg^{2+}-binding site.

DNA polymerase I can be cleaved by limited proteolysis into a 35,000-dalton fragment containing all the original 5′→3′ exonuclease activity, and a 68,000-dalton fragment (Klenow enzyme) with all the polymerase and 3′→5′ exonuclease activities. The exonuclease activities play a role in editing out mismatches of nucleoside units and in removing modified pyrimidine bases (damaged by UV light for example; 5′→3′ exonuclease activity only). A schematic drawing of the three domains of DNA polymerase I, each containing one of the three activities, is shown in Figure 11.

The portion of structural gene for *E. coli* DNA polymerase I Klenow fragment has been cloned into an expression vector (25), permitting production of large amounts of this segment of the polymerase. X-ray diffraction data indicate that the Klenow fragment has the tertiary structure shown in Figure 12 (23). The N-terminal residues 1-323 (not shown) of DNA polymerase I are thought to contain the 5′→3′-exonuclease activity (Fig. 11). Residues 324-517, which provide the 3′→5′-exonuclease activity, consist of six α-helical regions (A-F) and five β-sheets (1-5), which are mostly parallel. The domain has a central core of β-pleated sheets (1-4) with α-helices on both sides. In crystal form, the enzyme binds one molecule of dTMP and a divalent metal ion in this domain. The divalent metal ion is bound to the protein by the carboxyl groups of Asp335, Asp501 and Glu357 and the 5′-phosphate of dTMP.

The remainder of the molecule (residues 518-928) is a domain with a very deep cleft, about 20-24 Å wide and 25-35 Å deep (23). The bottom of the cleft is formed from six antiparallel β-sheets and the walls of α-helices. Steitz and Joyce (23) compared the cleft to a structure similar in shape to a right hand grasping a rod. "Thus, one side of the cleft forms a wall, 50 Å long, that can be compared with the curled-over fingers of a right hand. The other side of the cleft is formed primarily by two long α-helices, I and H, projecting from the protein like a thumb. At the tip of the thumb-like protrusion and hanging over the crevice are 50 amino acid residues (not shown in Fig. 12) that appear to be partially disordered in the crystal." Evidence that this is the binding site for double-stranded DNA comes from model building based on the X-ray data (23) and from two mutations, *pol5* and *pol6*, of DNA polymerase I that change amino acids in the cleft region and change binding of DNA to the enzyme (26).

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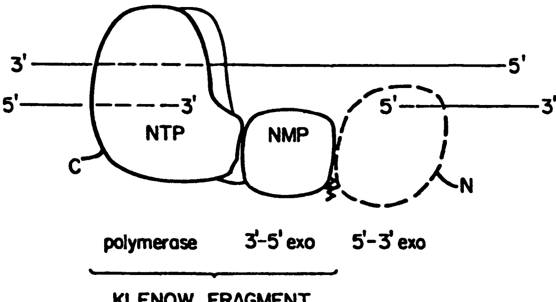

Figure 11. Schematic drawing of the three-domain structure of *E. coli* DNA polymerase I. (See text for details.) (Reproduced with permission from Ref. 23. Copyright 1987 Alan R. Liss, Inc.)

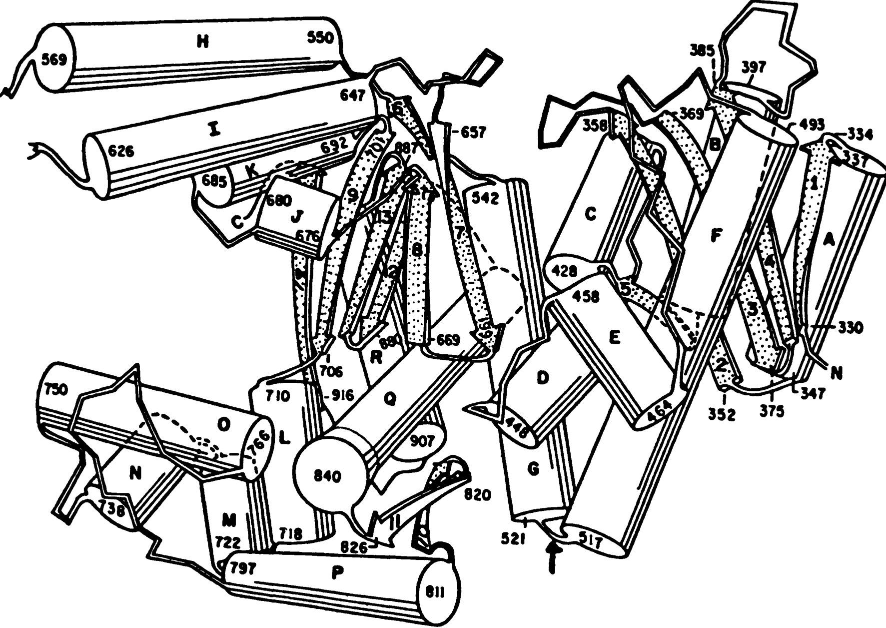

Figure 12. Tertiary structure of the Klenow fragment of *E. coli* DNA polymerase I, based on X-ray crystallographic data. The α-helices are represented by tubes (lettered) and β-sheets by arrows (numbered). (Reproduced with permission from Ref. 23. Copyright 1987 Alan R. Liss, Inc.)

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E. coli DNA polymerase I catalyzes the elongation reaction shown in Equation 1.

$$(\text{Deoxynucleotide})_n + \text{dNTP} \xrightleftharpoons{Mg^{2+}} (\text{Deoxynucleotide})_{n+1} + PP_i \qquad (1)$$

As indicated above, the enzyme also possesses 3′→5′ and 5′→3′ exonuclease activities (24); the 5′→3′ exonuclease activity is not present in the large proteolytic (Klenow) fragment (25).

For DNA synthesis, all four deoxyribonucleoside 5′-triphosphates (dATP, dGTP, dCTP and dTTP) must be present, as well as Mg^{2+}, a primer molecule ($(\text{deoxynucleotide})_n$ shown in Equation 1) and a DNA template. A detailed mechanism of the reaction must consider that the enzyme binds at least four compounds simultaneously, these being a deoxynucleoside 5′-triphosphate, Mg^{2+}, a deoxynucleotide primer molecule and a DNA template. The enzyme is thus able to convert an impossible five component reactant system into a unimolecular reaction, contributing substantially to the efficiency of the catalyzed reaction.

More detail of the elongation reaction catalyzed by DNA polymerase I is shown in Equation 2.

```
        OH
5′-C-C-G/                                      5′-C-C-G-A-T-A-G-C-T...
   : : :              2dATP    Mg2+               : : : : : : : : : :
   : : :          +   dCTP   ------>              : : : : : : : : : :      (2)
3′-G-G-C-T-A-T-C-G-A...  dGTP      |           3′-G-G-C-T-A-T-C-G-A...
                      2dTTP      6PPi
```

The substrate is a DNA template (bottom strand) annealed to a DNA primer possessing a 3′-OH terminus. The enzyme adds the nucleoside triphosphates to the 3′-OH terminus of the DNA primer in the order specified by the DNA template.

The chemistry of the elongation reaction catalyzed by DNA polymerase I is shown in Figure 13. The enzyme catalyzes the nucleophilic attack of the 3′-OH terminus of the primer molecule on the α-phosphorus of the deoxyribonucleoside triphosphate to form a new phosphodiester bond with release of pyrophosphate. Elongation of the DNA chain proceeds in the 5′→3′ direction at a rate of approximately ten nucleotides per second per molecule of DNA polymerase I. It is thought that the reaction is processive, in that many nucleotide units are added without release of the enzyme from the template.

Other DNA polymerases are known. DNA polymerase II and III are produced by the *polB* and *dnaA* genes of *E. coli*. They are similar to DNA polymerase I in most properties. They differ however, in template preferences. While DNA polymerase I acts best to fill in extended single-stranded regions near double-helical regions, DNA polymerases II and III act optimally on double-stranded DNA templates that have short gaps.

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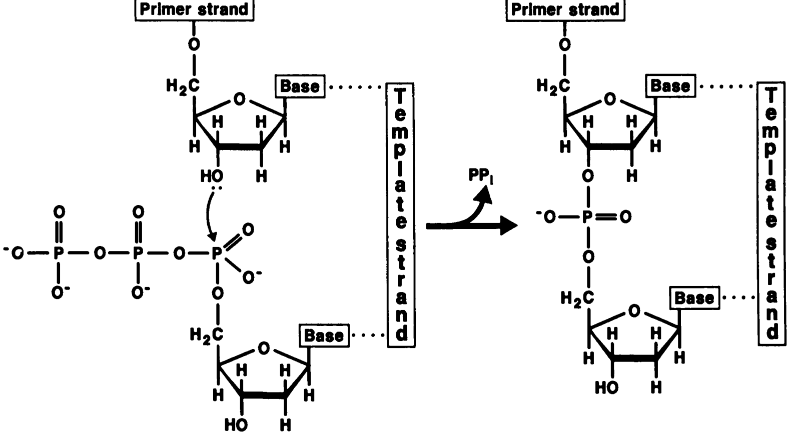

Figure 13. Proposed mechanism of the reaction catalyzed by DNA polymerase I.

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Chapter 5

Plant Cells for Secondary Metabolite Production

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Plant cell cultures represent a potentially rich source of secondary metabolites of commercial importance and have been shown to produce them in higher concentrations than the related intact plants. However, plant cell cultures often produce metabolites in lower concentrations than desired and commonly store them intracellularly. These limitations can be overcome by product yield enhancement procedures, including immobilization of cultured cells, and permeabilization, or ideally using a combined immobilization/ permeabilization process with retained plant cell viability. Complex coacervate capsules consisting of chitosan and alginate or carrageenan proved to be effective biomaterials for entrapment, controlled permeabilization of cells and to allow control of capsule membrane diffusivity.

Using immediate precursors of desired food aroma compounds also increased metabolite yields. For example, by applying ferulic acid to cultured *Vanilla planifolia* cells, vanillin concentration could be enhanced as compared to untreated cells. Vanilla concentration was also increased in green vanilla bean extracts when the cells were treated with β-glucosidase.

Microbial polysaccharides have been shown to stress plant cells, resulting in 'elicitation' (induction) and increased metabolite synthesis. Induction of various enzymes has been reported. Chitosan successfully elicited chitinase production in carrot (*Daucus carota*) cell cultures and elicitation of desired food ingredients and processing aids via chitosan has been attempted.

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The basic concept of totipotency leading to the recent advances of plant cell culture was formulated in 1838 (1) and 1839 (2). In 1902 Haberlandt (3) reported his pioneering experiments on maintaining plant cells in a viable state away from the parent body by bathing them in simple nutrient solutions. Since then, the synthetic potential of plant cell cultures was established (4). Large scale production of plant biomass from cultured cells was suggested as early as 1962 (5). Plant cell cultures can now be established for a wide range of higher plants species (6) and represent a potentially rich source of commercially important secondary metabolites (7,8).

Plant cell and organ cultures can produce higher metabolite concentrations than found in the corresponding intact plant organs (6, 9). However, plant cells grown in culture may also produce lower quantities of the desired secondary metabolites which are commonly stored intracellularly. The challenges to increase product yield and to enhance the release of secondary metabolites can be met in various ways (7). These include immobilization (9), permeabilization (10,11), the use of precursors (12,13), and the induction of secondary metabolite production via elicitors (14).

Immobilization of Cultured Plant Cells

Nunez and Lema (15) viewed the main objective in immobilizing a biologically active agent as the concentration of its activity in as small a volume as possible. This requires a procedure (16) which confines a catalytically active enzyme or cell inside a reactor system that prevents its entering the mobile phase carrying the substrate and the product. Some of the advantages of plant cell immobilization are listed in Table I. The advantages of cell immobilization have long been recognized including its importance in nitrogen fixation in legumes, the adherence of fluvial microorganisms to sand and stone or the production of vinegar using woodshavings as support medium (15).

Table I. Some advantages of plant cell immobilization[a]

Advantage	Reason/Consequence
Reuse of biocatalyst	entrapment
Maintenance of stable and active biocatalyst	elimination of non-productive growth phase; reduced risk of declining productivity from a selected cell line
Protection of cells by matrix	simplification of scale up; protection from physical damage
Employment of continuous process	continuous removal of metabolic inhibitors; improved process control and product recovery
Increased productivity	increased cell densities; elimination of growth phase

[a] Adapted from (9, 17)

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Polymers commonly used for plant cell immobilization include agar, agarose, alginate, carrageenan, chitosan, gelatin, polyacrylamide, prepolymerized polyacrylamide (17,18) and polyurethane foam (19). Complex coacervate gel capsules have also been developed (8,10,20-22) consisting of coupled chitosan-alginate or chitosan-carrageenan networks (23).

Entrapment methods have been used almost exclusively to immobilize plant cells (17). They can be classified into three general groups (24,25): a. gel formation by ionic crosslinking of a charged polymer (ionotropic); b. gel formation by cooling of a heated polymer (thermal), and c. gel formation by chemical reaction (cross-linking, radical polymerization).

Ionic crosslinking involving marine polysaccharides, such as alginate, carrageenan, or chitosan, as charged polyelectrolytes with calcium, potassium or polyphosphate ions as low molecular weight counterions, have received much attention (9). The polycationic nature of chitosan, as opposed to the polyanionic properties of most other marine polymers, provides various unique properties and offers a wide range of applications in food biotechnology (26,27). The polycationic properties of chitosan permit its use as a high molecular weight counterion in polyelectrolyte complex formation resulting in a coupled network consisting of chitosan and one or more polyanionic polymers (Figure 1). Mechanical stability of the complex coacervate capsules (with a liquid core) was improved by a combination of high and low molecular weight counterions, as well as by the addition of glucose (Table II). The use of glucose has additional practical relevance allowing shorter reaction times for bead formation which is essential for the viability of the entrapped cells, and lower alginate concentrations, which - due to the decreased viscosity of the alginate solution - provides better conditions for capsule formation. The mechanical stability of the chitosan/alginate gel capsules decreases with increasing esterification of alginate but gel stability was improved by autoclaving the capsules in Trizma (tris(hydroxymethyl)aminomethane) buffer solution (Table III). This is of importance for food biotechnology applications of the complex coacervate capsules (e.g. microencapsulation) where products require autoclaving.

Since the gel matrix provides a mass transport barrier, it can affect viability of entrapped plant cells as well as the release of desired plant metabolites. Consequently, adequate diffusivity of the polymer network is essential for development of immobilized plant cell reactors. Capsule wall properties of chitosan-alginate coacervate gel capsules are affected by pH, buffer treatment including phthalate, borax and tris(hydroxymethyl) amino- methane buffer, as well as by various types of polymers (20, 21, Pandya, Y. and Knorr, D., Univ. of Delaware, unpublished data). For example, degree of esterification of alginates used (20), chitosan concentration and degree of deacetylation of the chitosan (resulting in chitosan products of different molecular weight and viscosity) were shown to alter gel diffusivity. The effects of chitosan concentration and viscosity of alginate-chitosan coacervate capsules on the release of low molecular weight food dyes from such capsules, is shown in Figure 2. Also, significant differences in gel permeability occurred when the alginate of the complex coacervate gel

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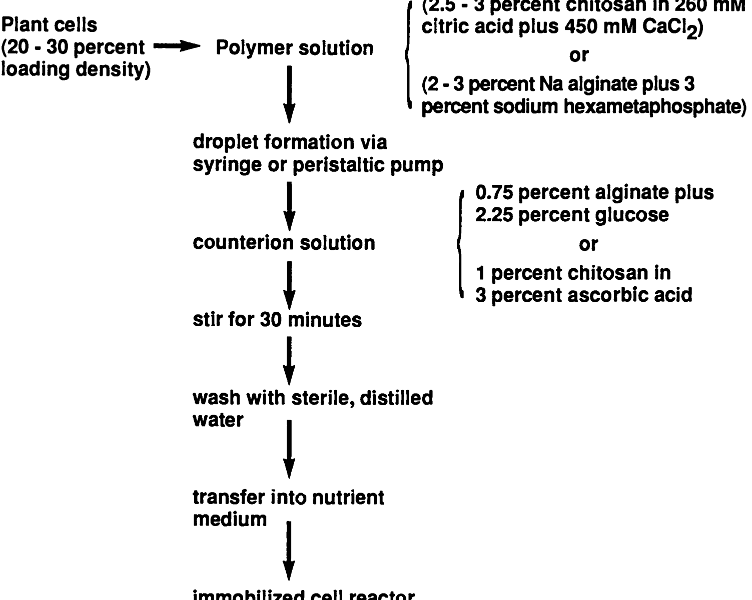

Figure 1. Simplified protocol of chitosan/alginate or alginate/chitosan complex coacervate capsule formation (adapted from 10,20,22).

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Table II. Effect of Calcium Chloride and Glucose on Mechanical Stability of Chitosan-alginate Coacervate Gel Capsules[a]

Polyelectrolyte (capsule core)	Counterion (outer membrane)	Reaction Time (min)	Setting Time (hrs)	Force to Burst (Newtons)[b]
3.0% chitosan	2.0% Na-alginate	20	3.0	0.73 ± 0.30
3.0% chitosan + 460 mM $CaCl_2$	2.0% Na-alginate	5	3.0	0.10 ± 0.05
3.0% chitosan + 460 mM $CaCl_2$	2.0% Na-alginate	15	3.0	0.97 ± 0.61
3.0% chitosan + 460 mM $CaCl_2$	2.0% Na-alginate	20	3.0	10.8 ± 5.70
3.0% chitosan + 460 mM $CaCl_2$	2.0% Na-alginate + 6.0% glucose	10	0.5	9.8 ± 6.10
3.0% chitosan + 460 mM $CaCl_2$	2.0% Na-alginate + 6.0% glucose	15	0.5	16.5 ± 3.00
3.0% chitosan + 460 mM $CaCl_2$	2.0% Na-alginate + 6.0% glucose	20	0.5	11.6 ± 5.70
3.0% chitosan + 460 mM $CaCl_2$	1.0% Na-alginate + 3.0% glucose	15	0.5	24.5 ± 2.40
3.0% chitosan + 460 mM $CaCl_2$	1.0% Na-alginate + 3.0% glucose	15	3.0	24.8 ± 10.50
3.0% chitosan + 460 mM $CaCl_2$	0.5% Na-alginate + 2.25% glucose	15	0.5	19.4 ± 7.10
3.0% chitosan + 460 mM $CaCl_2$	0.5% Na-alginate + 2.25% glucose	15	24.5	17.2 ± 10.20

[a] Average capsule diameter 5.7 mm; adapted from (20)
[b] Mean ± standard deviation (N > 5)

capsules was replaced by kappa-carrageenan (Pandya, Y. and Knorr, D., Univ. of Delaware, unpublished data).

Table III. Effect of buffer treatment and esterification of alginate on the mechanical stability of chitosan/alginate coacervate capsules[a]

Setting solution	Percent of alginate carboxyl groups esterified[b]		
	0	5	12.5
	Pressure to burst[c]		
Distilled water	-	23.6	6.0
100 mM $CaCl_2$	172.5	119.6	37.2
100 mM Trizma[d] + 100 mM $CaCl_2$	149.9	108.9	37.8
100 mM Trizma[d] + 100 mM $CaCl_2$[e]	189.3	176.6	100.1

[a] Adapted from (21)
b Esterification achieved by substituting 50% esterified propylene glycol alginate for 0, 10 and 25% of sodium alginate
[c] x 10^2 Pascal; Per initial cross-sectional area of capsule
[d] Tris (hydroxymethyl)aminomethane
[e] Capsules were autoclaved

Permeabilization of Plant Cells

Secondary metabolites produced by plant cell culture are commonly accumulated in the cells. With few exceptions such as *Capsicum frutescens*, *Thalictrum minus* (9) and *Vanilla planifolia* (Knorr, D. and Romagnoli, L., Univ. of Delaware, unpublished data) cultures, which release valuable compounds such as capsaicin, berberine and vanillin, respectively, into the medium, procedures to induce product release are required to develop continuous production processes. Reported permeabilization methods include treatment with dimethylsulfoxide (DMSO), isopropanol, toluene, phenethyl alcohol or chloroform (9, 28). But as Fontanel and Tabata (9) pointed out, such treatments with organic solvents are severe and other methods of permeabilization need to be developed.

Based on the pioneering work on chitosan and induced plant membrane permeability in cultured *Glycine max* cells conducted by Young et al. (29), the release of secondary metabolites from various cell cultures into the related nutrient medium was made possible in the presence of chitosan (18, 22, 30, 31, Beaumont, M. and Knorr, D., Univ. of Delaware, unpublished data). Leuba and Stossel (32) suggested that the release of Ca^{++} and subsequent efflux of

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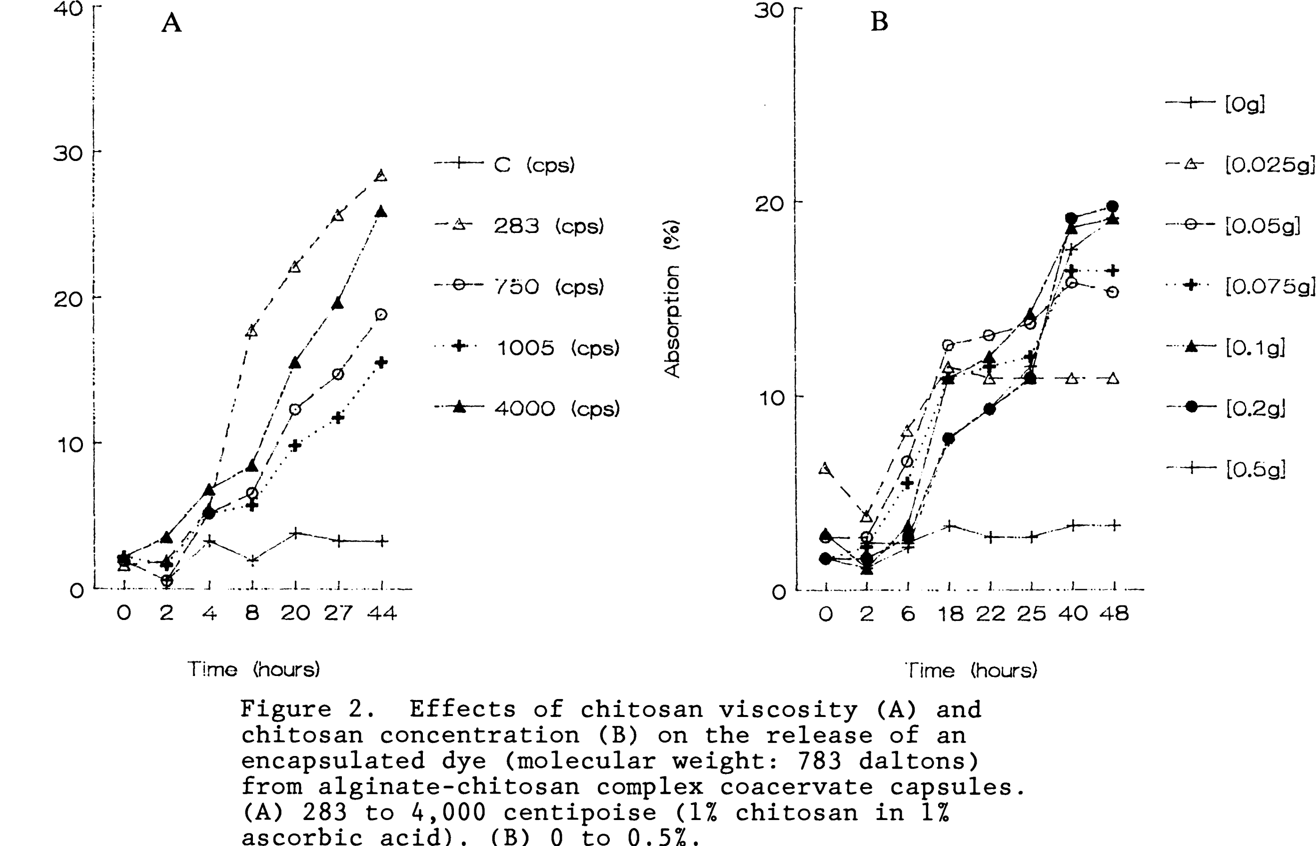

Figure 2. Effects of chitosan viscosity (A) and chitosan concentration (B) on the release of an encapsulated dye (molecular weight: 783 daltons) from alginate-chitosan complex coacervate capsules. (A) 283 to 4,000 centipoise (1% chitosan in 1% ascorbic acid). (B) 0 to 0.5%.

proteinaceous and UV-absorbing material from cultured Glycine max cells, as reported by Young et al. (29) and Young and Kauss (33) as well as their own data (32), confirmed the nonspecific action of polyamines on membrane integrity. Work by Hadwiger et al. (34) indicated that chitosan interacts with cellular DNA of plants. A linear relationship between the concentration of chitosan added to Daucus carota cells cultures and release of total protein was established in our laboratory (Figure 3) up to a chitosan concentration of 2,000 µg/mL of medium (cell concentration: 40 mg cells, fresh weight/mL medium).

Beaumont and Knorr (10) described the detrimental effect of chitosan on cell viability of Apium graveolens. Later it was found that at chitosan concentrations <250 µg/mL (Beaumont, M. and Knorr, D., Univ. of Delaware, unpublished data), plant cell viability was retained. Development of complex coacervate capsules consisting of alginate chitosan (22) and kappa-carrageenan-chitosan (10) allowed the concurrent release of secondary metabolites while still maintaining reasonable cell viability. Chitosan comprised the outer layer of the gel capsule and chitosan diffusivity could be controlled via capsule membrane permeability.

Data in Table IV show the permeabilizing effect of chitosan expressed in release of total proteins when chitosan was used as the immobilizing agent in complex coacervate capsules, but suggest low cell viability as measured by respiration. However, Beaumont and Knorr (10) reported that fresh weight of cells recovered at the end of a culture period in the growth medium is a better indicator of actual cell viability than respiration data.

Table IV. Concentration of total protein in culture medium and viability of carrot (Daucus carota) cell cultures entrapped in complex coacervate capsules[a]

Entrapment procedures	Total protein (mg/g fresh weight)	Respiration (% O_2/min)
Freely suspended cells	6.3 ± 1.7	40.9 ± 5.1
kappa-carrageenan-KCl[b]	15.3 ± 4.8	26.7 ± 26.7
kappa-carrageenan-KCl-chitosan (0.5%)[b]	33.7 ± 8.1	4.1 ± 3.6
kappa-carrageenan-KCl-chitosan (1.0%)[b]	38.6 ± 5.5	6.0 ± 1.6

[a] Two weeks after treatment (N=6); adapted from Beaumont, M. and Knorr, D., Univ. of Delaware, unpublished data

[b] 20% cell density

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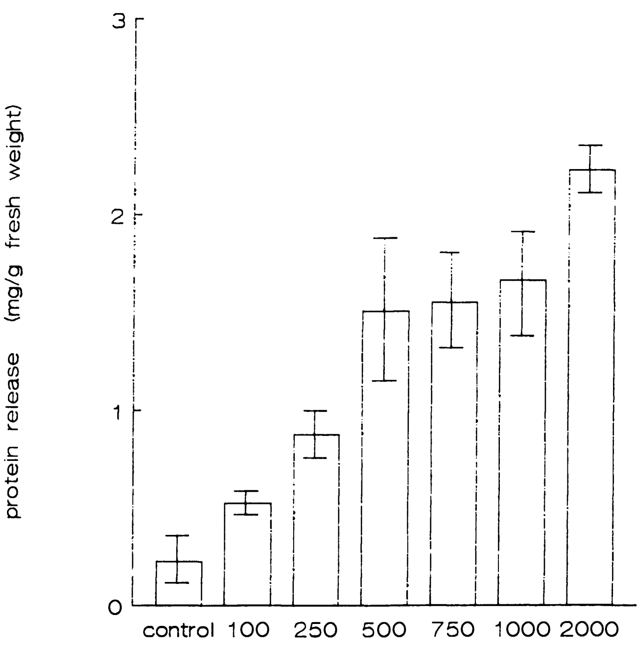

Figure 3. Relationship between addition of chitosan tc cultured Daucus carota cells and release of total protein into the culture medium (adapted from Beaumont, M.D. and Knorr, D., Univ. of Delaware, unpublished data).

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Other experiments on the chitinolytic activity of immobilized cells and culture medium of carrot cells (Table V) indicate that chitosan, in concentrations available to plant cells during the preparation of kappa-carrageenan-chitosan coacervate capsules, is detrimental to chitinase activity in plant cells and culture medium. This is also of interest in light of the data presented on the elicitor effect of chitosan on chitinase production at minute chitosan concentrations (see Table IX).

Table V. Chitinolytic activity of immobilized cells and culture medium of carrot (Daucus carota) cell cultures[a]

Cell Immobilization Procedures	Chitinase concentration (μkat/g fresh weight[b])	
	cells	medium
Freely suspended cells	5.00 ± 7.00	11.17 ± 9.00
kappa-carrageenan-KCl[c]	3.17 ± 4.33	7.17 ± 9.67
kappa-carrageenan-KCl-chitosan (0.5%)[c]	0.0	0.0
kappa-carrageenan-KCl-chitosan (1.0%)[c]	0.0	0.0

[a] Adapted from Beaumont, M.D. and Knorr, D., Univ. of Delaware, unpublished data
[b] Two weeks after immobilization
[c] Cell density 20% (w/v)

Metabolite Yield Enhancement: Treatment of Cells with Precursors and Elicitors

Secondary metabolite production has been increased by applying immobilization procedures alone (9, 19). Development of immobilized cell reactors, where cells remain viable and fully active biosynthetically after permeabilization treatment, would allow the continuous production of valuable secondary metabolites from a combined immobilization/permeabilization system as previously attempted by us (10, 18, Beaumont, M.D. and Knorr, D., Univ. of Delaware, unpublished data). This would also give higher product yields as compared to utilizing freely-suspended non-permeabilized cells.

Other approaches to increase desired product yield include optimizing culture conditions, treatment with precursors, or the use of elicitors to induce or increase biosynthesis. The strategies of selection and screening for high producing cell lines and optimization of media did not prove entirely successful (9); therefore, the use of precursors and elicitors seems to be a more promising route.

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Yeoman et al. (35) demonstrated that supplying immediate precursors of capsaicin, the hot aroma compound of pepper, to Capsicum frutescens cultures increased the amount of capsaicin produced. Feeding more general precursors such as amino acids, also increased capsaicin production but to a lesser extent than when immediate precursors were applied. Feeding intermediate precursors to Allium cepa L. callus, enhanced levels of aroma components of onion flavor (36).

Vanilla is one of the most important food flavors (37). Sahai et al. (38) suggested that ferulic acid is an immediate precursor to vanillin and vanillic acid, two key components of vanilla flavor (38). Applying a 1 mM ferulic acid solution to Vanilla planifolia callus increased vanillin concentration, as compared to the untreated samples (Table VI). Concentration of key vanilla flavor components

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Table VI. Production of vanillin in Vanilla planifolia callus cultures as affected by precursor treatment with ferulic acid[a]

Day of culture	Untreated sample	Ferulic acid treated sample
	(vanillin μg/g cells fresh weight)	
4	0.0	4.43 ± 0.19
8	1.67 ± 0.00[b]	6.08 ± 0.79
12	2.02 ± 1.20	4.78 ± 3.88
20	1.42 ± 0.17[c]	9.57 ± 0.01
24	2.08 ± 0.00	5.72 ± 0.00
28	2.26 ± 1.21[d]	7.40 ± 0.01

[a] Topical application of 1 mM ferulic acid solution as compared to untreated callus distilled water, N = 3; (adapted from (13))
[b] 9 days
[c] 21 days
[d] 27 days

(38) was also increased when β-glucosidase was added to green vanilla bean extracts (Table VII) to aid the biotransformation of gluco-vanillin (Knorr, D. and Romagnoli, L.G., Univ. of Delaware, unpublished data).

DiCosmo and Misawa (14) suggested the immense potential of plant cell culture-'elicitor' (inducer) interactions to the large scale production of secondary metabolites with the induction of shikonin formation by agar in Lithospermum erythrorhizon cell suspension cultures (39); this is so far one of the most successful examples of elicitor effects. Some reports on the induction of enzymes of plant

Table VII. Concentrations of flavor components in green vanilla bean extracts[a] after treatment with β-glucosidase

β-glucosidase treatment (mg/100 ml)	Vanillin	Vanillic Acid	4-Hydroxy-benzoic acid	4-Hydroxy-benzaldehyde
	(mg/100 ml)			
0	0.3	0.02	0.02	0.05
1	3.0	0.2	0.05	0.1
10	7.0	0.4	0.1	0.3
100	8.0	0.7	0.2	0.5

[a] 40% ethanol solution; Adapted from Romagnoli, L.G. and Knorr, D., Univ. of Delaware, unpublished data.

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cell cultures are presented in Table VIII. These data and other publications (14) indicate that cell stress commonly originates from cell wall-polysaccharides of phytopathogenic fungi. Experimental results on the effects of chitosan on the chitinolytic activity of Daucus carota cells (Table IX) suggest induction of chitinase by chitosan. At a chitosan concentration of 80 μg/mL, plant cell viability has been retained (Beaumont, M.D., Pandya, Y. and Knorr, D., University of Delaware, unpublished data). Since these experiments were carried out with chitosan obtained from crab shells, further experiments with chitosan isolated from Mucor rouxii (47) will prove whether microbially produced chitosans are more effective elicitors for the biosynthesis of certain phytochemicals than those obtained from marine organisms.

Conclusions

To date, progress achieved clearly demonstrates the potential of cultured plant cells for secondary metabolite production. Use of concurrent immobilization/permeabilization procedures, as well as precursor and elicitor treatments, may open new avenues of increasing product yields and will consequently affect the economic aspects of plant cell culture in a positive manner. However, our understanding of the many biosynthetic pathways of desired secondary metabolites is incomplete and successful industrial scale plant cell culture processes are still limited. Results of research in the area of plant cell culture will increase our understanding of the biosynthesis of plant metabolites, enhance our knowledge of plant-microorganism or plant-plant interactions and can lead to entirely new products or product lines of desirable compounds currently not available to use. Such work can also lead to development of industrial scale production processes for products now produced and recovered by conventional methods. Also, the genetic variety of the 250,000 to 750,000 plant species available remains to be explored. Presently only 5 to 15% of these species have been subject to even

Table VIII. Effects of elicitors on induction of enzymes of plant cell cultures

Elicitor	Plant cell culture	Induced enzyme	Reference
Cell wall preparations of Colletrichum lindemuthianum	Phaseolus vulgaris	phenylalanine ammonia lyase; cinnamic acid 4-hydrolase; chalcone synthase; chalcone isomerase	(40)
Cell wall preparation of yeast	Glycine max.	phenylalanine ammonia lyase	(41)
Ethylene	Phaseolus vulgaris	chitinase	(42)
Cell wall preparations of Chaetomium globosum	Daucus carota	chitinase; phenylalanine ammonia lyase	(43)
Cell wall preparations of Phytophthora megasperma	Glycine max.	phenylalanine ammonia lyase; chalcone synthase	(44)
Germ tubes of Puccinia graminis tritici	wheat plants (not in culture)	lipoxygenase	(45)
Cell wall phosphoglyco-peptides from Colletotrichum lagenarium	melon tissue	chitinase	(46)

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Table IX. Chitinolytic activity in cells and culture medium of carrot (Daucus carota) cell cultures after various treatments[a]

Treatment	Chitinase (μkat/g fresh weight)[b] cells	medium
Chitosan (5μg/mL)	10.84 ± 2.17	270.05 ± 145.20
Chitosan (80 μg/mL)	12.50 ± 4.50	281.72 ± 200.37
Freely suspended	8.67 ± 2.17	41.68 ± 8.84
DMSO (5%)	8.50 ± 3.33	43.34 ± 18.67

[a] Adapted from Beaumont, M.D. and Knorr, D., Univ. of Delaware, unpublished data
[b] 36 hours after treatment

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the most rudimentary screening for useful compounds (48). The production of new compounds is also more attractive from the scientific point of view, as well as for economic and regulatory considerations, than attempting by plant cell culture methods to "imitate" products already obtained from plants via traditional processes.

Plant cell culture methods constitute a most exciting part of food biotechnology research and development with many areas for food improvement, involving plant production, plant resistance and functionality, nutritional quality of plant foods and production of natural food ingredients (49-51).

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Chapter 6

Regulation of Starch Synthesis

Biochemical and Genetic Studies

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A key site in the regulation of starch synthesis is the enzymatic step where ADPglucose (ADPGlc) is synthesized. 3-P-Glycerate activates and orthophosphate (P_i) inhibits ADPGlc synthetase (EC 2.7.7.27) activity. Spinach leaf ADPGlc synthetase is composed of two subunits, of 51 and 54 kilodaltons (kd) mass. Their N-terminal amino acid sequences and tryptic peptide maps are different and they are antigenically dissimilar. Pyridoxal-P (PLP), an activator analog, when reduced onto ADPGlc synthetase causes the enzyme to be more active in the absence of activator. The covalently modified enzyme is very resistant to P_i inhibition suggesting that PLP binds at or close to the activator site. The activator binding site sequence is close to the carboxyl terminus of the enzyme. Starchless mutants of *Arabidopsis thaliana* have been isolated that lack or contain low ADPGlc synthetase activity. Genetic analysis showed that the deficiency of both starch and ADPGlc synthetase activity in the mutants were attributable to a single, nuclear, recessive gene demonstrating that *in vivo* leaf starch synthesis is entirely dependent on a pathway involving ADPGlc synthesis. Recent results show that the photo affinity substrate analogue, 8-azidoADPglucose ($8-N_3ADPGlc$) inactivates the spinach leaf enzyme when irradiated with UV light. ADPGlc can protect the enzyme from inactivation. When labeled $8-N_3ADPGlc$ is used, incorporation is seen mainly if not solely in the 54

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kd subunit. A partial deduced amino acid sequence of the spinach leaf ADPGlc synthetase 51 kd subunit has been obtained from two cDNA clones and compared with the known deduced amino acid sequence of the Escherichia coli and rice endosperm ADPGlc synthetases.

ADPGlc synthetase (EC 2.7.7.27) is a key enzyme involved in the synthesis of starch (1). Maize endosperm mutants, shrunken-2 and brittle-2 are deficient in ADPGlc synthetase activity. The enzyme activity is less than 10% of wild type and the mutants accumulate 25% of the starch observed in the normal endosperm (2,3). Recent results from our laboratory have described the isolation of a mutant, TL25, of Arabidopsis thaliana lacking ADPGlc synthetase activity (less than 2% of normal activity) and containing no detectable levels of starch (4). Thus at least in Arabidopsis the ADPGlc pathway accounts for 98% or more of the starch synthesis occurring both in leaf and root cap. These results also strongly indicate that both UDPglucose synthetase and starch phosphorylase play a negligible, if any, role in starch synthesis. The mutant also was not able to grow well in a 12 hour light/12 hour dark photoperiod cycle but could grow at rates similar to the normal plant under continuous light (4). This suggested that starch may be necessary for optimal growth under normal physiological conditions. It is quite possible that under growth conditions in the absence of light that the presence of chloroplast starch is required for its metabolism to provide energy and carbon for export to other parts of the plant.

Regulation of starch synthesis occurs at the ADPGlc synthetic step (1). Activation of ADPGlc synthesis is affected by 3-P-glycerate (3-PGA) and inhibition is caused by orthophosphate (P_i). These effector metabolites interact in that P_i can reverse the activation of ADPGlc synthesis caused by 3-PGA and increasing concentrations of 3-PGA can overcome P_i inhibition.

Because of the importance of ADPGlc synthetase for plant starch synthesis, efforts have been made to determine the structure of the enzyme and to relate catalytic and allosteric function to structure. The spinach leaf enzyme is composed of two subunits of 51 and 54 kd mass (5,6). The molecular mass of the native enzyme is 206,000 and presumably is a tetramer composed of two of each subunit. The subunits are antigenically dissimilar, exhibit different peptide patterns on HPLC after trypsin digestion and their N-terminal amino acid sequences are different (7). Thus it is likely that the peptide subunits are products of different genes.

Pyridoxal-5-phosphate (PLP) has been shown to be an activator analogue of 3-PGA (7,8). It activates ADPGlc synthetic rates about 5- to 6-fold and can overcome P_i inhibition as does the activator 3-PGA (7,8). Reductive phosphopyridoxylation of the spinach leaf enzyme with labeled PLP gives incorporation into both subunits in equimolar amounts (7,9). The PLP modified enzyme is less dependent on the presence of activator (3-PGA) and is more resistant to inhibition by the allosteric inhibitor, P_i (7-9). The activator,

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3-PGA, when present during the reductive phosphopyridoxylation inhibits the incorporation of PLP (7,9).

These data suggest that PLP is binding at the allosteric site. Thus the allosteric binding site may be on both the 51 and 54 kd subunits. For the 51 kd subunit, the amino acid sequence of the region where PLP binds to an epsilon amino residue of lysine has been determined (7,9). Of interest is that this sequence is very similar to a deduced amino acid sequence obtained from the nucleotide sequence of a cDNA clone of the rice endosperm ADPGlc synthetase gene (7,9). The activator binding site is situated close to the carboxyl terminus of the 51 kd subunit (9). In the case of the bacterial ADPGlc synthetases the activator binding site is at the amino terminal region of the subunit peptide (10).

In order to obtain further information on the structure-function relationships of the plant ADPGlc synthetase with respect to the substrate site we employed the use of a photo affinity substrate analogue, 8-azido-ADPGlc (11). Our preliminary results are reported here. We have also recently been able to isolate two cDNA clones of the spinach leaf ADPGlc synthetase 51 kd gene and these results are also reported.

RESULTS

Studies with 8-azido Nucleotide Substrates. Figure 1 shows that 8-azido-ATP (8-N_3ATP) is a substrate for the spinach leaf ADPGlc synthetase. The product formed was determined to be 8-N_3ADPGlc by procedures used previously (11). The isolated 8-N_3ADPGlc showed a typical spectrum of an 8-azido adenine nucleotide with an absorption maximum at 281 nm which was lost upon photolysis (12). The K_m value for 8-N_3ATP was 0.81 ± 0.05 mM, about 7-fold higher than the K_m value of 0.12 mM for the natural substrate ATP. In the absence of activator, 3-PGA, the K_m for 8-N_3ATP was increased to about 3 mM. Moreover, the V_{max} of the spinach leaf enzyme was only 0.48 $\mu mol\text{-}min^{-1}\text{-}mg^{-1}$ of product formed, only about 1% of that observed with ATP (50 $\mu mol\text{-}min^{-1}\text{-}mg^{-1}$). This relative activity seen with the azido analogue is similar to what was observed for the *Escherichia coli* enzyme (11) where the maximal velocity rates observed with 8-N_3ATP and 8-N_3ADPGlc were only 0.3 and 0.9% of those observed with the natural substrates, respectively. For the spinach leaf enzyme however, 8-N_3ADPGlc has a K_m of 80 µM compared to the K_m for ADPGlc of 225 µM. The maximal velocity for 8-N_3ADPGlc, however, is only 0.25 $\mu mol\text{-}min^{-1}\text{-}mg^{-1}$, 0.6% of that observed with ADPGlc.

Figure 2 shows that irradiation of the spinach leaf ADPGlc synthetase in the presence of 8-N_3ADPGlc, 3-PGA and Mg^{2+} resulted in loss of enzyme activity. No or slight inactivation of the enzyme, however, was observed when the enzyme was exposed to UV light without 8-N_3ADPGlc or when the enzyme was incubated with the analog in the dark. This may be due to a slight instability of the enzyme activity during the incubation period. Almost 70% inactivation was observed in the presence of 1.5 mM 8-N_3ADPGlc. Effective protection was observed with 10 mM ADPGlc. Neither UDPglucose (UDPGlc) nor ADP were as effective as ADPGlc. Figure 2A

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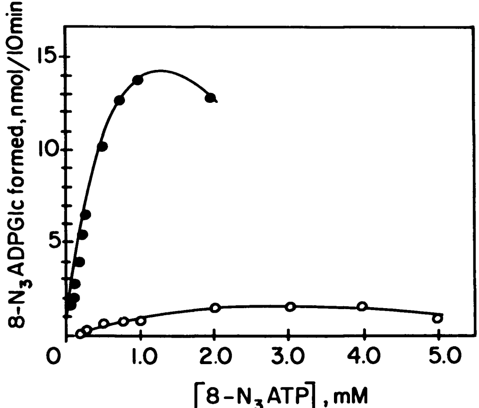

Figure 1. 8-azido-ATP saturation curves of ADPGlc-synthetase in the presence (•) or absence (o) of 1 mM 3-PGA. Enzyme was incubated 10 min at 37° at indicated concentrations of 8-N_3ATP in 100 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM [^{14}C]Glc 1-P, BSA (50µg/reaction), and pyrophosphatase (0.1 µL/reaction) in a volume of 200 µL as previously described (in ref. 5).

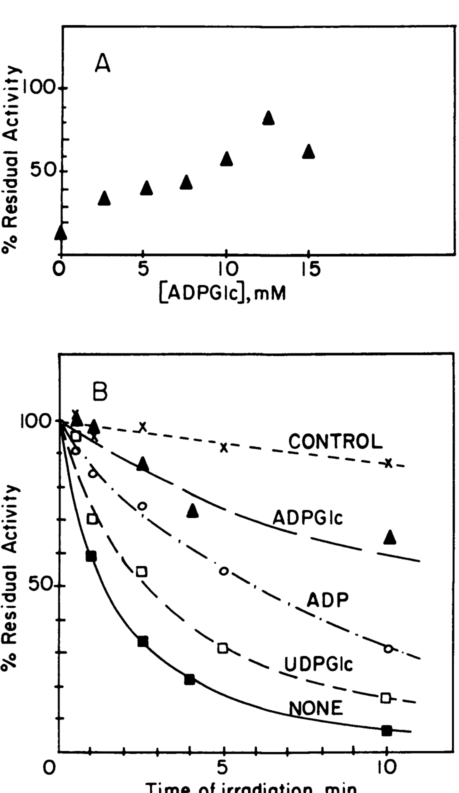

Figure 2. Photoinactivation of ADPGlc-synthetase at 1.5 mM 8-N_3ADPGlc. The mixture containing 1 μM (subunits) enzyme, 10 mM HEPES (pH 7.5), 1 mM 3-PGA, 5 mM $MgCl_2$, 1.5 mM 8-N_3ADPGlc and indicated nucleotide, was irradiated in the wells of Coor's plate with UVS-54 lamp, at a distance of 5 cm as previously described (11). The time of irradiation in experiment A was 3 min and in B it was varied as indicated. A - Effect of the varied concentrations of ADPGlc on photoinactivation. B - Effect of 10 mM ADPGlc (▲), ADP (o) and UDPGlc (□) on the rate of photoinactivation. (x) is a control sample, irradiated without 8-N_3ADPGlc. (■) is a sample photoinactivated without protecting nucleotide.

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shows that the protection by ADPGlc is concentration dependent reaching maximum protection between 10 to 15 mM. Thus, the azido nucleotides are substrates and the substrate ADPGlc is most effective in protecting from photoinactivation. This would strongly suggest that 8-N_3ADPGlc is binding at the substrate binding site and photoinactivation is occurring at or close to that site.

Labeled 8-N_3ADPGlc was prepared with uniformly labeled [^{14}C] glucose-1-P as previously described (11) and used to photoinactivate the spinach leaf ADPGlc synthetase. About 1.33 nmol of analogue was incorporated per nanomole of the native heterotetramer enzyme. The enzyme was then subjected to sodium dodecyl sulfate gel electrophoresis. As seen in Figure 3 the incorporation of radioactivity is mainly if not solely in the 54 kd subunit (lane 3). Lane 2 shows that ADPGlc partially inhibits the incorporation as the autoradiographic spot is less intense. The incorporation was 67% less in the presence of ADPGlc as indicated by the amount of label precipitated with 10% trichloroacetic acid. This would suggest that the substrate binding site is on the 54 kd subunit. There is still a possibility, however, that the catalytic/substrate site is shared between the 2 subunits but that the reactive nitrene radical reacts with a reactive nucleophile residue on the larger subunit causing most of the label to reside on that subunit. Nevertheless, the data do suggest a function for the 54 kd subunit.

cDNA Clones of the Spinach Leaf ADPGlc Synthetase Gene. Using antibodies prepared with the subunits of the spinach leaf ADPGlc synthetase and with the native enzyme, two cDNA clones, SL1 and SL5, were isolated from a λgt11 spinach leaf library prepared by Clonetech Laboratories, Inc., Palo Alto, CA. Both clones gave expression of proteins that reacted with antibody made with the 51 kd subunit and were sequenced via the dideoxy nucleotide technique (13). SL1, found to be 382 base pairs in size, and SL5, about 1176 base pairs in size, were sequenced. Unfortunately, there is no overlap with these cDNA clones. Presently, about 18 nucleotide base pairs of SL5 have not been sequenced at the Hind III region as fragments of the DNA were prepared from SL5 for sequencing at that region.

Figure 4 shows the deduced amino acid sequence from the nucleotide sequence of the two clones and compares it with the complete rice seed enzyme deduced amino acid sequence (9). There is a large amount of identity between the amino acid sequences, corresponding to about 76%. Most notable is the sequence between residues 424-434 in spinach leaf where it has been shown that Lys 431 is the site of chemical modification by PLP (7,9). There is complete agreement of this sequence in the same area with the rice seed enzyme sequence 462-472. Moreover, there is complete identity of the deduced amino acid sequences of amino acids 408-434 in the spinach leaf enzyme 51 kd subunit with amino acids 446-472 of the rice endosperm enzyme subunit.

Present studies are now involved in determining the site of labeling of the 54 kd subunit by 8-N_3ADPGlc and isolation of the 54 kd subunit cDNA clones.

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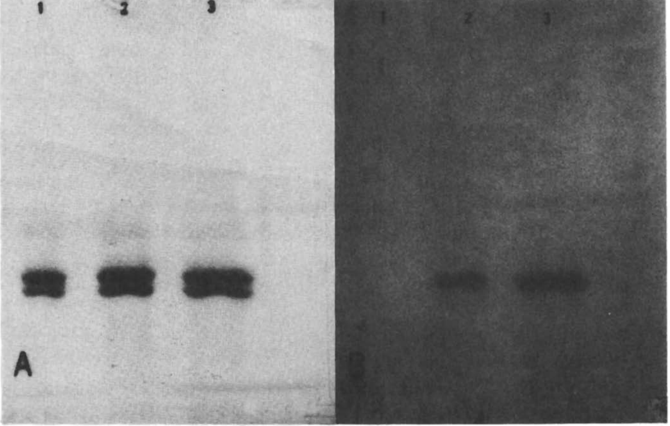

Figure 3. SDS-Electrophoresis of ADPGlc-synthetase photoinactivated with [^{14}C] 8-N_3ADPGlc. 1.8 μM (subunits) enzyme in 20 mM HEPES, 1 mM 3-PGA, 5 mM $MgCl_2$ and 1.5 mM [^{14}C] 8-N_3ADPGlc (specific activity, 1.8 x 10^7 cpm per μmole), was irradiated 10 min with UVS-54 lamp from a distance of 5 cm. Lane 1 - unirradiated control; Lane 2 - enzyme photoinactivated in the presence of 10 mM ADPGlc; Lane 3 - photoinactivated enzyme. A-Coomassie stained gel; B-autoradiograph of the same gel.

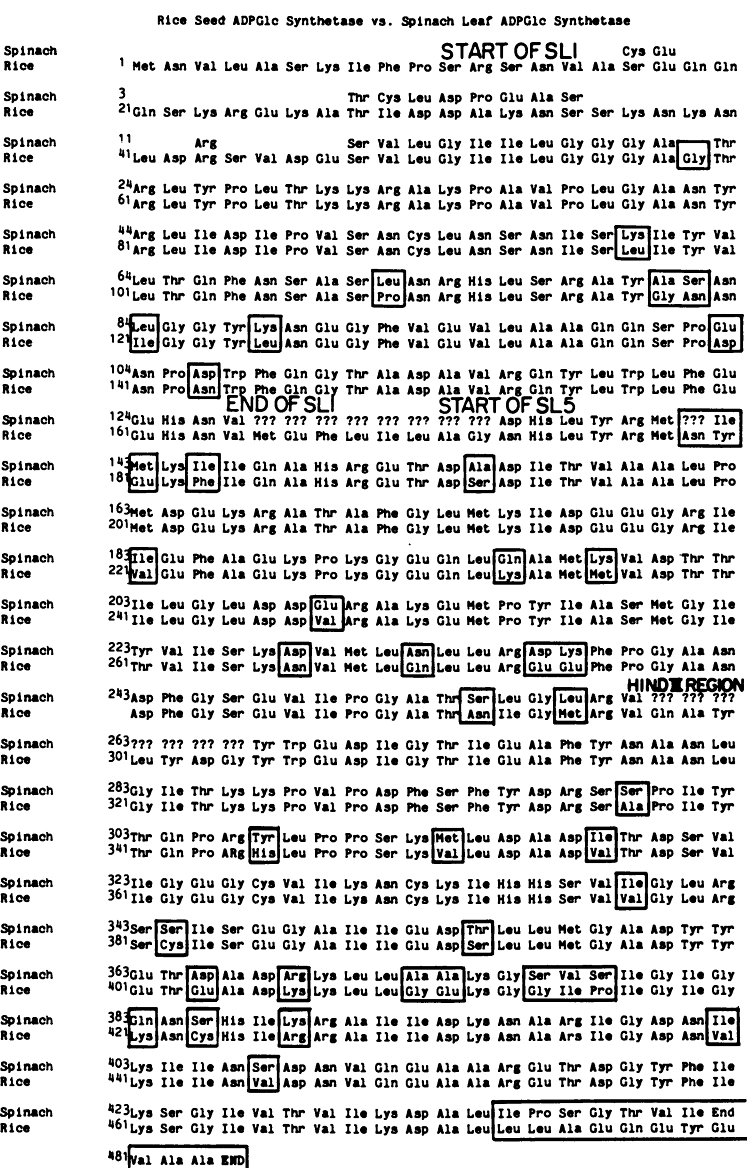

Figure 4. Comparison of the primary sequence of the spinach leaf ADPGlc synthetase with the rice endosperm enzyme deduced amino acid sequence. The non-identities of amino acids between the two enzymes are enclosed in boxes. The start and beginning of the SL1 and SL5 clones are indicated with respect to open reading frame as well as the Hind III restriction nuclease region where the nucleotide sequence has not been determined.

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Chapter 7

Pectin-Degrading Enzymes in Fruit and Vegetable Processing

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Pectic substances are important structural components of the middle lamellea and the primary cell walls of higher plants, in particular fruits and vegetables. The extent and mode of their degradation by enzymes affect many aspects of the processing of fruits and vegetables and many quality attributes of fruit and vegetable products.
Pectic enzymes occur as endogenous enzymes in higher plants; they are also added as processing aids during processing. Technical pectinase preparations are mainly derived from Aspergillus niger and contain next to pectic enzymes many other carbohydrases.
The classification of pectic enzymes in general, their occurrence in higher plants and micro-organisms and the properties of pectic enzymes from some plants and food grade micro-organisms are described with special emphasis on their substrate specificity. Their technological roles and applications, also in combination with (hemi-)cellulases, in a variety of processes are discussed. Evidence is presented for the existence of a new type of pectic enzyme which acts specifically in the hairy regions of pectic substances.

Pectins are important constituents of the cell walls of edible parts of fruits and vegetables. They are the main components of the middle lamellea which has led to the classical view that they function as intercellular adhesive. The primary walls are considered as a firm-gel composed of cellulose microfibrils embedded in a matrix of pectins, hemi-cellulose and protein with lignin virtually absent. In the secondary walls, which usually occur, cellulose and hemi-cellulose prevail (1-4).

The processing of fruits and vegetables cause changes in pectic substances. Changes in texture of fruits and vegetables during

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ripening and post harvest storage, and changes in consistency and cloud behavior of products derived from fruits and vegetables during processing or storage are in general ascribed to changes brought about by endogenous pectic enzymes (5,6). Technical pectinase preparations, commonly derived from Aspergillus niger or related fungi, are produced industrially as processing aids, mainly for fruit juice extraction and clarification (7-9). With increasing knowledge of the fine structure of pectins as cell wall components and of the properties of pectic enzymes and their technological roles, the management of endogenous enzyme systems and the conventional applications of technical pectinase preparations have been improved and new applications have been developed.

Pectin structure

Pectins are composed of a rhamno-galacturonan backbone in which 1,4-linked α-D-galacturonan chains are interrupted at intervals by the insertion of 1,2-linked α-L-rhamnopyranosyl residues. Other constituent sugars are attached in side chains and include predominantly D-galactose, L-arabinose and D-xylose.

Part of these sugars occur in short side chains, one to three units long, glycosidically linked to C-4 and/or C-3 of L-rhamnose or C-2 or C-3 of some of the galacturonate residues. The major sugars D-galactose and L-arabinose are present in more complex chains of considerable length with structures similar to arabinans and (arabino-)galactans. Part of the galacturonic acid residues carry methylester groups; acetyl groups may occur as substituents to the hydroxyl groups at C-2 and C-3 (1,2). Pectins from sugar beet and spinach have been found to carry ester linked feruloyl-groups at terminal arabinosyl and galactosyl residues of the side chains (10-12). By degradation of extracted, purified pectins from various sources specifically in the galacturonan backbone by β-elimination or enzymatically with depolymerases, essentially pure, degraded galacturonan fractions and pectin fractions of higher molecular weight in which virtually all of the neutral sugars were concentrated have been obtained (13-18).

From these results it was concluded that there is an intramolecular distribution in which the neutral sugar residues are concentrated in blocks of more highly substituted rhamno-galacturonan regions ("hairy"), separated by unsubstituted ("smooth") regions containing almost exclusively D-galactosyluronic residues (16) (Fig.1). The isolation of rhamnogalacturonans rich in neutral sugars from cell walls using pectolytic enzymes is in agreement with this concept (19). For apple pectin fractions De Vries et al. (20) established that the galactosyluronic residues in the hairy regions were almost completely methylated; in the smooth regions 70% of them were methylated.

Pectic enzymes; classification and substrate specificity

Pectic enzymes are defined and classified on the basis of their action toward the galacturonan part of the pectins. Two main groups are distinguished: pectin methylesterases (pectin esterase, PE;EC 3.1.11.1), which split off methoxy groups from pectin, transforming

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it gradually into low ester pectins and pectic acid, and pectin-depolymerases, which split the glycosidic linkages in the galacturonan backbone. Three groups of pectin depolymerases exist: polygalacturonases (galacturonases, PG;EC 3.2.2.15 and 3.2.1.67) split glycosidic linkages next to free carboxyl groups in the galacturonan chain by hydrolysis; pectate lyases (PAL;EC 4.2.2.2 and 4.2.2.9) degrade glycosidic linkages next to free carboxyl groups by β-elimination (Fig.2); they have an absolute requirement for calcium ions. Both endo and exo types of PGs and PALs are known. The endo types (EC 3.2.1.15, EC 4.2.2.2) split the pectin chain at random; a strong reduction in viscosity of the substrate solution will be accompanied by a small increase in reducing end groups. Exo-PGs (EC 3.2.1.67) split off mono- or dimers from the non-reducing end of the chain; exo-PALs split off unsaturated dimers from the reducing end. This means that the viscosity of a substrate solution is reduced only very slowly. For polygalacturonases, as well as for pectate lyases, pectate and low methoxyl pectins are the preferred substrates, while they are hardly active on highly methylated pectins. In contrast pectin lyases (PL;EC 4.2.2.10), another group of eliminative splitting enzymes, are specific for highly methylated pectins. Of this group only the endo type has been described. Naturally occurring pectins can be efficiently degraded by the combination of pectin esterase and polygalacturonase or pectate lyase, or else, if they are sufficiently highly methylated by pectin lyase alone (19,26).

A third group of hydrolytic enzymes specific for highly methylated pectins (polymethylgalacturonase, EC 3.2.1.41) has been described particularly in the older literature prior to the discovery of the pectin lyase by Albersheim et al. (21). We have never been able to find either exo-PL nor endo- and exo-PMG in our extensive screening of commercial preparations and micro-organisms. Many authors now agree that enzymes described as endo-or exo-PMG may very well have been a combined PE-PG activity, or a PL activity or even PG activity alone in cases where only initial velocities were measured. Extensive reviews of pectic enzymes have been published (22-26).

Pilnik et al. (27) and Rombouts (28) have determined the kinetic parameters K_m and V_{max} and degradation limits of purified endo-PG from Kluyveromyces fragilis, endo-PL from Aspergillus niger and various endo-PALs on pectins with different contents of methoxyl ester groups which were randomly distributed along the galacturonan chain. These pectins were prepared by partial alkaline saponification of a highly (95%) methylated pectin at low temperature to avoid chemical trans-eliminative degradation. Figure 3 shows the relation found between degree of methylation and initial activity for fungal endo-PL and endo-PAL from Bacillus polymyxa, Pseudomonas fluorescens and Arthrobacter 370 under conditions of saturation with substrate. For endo-PAL, low methoxyl pectins are, surprisingly, the best substrates and optima have been found at methylation levels of 26%, 35-40% and 44%, respectively. Also the degradation limits and the affinity (measured by $1/K_m$) of the enzymes for the substrates showed similar patterns. Results obtained by Rombouts (28) for PAL from Pseudomonas fluorescens indicate that the affinity is not only influenced by the degree of esterification but also by the calcium concentration. For exo-PAL the best substrate is pectate.

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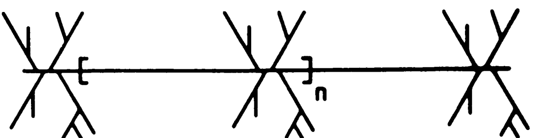

Figure 1. Schematic structure of apple pectin; rhamno-galacturonan backbone with regions rich in neutral sugar side chains (hairy regions).

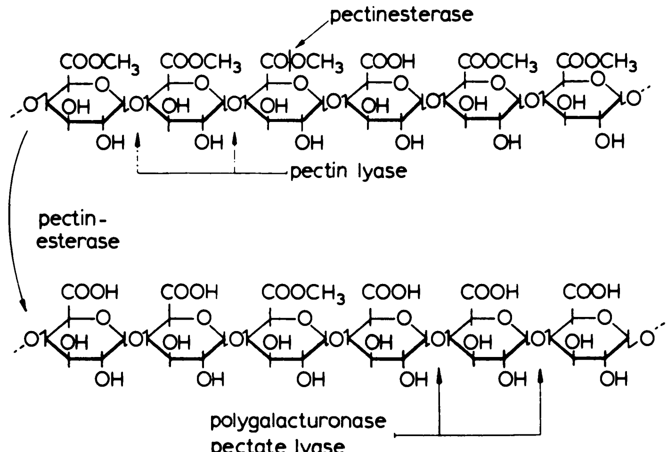

Figure 2. Fragments of a pectin molecule and points of attack of pectic enzymes.

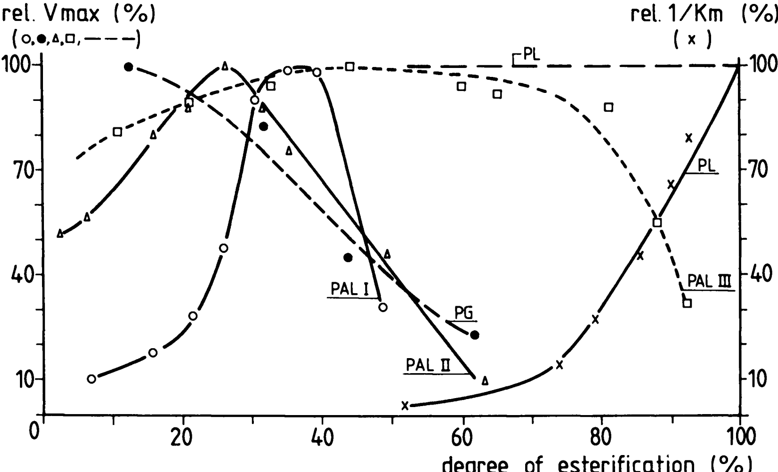

Figure 3. Influence of degree of esterification of pectin substrate on maximal velocity (relative V_{max}, %) of PG from Kluyveromyces fragilis, of endopectate lyases from Pseudomonas fluorescens (PAL I), Bacillus polymyxa (PAL II) and Arthrobacter 37D (PAL III) and of endo pectin lyase from Aspergillus niger (PL). For PL also the effect on the Michaelis-Menten constant ($1/K_m$) is shown (PL x-x).

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The curve for yeast endo-PG (Fig. 3) was obtained from activity measurements via viscosity on pectins esterified with glycol. The rate and the degree of hydrolysis were found to decrease rapidly with increasing degree of esterification. Similar results were obtained for endo-PGs from yeast and several fungal sources on pectic acids acetylated to various degrees. Rexova-Benkova et al. (29) have shown that acetyl groups decrease the extent of degradation by lowering the affinity of endo-polygalacturonase for its substrate through blocking of binding sites. Rombouts and Thibault (11) found that the degradation of sugar beet pectin fractions was severely limited by the presence of acetyl groups.

Endo-PL showed the same initial activity on pectins (Fig. 3) with decreasing degree of methylation under conditions of substrate saturation; completely esterified pectin was the best substrate for the fungal PL in as much as $1/K_m$ and the degradation limit for this substrate was maximal (27). However, this general picture was shown to be affected by reaction conditions, such as pH and calcium concentration. At pH's below the optimal pH range for 95% esterified pectin (<5) PL showed a higher affinity ($1/K_m$) for less highly methoxylated pectins. In the presence of calcium and strontium a second pH optimum was found at ca pH 8.5 (30). Most pectin lyases are markedly stimulated by calcium and other cations, the stimulation being dependent on pH value and the degree of esterification of the substrate (30-33). Because of these properties PL can play an active part in fruit and vegetable processing.

Endo-PL was found to be inactive on pectate, pectic acid amide and glycolester of pectate; endo-PALs have similar activity on glycolesters of pectate as on the methylesters. Activity on glycolesters of pectate was therefore suggested as a criterion to distinguish between pectin and pectate lyases (27,34).

From kinetic measurements on pectins with different degrees of esterification Versteeg (35,36) concluded that PE isoenzymes from orange differ in their affinity for these substrates and that the affinity strongly increased for substrates with lower degree of esterification. They also differed strongly in inhibition by polygalacturonic acid. Fungal pectin esterases have a higher affinity for highly esterified substrates (37). Pectin esterases which are inhibited by pectate can be purified on cross-linked pectate (38).

Pectin esterases are highly specific for methylesters of pectic acid; the methylester of polymannuronic acid could not be de-esterified by orange PE (39). The rate of hydrolysis was found to decrease with decreasing chain length and dropped to zero on trimethyl-trigalacturonate (40). Irregularities in the galacturonan chain such as acetylated galacturonate units, ester groups reduced to primary alcohol groups or transformed into amide groups, neutral sugar side chains or inserted rhamnosyl units inhibited pectin esterase activity (35,41). Plant pectin esterases are thought to attack either at the non-reducing end or next to a free carboxyl group and then to proceed along the molecule by a single chain mechanism creating regions of free polygalacturonic acid (42). Such regions are extremely sensitive to calcium (43). Fungal PE was found to remove methoxyl groups at random and a multichain mode of attack by *Aspergillus niger* PE has been suggested (44).

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Occurrence and technological roles

Pectic enzymes occur naturally in many fruits and vegetables (endogenous enzymes); they are also added as processing aids (exogenous enzymes, mostly derived from Aspergillus niger). Only pectin esterases and polygalacturonases, both endo and exo, have been found in higher plants (Table I). The occurrence of pectic enzymes in micro-organisms is summarized in Table II. Numerous multiple forms and isoenzymes have been detected. For chemical and physical properties, kinetic parameters and pattern of action of the various types of pectic enzymes and for regulatory aspects of their synthesis by micro-organisms we refer to the comprehensive review by Rombouts and Pilnik (26) which deals extensively with these aspects. The technological roles and applications of pectic enzymes are summarized in Table III. In Table IV properties of some plant and food grade microbial enzymes are listed.

Table I. Occurrence of pectin esterase and polygalacturonase in higher plants (23,26,35,45)

Plants	PE[a]	PG			Plants	PE	PG		
		endo	exo	n.s.			endo	exo	n.s.
Apple	+		+		Passion fruit	+	+	+	
Apricot	(+)			+	Peach		+	+	
Avocado		+			Pear	+	+	+	
Banana	+	+			Plums	+			
Berries	+			+	Beans	+			
Citrus: lime	+				Carrots	+		+	
orange	+				Cauliflower	+			
grapefruit	+		+		Cucumber	+	+		
mandarin	+				Leek	+			
Cherries	+	+			Onions	+			
Current				+	Pea	+			
Grapes	+			+	Potato	+		+	
Mango	+	+			Radish	+			+
Papaya	+	+	+		Tomato	+	+	+	

a) PE, pectin esterase; PG, polygalacturonase; n.s., not specified

Pectin esterases

Endogenous pectin esterases play an important role in citrus processing; they have therefore been studied intensively. They cause cloud loss in citrus juices, which is due to the calcium precipitation of enzymatically de-esterified pectin. This is desirable in the production of lemon and lime juices but undesirable in the production of orange juice. In orange juice concentrates strong calcium pectate gels may form which cannot be reconstituted

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Table II. Occurrence of pectic enzymes in micro-organisms (9)

Organism	PE[a]	PG		PAL		PL
		Endo	Exo	Endo	Exo	Endo
Fungi						
Aspergillus (niger)	++[b]	++	++			+
Penicillium	+	+				+
Fusarium	+	+		+		
Rhizopus	+	+	+			
Sclerotinia	+	+				+
Collectotrichum		+	+			
Yeasts						
Kluyveromyces		++				
Bacteria						
Bacillus (polymyxa)				+++		
Clostridium	+				+	
Erwinia		+	+	+++	+	+
Pseudomonas				+++		
Arthrobacter				+		

a) PE, PG; see Table I. PAL, pectate lyase; PL, pectin lyase
b) +++ means produced in high levels, no data means activity absent

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Table III. Technological roles and applications of pectic enzymes

Enzyme(s)	Technological roles/applications	
	Endogenous	Exogenous
PE	- Cloud stability in citrus juices - Pectin manufacture from citrus pomace - Distillates from fermented fruit pulps - Enzymatic maceration of fruits and vegetables - Self-clarification of lemon/lime and apple juices - Pressing characteristics of citrus pomace - Ca^{2+}-firming of fruits and vegetables	- Manufacture low-ester pectins with reduced Ca^{2+} sensitivity - Apple juice clarification
PG	- Softening during ripening (pears, peaches, avocado) - Texture loss of canned or bottled fruit products	- Citrus juice stabilization - Maceration (concentrated nectar bases, baby food, vegetable juices) - Viscosity reduction of citrus concentrates - Manufacture of low viscosity pectins
PE + PG	- High viscosity tomato juices and pastes (hot break) - Low viscosity tomato juices and pastes (cold break) - Vegetable juices in combination with tomatoes	- Clarification (apple juice, pear juice, grape musts or wines) - Enzymic juice extraction (apples, soft fruit, stone fruit) - Enzymic oil extraction (olives, palm fruit, coconut flesh - Depectinizing citrus pulp wash - Clouding agents from citrus peels - Recovery of oil from citrus peel
PL		- Maceration - Clarification; apple juice - Enzymic juice extraction
PAL		- Maceration
PG (+PE and/or PL) + (hemi)-cellulases		- Liquefaction; clear and cloudy juices (fruits + berries, tropical fruit, vegetables - Cell wall destruction for isolation of interesting cell constituents - Liquefaction and saccarification of biomass

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Table IV: Properties of pectic enzymes from some plants and food grade micro-organisms

Enzyme Type/source	Molecular weight	Iso-electric point	Spec.act. (units/mg)	Optimum pH	K_m value (mg/ml)	Ref.
PE						
Orange I[a]	36,000	10.0	694	7.6	0.083	50
II[a]	36,200	11.0	762	8.8	0.0046	50
III[a]	54,000	10.2	>180	8	0.041	50
Aspergillus niger	39,000	3.9	406	4.5	3	37
PG						
Tomato I[a]	100,000	8.6		4.5		5
II[a]	44,000	9.4		4.5		5
Kluyveromyces fragilis	46,000-48,000	6.1	89	4.0		b
Aspergillus aculeatus[a]	47,000	4.7-4.8	67	4.0-5.0		b
Aspergillus niger[a]	42,000-49,000	6.0-6.4	275	4.0		b
PL						
Aspergillus niger I	35,400	3.65	17	6	5.0	92
Aspergillus niger II	33,100	3.75	44	6	0.9	92
PAL						
Bacillus subtilis	33,000	9.85		8.5		93

a) Multiple molecular forms (iso-enzymes)
b) Unpublished results of our laboratory

(8,46). The problem can be overcome by either heat inactivation of PE which can harm the flavor or by storing at -20 °C. Another possibility is the addition of endo-polygalacturonase which breaks down the low ester pectin formed, before it can coagulate with calcium (47,48). By measuring the formation of methanol during storage of orange juice, Versteeg (35) has shown that the addition of PG accelerates PE action by degrading the pectate which is a strong inhibitor for PE. Termote et al. (49) found that degradation products of pectic acid with a degree of polymerization of 8-10 still had an inhibitory effect on citrus PE and, unlike pectate, do not coagulate with calcium. Addition of such preparations indeed delayed clarification.

Versteeg et al. (50) have isolated various multiple forms of pectin esterases in oranges and shown that they differ in affinity to pectins and pectates and in heat stability and therefore may play different rôles in cloud loss phenomena. One form (about 5% of the total PE activity) was found to be much more heat stable than the other forms; it was also active at low pH (2.5) and at low temperatures. Another form did not cause self-clarification; for this enzyme a similar mode of attack was proposed as for fungal pectin esterases which produce low-ester pectins less sensitive to calcium. Multiple forms of pectin esterases are present in the fruits of all of the orange varieties and citrus species tested. The two isoenzymes known to be responsible for cloud loss and gelation in citrus products were found to occur in all of the component parts of the orange fruit (51). In the French cider industry the endogenous PE of apple is used for the self-clarification of apple juice (52).

The calcium pectate coagulation phenomenon as a result of pectinesterase action improves the pressing characteristics of ground citrus peel and lowers costs when the peel has to be dried for cattle feed. When the pomace has to be dried for pectin manufacture pectin de-esterification must be prevented since otherwise calcium sensitive pectin is obtained. This is achieved by immediate blanching (8).

Native pectin esterases can be used for protecting and improving the texture and firmness of several processed fruits and vegetables; these include apple slices (53), canned tomatoes (54), cauliflower (55), carrots (56), potatoes (56), beans (57) and peas (91). Blanching temperatures, holding times, sometimes with the aid of calcium ions or pH shift are manipulated in such a way that the esterases are activated, causing a partial de-esterification of the pectins which then react with (added) calcium ions resulting in stronger intercellular cohesion. To prevent accumulation of methanol in distillates from fermented fruit pulps pectin esterases should be inactivated (59).

Fungal pectin esterases have lower pH optima (ca 4.5) than plant pectin esterases (7,8) and are less heat stable (35,26). A commercially available fungal PE preparation has been introduced for industrial application in cider and lemon juice clarification (52,60). A process for the manufacture of low-ester pectins with a lower sensitivity for calcium has been patented by Ishii et al. (61).

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Polygalacturonases

Polygalacturonases are widely distributed in higher plants (Table I). They have recently been reviewed by Pressey (5). The richest plant source of PG is ripe tomato fruit. Pressey identified two forms in extracts of tomatoes (PGI and PGII) which differed markedly in molecular size (100,000 and 44,000 respectively) and stability to heat (5 min at 90°C and 5 min at 60°C for inactivation respectively). Also, a protein (PG converter) has been isolated from tomatoes that reacts with PGII to form PGI (62-64). By heating PGI with mild alkali PGII could be recovered. Pressey (5) found that polygalacturonases appeared after ripening of the tomatoes began, but PG converter was present in the fruit at the green stage. PGs are considered to be closely related with softening and ripening. Some fruits that soften markedly during ripening such as pears and freestone peaches contain not only endo-PG but also exo-PG. Fruits with slow softening characteristics such as apples and clingstone peaches contain only exo-PG (5).

Polygalacturonases are also produced by a wide range of microorganisms (Table II). Many multiple forms and isoenzymes of endo-polygalacturonase have been investigated; some characteristics are given in Table IV. Pectate is their best substrate; mono-, di- and trimers accumulate as end products. These may appear quite rapidly by a single chain multiple attack mechanism (*Colletotrichum lindemunthianum*, 65) or only at a later stage if the mechanism of multichain attack applies (*Kluyveromyces fragilis* PG, 66). The pH optima of *Aspergillus niger* PGs are generally quite low (pH 4,5) which explains their widespread use in fruit processing. It has been found that fungal endo-PGs are inhibited by proteinaceous material in extracts of some fruits and vegetables (67,68) which may render them useless. Also microbial endo-PLs and endo-PALs were found to be inhibited by extracts of onions, peppers and french beans (69). Fungal pectinase preparations containing predominantly endo-polygalacturonase and free of pectin esterase are used successfully as macerating enzymes for the production of pulpy nectars (70,71). These comminuted fruit juices are viscous, pulpy drinks and are usually prepared by a mechanical-thermal dispersion process. However, by the use of enzymes products can be obtained which are superior in cloud stability and smooth consistency and have higher contents of soluble solids and pigments. These suspensions of loose cells from fruit and vegetable tissues are obtained by weakening the cell cohesion by a limited pectin breakdown particularly in the middle lamella. Nutrients such as vitamin A or proteins are protected inside the intact cells. Enzyme preparations which have only one depolymerase system are chosen (8). The presence of PE can easily transform the macerating activity of a pure PG into cell disintegration activity because of the general depolymerizing activity of the PE/PG combination (72). When endogenous pectin esterase is present blanching is indicated. Maceration can also be achieved with endo pectin lyase (73), or with bacterial pectate lyase (74,75). Rombouts (28) explained the potential of pectate lyase for maceration of potatoes and vegetables by their optimal activity on intermediate esterified pectins.

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In recent studies on the action of pure endo-PGs from various sources (Kluyveromyces fragilis, Aspergillus aculeatis and Aspergillus niger) on vegetable cell walls (water insoluble solids, WIS) we observed that equal amounts of enzyme units of each PG (0.5 U/40 mg WIS) released cell wall pectin at different rates and quantities when incubated under the same conditions (pH 4.0, 40 °C, rotary shaker at 150 RPM). Figure 4 shows this for parsley WIS; similar observations were made for WIS of french beans and celery roots.

Yeast PG showed the highest initial rate, but Aspergillus niger PG released the highest amounts of pectin (up to 95% on celery WIS) after 20 h incubation. From Table IV it can be seen that the PGs studied here had similar molecular weights (ca 45,000), and pH optima (4.0); they were different in their iso-electric points. It appears that apart from differences in cell wall composition and fabric PGs act differently on cell walls. Endo-polygalacteronases bind in a specific way to cross-linked pectate (76) which is, however, slightly biodegradable by pectic enzymes. This is not the case for crosslinked alginate and calcium alginate beads which also bind endo-PG, probably by biospecific and nonspecific ionic interactions. Adsorption characteristics can be influenced by changing the pH and ionic strength of the system (77). A fluidized bed reactor containing calcium alginate beads is under investigation for isolation of PG from fermentation liquors on a technical scale (78).

Pectin esterase and polygalacturonase

Pectin esterase and polygalacturonase activity are abundantly present in tomatoes, where they to a large extent determine the consistency of tomato juices and pastes. High viscosity juices are obtained when the tomatoes are heat treated during disintegration to inactivate the enzymes instantaneously (hot break process); thin juices are obtained when there is a holding time between disintegration and pasteurization (cold break process, 79). Bock et al. (80,81) have patented a process of making vegetable juices using the enzyme system present in tomato juice.

Technical pectinase preparations, originally introduced for the clarification of turbid juices, contain pectin esterases, polygalacturonases, and pectin lyases in varying amounts along with other enzymes such as arabinanases, galactanases, xylanases, cellulases, glycosidases and proteases. It has been shown that through the combined activity of pectin esterase and polygalaturonase the pectin dissolved in the juice, as well as the negatively charged pectin coating of the suspended particles, are degraded. Subsequently the destabilized particles flocculate through electrostatic interaction and precipitate (82,83). Apple juice clarification is also possible with pectin lyase for which the highly esterified apple pectin is an ideal substrate (84). Clarification and complete depectinization enables concentration of fruit juices and avoids gelling of the concentrates. Pectinases are also used, for a few decades already, to facilitate pressing and to ensure high yields of juice and pigments from soft fruits and more recently from apples and grapes (8). For apples it was shown that enzyme mixtures capable of rapidly degrading highly methylated pectin will perform well

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(85). Cell wall pectin can be effectively solubilized and degraded by the combined action of pectin esterase and polygalacturonase as was demonstrated for apple and apricot tissue (86).

Pectinases and (hemi-)cellulases

Through the use of pectic and cellulolytic enzymes the cell walls of fruit pulps can be degraded to the stage of almost complete liquefaction which has led to the introduction of the enzymatic liquefaction process for (tropical) fruit and vegetable pulps (87). Juice yields exceeding 90% can be obtained and, as a result of the solubilization of cell wall polysaccharides, the dry matter content of the juices is also sensibly increased. The presence of both endo- and exo-β 1,4-glucanases (cellulases) together with pectic enzymes is essential (19,88,89). New brands of pectic enzymes with broader spectra of cellulose and hemi-cellulose degrading activities (arabinases, galactanases) have been introduced.

Treatment of pulps with such enzymes causes the release of cell wall polysaccharides in the juice as mono-, oligo- and polysaccharides. Table V shows amounts and composition of high molecular weight fragments present in apple juices prepared by conventional pressing, pulp enzyme treatment or enzymatic liquefaction using four technical enzyme preparations which differed in level and type of pectic and (hemi-)cellulolytic activities. The polysaccharide fractions were isolated as freeze-dried dialysis retentates from raw juices clarified with bentonite, gelatin and Klar-Sol 30. The effect of enzyme treatment is unequivocal; the largest amounts were released in the liquefaction process. The amounts and compositions of the polysaccharide fractions also depended on the technical preparations used; distinct variations in arabinose, galactose, galacturonic acid and xylose contents were found. Also variations in the degree of methylation were observed; fragments with increased acetyl group contents accumulated in the liquefaction juices.

We isolated and characterized the polysaccharide fraction released by preparation D in a liquefaction process and have shown that it consists of an almost pure, arabinan fraction and a pectin fraction rich in branched arabinose side chains (86,90). The hypothetical structure of the pectin fraction is shown in Figure 5; it resembles the structure of hairy regions of pectins as described by de Vries et al. (13). Its composition deviates particularly in the lower galactose content, obviously due to the galactanase activity present in D. Fragments rich in arabinose were also released by C; preparation B on the other hand released fragments low in arabinose and galactose. This preparation is characterized by a complete spectrum of arabinolytic enzymes. The arabinans and arabinan-rich pectic material solubilized in the juice are potential precursors for the formation of arabinan hazes in apple and pear juice concentrates. This is particularly the case when they are debranched by exposure to arabinofuranosidase activity in the absence of endo-arabinase and/or chemically debranched by heat treatments as a result of juice acidity (90).

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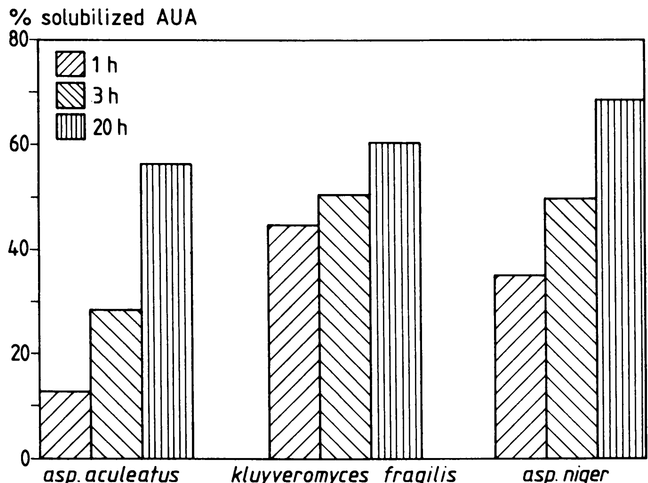

Figure 4. Pectins solubilized (AUA) from parsley WIS by pure microbial endo polygalacturonases after 1, 3 and 20 hours incubation (0.5 Unit PG/40 mg WIS, pH 4.0, 40°C, shaking).

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Table V. Amounts and characteristics of polysaccharides isolated from apple juice prepared by different processes and different enzymes

Analysis	Pressing	Pulp enzyme treatment			Liquefaction	
		A[a]	B[a]	C[a]	C[a]	D[a]
Polysaccharides						
Content (mg/kg pulp)	155	1075	1860	1075	3000	4300
Sugar composition						
Rhamnose	3	5	3	5	4	4
Arabinose	21	38	6	52	43	54
Xylose	2	5	2	5	6	8
Galactose	19	9	4	7	6	8
Glucose	2	1	1	1	1	0
Anh.-galacturonic acid	53	42	84	30	40	26
DM[b]	52	72	76	31	59	39
DA[c]	15	18	6	13	28	29

a) Different enzyme preparations used. A, B, and D were experimental preparations from Gist-Brocades, Delft, The Netherlands; A was a pectinase; B a pectinase rich in hemi-cellulase activity and D a pectinase-cellulase mix. C was ultra SP-L from Novo Ferment AG, Basel, Switzerland

b) Degree of methylation

c) Degree of acetylation calculated on anhydrogalacturonic acid content

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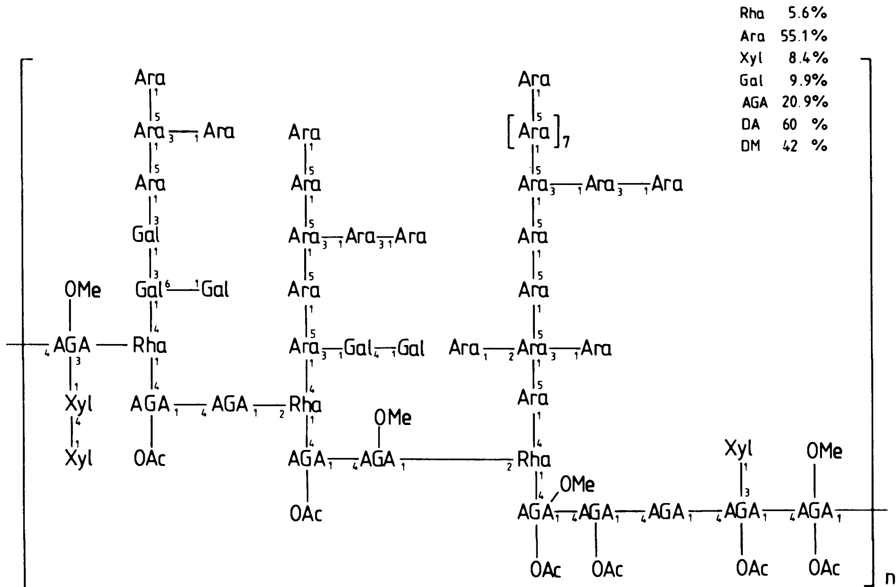

Figure 5. Tentative structure of pectin fragments released in apple juice by enzymic liquefaction (modified hairy regions; MHR). (Reproduced with permission from reference 90, copyright 1987 Food Hydrocolloids).

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Rhamnogalacturonase

In a study on the enzymatic degradability of the polysaccharide fraction released by enzyme D (from now on indicated as MHR, modified hairy region) we observed that Ultra SP (preparation C) was able to cause a shift in the molecular weight distribution of MHR as shown in Figure 6. The curve obtained for Rapidase C80 (Gist-Brocades, Delft, The Netherlands) is typical for all other technical preparations available to us. By an involved purification procedure using high performance gel permeation chromatography (HPGPC) for assaying enzyme activity we were able to isolate an electrophoretically pure enzyme active on MHR and de-esterified MHR (treated with cold, 0.1 N sodium hydroxide) as shown in Figure 7. The enzyme had a molecular weight of 63,000 D and was optimally active in the pH range 4-5. The enzyme showed no activity on low and high esterified pectins, arabinan, galactan and various para-nitro phenylglycosides. From the chromatograms it is evident that the enzyme activity is enhanced by removal of methoxyl and/or acetyl groups. Removal of arabinan side chains by an acid treatment stimulated the enzyme action even more.

In an effort to characterize the reaction products of the enzyme we treated arabinose-free MHR with this enzyme and the reaction mixture was submitted to gel permeation chromatography on a Sephadex G 50 column. The chromatograms are shown in Figure 8. Fractions containing sugars were pooled as indicated and analyzed for sugar composition. It can be seen that rhamnose and galactose are mainly present in the oligomeric fraction (II), while xylose remains in the polymeric fraction (I). The elution pattern and the sugar composition of the arabinan free MHR is shown in Figure 8a. These results indicate that MHR has subregions rich in rhamnose and galactose and sub-regions rich in xylose; the hypothetical structure of MHR has therefore to be revised. In preliminary experiments we observed that monomeric rhamnose was released from the oligomeric fraction when it was treated with an enzyme preparation containing glycosidase activities. Preliminary results of DCI-mass spectroscopy measurements on the oligomeric fraction indicate that rhamnose is present at the non-reducing end.

From these results we conclude that the enzyme isolated from Ultra SP is able to split glycosidic linkages in the rhamno-galacturonan backbone of modified hairy regions of apple pectins probably next to a rhamnosyl residue producing a range of oligomers, composed of galacturonic acid, rhamnose and galactose, with an estimated degree of polymerization between 6 and 10 which have rhamnose at the non-reducing end and polymeric fragments rich in galacturonic acid and xylose. We propose the name rhamnogalacturonase (RG) for this enzyme. RG is evidently hindered by the presence of ester groups and arabinan side chains and might act synergistically with arabinanases and pectin- and acetyl esterase and play a role in the disintegration of cell walls and the release of arabinans.

More knowledge of physical and chemical properties of pectic enzymes, their substrate specificity, pattern of action, action on plant tissues, interaction with each other and other polysaccharide degrading enzymes is essential to establish their technological roles and to improve existing applications and to develop new applications.

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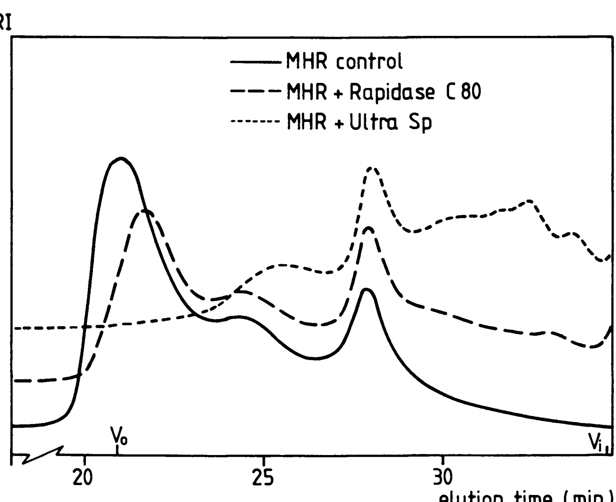

Figure 6. High performance gel permeation chromatography (HPGPC) profiles of MHR treated with technical enzyme preparations (30°C, 3 hours). HPGPC conditions: BioGel TSK 40xl, 30xl, 20xl in series, eluted with 0.4 M NaAc, pH 3.4, at 30°C.

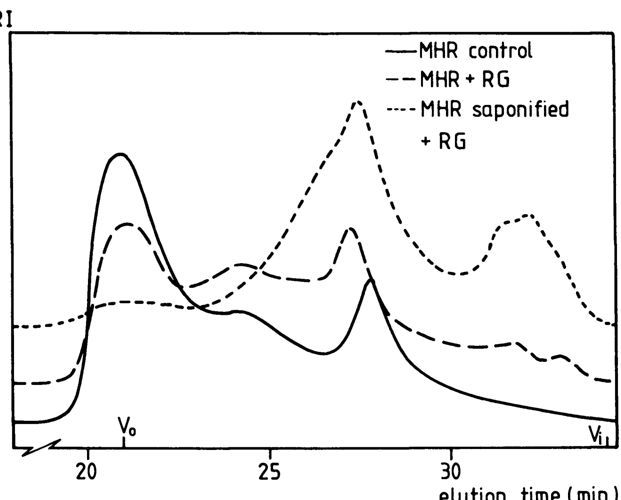

Figure 7. HPGPC profiles of MHR, MHR treated with "Rhamnogalacturonase (RG), saponified MHR treated with "Rhamnogalacturonase". Enzyme treatment at 30°C for 24 h. HPGPC conditions as in Figure 6.

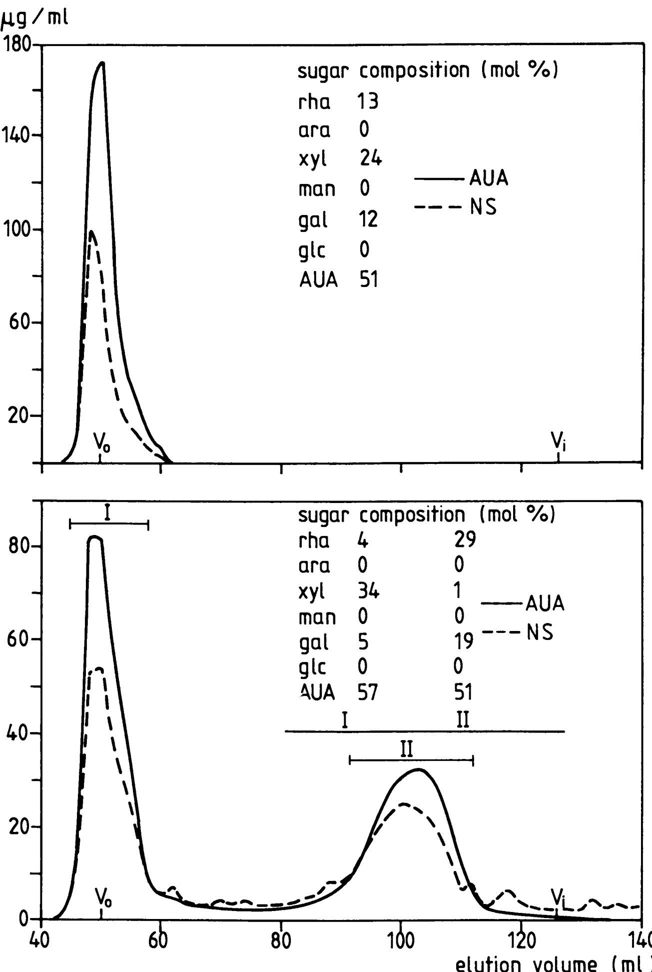

Figure 8. Elution profiles of arabinose-free MHR treated with RG (30°C, 24 hours) obtained by gel permeation chromatography over Sephadex G 50. The top chromatogram is the control. AUA is anhydrogalacturonic acid concentration in eluate (——), NS is concentration of neutral sugars (---).

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This creates the need for the selective isolation of specific enzymes on a technical scale. Identification, purification and molecular characterization of technological relevant enzymes are necessary for improving or blocking their synthesis by genetic manipulation.

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Chapter 8

Chitinolytic Enzymes and Their Applications

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Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. The role of the chitinolytic enzymes in nature is to degrade the huge quantities of chitin for recycling. Renewed commercial interest in the production and utilization of chitin and chitinolytic enzymes has demonstrated the need for inexpensive reliable sources of stable chitinase and chitobiase. Presently, commercially available purified and semi-purified chitinases are obtained from microorganisms at a high cost, yet of unreliable specific activity. It is imperative to increase the supply of active chitinase while reducing the cost of production. This challenge may be met by extracting both chitinase and chitobiase from low cost readily available non-microbial sources (such as soybean seeds), and by recombinant DNA technology to develop chitinase/chitobiase - overproducing microbial strains. Furthermore, this paper will consider the important role of chitinases for the biological control of soil-borne plant pathogenic pests as well as biomass recovery from shellfish and cheese manufacturing industries.

Chitin, a high molecular weight insoluble homopolymer of β-1, 4-N-acetyl-D-glucosamine (GlcNAc), is one of the most abundant substances of biological origin found on earth, second only to cellulose (1).Chitin is the main structural material in the exoskeleton of a large number of organisms; it is the principal cell wall polymer in a variety of fungi and yeasts (1-3); and it is in the shells or cuticles of arthropods including crustaceans and insects (2-4). Estimates of worldwide annual chitin bioproduction by microorganisms, marine invertebrate, and insects approach the estimated 10^{11} metric tons of cellulose produced annually (5). Purified chitin is a colorless amorphous solid which is insoluble in water, dilute acids, dilute and concentrated alkalis, alcohols, and all organic solvents. Chitin is soluble in concentrated mineral acids, but these degrade it. Depending on the source, chitin varies in molecular weight, specific rotation, solubility, degree of acetylation and other characteristics. Recently, two chitin solvent systems have been developed that dissolve chitin and permit the regeneration of crystalline

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orientable films and fibers which may be used for surgical suture, wound coverings, and other applications (6). The first system consists of trichloroacetic acid (40%), chloral hydrate (40%), and methylene chloride (7) while the second is an aprotic solvent system of mixture of N, N-dimethylacetamide and LiCl (8).

The abundance of this renewable resource as well as its unique properties (including toughness, bioactivity, biodegradability, and non-allergenicity), have spurred renewed interest in the broad field of chitin chemistry and applications (4,6,9,10). One area of interest has been the study of the enzyme system that degrades chitin. In this paper, I will consider the distribution of chitinases and chitin degradation. Also, I will discuss the various sources of purified and semi-purified chitinases and some of the problems associated with unreliable specific activity and cost of commercially available chitinases. Furthermore, I will consider the important role of chitinolytic enzymes for the biological control of soil-borne plant pathogenic fungi and nematodes, as well as the bioprocessing and bioconversion of shellfish and cheese whey wastes in animal nutrition.

Distribution of Chitinolytic Enzymes

Microorganisms. Chitinases are widely distributed among bacteria, molds, and fungi (2,4). Their existence may be constitutive or inducible in the presence of chitinous substrate (11). The distribution of microbial chitinases has been reviewed by others (12,13). Chitinolytic bacteria have been isolated from soil, fresh water, marine water and mud, and the digestive tracts of animals (2,13,14). Ohtakara (15-17) has done extensive research on the chitinolytic enzymes of *Aspergillus niger*. Leopold and Samsinakova (18) have reported substantial amounts of a constitutive extracellular chitinase produced by the insect pathogen fungus *Beauveria bassiana*. Chitinase hydrolyzes the insect exoskeleton, allowing the fungus to invade its host. Ohtakara and Mitsutomi (19) have obtained homogeneous chitinase and chitobiase from the fungus *Pycnoporus cinnabarinus*.

The chitinolytic system of *Streptomyces griseus* has been described by Berger and Reynolds (20), Jeuniaux (21) and Ohtakara (22) who have isolated several chitinase fractions during purification. Furthermore, Monreal and Reese (23) have found an inducible extracellular endochitinase in the culture filtrate of *Serratia marcescens*. Recently, Correa et al. (24) have purified a mannan-associated endochitinase from the yeast *Saccharomyces cerevisiae*. They proposed that the chitinase is covalently linked to mannan through a GlcNAc-asparagine bridge. This sugar may play a role in the formation of high molecular weight chitinase polymers of varying size following extraction.

Plants. Even though chitin has not been found in higher plants, chitinases are widely distributed in plants. Chitinolytic enzymes do not participate in the metabolism of plants. Recent evidence suggests that these enzymes may serve to protect higher plants against fungal pathogens. The presence of chitinase in the extract of sweet almond was first reported by Grassman et al. (25). Chitinase and chitobiase activity has been reported in the seeds of beans and other plants by Powning and Irzykiewicz (26) who first speculated on the possible fungicidal role of chitinase in plants. Pegg and Young (27) have purified and characterized chitinase from the stems of healthy and infected tomato plants while Worgo (28) isolated the enzyme from forest trees. The enzyme has been purified and characterized from the latexes of papaya and fig (29,30). Boller et al. (31) have assayed for chitinase the leaves of plants from six different families and reported activity in all samples tested. Activity varied considerably and was highest in tomato and lowest in cucumber leaves. Molano et al. (32) obtained a homogeneous preparation of chitinase from wheat germ and more recently Wadsworth and Zikakis (33) purified several chitinases from soybean seeds.

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The majority of the recent literature on plant chitinases has dealt with its role as a defense against fungal pathogens. The cell walls of many fungi contain chitin and there is evidence that the growth of hyphal tips depends on a delicate balance between cell wall synthesis and wall lysis. Chitinase can alter the balance. Later on, I will discuss this important role of chitinase for controlling soil-borne plant pathogenic pests.

Animals. In invertebrates, chitinases are produced by certain protozoans and by various glandular tissues of many coelenterates, nematodes, polychaetes, oligochaetes, molluscs and arthropods (11). Jeuniaux (34) has extensively reviewed the evolution of the biosynthesis of chitinase in animals. While many microorganisms synthesize chitinase in both ectodermal and endodermal tissues, there is a marked trend toward the loss of chitinases of ectodermal origin in higher animals. In deuterostomes, particularly the vertebrates, there is a tendency towards the loss of chitinase synthesis altogether (34).

Animal chitinases are predominantly synthesized in the digestive tract and glands. Their presence or absence is closely related to the nature of the normal diet of the species and synthesis cannot be induced by addition of chitin to the feed (3). It appears as though chitinase secretion is a result of genetic adaptation of a species to a chitin-containing diet (2). The most remarkable exception in this model of regressive evolution are the arthropods. Arthropods (except adult insects) retain the ability to synthesize chitinase in the epidermis. The periodic stimulation of chitinase secretion by this tissues facilitates the molting process (2). Kimura (35) has separated several fractions containing chitinase and chitobiase activity from the molting fluid of the silkworm *Bombyx mori*. Bade (36) has described the digestion of cuticle chitin during the molt of the tobacco hornworm *Manduca sexta*. A number of sequential steps are involved in the process, which apparently requires a heterogeneous complex of chitinases to split both chitin and chitin-protein bonds.

The presence of endogenous chitinase in the digestive tract of vertebrates was first described by Jeuniaux (37). Chitinase activity was identified in the digestive juices and glandular extracts of certain fishes, lizards, birds and mammals. Micha et al. (38) have identified a gastric chitinase in two species of reptiles. The enzyme exhibited a pH optimum of about 3.0 and retained much of its activity at pH 1.5. Okutani (39) reported conspicuous differences between endogenous chitinase and chitinases produced by bacteria in the digestive tract of marine fish. The enzymes differed in their location in the digestive tract and in their response to changes of pH and temperature. Jeuniaux and Cornelius (3) have reviewed the distribution and activity of chitinase in the digestive tract of a variety of birds and mammals. The highest activity in birds was found in the glandular stomach of the sparrow and for mammals in the gastric mucosa of the mole. *In vivo* chitin digestion was demonstrated in mice, Japanese nightingale and chicken. No chitinase activity was found in the gastric mucosa or pancreas of sheep, rabbit, guinea pig, cat, stoat, ferret, marten, sloth or man(3). Smirnoff has reported considerable chitinolytic activity in the gastric juice and intestinal chyme of the chicken and has developed a large scale method for the isolation of the chitinase system (40,41).

Degradation of Chitin. The pathway for the complete hydrolysis of chitin to GlcNAc is facilitated by two enzymes, which are often found together as an enzyme system. Chitinase (chitin glycanohydrolase, E.C. 3.2.1.14) randomly attacks free strands of chitin yielding chitooligosaccharides, chitobiose and eventually degrading all oligomers to end product chitobiose. On the basis of reaction products formed, this is an endochitinase. Chitobiase (chitobiose acetylaminodioxyglucohydrolase, E.C. 3.2.1.30) completes the hydrolysis by converting chitobiose molecules into GlcNAc. Chitobiase can also hydrolyze chitooligosaccharides up to the hexamer at decreasing rates as the size of the chitin

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oligosaccharide increases (2). Chitobiases sometimes are referred to as exochitinases. However, since they produce only GlcNAc, they are β-hexosaminidases and should be considered as glycosidases (42). One of the most widely used chitinase assay is the colorimetric method of Reissig et al. (43). This assay measures free GlcNAc and for this reason it requires the presence of an excess amount of chitobiase. In order to reduce cost, some investigators (11,44,45) have substituted chitobiase with β-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21) preparation in the chitinase assay. β-Glucosidase is substantially less expensive than chitobiase (about $40/g vs. $38/mg). However, we have observed that a β-glucosidase preparation sold by Sigma Chemical Co. readily hydrolyzed chitobiose, but unfortunately, it also degrades chitin substrates (46). These observations suggest that the "purified" β-glucosidase preparation contained chitinase or further work is needed to determine the usefulness of β-glucosidase as a substitute for chitobiase in chitinase assay systems.

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Sources of Purified and Semi-Purified Chitinase. The success of applications utilizing chitinase would depend on an ample supply of high specific activity purified chitinase at a reasonable cost. Presently, all commercially purified chitinases are isolated from microorganisms. In addition to the high cost, some of these preparations contained unreliable level of specific activity (33,47,48). Recently, researchers have purified chitinases from other sources and from genetically engineered chitinase-overproducing microbial strains (14,49-51). However, none of these methods and procedures have as yet been utilized for the commercial production of chitinases. Non-microbial chitinases have been purified from wheat germ (32), bean leaves (31) and soybean seeds (33). More recently, Zikakis and Castle (47) described a rapid and inexpensive method for extracting highly active semi-purified chitinase from selected puffball species. Smirnoff (40,41) developed a semi-industrial scale procedure for the isolation of chitinase from chicken entrails.

Presently, the most promising raw material for commercial production of chitinase (in terms of cost per unit activity and ready availability to satisfy present and future demand) is soybean seeds. Table I shows a comparison of cost (based on the retail prices) for the production of chitinase from microbial sources by three commercial suppliers and from soybean seeds. The cost is very high for the commercially prepared enzyme. The method used to purify the soybean enzyme (33) has an average yield of 3.6 I.U./kg or 3600 I.U./metric ton soybean seeds. The cost for producing 3600 I.U. chitinase from the three suppliers has been calculated and shown in Table I. The Sigma and Calbiochem-Behring preparations contained the lowest specific activities. The precise cost of production for soybean chitinase is not known. However, we know the cost of soybean is about $230/metric ton. A rough estimate of the cost for producing 3600 I.U. of chitinase from soybean would be in the range of $60,000 - 80,000. With additional improvements in the methodology, one should be able to increase recovery and reduce even more the production cost. Furthermore, since only about 2% of the soybean protein is removed (as crude chitinase extract), all the soybean oil and the rest of the soybean meal can be sold, thus reducing the cost of the raw material and the overall production of the enzyme.

For smaller quantities, chitinase can be extracted easily from puffballs (47). In the future, a good source for chitinolytic enzymes will be from chitinase-and chitobiase-overproducing microorganisms obtained by molecular cloning of the genes encoding chitinase and chitobiase (14,50,51).

Biological Control of Pathogenic Fungi and Nematodes. The cell walls of many plant pathogens (e.g., fungi, nematodes) contain considerable amounts of chitin, β-glucan and in many cases a small amount of chitosan. Higher plant tissues contain high amounts of chitinase and β-glucanase, but contain no chitin and only limited

Table 1. Comparison of production cost for chitinase from various commercial sources and from soybean seeds

Enzyme source	Cost/g	Spec. Activity (ImU/mg)	Cost/3600 I.U.
U.S. Biochemicals	$198	284	$257,000
Calbiochem-Behring	$240	61	1,416,000
Sigma Chemical Co.	$232	17	4,912,000
Soybean seeds	?	89	60,000-80,000[a]

[a]Estimate

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quantity of β-glucan. Since plants have no metabolic need for chitinase and only limited need for β-glucanase, what is the function of these enzymes in higher plants? This question led to the hypothesis that plant chitinases and β-glucanases may play an important role in defense against pathogenic pests (53-58).

Plants with viral, bacterial and fungal infections evolve greater amounts of ethylene, as a defensive response, than do similar healthy plants (55,56). Abeles et al. (55) demonstrated an increase in chitinase and β-glucanase activities in red kidney bean leaf extracts from plants treated with ethylene to simulate infection. They suggested that chitinase serves as an endogenous protective mechanism against pathogenic pests. Pegg and Vassey (58) have shown the *in vivo* lysis of fungal haphae by chitinase and β-glucanase in tomato plants. Pegg (53) found increased chitinase and β-glucanase activity in potato plants infected with a fungal pathogen when compared to uninfected controls. Similar observations have been made by Wargo (28) in forest trees, Rabenantoando et al. (59) in melons, Nichols et al. (60) in pea tissue and Boller et al. (31) in leaves of six different families of plants.

All the above cited evidence for the hypothesis of a defense function of chitinase and β-glucanase is indirect. Very recently, Mauch and collaborators (Mauch, F. C., University of Colorado, personal communication, 1988.) have obtained the first direct evidence that chitinase and β-glucanase strongly inhibit fungal growth. They demonstrated that crude protein extracts from infected young pea pods as well as combinations of purified chitinase and β-glucanase (at similar concentration found in the infected tissue) had strong, antifungal activity. Because newly formed single strand chitin (nascent chitin) is less organized than preformed chitin (62), chitinase action was most effective on nascent chitin emerging at the hyphal tip. However, lysis of the hyphal tips could occur only in the presence of chitinase and β-glucanase, indicating that hyphal tips contained both chitin and β-glucan (Mauch, F. C.).

When chitin and β-glucan in the fungal and bacterial cell walls are hydrolyzed by their respective enzymes, they yield soluble monomers and oligosaccharide fragments. Alberheim and associates (62-64) were the first to show that fragments from the fungi's cell walls can act as defensive signals (elicitors) in plant tissues and induce production of phytoalexins in plants. Phytoalexins are not present in healthy plants (65). A β-glucan elicitor has been isolated from the cell walls of the fungus *Phytophthora megasperma* f. sp. soyja (63,66) and it is capable of inducing production of the phytoalexin glyceollin. Hadwiger and co-workers (67,68) have found that defensive responses in plants can also be induced by fragments of the chitin and chitosan

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components. They demonstrated that extracts of pea endocarp containing chitinase and chitobiase could lyse Fusarium solani f. sp. pisi and f. sp. phaseoli cell walls (which contain chitin, chitosan and β-glucan) to produce carbohydrate fragments which were strong elicitors of the isoflavinoid phytoalexin pisatin in the pods. Since chitosan was found to be both antifungal and able to increase host resistance responses, chitosan will be useful in agricultural applications to control pathogens. In fact, Hadwiger et al. (57) utilized chitosan as a soil additive, a seed treatment, and a foliar spray. Chitosan treated seeds provided a level of protection to the seed piece and to the seedling in the first 2-6 weeks following germination. Over a five year test period with winter wheat, spring wheat, peas and lentils, chitosan seed treatment increased yield 10-30%. The chitosan cost versus the benefit is very favorable if chitosan is applied on the seeds. The mode of entry of chitosan and its oligomers into the wheat seedling cell appears to be similar to that reported for pea endocarps and funcal cells (57). Chitosan in direct contact with seeds inhibits fungal pathogens and its entry into the emerging seedling induces resistance responses (57).

Tanaka et al. (69) and Ogasawara et al. (70) prepared an antimicrobial mixture containing β-glucanase, chitinase, isopropanol and polyoxyethylenelaurylether and sprayed it over a rice field. The rice plants were inoculated with Pyricularia oryzae (causing rice blight). A week later, they found that the enzyme-mixture treatment had provided significant protection against rice blight. Chitinase is also known to increase the insecticidal activity of microorganisms. Smirnoff (71) sprayed balsam fir trees infected with spruce budworms with a combination of Thuricide and chitinase. The chitinase-Thuricide combination was more effective over the Thuricide control in arresting feeding of larvae and in increasing the mortality rate.

It is known for nearly 50 years that the addition of ground crustacean shells (from crab, shrimp) to soil depresses the growth of some soil fungi and root-rot nematodes (72,73). Furthermore chitin, extracted from shellfish processings, when added to soil induces growth of chitinase-producing microorganisms. In turn, microbial chitinases attack and degrade pathogenic fungi such as Fusarium which causes root-rot of beans (74). More recently, McCandlis et al. (75) developed a process for converting shellfish processing waste into a chitin-protein complex. They claim that this complex is different from chitin and chitosan and has useful nematostatic and nematocidal activities suitable for use in agricultural and horticultural applications. The chitin-protein complex is insoluble in neutral and dilute acid solutions, soluble in concentrated mineral acids with considerable decomposition, low in ash and high in bound nitrogen (it contains about 10% nitrogen). Addition of the complex to nematode cultures in vitro resulted in a significant reduction in the number of living nematodes (75). The recommended application rates of the complex range from 1 to 50% (W/W) in admixture with a plant growth medium. The preferred rates are in the range of 2 to 20% in a potting mixture with soil or vermiculite used in greenhouse plants or nursery stock (75).

Biomass Recovery from Seafood and Cheese Manufacturing Industries

Bioprocessing of Shellfish and Whey Wastes. The United States shellfish processing and cheese manufacturing industries are producing huge quantities of wastes. These industries are being confronted increasingly with problems of proper disposal of their byproducts and the profitable return of such materials into the food chain and other markets. In recent years, some progress have been made in utilizing byproducts of shellfish (crab, shrimp, crawfish) and cheese (whey) industries. Nonetheless, the problems associated with disposal of shellfish and whey wastes worsen each year because the demand for shellfish and cheese is high (76). Consequently, about 75% of the more than 350 million kg of the annual U.S. production of shellfish processing waste are disposed of into municipal sewage systems, streams, landfill

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disposal sites and coastal zone areas (77,78). In 1981, nearly 10 billion kg of whey were produced in the United States as a byproduct of cheese manufacturing (79). Approximately 50% of production was used in a number of markets while the balance was released into the environment (79). In addition to the economic and nutrient losses, the dumping of whey is considered the strongest environmental pollutant of food origin (80,81). Whey is rich in nutrients for it retains about 55% of the nutrients and all the water soluble vitamins in whole milk. Dried whey contains nearly 13% protein of high biological value and about 70% lactose. This high quantity of lactose in dried whey is the reason for its under-utilization as a food source since the prevalence of lactose intolerance and malabsorption ranges from 70-90% in some populations in Africa, Asia, Latin America, and the United States (82-86). A similar incidence of intolerance exists in most adult animal species. The problem of lactose intolerance begins shortly after weaning when consumption of lactose-containing food is reduced. This event is followed by a similar reduction in the synthesis of lactase in the gastrointestinal (GI) tract. Unlike in most microorganisms, lactase is not inducible in humans and most animals. The question, then is: How can we increase the ability of an individual (animal or human) to digest larger amounts of lactose in the diet?

We accomplished this by stimulating the growth of certain beneficial lactase-producing bacteria in the GI tract, thereby supplying lactase to the individual indirectly (87-92). This approach is based on earlier discoveries by Gyorgy and associates (93,94) who found that GlcNAc glycosides (and some oligosaccharides) promote the growth of bifidobacterium bifidus var. pennsylvanicus. The growth promoters serve as a source of glycosidically-bonded GlcNAc residues for bacterial cell wall biosynthesis. The working hypothesis states that chitin is hydrolyzed by chitinolytic enzymes releasing alkyl GlcNAc glycosides and oligosaccharides; these bioactive compounds stimulate the growth of bifidobacteria in the GI tract; as bifidobacteria growth increases, so does production of bacterial lactase, thereby elevating the lactose digestive capacity of the individual. Only the β-anomers of GlcNAc glycosides (and their oligosaccharides) are bioactive. Therefore, the stimulation of bifidobacteria for individuals without chitinolytic enzymes in their GI tract (such as the rat and human), may only be made by feeding the β-anomer of GlcNAc glycoside. Our hypothesis was first tested in rats using 1-propyl-GlcNAc glycoside as chitinous supplement to whey-rich diets. Rats fed a diet containing 30% dried whey and 1.2% chitinous supplement, gained more body weight in eight weeks than rats on the same diet without the supplement (87). The latter group of rats lost their appetite, developed severe diarrhea and cataract and eventually died from dehydration and malnutrition. These results support the hypothesis, but the high cost of the 1-propyl-GlcNAc glycoside (unavailable commercially) would discourage large-scale application. Cost reduction of the supplement was accomplished by using the chicken as the animal model to test our hypothesis. Since the chicken possesses the chitinolytic enzyme system, we were able to feed chitin and thus reduce the cost of the chitinous supplement by more than 180-fold (1-propyl- GlcNAc glycoside was prepared at $1500/kg; chitin is sold at $8/kg).

We conducted several nutritional experiments with broiler chickens using whey-rich diets and chitin or double-sheared chitin (88). Isonitrogenous/ isocaloric diets containing 20% whey and 2% chitin produced chickens which were significantly heavier ($P<0.05$-$P<0.01$) and had better feed efficiency ($P<0.01$) than control birds on the same diet without chitin. Also, birds on the test diet contained less fat ($P<0.01$) in their abdominal fat pads than all control groups including the group fed the commercially formulated diet. In addition to the poor growth, chickens on whey without chitin developed severe diarrhea. In summary, whey alone in the diet depressed weight gain and induced diarrhea. These effects are overcome when both chitin and whey are added to the same diet.

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Further reduction in cost of the chitinous supplement was achieved in two experiments with young ruminating heifers where we supplemented whey-rich rations with raw and demineralized crab meals (91). Statistical analysis showed no significant dietary effect on net weight gain or feed efficiency for either experiment. Diarrhea increased by including whey alone in the ration. In contrast, the inclusion of either raw or demineralized crab meal alone in the ration induced constipation. The addition of crab meal and whey to the ration restored feces to normal. Whey or raw crab meal alone depressed the nutrient digestibility of the ration, but demineralized crab meal alone showed nutrient digestibilities equal to the digestibilities observed in the commercially formulated ration. The addition of either crab meal to the experimental rations yielded nutrient digestibilities similar to those observed in commercial ration. We believe that it is possible to increase the amount of whey (40-60%) and crab meal (20%) in the heifer's ration without adverse effect on growth. Similar results were obtained from a heifer nutritional experiment conducted in Panama under tropical conditions (92). Animals on the experimental ration (which contained 21% domestically grown hay, 22% citrus pulp, 35% dried whey, 16% shrimp meal, and 6% sugar cane bagasse) out-performed all other animals on other rations (including the positive control which contained 27% corn, 33% hay, 20% pulidura rice, and 20% citrus pulp) with an average daily weight gain of 0.84 kg/heifer.

Application of this technology will increase the food supply (especially in third world countries) thereby helping to meet food shortages projected for the latter part of this century and beyond (95), reduce the cost of food production and alleviate a growing waste disposal problem.

Bioconversion of Shellfish Chitin Waste. In the quest of finding ways to utilize the nutrients in shellfish processing waste, another avenue has been discovered. A process has been developed which bioconverts shellfish chitin to yeast single-cell protein (96-98). The product of this process is a protein-rich material which can be used as an animal and aquaculture feed supplement. Since chitin's chemical structure is nearly similar to cellulose, the concept of this process was inspired from the bioconversion process of cellulose to ethanol and single-cell protein (99,100). Carroad and associates (96-98) designed a four-step process for chitin waste bioconversion where chitin is hydrolyzed in a cell-free enzyme environment. Briefly, in the first step shrimp shell waste is reduced in size, deproteinized and demineralized. In the second step, the shrimp shell chitin waste is used to induce the synthesis of the chitinase enzyme system in submerged cultures of *Serratia marcescens*. Hydrolysis of chitin in the shell waste occurs in the third step where the chitinase system produced during the second step is combined with the chitinous waste material. The hydrolysate is filtered and the filtrate is fed to product generation (fourth-step) where it is fermented in submerged culture to yield yeast single-cell protein. Cosio et al. (98) conducted an economic analysis of the bioconversion process and found a negative after-tax cash-flow of $0.06/kg of wet shrimp shell waste. The data used in their study were for the State of California where seafood processors have zero-cost arrangement for disposing of the waste (98). However, in many states, seafood processors pay for the cost for transporting and disposing of their shellfish processings. For example, in Florida, the present cost for shellfish processings disposal for a major processor is $0.224/kg of wet shells (Prebluda, Harry J., Miami FL. 33140, personal communication, 1988). If we enter the cost of disposing of the waste ($0.224/kg of wet shells) in the economic analysis, the bioconversion process becomes profitable with a positive cash-flow of $0.164/kg. Furthermore, additional improvements in reducing equipment cost and favorable changes in federal tax policy would improve even more the economics of the process. Aside from the economic considerations, a successful bioconversion process for the utilization of shellfish processing waste will reduce significantly the environmental damage (by dumping this material) and provide a means by which nutrients in the shellfish waste can be returned into the food chain.

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Chapter 9

Extracellular Peroxidases Involved in Lignin Degradation by the White Rot Basidiomycete *Phanerochaete chrysosporium*

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The white rot basidiomycete Phanerochaete chrysosporium secretes two soluble H_2O_2-requiring peroxidases which are involved in lignin degradation. Both lignin peroxidase (LiP) and manganese peroxidase (MnP) form the oxidized intermediates compound I and compound II. LiP catalyzes the one-electron oxidation of a variety of substrates to generate aryl cation radicals. These radicals undergo subsequent nonenzymic reactions to yield a multiplicity of final products. MnP oxidizes MnII to MnIII which acts as a unique redox couple, and in turn oxidizes phenolic substrates to phenoxy radicals, which undergo subsequent reactions to yield the final products.

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Lignin is the second most abundant natural polymer in the biosphere and the most abundant renewable aromatic material. It comprises 15-30% of woody plant cell walls, forming a matrix surrounding the cellulose. This encrusting matrix (1,2) significantly retards the microbial depolymerization of cellulose and thus lignin plays a key role in the earth's carbon cycle (1-3).

Lignin is a phenylpropanoid polymer (Fig. 1) synthesized from p-hydroxycinnamyl (coumaryl) alcohol, 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol precursors (2,3). Free radical condensation of these alcohols, initiated by plant cell wall peroxidases, results in the formation of a heterogeneous, amorphous, optically inactive, random and highly branched polymer containing at least 12 different interunit linkages connecting the aromatic nuclei. The most abundant linkage is the β-O-4 type (between aromatic nuclei 2 and 1 in Fig. 1). The β-1 linkage type (between aromatic nuclei 11 and 12 in Fig. 1) has served as a model in several biodegradation studies.

White-rot basidiomycetes are the only known organisms which are capable of degrading lignin extensively to CO_2 and H_2O in pure culture. These organisms invade the lumen of woody plant cells and secrete extracellular enzymes which attack all of the polymers in

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Figure 1. Schematic structure for conifer lignin.

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wood. Early work indicated that lignin degradation by the best-studied lignin degrading fungus, Phanerochaete chrysosporium, is oxidative and nonspecific (4-6). Chemical analyses of white-rotted residual lignin (7,8) and of lignin model compound degradation products (9-11) indicated that sidechain cleavage and ring opening reactions occur simultaneously.

Since lignin is such a complicated insoluble polymer, it is difficult to elucidate the mechanism of its oxidation by analysis of its degradation products. Therefore, starting in the late 1970's, a number of studies in our own laboratory and from the laboratories of T. Higuchi and T. K. Kirk focused on the metabolism of dimeric model lignin substructures (9-16), whose oxidation products are easier to analyze than the degradation fragments of the polymeric substrate. Dimeric model compounds were shown to be degraded under the same physiological conditions necessary for polymer degradation (4,5,12-18), suggesting that the lignin degradation system was responsible for the oxidation of these model compounds. Work with β-O-4 and β-1 dimeric model compounds indicated that a multiplicity of oxidative reactions were catalyzed by the nonspecific lignin degradative system of P. chrysosporium (9-17). Indeed, the metabolic studies with dimeric lignin model compounds led to the rational design of assays for the enzymic components of the lignin degradative system.

In 1983, our laboratory and that of T. K. Kirk announced the simultaneous discovery in P. chrysosporium of an extracellular H_2O_2-requiring enzyme involved in lignin degradation (19-22). This enzyme is now referred to as lignin peroxidase (LiP) or ligninase. Subsequently, a second P. chrysosporium enzyme involved in lignin degradation, manganese peroxidase (MnP), was isolated in our laboratory (23). These two enzymes, LiP and MnP, along with an H_2O_2 generating system (24) appear to be the major components of the P. chrysosporium lignin degradation system. This chapter reviews some of the work on the spectroscopic and mechanistic properties of these peroxidases. For more exhaustive reviews of lignin degradation, see Literature Cited (24,25).

Lignin Peroxidase and Manganese Peroxidase: General Properties

LiP has been purified to electrophoretic homogeneity by a combination of anion exchange chromatography and gel filtration (26-28). The enzyme is present as a series of isozymes (28,29) with pI ranging from 3.2 to 4.0. LiP contains one mole of iron protoporphyrin IX per mole of enzyme and is a glycoprotein of molecular mass 41,000. MnP has also been purified to electrophoretic homogeneity (30,31). The enzyme exists as a series of isozymes with pI ranging from 4.2 to 4.9, contains one mole of iron protoporphyrin IX per mole of enzyme and is a glycoprotein of molecular mass 46,000 (29-31).

Spectroscopic Properties of Lignin Peroxidase and Manganese Peroxidase

Electronic absorption maxima for LiP, MnP, horseradish peroxidase (HRP) and various liganded forms of these enzymes are shown in Table I. The spectra of native LiP and MnP are characteristic of high-spin ferric heme proteins, with Soret and visible maxima (407, 502 and

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632) (26,30) similar to those of HRP and myoglobin. Like HRP, the ferric forms of LiP and MnP form typical low-spin hexacoordinate complexes with CN^- and N_3^- (26,30), with spectra distinct from those of P-450 low-spin complexes (32). The spectra of the reduced enzymes are typical of high-spin pentacoordinate ferrous heme. The ferrous enzymes also form complexes with CO (Table I) which have spectra typical of peroxidases and rule out a cysteinate fifth ligand as is found in cytochrome P-450 (32,33).

Table I. Electronic Absorption Spectral Maxima of Lignin Peroxidase, Manganese Peroxidase, and Horseradish Peroxidase

System	nm				Ref.
Ferric, high spin					
LiP, pH 4.5	407	500		632	26
MnP, pH 4.5	406	502		632	30
HRP, pH 6.0	403	500		641	39
Ferric, low spin					
CN^--LiP	423		540		26
CN^--MnP	421		546		30
CN^--HRP	422		539		39
N_3^--LiP	418		540	575	26
N_3^--MnP	417		542	580	30
N_3^--HRP	416		534	565	39
Ferrous					
LiP	435		556		26
MnP	433		554		30
HRP	437		556		39
Ferrous CO					
CO-LiP	420		535	568	26
CO-MnP	423		541	570	30
CO-HRP	423		541	575	39

The EPR spectra of native LiP (34) and MnP (35) are also typical of high-spin ferric heme, with g values 5.83 and 1.99 (LiP) and 5.79 and 1.99 (MnP). These values are essentially identical to those of aquometmyoglobin but differ somewhat from those of HRP for which a large rhombic component is observed. Resonance Raman (RR) studies (34-37) also indicate that native LiP and MnP are high-spin ferric enzymes which are predominantly pentacoordinate at room temperature. RR studies confirm that the native enzymes form low-spin complexes with CN^- and N_3^- and that the reduced enzymes have high-spin

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pentacoordinate ferrous hemes. All of these studies indicate that the fifth ligand to the heme iron is a histidine as found for other plant peroxidases (38,39).

Oxidized Intermediates of LiP and MnP

Optical spectral data for the oxidized intermediates of LiP, MnP and HRP are shown in Table II (38-41). The primary reaction product of peroxidases with H_2O_2 is the oxidized intermediate compound I. This intermediate has absorption maxima at ~407, ~550 and ~650 (Table II). The absorption maxima of LiP and MnP compounds I are very similar to those of HRP compound I, indicating that they contain two oxidizing equivalents over the native ferric enzyme. The first oxidizing equivalent resides in the FeIV=O state of the iron (38,39). The second equivalent resides in a porphyrin π-cation radical $[P^{\dot{+}}]$. Reduction of compound I by one molar equivalent yields compound II (maxima at ~420, ~525 and ~555). A variety of spectroscopic studies indicate an FeIV=O structure for HRPII (38,39). Recent RR evidence supports an FeIV=O structure for LiPII (36). Finally, addition of excess H_2O_2 to HRP, LiP or MnP yields the complex compound III (maxima ~419, 545, 579) which is believed to be a ferric superoxide complex ($FeIIIO_2^{\dot{-}}$) (39-41). While compound III is not part of the normal catalytic cycle of peroxidases, LiP is unusual in that it is easily oxidized to LiPIII at its pH optimum (3.0) in the absence of reducing substrate. This results in the inactivation of the enzyme (Wariishi and Gold, in preparation, 42).

Table II. Absorption Maxima (nm) of Oxidized Intermediates of Several Peroxidases

Peroxidase	Compound I	Compound II	Compound III	Ref.
LiP	408, 550, 608, 650	420, 525, 556	419, 543, 578	40
MnP	407, 558, 605, 650	420, 528, 555	417, 545, 579	41
HRP	400, 557, 622, 650	420, 527, 554	413, 546, 583	38,39

Lignin Peroxidase Kinetics and Catalytic Cycle

LiP catalyzes the oxidation of 3,4-dimethoxybenzyl alcohol (veratryl alcohol) to veratryl aldehyde. Since this reaction can be easily followed at 310 nm, it is the basis for the standard assay for this enzyme (26,27). The enzyme exhibits normal saturation kinetics for both veratryl alcohol and H_2O_2 (28,43). Steady-state kinetic results indicate a ping-pong mechanism in which H_2O_2 first oxidizes the enzyme and the oxidized intermediate reacts with veratryl alcohol (43). The enzyme has an extremely low pH optimum (~2.5) for a peroxidase (43,44); however, the rate of formation of compound I (k_1, Fig. 2) exhibits no pH dependence from 3.0-7.0 (45,46). Addition of excess veratryl alcohol at pH 3.0 results in the rapid conversion of

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LiPI to LiPII (~20 msec) followed by a slower conversion of LiPII to the native enzyme (~500 msec) (46). Thus with veratryl alcohol as the reducing substrate, the normal catalytic cycle is completed via two single electron steps (40,46) (Fig. 2). In contrast to LiPI formation, the rate of conversion of LiPI to LiPII (k_2) and LiPII to the native enzyme (k_3) are strongly dependent on pH with optimum activity at low pH. Apparently the pH dependence of k_2 and k_3, rather than of k_1, dictate the overall pH dependence of the enzyme (46).

Manganese Peroxidase Catalytic Cycle

The oxidation of phenols and other organic substrates by MnP is dependent on MnII (23,30). Apparently the enzyme first oxidizes MnII to MnIII, and MnIII subsequently oxidizes the organic substrates (30,31,47). As shown in Figure 3, addition of one equivalent of MnII rapidly reduces MnP compound I to compound II (41). A second equivalent of MnII reduces MnP compound II to the native ferric enzyme. Similarly, MnP compound I is reducible by phenolic substrates, albeit at a slower rate. However, phenolic substrates are not able to reduce MnP compound II efficiently (41). Thus the enzyme is unable to complete its catalytic cycle efficiently in the absence of MnII. This would seem to explain the absolute MnII requirement for catalytic activity. In the conversion of MnP compound I to compound II, the porphyrin π-cation is reduced back to a normal porphyrin. This suggests that the porphyrin radical is exposed in a peripheral site as recently suggested for HRP (48) and that this site may be available to organic substrates and to MnII. In contrast, the FeIV=O center of MnP compound II may be partially buried and only available to MnII ions.

Reactions Catalyzed by Lignin Peroxidase

Lignin peroxidase catalyzes the H_2O_2-dependent oxidation of a wide variety of nonphenolic lignin model compounds to yield numerous products (Figs. 4 and 5). These reactions include but are not limited to (a) benzylic alcohol oxidations, (b) $C\alpha$-$C\beta$ bond cleavage, (c) α-methylene hydroxylation, (d) β-aryl ether bond cleavage and demethylation, (e) rearrangements, and (f) ring-opening reactions (24-27,49-51). All of these reactions are consistent with a mechanism involving the initial one-electron oxidation of the substrate by the oxidized enzyme intermediates LiPI and LiPII to form an aryl cation radical followed by a series of nonenzymatic reactions to yield the final products (28,52-54). This is illustrated in Figure 5 for the oxidation of a β-1 diarylpropane dimeric model compound (54). In this pathway the oxidized enzyme in turn oxidizes the diarylpropane to form an aryl cation radical. Direct EPR evidence for formation of aryl cation radicals by LiP has been presented (53). The unstable aryl cation radical undergoes $C\alpha$-$C\beta$ cleavage to form the benzaldehyde **(2)** and a C_6C_2 benzylic radical intermediate. EPR evidence for such carbon-centered radical intermediates in LiP oxidation has also been presented (55). The benzylic radical intermediate is attacked preferentially by O_2 under aerobic conditions and the resulting hydroperoxy radical would decompose to form the phenylglycol **(3)**

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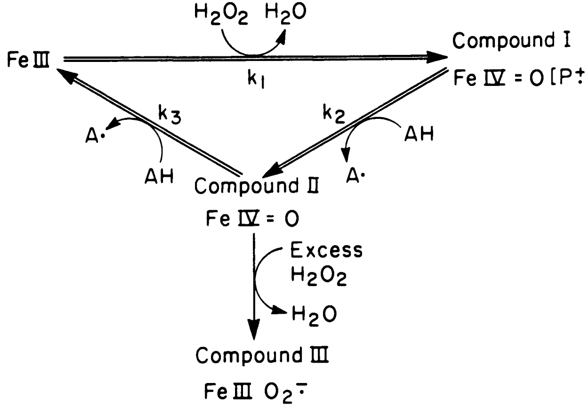

Figure 2. Catalytic cycle of lignin peroxidase.

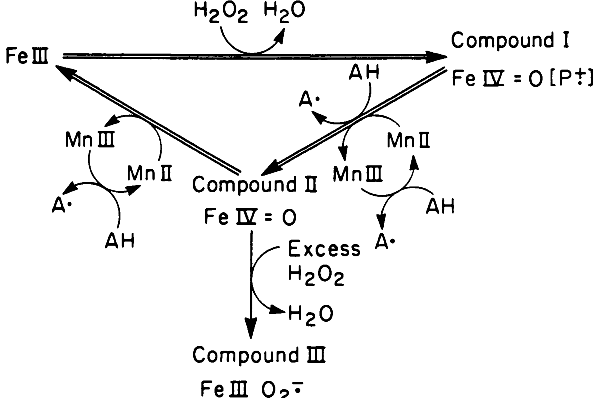

Figure 3. Catalytic cycle of manganese peroxidase.

Figure 4. Reactions catalyzed by lignin peroxidase. Me = methyl, Et = ethyl.

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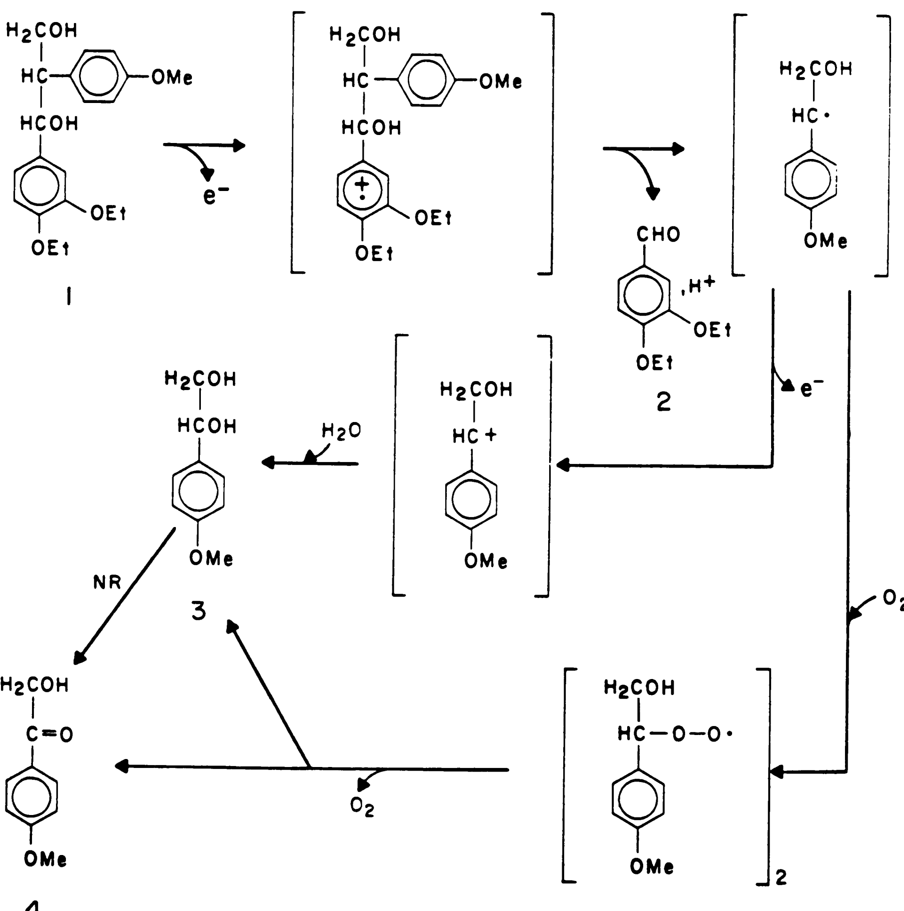

Figure 5. Mechanism of C_{α}-C_{β} cleavage by lignin peroxidase. (Reprinted with permission from ref. 54. Copyright 1986 Academic.)

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and phenylketol **(4)** final products. In the absence of O_2, however, the C_6C_2 benzylic radical is oxidized by the enzyme to the corresponding carbonium ion which is subsequently attacked by H_2O yielding the phenylglycol **(3)**. This pathway is consistent with $^{18}O_2$ and $H_2{}^{18}O$ incorporation studies (54).

Thus, the key reaction of LiP with lignin model compounds is a one-electron oxidation of the substrate to an aryl cation radical and this is partially dependent on the oxidation potential of the aromatic ring. Strong electron withdrawing groups such as benzylic carbonyls suppress the formation of aryl cation radicals (56) while electron donating alkoxy groups tend to activate aryl cation radical formation (51). Once the aryl cation radical is formed, in most cases it can diffuse away from the enzyme active site, where it can undergo C_α-C_β sidechain cleavage as shown in Figure 5, or react with H_2O, O_2 or other radicals to yield the other oxidized, hydroxylated, β-ether-cleaved, or ring-opened products seen in Figure 4.

The rational design of substrates has allowed us to study sidechain cleavage and rearrangement as well as ring-opening reactions in greater detail (50,51,57). The β-ether substructure **(12)** (Fig. 4) has no alkoxy groups on ring C, one alkoxy group on ring A, and three alkoxy groups on ring B. Since at least two ring alkoxy groups are usually required to form a radical cation, only ring B will be oxidized. Formation of a radical cation on ring B results in its subsequent attack by H_2O or the γ or α sidechain hydroxyls followed by coupling with O_2 to yield several possible cyclic peroxide intermediates (49,50). The unstable cyclic peroxides undergo a variety of cleavage reactions to yield the final products **13** through **19** (Fig. 4) (50). The cyclic carbonates 14 and 15 were originally isolated by Higuchi's group (11,49,58). Oxidative aromatic ring cleavage had heretofore been thought to be solely catalyzed by dioxygenases; however, in the LiP-catalyzed reaction, ring cleavage is initiated by a one-electron oxidation.

Reactions Catalyzed by Manganese Peroxidase

MnP was originally characterized as a manganese-dependent lactate-activated peroxidase which could oxidize a variety of phenols and dyes (23). Later it was reported that the enzyme could readily oxidize MnII to MnIII (30,31,47) and that α-hydroxy acids such as lactate activate the system, probably by chelating MnIII to form stable complexes with a high redox potential (41,47). Recently, we showed that the enzyme could not readily complete its catalytic cycle in the absence of MnII (41) explaining MnP's absolute requirement for MnII. At low pH the MnIII/MnII oxidation reduction potential is 1.5 volts and exogenous MnIII is capable of oxidizing the phenols and amines oxidized by the enzyme system (30,31,41,47). All of these results indicate that this enzyme system utilizes freely diffusible MnII/MnIII as an obligatory redox couple to oxidize the terminal phenolic substrate, lignin. As shown in Figure 1, about 10-15% of the phenolic groups in lignin are free. Presumably, the MnIII can diffuse into the polymeric matrix and oxidize the polymeric free phenolic groups. The MnP-catalyzed oxidation of a free phenolic β-1 lignin substructure is shown in Figure 6 (Wariishi et al., in

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Figure 6. Oxidation of a free phenolic β-1 dimer by manganese peroxidase.

preparation). The initial reaction is a one-electron oxidation of the phenol to form a phenoxy radical intermediate. Subsequently, alkyl phenyl cleavage of the radical intermediate would yield products **3** through **5**, dehydrogenation would yield the ketone **(2)** and finally, C_α-C_β would yield the products **6** through **8**. With this substrate, radical coupling is inhibited by the presence of a methoxy group at the 5 position on ring A.

Conclusions

The white rot basidiomycetous fungus P. chrysosporium produces two extracellular nonspecific peroxidases which catalyze the oxidative degradation of lignin. Both of these enzymes are oxidized by H_2O_2 to form the oxidized intermediate compound I. Depolymerization is initiated by the one-electron oxidation of the substrate either directly or through the redox mediation of Mn, leading to the formation of substrate free radicals. The free radical intermediates in turn undergo further reactions, dependent on their structure, ultimately yielding the diversity of final products observed.

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Chapter 10

Peroxidase-Catalyzed Polymerization of Phenols

Kinetics of *p*-Cresol Oxidation in Organic Media

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Horseradish peroxidase is up to 5.4 times more active in high concentrations of dioxane and methanol than in aqueous buffer, as determined by values of k_{cat} (the catalytic turnover number), when the intraparticle and external diffusional limitations, normally associated with enzymatic catalysis in monophasic organic solvents are eliminated. In model reaction systems consisting of *p*-cresol, hydrogen peroxide, and dioxane, methanol, or acetone (each in concentrations ranging from 60-95% v/v), horseradish peroxidase catalyzed the initial oxidation of *p*-cresol to 2,2'-dihydroxy-5,5'-dimethylbiphenyl (biscresol) as a major product. At longer reaction times, polymeric material was formed. Effect of the organic solvents on peroxidase was primarily to increase the K_m of *p*-cresol. This increase was often dramatic with K_m of *p*-cresol in 80% methanol over 2 orders of magnitude larger than in aqueous buffer.

Phenol-formaldehyde resins find numerous applications in such areas as wood composites, fiber bonding, laminates, foundry resins, abrasives, friction and molding materials, coatings and adhesives, and flame retardants (1). From a specialty chemicals standpoint, they are also used as developer resins in carbonless papers (2). Conventional methods of preparation involve condensation of a phenol with formaldehyde under either acidic (novolak) or basic (resole) conditions (3). Their typical molecular weight range is from 800-4000 daltons (D) and includes a wide variety of alkyl or aryl substituted phenols (4). The

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final high molecular weight resinous material is produced via thermal curing.

Recently, serious concern has been raised over the continued use of phenol-formaldehyde resins due to the various toxic effects of formaldehyde, both in resin manufacture and use (5-6). An additional concern is that the simple acid- or base-catalyzed condensation of phenols with formaldehyde is not conducive to achieving rigorous control over the desired physicochemical characteristics of the final polymer. Such characteristics include molecular weight and polydispersity, degrees of cross-linking and crystallinity, nature of interunit bonding, and melting/softening temperature, all of which contribute to the structural and functional properties of a desired phenolic polymer. For these reasons, alternatives for the production of phenolic resins are needed.

Horseradish peroxidase-catalyzed polymerization of phenols may offer a potential solution to this problem. In nature, peroxidases catalyze the formation of lignin in plants (7). Peroxidases, therefore, have the inherent ability to synthesize high molecular weight phenolic polymers that have the potential to act as general substitutes for conventional phenol-formaldehydes without the need for formaldehyde. Attempts to reproduce high molecular weight lignin-type compounds (m.w.'s above 1500), using horseradish peroxidase, *in vitro* in aqueous media, however, have failed (8) apparently because of the poor solubility of the growing polymer chain in water. An alternative approach is to carry out peroxidase-catalyzed polymerization of phenols in non-aqueous media. As opposed to water, a wide variety of organic solvents will solubilize high molecular weight phenolic polymers; the growing polymer chain, therefore, will not precipitate and polymerization can continue.

We have shown that horseradish peroxidase can oxidize phenols in a wide variety of organic solvents (9-10). Using *p*-phenylphenol as an example, we have shown that peroxidase-catalyzed polymerization in dioxane (containing 15% aqueous buffer) yields polymers with molecular weights over 50-fold higher than in aqueous media. A wide variety of alkyl, aryl, and halo phenols were polymerized by a similar approach yielding polymers with molecular weights ranging from 375 - 2.6×10^4. Water-miscible solvents supported high polymer formation, whereas reactions in water-immiscible solvents yielded only low molecular weight oligomeric products. Perhaps a partitioning of the growing polymer chain away from the enzyme (which acts as a phenoxy radical initiator) and into the bulk solvent dilutes the local concentration of the oligomeric polymer building blocks in the vicinity of the enzyme thereby preventing high polymer formation. Such an effect would not occur in dioxane.

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This initial study was highly phenomenological. Peroxidase is capable of polymerizing a number of phenols in organic solvents but information regarding the effect of the solvent or the nature of the phenolic substituent on the progress of polymerization is lacking. Such information is vital to the development of rational approaches for the commercial synthesis of peroxidatively-produced phenolic resins. From a broader perspective, the elucidation of enzyme kinetics in organic solvents is a challenge which has rarely been addressed. Perhaps the most sought after question which has yet to be answered is - what is the effect of organic solvent on the intrinsic kinetics of enzymatic catalysis?

Evaluating the catalytic "efficiency" in organic solvents presupposes a reference point. It is argued, herein, that the most logical reference point, for most enzymes, is enzymatic catalysis in aqueous solutions. Because water is the natural solvent for all non-membrane associated enzymes, the efficiency of enzymatic catalysis in organic solvents can only be determined by direct comparison to the kinetics in water. While this approach appears logical and straightforward, two factors have prevented enzyme kinetics in organic media from being directly compared to that in aqueous solutions: a) the insolubility of enzymes in nearly all organic solvents; and b) the alteration in the enzymatic reaction mechanism in organic media as opposed to water. The former factor is similar to comparing free versus immobilized enzymatic catalysis in aqueous solutions and stems from the difference in observed versus intrinsic kinetics brought about by significant diffusional resistances caused by immobilization. The latter factor results from the fact that in low water environments, many enzymes carry out reactions completely distinct from the natural, aqueous-based reactions.

Horseradish peroxidase is an excellent candidate with which to elucidate enzymatic kinetics in organic solvents. It is an active enzyme with turnover numbers exceeding 320 s^{-1} in organic media (11) and hence is susceptible to diffusional limitations which must be overcome. Peroxidase also catalyzes mechanistically identical reactions in aqueous and organic media. Therefore, direct kinetic comparisons between aqueous and organic reactions can be made and the effects of the organic solvent on reactivity and substrate specificity can be directly compared to aqueous-based catalysis.

In this study, the kinetics of horseradish peroxidase-catalyzed oxidation of *p*-cresol (4-methylphenol) is evaluated in a number of representative water-miscible organic solvents. Cresol is one of the most common phenols used in the phenolic resin industry (1) and is an excellent substrate of peroxidase (12). The stoichiometry of peroxidase catalysis is described in Equation 1. The predominant products in aqueous solutions are

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2 [p-cresol: OH, CH_3] + H_2O_2 ⟶ 2 [phenoxy radical: O•, CH_3] + $2H_2O$ ⟶ Polymer (1)

2,2'-dihydroxy-5,5'-dimethylbiphenyl (biscresol), 4',6-dimethyldibenzofuran-2-one (Pummerer's ketone), and low molecular weight oligomeric coupling products (13). At initial reaction times, only the first two products will be significant and they can be easily followed by HPLC.

Experimental

Materials. Horseradish peroxidase (type II, 200 purpurogallin units per mg protein) and non-porous glass beads (75-150 μ diameter) were obtained from Sigma Chemical Co. (St. Louis, MO). *p*-Cresol, hydrogen peroxide (as a 30% solution in water) and dioxane (HPLC grade) were obtained from Aldrich Chemical Co. (Milwaukee, WI). The actual H_2O_2 content was determined spectrophotometrically at 240 nm. All other solvents used in this work were HPLC grade.

Purification of Dioxane. Dioxane was purified (to remove unwanted peroxides) as follows (14): 300 mL water, 40 mL concentrated HCl, and 3 L dioxane were refluxed for 12 h, maintaining a slow nitrogen purge through the solution. The solution was cooled, KOH pellets were added to saturation, and the dioxane was decanted from the resulting upper layer. The dioxane was dried with fresh KOH. Finally, the dry dioxane was refluxed over sodium metal for 12 h and distilled affording dry, peroxide free dioxane. The dioxane was stored in the dark under a nitrogen atmosphere.

Peroxidase-Catalyzed Polymerization of *p*-Cresol. Large scale polymerizations were carried out in a volume of 250 mL in a 500 mL round bottom flask at 25°C with stirring at ca. 250 rpm. *p*-Cresol (688 mg, 25 mM) was dissolved in 213 mL dioxane and 37 mL aqueous buffer, pH 7 (0.01 M phosphate) added to give a solution consisting of 85% (v/v) dioxane. Horseradish peroxidase (25 mg, free powder) was added and the reaction was initiated by the addition of 0.28 mL of 30% H_2O_2 (10 mM). The suspension (peroxidase is insoluble in 85% dioxane) immediately turned yellow and the reaction was allowed to proceed 15 min. The concentrations of *p*-cresol and reaction products were determined by high performance liquid chromatography (HPLC) with a C_{18}-reverse phase column (Waters Associates, Milford, MA). The isocratic solvent used was acetonitrile:water (56:44) with

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a flow rate of 1.5 mL/min. Under these conditions, the *p*-cresol eluted at 3.28 min and the dimeric product at 5.88 min. Detection was performed with a Model 440 Absorbance (Waters Assoc.) detector at 280 nm. Molecular weight determination of the reaction products of *p*-cresol oxidation was performed using gel-permeation (GPC) HPLC (100 A Ultrastyragel from Waters) with tetrahydrofuran as eluant (flow rate of 1 mL/min). Under these conditions the *p*-cresol eluted at 8.80 min and the dimer at 7.34 min. Molecular weight standards were polystyrenes with molecular weights of 517, 1000 and 2000.

Product identification was carried out as follows. The reaction mixture was filtered to remove the enzyme particles and the solvent evaporated on a rotary evaporator yielding a yellow oil. The oil was dissolved in 100 mL diethyl ether and the low molecular weight phenolic products were extracted into an equal volume of 5% NaOH solution. The residual ether soluble fraction contained primarily Pummerer's ketone and higher molecular weight phenolics while the base-soluble fraction contained the *p*-cresol and the biscresol. Further purification was performed by preparative thin layer chromatography (TLC) (1 mm silica gel G plates, Analtech, Newark, DE) with a solvent system of diethyl ether:heptane (2:1). R_f values were 0.81 for biscresol and 0.63 for Pummerer's ketone.

<u>Adsorption of Peroxidase onto Glass Beads</u>. Glass beads were washed with 10% (v/v) nitric acid prior to use. Peroxidase was deposited (adsorbed) onto the beads in the following manner. One mL of the enzyme solution in 0.01 M phosphate buffer, pH 7, was added to 2 g glass beads. The slurry was gently mixed, spread on a watch glass, and left to dry at room temperature with occasional mixing until visibly dry and freely flowing beads were obtained. This approach enables variable enzyme loadings onto the glass beads to be attained and is especially pertinent for kinetic studies.

<u>Determination of Initial Rates of Peroxidase Catalysis in Organic Media</u>. As a typical oxidation reaction, the following example with dioxane as solvent is discussed. The peroxidase, adsorbed onto glass beads, was added to 5 mL dioxane (containing from 5-40% v/v aqueous buffer). [In independent experiments it was determined that the enzyme remained completely adsorbed to the glass surface in dioxane concentrations of 70% and greater. At 60% dioxane, roughly three-quarters of the enzyme had desorbed from the glass (this manifests itself as completely soluble enzyme in the reaction supernatant). A similar effect was observed with acetone, while with methanol, complete retention of adsorbed peroxidase requires 80% of the organic solvent. In aqueous solutions, complete desorption of the enzyme takes place with full retention of activity.]

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p-Cresol (2.5-50 mM) was added and the reaction was initiated by the addition of 0.25 mM H_2O_2. The mixture was shaken at 250 rpm and 30 °C, and periodically 100 µL aliquots were removed and initial rates of reaction determined by the formation of the dimeric product as a function of time by HPLC.

Results and Discussion

Cresol Oxidation Products in Organic Media. The oxidation of *p*-cresol was initially carried out in dioxane (containing 15% aqueous buffer, pH 7). Dioxane was previously shown to be an ideal solvent for synthesizing high molecular weight phenolic polymers. It was of interest, therefore, to examine the oxidation products of peroxidase-catalyzed oxidation of *p*-cresol in this solvent, particularly under initial rate conditions. To that end, a semi-preparative scale reaction was performed (See Experimental). After 15 min, the reaction was terminated and the products worked up as described in the Experimental Section. Roughly 40% of the *p*-cresol had reacted, mainly to dimeric material as determined by GPC. This was expected as the reaction time and hence conversion of the *p*-cresol was kept purposely low in order to prevent polymeric material from forming. The products were separated by preparative TLC and two major components, other than the starting material, were identified as biscresol and Pummerer's ketone (253 mg, 37% yield, and 15 mg, 2% yield, respectively). These products were expected based on the well-established mechanism of *p*-cresol oxidation catalyzed by peroxidase in aqueous solutions (13). At least at early reaction times, where conversion to polymeric material has not occurred, the oxidation reaction in organic solvents is similar to that in water. This is crucial in order to directly compare the intrinsic kinetics of peroxidase catalysis in organic versus aqueous media.

Reaction Optimization. The use of enzymes in organic media is often not a straightforward endeavor. Numerous conditions must be satisfied in order to obtain optimal catalysis including the maintenance of the proper ionogenic state of the enzyme and elimination of diffusional limitations (via proper biocatalyst preparation).

One intriguing aspect of enzymatic catalysis in low water environments (this includes 85% dioxane where the water activity is substantially less than unity) is the effect of reaction pH. In aqueous solutions, the ionogenic functional groups of an enzyme respond to the pH of the solution. Definitive pH optima exist for all enzymes. In organic solvents, however, the lack of water as a bulk solvent makes pH an unmeasurable variable; although there will be a small concentration of protons in the vicinity of

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the enzyme, pH measurement is technically not feasible. While the determination of pH in organic solvents is not possible, the pH of the aqueous solution in contact with the enzyme can be easily controlled.

To elucidate the behavior of peroxidase in dioxane/water solutions at different pH's, the following experiment was designed. Horseradish peroxidase (1 mg) was suspended in 8.5 mL anhydrous dioxane containing 5.5 mg *p*-cresol (5 mM) and 1.5 mL aqueous buffer was added with pH ranging from 3-9. The buffers (0.01 M) used were tartrate (pH 3), phosphate (pH 5-7), and borate (pH 9). The enzymic reaction was initiated by adding 0.25 mM hydrogen peroxide and the suspension shaken at 250 rpm and 30 ^{o}C. As depicted in Figure 1, a sharp pH optimum for *p*-cresol oxidation was obtained with a maximum at pH 7. Published reports of peroxidase catalysis in aqueous solutions have shown that the enzyme exhibits a broad pH optimum from pH 4-9 (15). Hence, the organic solvent appears to constrict the operational pH range of peroxidase, although a shift in pH optima in dioxane as compared to aqueous solutions is not evident.

The insolubility of enzymes in monophasic organic systems has a controlling influence on the kinetics of enzymatic catalysis in organic media. Insolubilized enzymes are subject to intraparticle and external diffusional limitations which can mask the true, intrinsic kinetics of catalysis. These limitations are particularly severe for highly active and purified enzymes such as horseradish peroxidase. One way to overcome this problem is to increase the surface area of the enzyme in contact with the organic solvent.

We have opted to use adsorption of the peroxidase onto glass beads (9). This method is quick and simple and is based on the inability of enzymes, once adsorbed onto a solid support, to desorb in an organic solvent unless significant water is present (i.e., dioxane containing greater than 30% v/v water). Furthermore, it is expected that until monolayer coverage of the peroxidase on the bead surface is reached, internal diffusional limitations are negligible. We set out to prove this hypothesis by performing the following experiment. A solution of horseradish peroxidase in 0.01 M phosphate buffer, pH 7, was dried onto glass beads (75-150 μ) in the range from 0.05 mg peroxidase per g bead to 20 mg peroxidase per g bead. One hundred micrograms of peroxidase adsorbed to different amounts of glass beads were then added to 10 mL dioxane solutions containing 5 mM *p*-cresol and 15% v/v phosphate buffer (0.01 M, pH 7). The reactions were initiated by adding 0.25 mM hydrogen peroxide and the suspensions shaken at 250 rpm and 30°C. The effect of enzyme loading onto glass beads is depicted in Figure 2 and the results are dramatic. As the enzyme loading on the glass surface is decreased from 20 mg peroxidase/g bead to

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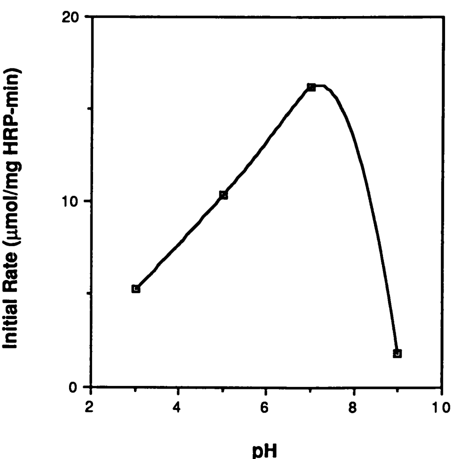

Figure 1. Effect of aqueous component pH on peroxidase catalysis in 85% dioxane. See text for details.

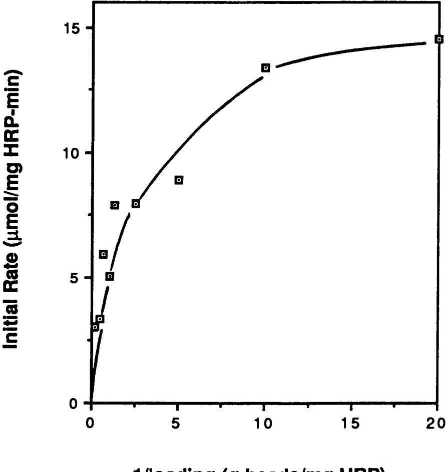

Figure 2. Elimination of intraparticle diffusional limitations by peroxidase deposited onto glass beads (diameter of 75-150 µ). See text for details.

0.05 mg peroxidase/g bead, the specific initial rate of catalysis, as determined by the rate of biscresol formation, increased over 7 fold. The specific activity of peroxidase leveled off at approximately 0.1 mg peroxidase/g beads suggesting that maximum monolayer enzyme coverage on the glass beads was achieved. At this coverage, internal diffusional limitations are expected to be minimal.

In order to ascertain whether maximum monolayer coverage of the enzyme onto the glass surface had occurred, a theoretical evaluation of surface area coverage of peroxidase on the glass surfaces was made. The bead surface is easily calculated from the bead radius and gives the maximum area the peroxidase (assumed to be spherical) can project onto the glass surface thereby defining the effective monolayer coverage. For proteins, the hydrated radius, r_h, which determines the size of the molecule (assuming a spherical shape) is give by Equation 2 (16) where M is the protein molecular weight (42,000 for horseradish peroxidase), N_a is Avogadro's

$$r_h = [0.75/\pi(M/N_a)(V_2 + \partial V_1)]^{0.33} \qquad (2)$$

number, V_2 is the specific hydration volume (0.75 cm^3/g, assuming peroxidase has a similar value to that of hemoglobin), ∂ is the degree of hydration of the protein (0.36 g H_2O/g protein, assumed from comparison to published values from hemoglobin (15), and V_1 is the partial specific volume for pure water (1 cm^3/g). For horseradish peroxidase the value of r_h is 2.64×10^{-7}cm. This translates into a projected area of 2.19×10^{-13} cm^2/enzyme molecule. Table I shows the values of monolayer enzyme loading as a function of the glass bead size. Clearly a value of 100 μg/g peroxidase using 75-150 μ size beads is very similar to the predicted value range of 51-153 μg/g and indicates that peroxidase assumes a monolayer orientation on the glass beads' surface. External diffusional limitations can be minimized by increasing agitation. Unlike aqueous or liquid-liquid biphasic systems (17-18), high agitation in monophasic organic solvent systems does not lead to shearing of the enzyme molecules, perhaps because the enzymes are insoluble and incapable of dissolving and denaturing at an interface. In dioxane containing various concentrations of aqueous buffer ranging from 5-30%, pH 7, and 100 μg/g beads peroxidase loading onto glass beads, maximal catalytic rates were obtained at 250 rpm. The maximization of catalytic power of peroxidase in organic media is obtained upon the elimination of diffusional resistances. From the data above, this corresponds to 100 μg peroxidase per g bead and shaking at 250 rpm.

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Table I. Calculated Values of Critical Enzyme Loading, L_c, As a Function of Bead Size[a]

Bead Size (μ)	Area of Bead (cm^2/g bead)	L_c (μg HRP/g bead)
25	927	307
50	486	153
150	158	51
500	48.6	15.3
3000	8.0	2.5

[a]Bead density calculated to be 2.5 g/cm^3 using Sigma non-porous glass beads; sizes from 75-150 μ.

As a final optimization study, we evaluated the effect of hydrogen peroxide concentration on peroxidase catalysis. Unlike the phenolic substrate, H_2O_2 is a well-known inhibitor of peroxidase (12). We examined the effect of hydrogen peroxide concentration on the oxidation of *p*-cresol (5 mM) in 85% dioxane using the aforementioned optimized conditions. Apparent saturation kinetics were observed for H_2O_2 concentrations below 0.25 mM with an apparent K_m of 0.1 mM. Above 0.25 mM H_2O_2, severe substrate inhibition was evident. Because the enzyme is severely inhibited by high levels of hydrogen peroxide, all subsequent kinetic evaluations assume pseudo-single substrate kinetics at high concentrations of *p*-cresol.

Effect of Organic Solvents on Peroxidase-Catalyzed Oxidation/Polymerization of *p*-Cresol. It was of definite interest to determine whether peroxidase obeyed Michaelis-Menten kinetics for *p*-cresol oxidation at the maximal non-inhibitory concentration of H_2O_2 (0.25 mM). While peroxidase catalysis is complicated by the fact that it is bi-substrate dependent, the substrate inhibition at H_2O_2 concentrations above 0.25 mM precludes us from determining saturation kinetics at saturating levels of both substrates. Hence, saturation kinetics experiments were carried out by varying only the *p*-cresol concentration and keeping the H_2O_2 concentration fixed. To that end, thirty milligrams glass beads (containing 3 μg peroxidase) were suspended in 5 mL dioxane/aqueous buffer (85/15) containing different concentrations of *p*-cresol (2.5-50 mM), 0.25 mM H_2O_2 was added and the reactions shaken at 250 rpm and 30 ^oC. Initial rates of biscresol formation were

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determined using HPLC to measure product concentration. Saturation kinetics were evident (Fig. 3) with an apparent K_m for *p*-cresol of 59 mM and a k_{cat} of 250 s^{-1}.

Similar experiments were performed in different dioxane concentrations ranging from 60% to 100% v/v. No reaction was observed in pure dioxane or in dioxane supplemented with 1% aqueous buffer. All peroxidase reactions in dioxane concentrations below 95% v/v obeyed Michaelis-Menten kinetics (Fig. 3). The values of apparent K_m and k_{cat} are plotted in Figures 4 and 5, respectively. The maximum value of K_m at 80% dioxane remains poorly understood. It is possible that the enzyme undergoes a conformational change in dioxane concentrations above 80% which enhances the binding of the *p*-cresol (and hence a drop in apparent values of K_m above this concentration of dioxane). The significant drop in k_{cat} above 80% dioxane is consistent with this speculation as conformational changes in the peroxidase would almost surely lead to diminished catalytic activity.

In order to more fully understand the effect of dioxane on peroxidase catalysis, we evaluated the kinetics of *p*-cresol oxidation in aqueous buffer -- 0.25 μg/mL peroxidase solutions dissolved in 5 mL aqueous buffer, pH 7, containing from 0.25-9.5 mM *p*-cresol and 0.25 mM H_2O_2 were shaken at 250 rpm and 30 ^oC. Once again Michaelis-Menten kinetics were observed with an apparent K_m of 0.7 mM and a k_{cat} of 88 s^{-1}. Therefore, the K_m values in dioxane-water mixtures were from 20 to 80 fold higher than in aqueous buffer. On the other hand, the value of k_{cat} in aqueous solution was actually lower than the values of k_{cat} in 60-85% dioxane. Hence, peroxidase shows a stimulation in activity in dioxane as compared to conventional aqueous catalysis. The K_m effect is intriguing and the mechanistic basis for this effect can be speculated to be due to the diminished propensity of the hydrophobic *p*-cresol molecule to partition into the enzyme's active site. Thus an effectively larger concentration of *p*-cresol in dioxane-water mixtures is required to saturate the peroxidase as opposed to water. A similar phenomenon has been observed by Douzou and Balny (19) for trypsin catalysis in dioxane/water mixtures and hence precedence for this explanation does exist in the literature.

The maintenance of high catalytic activity in high concentrations of organic solvents may be surprising, but there is no precedence against it in the literature. A number of enzyme kinetic studies in organic solvents (for review see 20) have failed to take into account the often severe alterations in K_m values for catalysis. Apparently low reaction rates in organic media are often due to the high K_m values and not necessarily to low catalytic turnovers (k_{cat}). For example, if 10 mM p-cresol were used

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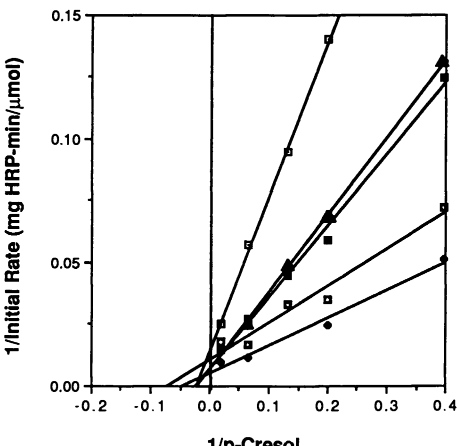

Figure 3. Saturation kinetics of peroxidase catalyzed oxidation of *p*-cresol in different dioxane concentrations. (◆) = 60% dioxane, (◘) = 70%, (■) = 80%, (▲) = 85%, and (□) = 95%. [HRP] = 0.6 µg/mL; [H_2O_2] = 0.25 mM.

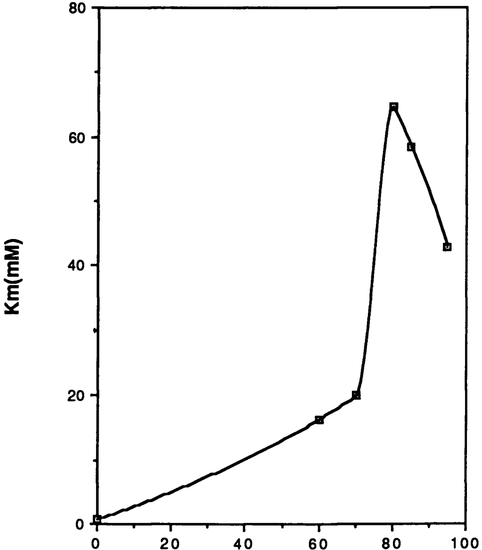

Figure 4. Alteration of K_m of *p*-cresol for peroxidase in different dioxane concentrations.

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in both aqueous buffer and 80% dioxane, the rate of oxidation catalyzed by peroxidase in water would be over twice that in dioxane. This belies the fact that the turnover number of peroxidase in 80% dioxane is 3.3 fold higher than in water. In fact, the value of k_{cat}/K_m, the so-called catalytic specificity constant is nearly 30 times lower in 80% dioxane than in aqueous buffer. It is suggested, therefore, that a more accurate description of catalytic activity in organic solvents is the value of k_{cat} rather than the lumped kinetic parameter of k_{cat}/K_m.

It was of further interest to examine peroxidase catalysis in other water-miscible solvents and to that end, *p*-cresol oxidation was studied in methanol and acetone. As with dioxane, peroxidase obeyed Michaelis-Menten kinetics over a wide range of methanol and acetone concentrations and the values of K_m and k_{cat} are depicted in Figures 6 and 7, respectively. Values of K_m are significantly higher in high concentrations of both solvents as compared to water, and is particularly high in methanol concentrations in excess of 60% v/v -- a maximum K_m of 247 mM is observed in 70% methanol. Once again, values of k_{cat} showed that the organic solvent stimulated peroxidase catalysis. The stimulation was dramatic - a 5.4 fold increase in the catalytic activity of the peroxidase was observed in going from aqueous buffer to 70% methanol. Above 70% methanol, both the K_m and k_{cat} fell dramatically, once again suggesting that a conformational change in the peroxidase had taken place. Such a conformational change is most likely the result of a denaturation of the peroxidase. Although no evidence for such a phenomenon has been obtained in this work, solvent-induced denaturation of proteins is a well-known fact (21). Most surprising, however, is that dioxane and methanol concentrations in excess of 70% and 80%, respectively, were required to elicit a structural change in the protein.

Peroxidase behaved differently in acetone. While an increase in the K_m of *p*-cresol was evident in high acetone concentrations, the catalytic activity of peroxidase was considerably lower as compared to dioxane or methanol.

Conclusions

Our work with horseradish peroxidase indicates that enzymes are often significantly activated under the proper conditions in organic as opposed to aqueous media. These conditions include the elimination of diffusional limitations which often plague enzymatic catalysis in heterogeneous systems, such as in organic media where the enzyme is insoluble. Adsorption of enzymes onto glass beads will eliminate internal diffusional resistances only if monolayer spreading onto the glass surface is obtained.

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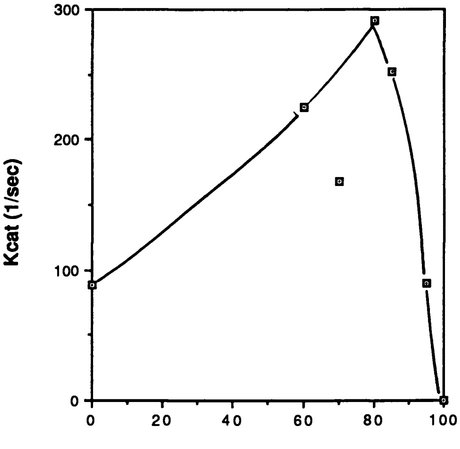

Figure 5. Effect of dioxane concentration on the catalytic turnover of peroxidase for *p*-cresol.

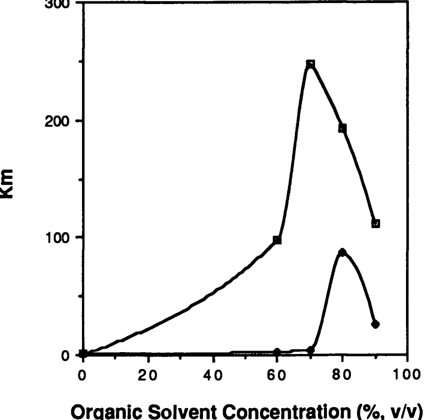

Figure 6. Alteration of K_m of *p*-cresol for peroxidase in methanol (□) and acetone (◆). See text for details.

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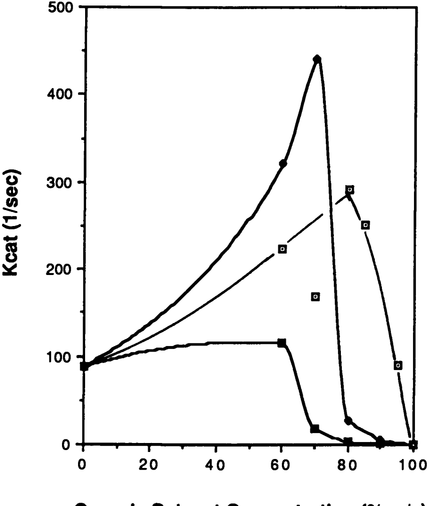

Figure 7. Effect of methanol (◆) and acetone (■) in comparison to dioxane (□) on the catalytic turnover of peroxidase for *p*-cresol. See text for details.

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The different catalytic responses of peroxidase in dioxane and methanol versus acetone are intriguing. It is clear that the effects of water-miscible solvents on enzymatic catalysis are not equivalent and for the first time quantitative kinetic data have been obtained which highlight this. However, the cause of this effect remains unresolved. We are continuing and expanding this kinetic study to include other solvents, both water-miscible and immiscible, and other phenols. This future study will enable rational and quantitative approaches for peroxidase-catalyzed phenolic polymerizations to be based on optimal solvent and phenol choices. From a more fundamental standpoint, this work has shown that enzymes may be more active in organic media than in water as long as optimal conditions are employed. There is no reason to believe peroxidase is unique in this respect.

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Chapter 11

A New Immobilized Positional Nonspecific Lipase for Fat Modification and Ester Synthesis

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The yeast Candida antarctica produces a positional non-specific lipase. The lipase preparation retained about 60% of its activity after 30 min of incubation at 70°C, pH 6.5. A highly thermostable and positional non-specific catalyst is formed when the lipase preparation is immobilized on a macroporous resin. The catalyst is efficient in ester synthesis and in interesterification. It is able to catalyze ester formation with both primary and secondary alcohols. The temperature optimum for interesterification is 85°C or higher, and the half-life in continuous acidolysis of soy bean oil with lauric acid at 60°C is above 2500 h. The non-specificity makes the catalyst useful in random interesterification of different fats. The catalyst has some saturated fatty acid specificity. Two lipase components (A and B) were purified. Lipase A is important for interesterification, and Lipase B is important in ester synthesis.

There is considerable worldwide interest in the application of lipases for fat modification and ester synthesis. In systems with low water activity, lipases are able to catalyze ester synthesis as well as interesterification reactions in fats. The lipase reactions under low water activity can be performed in several ways. We consider batch or continuous reactions in melted fats by use of immobilized lipases as the most relevant way for the industry.

The type of reaction depends on the positional specificity of the lipase (Table I).

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Table I. Reactions of Immobilized Lipases under Low Water Activity

Reaction	With 1,3-positional specific lipase	With positional non-specific lipase
Interesterification: Acidolysis (triglyceride + fatty acid); Transesterification (e.g. triglyceride + triglyceride)	Reaction with the fatty acids in the 1,3-positions only	Reaction with the fatty acids in all three positions
Ester synthesis (acid + alcohol)	Reaction preferably with primary alcohols	Reaction with primary and secondary alcohols

In the interesterification of fats, 1,3-positional specific lipases catalyze reactions in which only the fatty acids in the α-positions of the triglycerides take part, whereas positional non-specific lipases are able to catalyze reactions in which the fatty acids from both the α- and β-positions take part. In transesterification between two types of fat, the positional non-specific lipase is therefore able to randomize the fatty acids, resulting in the same fatty acid composition in the triglycerides as obtained in the commercially important chemical randomization process. In ester synthesis, positional non-specific lipases catalyze the reaction with both primary and secondary alcohols whereas positional specific lipases are more or less specific for primary alcohols.

For industrial applications, thermostable lipases are desirable in order to work in melted fat. Thermostable, positional specific lipases are well known. One example is Mucor miehei lipase which we have developed into an immobilized commercial product (Lipozyme) (1,2).

Some positional non-specific lipases are known (3-6), but they have insufficient thermostabilities for long-term applications in melted fats. We consider the lack of thermostable non-specific lipases to be one of the main reasons for the limited use of lipases in the fat and oil industry. The existence of such a lipase would open up for the use of lipases as catalysts for randomization in fats, and as general catalysts for ester synthesis.

As a result of a large screening program carried out in our laboratories in Japan, we have found a new non-specific lipase (7,8). It is produced by the yeast Candida antarctica.

In this paper, some properties of soluble and immobilized lipase preparations from C. antarctica are presented.

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Properties of the Soluble Crude Lipase Preparation

The thermostability of the crude lipase produced by Candida antarctica is demonstrated in Figure 1. The remaining activities after incubation for 30 min at different temperatures in 50 mM Tris-maleate buffer, pH 7.0, were measured using olive oil as substrate at 40°C, followed by end-point titration of the liberated fatty acids.

A significant remaining activity after incubation at 70°C and even 80°C was observed. This stability can be improved by a preheat treatment. The lipase solution was heat treated for 3 h at 60°C and pH 7 (Fig. 2).

The enzyme preparation does not only exhibit a good thermostability, but it is also active in a wide pH range, as demonstrated in Figure 3 where the activities are measured at 40°C. The pH optimum is at pH 7 to 8.

The Immobilized Crude Enzyme Preparation

Immobilization. We have worked with different carriers for immobilization. Active immobilized preparations have been obtained by adsorption on macroporous, weakly basic anion exchange resins, and on nonionic adsorbent resins. Resins of both acrylic and phenol-formaldehyde have been used (8). In the following examples we have used lipase immobilized on nonionic adsorbent acrylic resin.

When immobilized, the enzyme is further stabilized and can be used in batch and continuous processes at low water activities.

The immobilized enzyme is by addition of water hydrated to 10% (w/w) 24 h before use.

Activity Assays

To measure the activity in acidolysis, triolein was esterified with equimolar amounts of palmitic acid at 60°C by means of immobilized lipase (Fig. 4). The incorporation of palmitic acid into the triolein is measured by GLC of fatty acid methyl esters obtained after methoxylation of the triglycerides (8,9). One Batch Interesterification Unit (BIU) corresponds to 1 µmol of palmitic acid incorporated (initial activity) into triolein per min.

To measure the activity in transesterification, equimolar amounts of triolein and tripalmitin were reacted by means of immobilized enzyme (8). The transesterification was followed by HPLC analysis of the triglycerides. One Batch Transesterification Unit is the initial exchange of fatty acids in µmol per min.

Temperature Optimum and Stability

The relation between temperature and activity is demonstrated in Figure 5. The temperature optimum is at least 85°C in both acidolysis and transesterification. It is observed that the activity in transesterification is about three times higher than the activity in acidolysis. We have tested different lipases and found that the activity ratio between transesterification and acidolysis varies. The enzymes tested were all more active in transesterification.

In Biocatalysis in Agricultural Biotechnology; Whitaker, J., el al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1989.

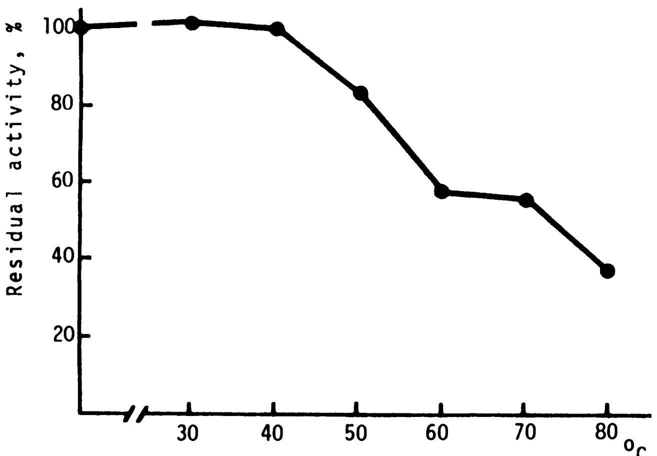

Figure 1. Thermostability of C. antarctica crude lipase. Remaining activities were measured after 30 min of incubation at pH 7.0.

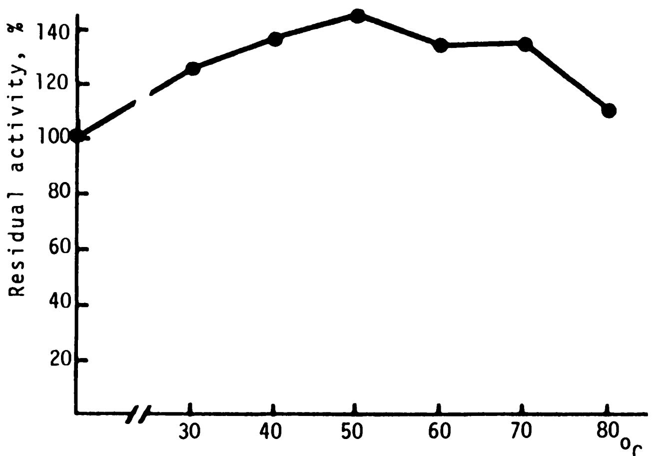

Figure 2. Thermostability of C. antarctica crude lipase after 3 h of pre-heating treatment at 60°C, pH 7.
Remaining activities were measured after 30 min at pH 7.

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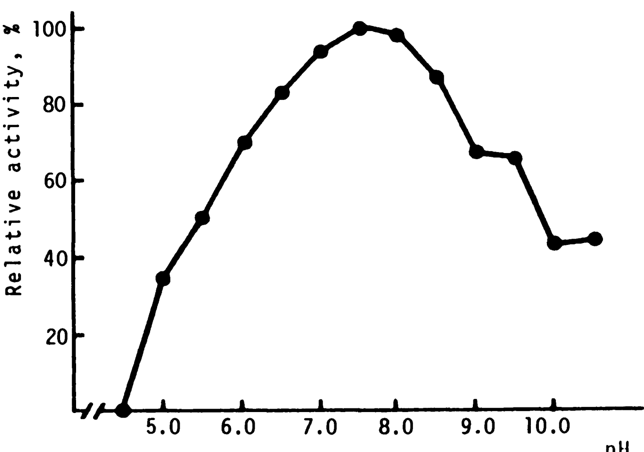

Figure 3. Activity on olive oil.

The activities were measured at pH 4.5-5.5 in 200 mM acetate buffer, at pH 5.5-8.5 in 50 mM tris-maleate buffer, and at pH 9.0-10.5 in 100 mM glycine buffer.

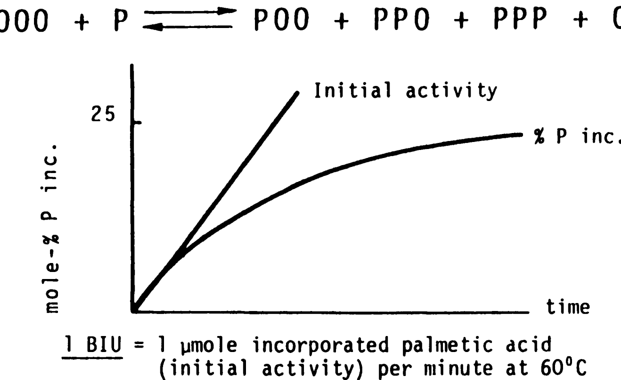

Figure 4. Principles of batch interesterification assay.

Please notice that PPO means OPP + POP + PPO, and POO means POO + OPO + OOP.

The thermostability of the immobilized lipase is most easily demonstrated in continuous interesterification. A general test system is shown in Figure 6. The immobilized enzyme is placed in the enzyme column. The precolumn is used to saturate the reactants with water as they are pumped through the system.

We have used a mixture of soybean oil and lauric acid in order to test the performance of the lipase in continuous acidolysis (8) (Fig. 7). The flow through the column was varied in order to maintain a constant incorporation of lauric acid. The half-life of the enzyme was approximately 500 h at 80°C. At 60°C, no significant deactivation was observed after 2000 h.

Non-specificity

The non-specificity of the immobilized lipase can be demonstrated in several ways. We used a convenient acidolytic test system where cocoa butter (stearic fraction) was interesterified with lauric acid (8). The test principles are outlined in Table II.

Table II. Non-specificity Index, Principles

Lipase acidolysis of cocoa butter (XOX) with lauric acid (La)		
Lipase	Triglycerides obtained after acidolysis	Oleic acid (O) content in triglycerides
1,3-specific	XOX + LaOX + LaOLa	unchanged
Non-specific	XOX + LaOX + LaOLa + XLaX + LaLaX + LaLaLa	declined

$$\text{Non-specificity Index (NSI)} = \frac{\text{3 x decline in oleic acid in TG}}{\text{lauric acid incorporation in TG}}$$

X means palmitic or stearic acid.

Cocoa butter (stearic fraction) contains about 33% oleic acid occupying the second position only. Consequently, positional specific lipases do not react with oleic acid. The Non-specificity Index (NSI) is based on the decline in oleic acid content in the triglycerides in relation to the lauric acid incorporation. A positional specific lipase will give a non-specificity index of 0 whereas a non-specific lipase will give a non-specificity index of 1.

In Table III, we have compared the non-specificity index of the immobilized C. antarctica lipase with the indices of the immobilized Mucor miehei and Candida cylindracea lipases.

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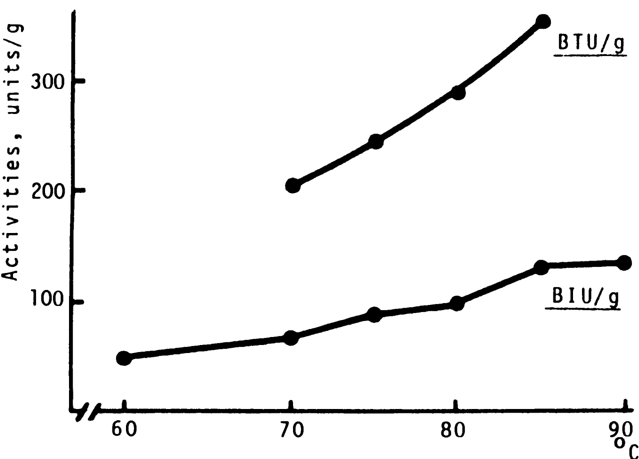

Figure 5. Activity in acidolysis (BIU) and transesterification (BTU) of the immobilized crude enzyme preparation.

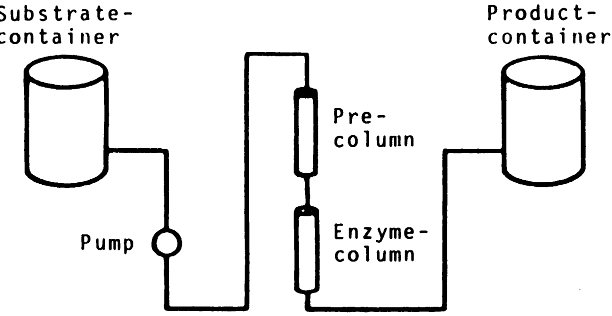

Figure 6. Simplified flow-sheet for continuous solvent-free interesterification with immobilized lipase.

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Table III. Non-specificity Index of Different Lipases

Source of Lipase	Composition of triglycerides: Lauric acid (mole-%)	Composition of triglycerides: Oleic acid (mole-%)	Non-specificity index
Candida antarctica	60.8	13.2	0.96
Candida cylindracea	53.0	15.0	1.04
Mucor miehei (Lipozyme)	45.5	33.0	0.02
Control (cocoa butter)	0	32.1	-

Candida cylindracea lipase is a commercial lipase produced by Meito Sangyo Co. in Japan. The lipase is known to be positional non-specific (6). It is evident that the Candida antarctica lipase is non-specific, like the non-specific reference lipase. As expected, the non-specificity index of the positional specific Mucor lipase is close to 0.

Fatty Acid Specificity

The fatty acid specificity in the acidolysis reaction has been examined (Table IV).

Table IV. Activity in Acidolysis of Tricaprylin with Different Fatty Acids

Acidolysis of Tricaprylin With:	Initial activity µmol incorporated fatty acid per min (U/g)
Lauric acid	116
Myristic acid	100
Palmitic acid	121
Stearic acid	134
Oleic acid	74
Linoleic acid	56

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The activities in acidolysis of tricaprin with different fatty acids were measured in separate experiments, each performed like the assay for batch interesterification units. It is observed that the enzyme has some specificity towards saturated fatty acids. This kind of specificity is unique, and has not previously been described.

Ester Synthesis

The activities of immobilized C. antarctica and Mucor lipases in ester synthesis of propanol and isopropanol were compared (Fig. 8). Both lipases have high activities in ester synthesis with the primary alcohol propanol, but only the positional non-specific C. antarctica lipase performs well in synthesis with the secondary alcohol.

The efficiency of the C. antarctica lipase in ester synthesis of both primary and secondary alcohols is demonstrated whereas the positional specific Mucor lipase only performs well in synthesis of esters of primary alcohols.

Ester synthesis stops at about 85% at which point the reaction equilibrium with the co-produced water has been reached. Further synthesis can be obtained if the produced water is removed. In Table V it is demonstrated that a yield of 98% of the wax ester myristylmyristate is obtained by esterification of myristic acid with myristic alcohol under vacuum.

Table V. Formation of Myristylmyristate[a]

	Enzyme dosage (dry weight)	
Reaction time	1 g	0.2 g
0.5 h	98%	57%
1 h	-	86%
2 h	-	97%
3 h	-	98%

[a] 0.05 mol myristic acid + 0.05 mol myristic alcohol, 60°C under vacuum.

We have made a number of wax esters by this technique in a stirred batch reactor under vacuum, thus demonstrating one of many important practical aspects of lipase applications.

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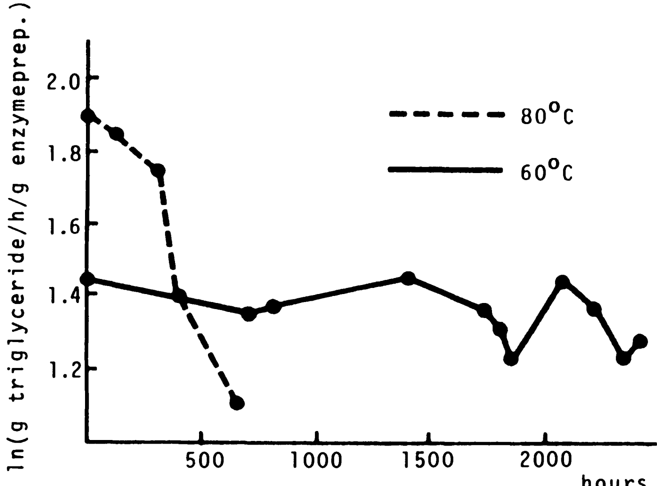

Figure 7. Continuous acidolysis of soybean oil with lauric acid.

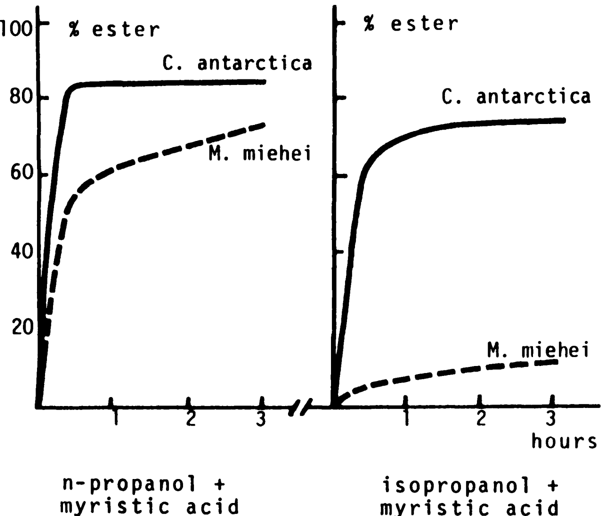

Figure 8. Ester synthesis at 60°C. 1 g of immobilized lipase and 0.05 mmoles of reactants.

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Lipase Components

Purification of the Lipase Components. Two lipase components, designated A and B, respectively, have been purified from the crude *C. antarctica* lipase preparation by well-known chromatographic techniques (8). The crude lipase preparation may contain other lipases, but we consider lipases A and B to be the important ones.

Characterization of Soluble Lipases A and B

The hydrolytic activities of the two lipase components on olive oil, diolein, and methylesters have been compared (Table VI).

Table VI. Activities of Soluble Lipases A and B

Activity on	Lipase A (%)	Lipase B (%)
Olive oil[a]	100	100
rac. Diolein[b]	25	480
Methyllaurate[b]	20	215
Methyloleate[b]	7	95

[a] Incubation at 40°C, end-point titration

[b] pH-stat measurement at 30°C.

The activities are given in per cent of the activity on olive oil.

Lipases A and B are compared on the basis of the same activity on olive oil, and it is observed that Lipase B has 10-20 times higher activity than Lipase A on diolein and on methylesters of lauric and oleic acids.

The thermostabilities of the two enzymes were compared after 30 min of incubation at 75°C in 50 m*M* Tris-maleate buffer, pH 7.0. The remaining activity of Lipase A was approximately 50% whereas the activity of Lipase B was almost inactivated. It is therefore assumed that Lipase A is responsible for the high thermostability observed in crude lipase preparations from *C. antarctica*.

Immobilization of Lipase Components

Lipases A and B have been immobilized on nonionic adsorbent resins. Comparisons have been carried out with a similar immobilization of the crude *C. antarctica* enzyme.

The activities of the immobilized lipases are shown in Table VII.

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Table VII. Acidolytic Activity of Immobilized Lipase Components

Lipase	Load of carrier[a] (IU/g)[b]	Acidolytic activity of the immobilized lipase (BIU/g)
Crude	30,200	41.9
Lipase A	28,200	21.4
Lipase B	29,200	4.4

[a] The carrier load is calculated as the difference in activity before and after adsorption to the carrier per gram dry weight of immobilized enzyme.

[b] IU is lipase units on tributyrin, Novo method No. AF 95/4.

The immobilized Lipase B had very low activity in the acidolytic assay whereas immobilized Lipase A has 50% of the activity found for the crude enzyme. The importance of the difference between Lipase A and the crude lipase should not be overestimated as the activity of immobilized lipases is often influenced by effects of co-immobilized proteins.

Positional Specificity of the Lipase Components

The non-specificity indices of the lipase components have been measured (Table VIII).

Table VIII. Non-specificity Indices of Lipase Components

Lipase	Lauric acid incorporation (%)	NSI
Crude[a]	58	0.87
Lipase A[a]	58	0.81
Lipase B[b]	14	0.65

[a] 2 h incubation with 150 mg enzyme and 0.4 μmol cocoa butter

[b] 4 h incubation with 250 mg enzyme and 0.133 μmol cocoa butter.

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Lipase A is positional non-specific, but the non-specificity index of Lipase A is somewhat lower than what is found for the crude lipase. This can be explained by the specificity towards saturated fatty acids observed for Lipase A, as will be demonstrated later.

The positional specificity of Lipase B is questionable. The low activity of the immobilized enzyme, and consequently the low incorporation of lauric acid, makes the specificity determination uncertain. It is also difficult to determine the positional specificity of Lipase B before immobilization by the traditional analysis of 1,2- and 1,3-diolein obtained by hydrolysis of triolein as the diolein is very quickly degraded by the enzyme.

Fatty Acid Specificity

The fatty acid specificity of the immobilized Lipase A has been compared with the specificity of the crude enzyme by the method described earlier (Table IX).

Table IX. Fatty Acid Specificity of Lipase A and the Crude Enzyme

	Activity (μmol fatty acid incorporated per min)	
	Lipase A	Crude enzyme
Acidolysis of tricaprylin with:		
Lauric acid	13 U/g	116 U/g
Oleic acid	3 U/g	74 U/g
Ratio:		
lauric acid / oleic acid	4.3	1.6

It is seen that Lipase A has a marked preference for lauric acid compared to oleic acid. As Lipase B has a very low acidolytic activity, it can be assumed that Lipase A is responsible for the specificity towards saturated fatty acids observed for the crude enzyme.

This fatty acid specificity may lead to the assumption that the activity in acidolysis of polyunsaturated fatty acids would be negligible. We have tested the incorporation of polyunsaturated fatty acids isolated from menhaden oil into trilaurin, and found that both immobilized Lipase A and the immobilized crude enzyme are quite active, and more active than the immobilized *Mucor miehei* lipase (Lipozyme).

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Ester Synthesis by Lipase Components

The activities of the immobilized lipase components in ester synthesis have also been tested. Myristic acid was esterified with propanol, isopropanol, and oleic alcohol, respectively (Table X).

Table X. Ester Synthesis by the Lipase Components[a]

Enzyme	Alcohol	Ester synthesis obtained after 90 min (%)
Lipase A	1-propanol	22
	2-propanol	16
	oleyl alcohol	52
Lipase B	1-propanol	83
	2-propanol	75
	oleyl alcohol	66
Crude lipase	1-propanol	84
	2-propanol	71
	oleyl alcohol	82

[a] Ester synthesis of myristic acid with long and short chain alcohols

Enzyme dosage: 1 g per 85 mmoles of reactants.

Results almost identical with the above ones were obtained when using oleic acid instead of myristic acid.

Even though the immobilized Lipase B has low activity in acidolysis, it is quite active in ester synthesis with short-chain alcohols where it seems to be responsible for the abilities shown by the crude enzyme. Lipase B functions almost equally well on 1-propanol and 2-propanol; but for the long-chain alcohol, oleic alcohol, Lipase B has somewhat lower activity, and Lipase A seems to contribute the activity observed for the crude enzyme.

In contrary to Lipase B, Lipase A has low activity in ester synthesis with short chain alcohols, and higher activity on long chain alcohols.

Actions of the Lipase Components - Overview

The given examples of the actions of the lipase components allow one to give a summary of the contribution of the components to the qualities of the immobilized crude enzyme (Table XI).

Table XI. Summary of the Actions of the Lipase Components

Process	Lipase A	Lipase B
Interesterification	High activity Specificity for saturated fatty acids	Low activity
Ester synthesis:		
With short chain alcohols	Low activity	High activity
With long chain alcohols	Good activity	Good activity

Lipase A seems to be responsible for the interesterification characteristics of the immobilized crude lipase preparation, including its unique specificity towards saturated fatty acids. Further, Lipase A can explain some of the activity found in ester synthesis with long-chain alcohols. On the other hand, Lipase B is responsible for the activity in ester synthesis of short-chain alcohols, and for some of the activity on long-chain alcohols.

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Chapter 12

Specialty Enzymes and Products for the Food Industry

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Consumer advocates, environmentalists, political protectionists, and the litigious nature of our society all create opportunities for the enzyme industry. Aside from the historically established hydrolytic enzymes that modify starch, pectin, protein, and fats to their component parts, and a few others like glucose isomerase and glucose oxidase, newly produced enzymes have become or are becoming commercially important in specific chemical transformations. Examples of these are the glucosyltransferases and glycosylating enzymes for the production of "new" carbohydrates and modification of stevioside to bring the sweetness closer to sucrose. Isomerases for increasing sweetness of invert sugar, phytase for sludge-free corn steep liquor, various antimicrobial enzymes, and other examples are discussed.

Applied enzymology has moved significantly beyond the early broad extracellular hydrolytic enzyme preparations such as the bacterial α-amylase, papain, pectinase, and pancreatin where the commercial "concentrate" might contain as much as "1%" of the active labeled principle. It has been some years now since the commercialization of intracellular enzymes like glucose oxidase, and even such enzymes as crystalline glucose isomerase by the ton! Although these represent early achievements, they constitute the bulk of current commercial enzyme usage. The newer technical applications now use purified enzymes for specific chemical transformations, transformation to make things that may not occur in nature, or to make a reaction take place in an environment where it usually doesn't react.

Much of the driving force behind the current opportunities for the diversity of enzyme applications comes from pressures by consumer groups, environmentalists, the litigious nature of our society, political protectionism and just plain competition in a free economy. Consumer groups want "all natural", "carcinogen-free", and "additive-free" foods, creating opportunities for enzymes replacing commonly used food additives such as anti-staling ingredients, sulfite, nitrite, or antibiotics. Environmentalists want zero effluent residues from industry and agriculture, even penalizing farmers for

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spreading phosphate-rich pig manure on fields that contaminate run-off water (see phytase); our analytical abilities, to now quantitate what used to be called "zero", (i.e. urethanes in alcoholic beverages) exacerbates the situation. <u>The litigious nature of our society</u> makes it cheaper to use more expensive enzymes to avoid hazardous additives, e.g. substituting enzymatic treatment for formic and other acids now used in many European countries for the production of high moisture silage to minimize injury to workers. <u>Political protectionism</u> results in skewed pricing situations such as high fructose corn syrup (HFCS) existing in the USA primarily because of an artificially high domestic sucrose price, while it is discriminated against in European countries producing beet sugar on legal and tax bases. <u>Competitive pressure</u> has intensified for product/process cost-reduction, and quality improvement at no added cost as commercial consumers have become more knowledgeable.

I've selected some examples below that are of broad interest or exemplify the diversity of what is now being done or is practical to do.

ENZYMES IN BREWING

The malting process used to produce fermentable sugars from grain is at the heart of the brewing process. Amylases are used to convert non-malt carbohydrates ("adjuncts") to fermentables, and papain is used as a chillproofing enzyme. More recently amyloglucosidase is used to convert the last dextrins to fermentable sugar for "light" beer, and ß-glucanase is used to aid mash filtration by reducing viscosity and filter-blocking ß-glucans. All ß-glucanases are not the same; *Trichoderma* ß-glucanase is stable through the mashing stage, while those from *Bacillus subtilis*, *Aspergillus niger*, and *Penicillium emersonii* are not.

The newest enzyme for use in beer is acetolactate decarboxylase, used to decrease the fermentation time, by avoiding the formation of diacetyl. Externally or internally produced α-acetolactate decarboxylase transforms the α-acetolactate to acetoin (acetylmethylcarbinol); without the enzyme, acetolactate goes to diacetyl, and then a secondary fermentation slowly reduces it to acetoin. Avoiding or reducing the secondary fermentation results in significant reduction in storage capacity and money tied up in inventory (<u>1</u>). Normally acetolactate forms by the thiaminepyrophosphate-catalyzed acyloin condensation of acetaldehyde and pyruvic acid (<u>2</u>) or by the condensation of two pyruvic acid molecules to yield acetolactate and CO_2. Acetolactate is important in the synthesis of isoleucine and valine by the yeast. The acetolactate left at the end of the primary fermentation is oxidized spontaneously in a <u>non</u>enzymatic reaction to diacetyl and CO_2 (Eqn. 1)

$$CH_3COCOH(COOH)CH_3 + \tfrac{1}{2}O_2 \rightarrow CH_3COCOCH_3 + CO_2 + H_2O \qquad (1)$$

Typically the level of diacetyl might be 0.10-0.15 mg/L, well above the taste threshhold level of 0.05 mg/L. The purpose of the secondary fermentation is to allow the excess acetolactate that diffused out of the yeast cells to diffuse back in and be reduced to acetoin, <u>before</u> it is spontaneously converted to diacetyl; that secondary fermentation can take several weeks (J. Power, J.E. Siebel & Sons, Personal Communication). If acetolactate decarboxylase is used it then decarboxylates the acetolactate to produce CO_2 and acetoin in

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days. The acetoin may be further reduced by the appropriate dehydrogenase to 2,3-butylene glycol, which doesn't affect the flavor of beer in the concentrations encountered (Eqn. 2).

$$CH_3COH(CO_2^-)COCH_3 + H^+ \rightarrow CH_3\text{-}CHOH\text{-}CO\text{-}CH_3 + CO_2 \qquad (2)$$

This can be done in a day or two. A unique twist to this has been added by The Technical Research Center of Finland (J. Knowles, personal communication giving details of Finnish Patent Application). The gene responsible for the acetolactate decarboxylase production was removed from *Aerobacter aerogenes* and spliced into a strain of *Saccharomyces* used for brewing; the result is conversion of the excess acetolactate to acetoin in the primary fermentation. With the engineered yeast, diacetyl levels of about 0.01 mg/L or less are achieved without a secondary fermentation. By making the secondary fermentation unnecessary, this engineered yeast reduces the overall beer production time from about 5 weeks to 2 weeks, with no other brewing properties affected. This same lab has also constructed two glucanolytic brewing yeasts by transfering a single ß-glucanase gene into the yeast; the resultant beer is virtually devoid of ß-glucans and filters much more easily (3).

ENZYMES USED IN FLAVOR MODIFICATION

Cheese/butter flavor. Pregastric lipases, have been used for years to intensify flavor in "enzyme-modified cheese", and for an intensified butter flavor in lipolyzed butter. Generally the fatty acid residues that need to be split off (to generate the right flavor) are the short chain fatty acids, especially the C_4 to C_{10} acids typical of Italian cheeses. The butyric acids are produced from butterfat more specifically by newly developed lipases (really esterases) from *Mucor meihei* and a very new one, from *Aspergillus oryzae*, especially for cheddar cheese flavor development. The latter enzyme is marketed under the name "Flavor Age" (4). Flavors produced in this manner are used widely in cheese-flavored snack foods; the value of the intensified cheese flavors is on the order of $50 million, but the value of the enzymes employed is only about $2-3 million.

Cheese flavors are not only developed by fat modification, or free fatty acids, but also by the modification of the milk proteins by endopeptidases. An aminopeptidase from *Lactobacillus lactis* is being used to halve the ripening time of hard cheeses, like cheddar (5).

Flavor Esters. Some of the newest applications of lipases/esterases involve the production of flavor esters, such as ethyl butyrate (pineapple flavor), which can be synthesized by lipases suspended in organic solvents such as heptane or longer alkanes (6). The water of hydration in the enzyme preparation is sufficient for the reaction to proceed, in most cases. The reaction is a reversal of the hydrolysis reaction lipases/esterases are traditionally used for; the reaction is an enzymatic condensation combining an organic acid with an alcohol. A preferred lipase is derived from *Candida cylindracea* (7).

Yeast-derived meaty flavor. Yeast extract is an important food ingredient used primarily for its meaty flavor. The conventional method for production from the yeast *Saccharomyces cerevisiae* is done by plasmolysis, followed by autolysis by the yeast's own enzymes, pasteurization, filtration, clarification, and drying. The typical yield is about 70% of the dry weight of the yeast. The enzymes thought to be active in the autolysis process are proteases; it has been

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common to supplement the native enzyme with papain, to accelerate the process. Other depolymerizing enzymes from the yeast are also involved. An *Arthrobacter luteus* preparation known as "Zymolase" (8) is reportedly effective in yeast lysing because it contains a ß-glucan laminaripentaohydrolase that has a high affinity for insoluble glucan. We know we have enzyme preparations that work, but we are not yet certain we know all of the enzymes present or involved. Addition of a preparation with high endo- and exo ß-glucanase activities, with protease activity too, from the plant pathogen *Rhizoctonium solani (Basidiomycete aphyllophorales)* raised the yield to 95% (9-10). The added yield probably comes from the yeast cell wall (15% of the dry weight of the yeast) and solubilization of other cell components. Of course the important thing about yeast extract is the flavor as well as the yield. Different processes, especially the pasteurization and drying that result in Maillard browning, give the various yeast extracts their individualities for food applications.

CYCLODEXTRINS

Cyclodextrins are non-reducing cyclic molecules with 6-8 glucose residues arranged in a doughnut shape. They effectively trap many different kinds of molecules in the hole of the doughnut, a process now called "inclusion complexation". Cyclodextrins are made from starch using cyclomaltodextrin glucanotransferase (CGTase) obtained from *Bacillus macerans* and other *Bacillus* sp. (11).

The process used by American Maize begins with potato or waxy maize starch conversion to maltodextrins and then subjects these to the actions of CGTase. This enzyme hydrolyzes off the 6-, 7-, or 8-glucosyl residues and then covalently attaches the reducing end to the non-reducing end. The three different cyclodextrins can be separated by either solvent fractionation or ultrafiltration. Whereas starch contains chains of between one and three million glucosyl residues, the maltodextrin subjected to CGTase action has only about 14-70 glucosyl residues (G. A. Reed, American Maize, personal communication), and the cyclodextrin has 6, 7, or 8 glucosyl groups.

These compounds offer substantial promise because of the hydrophilic-hydrophobic nature, and the ability to protect many different kinds of molecules against deterioration or evaporation.

LOW SWEETNESS SWEETENERS

Low sweetness sweeteners are of interest because they are generally metabolized in the body yet do not contribute to dental caries, and tend to have a lower effect on blood glucose in diabetics. Four examples are discussed here. Where sweetness equal to sucrose is desired, a high intensity sweetener can be mixed with it in most countries. However low sweetness caloric sweeteners are of particular interest for persons with kidney disease, where it is often difficult to get them to take enough calories. Use of glucose syrups to "stuff calories" with little water is often unacceptable to the patient because of the excessively sweet taste. Perhaps someone will develop a low sweetness metabolizable sweetener for these people.

Non-reducing fructose derivative sweeteners. A Hyashibara process (12) subjects a mixture of starch and sucrose to cyclodextringlucosyltransferase and amylopectin-α-1,6-glucosidase (such as pullulanase or isoamylase). Cyclodextrins are formed as intermediates, but then act as glucosyl donors for attachment to the glucose portion of the

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sucrose. This results in sweet non-reducing oligomers with a fructose end. The sweetness and the average number of glucosyl residues attached to the fructose will vary with the ratio of starch to sucrose, the enzyme level, and reaction time and conditions.

Isomaltulose. Isomaltulose is a reducing disaccharide comprised of glucose and fructose. It occurs naturally, but it is primarily obtained by the rearrangement of sucrose by α-glucosyltransferase derived from *Protaminobacter rubrum.* Conversion yields of 85% or more are not unusual. It is made in Japan by Mitsui Sugar Co. (13). Isomaltulose is 42% as sweet as sucrose, but fully as caloric. It offers advantages as a non-cariogenic nutritive sweetener, (and possibly anticariogenic as, like xylitol, it inhibits insoluble glucan formation by *Streptococcus mutans* from sucrose when consumed with sucrose. It is acid-stable, and appears to be useful in the manufacture of hard candies (14). Isomaltulose is a reducing disaccharide, with a free aldehyde group. Consequently, on hydrogenation it yields Isomalt, an equimolar mixture of α-D-glucopyranosyl-1,6-mannitol and α-D-glucopyranosyl-1,6-sorbitol. This is reported to be half as sweet as sucrose with 2 calories per gram, making it useful as a sucrose substitute in reduced calorie formulations where it is used with a high-intensity nutritive or non-nutritive sweetener. It is reportedly approved for food use in the EEC since 1985 (15).

Leucrose is 50-60% as sweet as sucrose and is made by the action of an α-1,6-glycosyltransferase on sucrose in the presence of fructose, whereby the glucose portion of the sucrose is transferred to the fructose, and fructose regenerated (16).

NORMAL & ENHANCED SWEETNESS SWEETENERS

Immobilized invertase. Hydrolysis of sucrose by immobilized invertase now is cheaper and better than hydrolysis by acidic resins. Invertase immobilized ionically on a *reusable carrier*, diethylaminoethyl-cellulose in a weighted polystyrene (17-18), is being used in countries where beet or cane sugar interests or governmental constraints make corn sugar prohibitively expensive as a starting material (H. Lommi, Finnish Sugar Co., personal communication). The immobilized invertase gives good conversion; at flow rates of 2-4 bed volumes per hour 99.5% conversion is achieved, while even at 40 bed volumes per hour 50% hydrolysis is achieved. The cost of enzymatic hydrolysis, at about $US 0.10-0.15/cwt dry basis, appears higher when only the hydrolysis step is considered but not when the overall process is considered. Overall, *immobilized invertase is cheaper; strong cationic hydrolysis costs more than immobilized invertase* because the acid method results in significant fructose polymerization forming trioses and tetroses, lowering the yield. The acid resin method requires a decolorization step because of the color formation; the acid method forms significant amounts of hydroxymethylfurfural. The mineral content resulting from the acidification-neutralization requires a demineralization step.

A second immobilized invertase, wherein the carrier must be discarded with the spent enzyme, without possibility of *in situ* regeneration, has also been described (19).

Glucose isomerase. Glucose isomerase for HFCS in the USA is a success story primarily because of the artificial difference in price between corn starch and the supported price of sucrose. This price difference

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does not exist in Canada, where world market sucrose prices prevail, and, for that reason, the incentive to switch to HFCS does not exist in Canada. Enhancing the sweetness of natural invert sugar is another interesting immobilized glucose isomerase (IGI) application which starts with invert sugar. This is based on labeling laws. Concentrated white grape juice is used as a sweetener, but appears on the label as "juice", not sugar. Jellies made with concentrated grape juice are often promoted as "no sugar added". It is now possible to make super-sweet concentrated grape juice by separating the glucose from the fructose, isomerizing the glucose and adding it back for a 60:40 fructose:glucose or even an 80:20 fructose:glucose (F. Hammer, Finnsugar Biochemicals Inc., personal communication). This gives higher sweetness per unit weight. The 80:20 would have a sweetness of as much as 25% more than the 50:50 product, allowing the use of 20% less concentrated juice. Label declaration requirements, and consumer perception of these labeled ingredients, may make this attractive for specific uses.

HIGH INTENSITY SWEETENERS

High intensity sweeteners include both natural and synthetic compounds. Saccharin and aspartame are synthetic. On the other hand both stevioside and ribaudioside-A are natural high intensity sweeteners derived from the *Stevia* plant. Aspartame and glycosylated steviosides are commercially produced with enzymes.

Aspartame. The success of L-aspartyl-L-phenylalanine methyl ester (aspartame) as a high quality low calorie sweetener has resulted in considerable research effort directed at a more efficient direct enzymatic coupling of the components. The original chemical process was done at very low temperatures at high cost. The first enzymatic process commercialized used immobilized thermolysin to couple N-protected aspartic acid with the methyl ester of phenylalanine, then removing the protective group by hydrogenation. Newer methods have been developed by several companies. The phenylserine ester has been synthesized (20) using serine hydroxymethyltransferase, followed by coupling with blocked aspartic acid using thermolysin; this is then hydrogenated to give hydroxyaspartame or aspartame. L-Aspartic acid has been directly coupled with L-phenylalanine (21), using unstated enzymes produced by *E. coli* or other mixed organisms in the fermentation broth, to which the reactants are added. The yields in this process are very low, but it is obvious that the direct process is being rapidly approached as the basic patents on aspartame have already, or will soon expire. A new entry in the aspartame field is said to employ immobilized enzymes (in immobilized cells) to make the phenylalanine from cinnamic acid and ammonia, prior to coupling directly to aspartic acid (22). The limitations of aspartame as a sweetener have spurred work on other clean-sweet high intensity sweeteners as well.

Steviosides and ribaudioside-A. Stevioside and ribaudioside-A (ß-glucosyl stevioside) both occur naturally in the plant *Stevia rebaudiana*, with the steviosides comprising 10-20% of the dried weight of the leaves. Ribaudioside-A may constitute as much as 60-70% of the mixture for some strains of the plant (23). While stevioside is about 150-300 times as sweet as sucrose, the flavor profile has a bitter

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component and a delayed sweetness onset that makes it not fully a sucrose-taste substitute. Ribaudioside-A, as well as α-glucosylstevioside, do not have the bitter taste, and more closely mimic the desirable taste of sucrose. The best sweeteners are made by converting the stevioside into the glycosylated derivative (24-26).

The Hayashibara process (25) consists of making the α-glucosyl derivative of stevioside by the use of an α-glucosyltransferase such as α-glucosidase, α-amylase, cyclodextringlycanotransferase, or α-1,6-glucosyltransferase, depending on the α-glycosyl donor. Usually a mixture of α-mono-, α-di-, and α-tri-glucosyl steviosides result, as well as α-glycosylribaudiosides, if ribaudioside was present. The α-glycosyl donor can be gelatinized starch, dextrins, maltose, or even sucrose, for example. The commercial donor used is dextrin.

In the Dainippon Ink & Chemical ("DIC") process (26), stevioside is reacted with a ß-1,3- or ß-1,4-glycosyl sugar compound in the presence of the appropriate ß-glycosyl transferase to make the ß-glycosyl stevioside (ribaudioside-A.) Donors of the ß-glycosyl group can be glucan from yeast cell wall, laminarin, lactose, cellobiose, or curdlan for example. Curdlan, a ß-1,3-glucan, is the preferred donor. Any ß-1,3- or ß-1,4-glycosyl transferase can be used, including cellulases, emulsin (the ß-glucosidase from almonds), depending on the ß-glucosyl donor, but the preferred glucosyl transferase for use with curdlan is a *Streptomyces* transglucosidase. This Streptomyces culture is also the source of the laminaripentaohydrolase (27) used to hydrolyze the curdlan into 5-unit fragments (M. Yamada, DIC, personal communication). In this process the endo- and exo- ß-glucanases with transferase activity are separated and immobilized; the laminaripentaohydrolase is also removed and added to the curdlan fermentation broth, cutting the curdlan into laminaripentose (5-glucose residues). The cells are removed and the entire mixture is added to the stevioside and passed over the immobilized ß-glucanases to produce the ß-glycosylated stevioside, ribaudioside-A. DIC, however, is working with plant improvement thru conventional and probably genetic engineering and cloning to produce a plant that makes primarily ribaudioside-A.

ENZYMES IN CORN WET MILLING

Wet milling of corn begins with a steeping step. Corn kernels are softened by absorbed water while soluble solids diffuse into the steep water. Steeping makes it easy to separate the starch granules, protein, fiber and germ. The corn kernel is steeped in water, with sulfur dioxide added, then ground slightly to break down the structure. Much of the starch is embedded in fibrous material, and bound to the gluten; as much as 5-10% of the starch may remain thus bound. The judicious addition of *Trichoderma reesii* cellulases rich in side activities can significantly improve the starch yield by freeing bound starch granules, which are then easily separated from the protein and other components based on density. (T. Vaara, Alko Ltd., personal communication, and J. Zelko, Genencor, personal communication).

One very important additional concurrent enzyme process has been developed by Alko and Dorr-Oliver (28). This is the steeping of corn with phytase in combination with a *T. reesii* cellulase rich in side activities. (This enzyme combination was originally developed under the Econase 434 name for use in pig feeds; the breakdown of phytate by phytase released the phosphorus bound by the phytate. This amounts to 50% of the phosphate in corn, for example.) Anyone working with Corn

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Steep Liquor as a microbiological nutrient or in animal feed has run into problems with the insoluble calcium phytate-phosphate sludge. The Alko/Dorr Oliver process involves a two-stage steeping, which increases the efficiency of the extraction, substantially shortens steeping time to 12 hours, and gives a concentrated corn steep liquor reduced in phytic acid and free of precipitate, making the calcium and phosphorus available to the organism or animal being so fed. Making the phosphate in the corn available to animals eating it avoids the need for addition of phosphate to the animal feed; in turn this results in reducing the amount of phosphorus excreted by the animal. In some countries farmers are penalized for adding phosphorus compounds to surface waters (when disposing of manure rich in phytate-phosphorus materials, for example) because of the eutrification of ponds and streams. It is for this same reason that phosphates have been removed from household detergents in many areas.

OXIDASES

There are many oxidases used in clinical and analytical chemistry for the specific determination of constituents of complex samples, based on the generation of hydrogen peroxide by the action of an aerobic oxidase on its substrate. Cholesterol, glucose, galactose and many other analytes are determined that way. These analyses are also important in food analysis. But the volume of oxidases consumed in food processing is primarily based on the protection afforded the food by the removal of either oxygen or the other substrate acted upon.

Glucose oxidase. Glucose oxidase has been used for forty years for the stabilization of egg products by conversion of glucose to gluconic acid, preventing the Maillard reaction that in this instance is undesirable. Glucose oxidase has also been used for the protection of foods against growth of aerobic organisms and against various oxidative reactions that cannot take place in the absence of oxygen. It now appears that some of the deoxygenation uses anticipated decades ago will be of commercial importance due to improvements in packaging materials, or changes in the form of products being marketed. For example, OVAZYME was promoted as a surface coating for film for packaging cheese, to serve as an oxygen barrier, over 30 years ago, but never really used because vacuum packing techniques were, generally, adequate. Now, 30 years later, cheese is being marketed in shredded form, a form that does not lend itself to vacuum packing. Instead 50% of the anticaking mixture dusted onto the shredded cheese has been replaced by a mixture of glucose or glucogenic material, with added pulverant glucose oxidase and catalase, which is activated by the moisture in the cheese and completely deoxygenates the sealed package, retarding molding (D. Scott and T. Szalkucki, Finnsugar Biochemicals Inc, unpublished data).

Oxalate oxidase. In some instances, as in dry wine, there is not sufficient glucose to effect deoxygenation. However there appears to be sufficient oxalic acid in many wines to act as the substrate for combination with oxygen by oxalate oxidase, derived from the discarded leafy tops of the sugar beet plant (29). The application may still not work well if it relies on catalase to decompose the H_2O_2 formed by the oxidase, as catalase is strongly inhibited by ethanol at the pH of wine.

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Alcohol oxidase. Another purified oxidase of particular interest is Provesta's alcohol oxidase, from the yeast *Pichia pastoris*. Described as "equally active on methanol and ethanol" (D. Banasiak and T. Hopkins, Provesta, personal communication) it is "one-third as active on ethanol than on methanol.." on an enzyme electrode (Hopkins and Muller (30)). The commercial product is virtually devoid of catalase and has a pH stability profile (5.5-8.5) that *seems* to rule out its use in beer or wines. Alcohol oxidase is still of interest because it acts on a highly volatile substrate. This permits including everything but the substrate in the cap of a wine bottle and activating it by delivering the substrate as the vapor when the cap is applied to the wine bottle. Alcohol oxidase has also been suggested as a means of making a *natural* acetaldehyde, though the trapping of acetaldehyde by bisulfite in a *Saccharomyces* fermentation, and subsequent recovery from that complex, seems more economical. Alcohol oxidase continues to be a very interesting enzyme, one that should find application in foods and in the transformation of flavor components for use in foods. Protein engineering should also be able to shift the pH activity-stability range.

Sulfhydryl oxidase. Developed for a use conceived some years earlier (D. Scott, Finnish Sugar Co.,unpublished, and 31), a new sulfhydryl oxidase from *Aspergillus niger* has been shown to be capable of strengthening "weak" doughs (made with low-gluten flour, or doughs treated with reducing compounds to reduce viscosity for mixing). Strong doughs can be made from weak doughs by treatment with sulfhydryl oxidase alone or in combination with glucose oxidase (S. Haarasilta and S. Väisänen, Finnish Sugar Co.,personal communication). Doughs so treated showed substantially better stability, and produced breads more symmetrical in shape, with higher specific volumes, more uniform pore size, and better texture.

Oxyrase. We must also consider the Oxyrase system for oxygen removal. This is presently derived from *Escherichia coli* or *Bacillus subtilis*, but could be derived from other sources too, such as various yeasts (J. Copeland, Oxyrase Inc., personal communication), making the system potentially suitable for use in foods. Because of the diversity of enzymes in this system, the presence of such substrates as lactic acid, succinic acid, formic acid, or α-glycerophosphate, found in virtually any biological tissue, can effect deoxygenation and attendant stabilization or restriction on the growth of aerobic organisms.

FEED ENZYMES

ß-glucanases. Barley is a cheap ingredient for poultry feed in many parts of the world, such as some Nordic countries, parts of Russia, and Western Canada. Barley content is limited because high amounts in feed mixtures may retard the growth rate of broilers, cause sticky droppings and reduce the quality of the straw bedding because of the "gummy" ß-glucans. The use of ß-glucanase in poultry feed was pioneered by Finnsugar (32). Levels as high as 70% barley can now be used in poultry feeds if ß-glucanase is added to eliminate these problems. There is a further advantage because the enzyme breaks down the walls surrounding the starch granules of the barley, making it more available. ß-Glucanase, with cellulase and hemicellulase, all put on a proprietary cereal-based carrier,is reported to result in an enzyme-

enriched feed mix containing 0.1% Avizyme (33) wherein the enzymes can better tolerate the heat of feed pelleting. Apparently a similar mix of enzyme activities is recommended (34) for use with triticale, rye and barley in poultry feeds.

Enzyme mixtures similar to the those used for poultry feeds are also useful in barley-based piglet feed. Digesting the oligosaccharides early enough in the digestive tract to make it available for the piglet avoids diarrhea resulting from complex oligosaccharides reaching the large intestines, where indigenous organisms utilize them as carbon and energy sources in a heterofermentative manner (35).

Silage enzymes. Silage is preserved by a natural lactic fermentation, but some crops ensiled, particularly grasses, do not have sufficient fermentable sugars to generate enough acid to drop the pH to the preserving level. This can become critical for high moisture silage, especially for crops like luzerne that are low in fermentables, yet high in buffering capacity. If the pH doesn't rapidly go to about 4.2 or lower the clostridial growth can give the silage a butyric odor and tends to be rejected by cattle. Cellulases are beneficial here. In some tropical grasses, such as Bermuda grass, there is a high starch content, which can readily be converted to fermentables for the Lactobacilli.

OTHER INTERESTING ENZYMES

Urease. Alcoholic beverages contain a urethane (ethylcarbamate), a known carcinogen. Levels reported range from 658 ppb in brandies to 13 ppb in grape wine, but vary substantially from brand to brand (36). Apparently arginine is metabolized by the yeast during fermentation to form urea. Ethylcarbamate is presumed formed by the reaction of urea with ethanol, especially when heated, as in distillation of brandies or whiskeys, or in cooking of sherry. The addition of urease to certain alcoholic beverages depletes the urea before it can react with the ethanol. Jack bean urease is not desirable because of the lectin (hemagglutinen) content of impure preparations and because this urease acts poorly in the pH range of most alcoholic beverages (pH 3-5).

A recently identified acid urease, from *Lactobacillus fermentum*, an organism used in the production of Grana, a hard Italian cheese (37), has been shown to be effective in reducing the urethane content of sake (38). This urease is reported as being used now in sake in Japan to reduce urethanes. The process seems to work well for sake and whiskey, but not for wine. Despite the demonstated reduction in urea when urease is added during the fermentation, the benefits of the use of urease in new production is somewhat questionable as "...the products with reduced urethanes, like aged wines and whiskeys, won't be available until the 21st century" (39). The risk is considered extremely low (40-42).

Soy-bean milk clotting enzymes. Soy milk, of course, differs from cow's milk, so that rennin (chymosin) is not the enzyme of choice. Microbial enzymes, particularly neutral and alkaline proteases seem to offer the likelihood of making cheese-like soybean protein products of the hard-, soft-, or cream-type, even yogurt-type (43-44). A process involving the use of plant (bromelain or ficin) or microbial (*Bacillus subtilis*, *Aspergillus oryzae*, or *Streptomyces*) endopeptidases, along with the addition of Ca^{++} has also been developed (45).

In Biocatalysis in Agricultural Biotechnology; Whitaker, J., el al.;
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α-Galactosidase for modified food gums. Locust bean gum is a galactomannan containing 23% galactose; it is sparingly soluble in cold water and interacts with carrageenan, xanthan and other polysaccharides. The higher-substituted guar-seed galactomannan has much more galactose (38%) and therefore has shorter unsubstituted D-mannan regions, and is cold water soluble, but doesn't react with other polysaccharides. The interaction is a strongly desired property for stabilizing suspensions. Modifying guar galactomannan with an α-galactosidase very low in endo-mannanase activity reduces the galactose substitution, thereby lengthening the average unsubstituted mannan region; this gives a product that mimics the locust bean gum, which is often in short supply and more costly than guar (46-47). Unfortunately, at the moment, guar gum prices are "out of sight", but this changes from year to year and this process gives greater flexibility.

Pentosanases in baking. Pentosans in grain are both blessings and problems. Pentosans are thought to have a role in dough development, loaf volume, and antistaling actions on the final loaf of bread. Good or bad is a matter of "how much". Strong wheats may have 2-3% of pentosans, with arabinoxylan predominating. About a third of the pentosan is water soluble. Adding a purified pentosanase, a 1,4-ß-xylanase free of protease, to wheat flour causes the dough to become sticky and "weak", resulting in decreased bread loaf height and and a crisp, brittle loaf instead of a springy one (46).

On the other hand rye flours contain 8% pentosans, which seriously restrict moisture uptake and dough development, as well as dramatically increasing power requirements in dough mixing. The use of a suitable pentosanase for rye bread products can have important advantages in decreased dough development time and power requirements, and yield a more moist, easily consumed bread.

Tannase. Immobilized tannase has been used to produce n-propylgallate from tannin and n-propanol in contact with a water-immiscible phase. The propyl gallate moves into the non-aqueous phase while the free gallic acid and sugar remain in the aqueous phase (48).

Phospholipase D. An improved biosurfactant and emulsifier is made from lecithin by phospholipase D. The enzyme transphosphatidylates lecithin, converting most of the phosphatidylcholine and phophatidylethanolamine to phosphatidylglycerol (49).

Phospholipase A. Phospholipase A hydrolyzes the C2 ester linkage, converting lecithin into a free fatty acid and a lysophospholipid. This lysophospholipid is reported (50-51) to improve the elasticity and extensibility of dough, resulting in better bread quality.

Glutathione-decomposing enzymes. Gamma-glutamyl transferase, with cysteinyl glycine dipeptidase and glutathione oxidase, decomposes glutathione into L-glutamic acid, glycine, and L-cysteine (52). Addition of these enzymes to dough, especially where low protein flour (less than 12%) has been used, results in improved dough elasticity and bread with increased loaf volume, improved crumb structure, and retention of bread softness on storage.

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5'-Adenylic deaminase. The deamination of 5'-adenylic acid by 5'-adenylic deaminase results in the formation of 5'-inosinic acid. This process, applied to mushrooms, intensifies the natural flavor (53).

Cyanidase. Cyanidase hydrolyzes cyanide to form ammonia and formic acid (54). This is of potential importance for eliminating cyanide from the waste water from cassava and bitter almond processing plants, as well as industrial spills.

ANTI-MICROBIAL ENZYMES

The trend away from "chemical" preservatives, such as sulfite, has expanded interest in enzymes as natural antimicrobials (preservatives.) The situation has been exacerbated by the modern sanitary practices that eliminate large numbers of harmless microflora that, when present, inhibit the growth of more fastidious but dangerous organisms, such as *Salmonella* and *Yersinia* (55-56).

Enzymes can function as antimicrobials by depriving an organism of a necessary metabolite, by generating a substance toxic to the organism, by attacking a cell wall component so as to alter cell membrane permeability or physically disrupting the cell, or by "killer enzymes" inactivating other enzymes (D. Scott, Food Biotechnology, in press).

Oxidases. These can function by generating H_2O_2, which can attach -SH groups, oxidize lipids, deplete NADH or NADPH, or form carbonyl groups. Oxidases can also function to limit microbial growth of obligate aerobes by depriving them of oxygen (C. Mannheim and I. Sagi, personal communication).

Xylitol phosphorylase. *Streptococcus mutans* converts xylitol to xylitol-5-phosphate, which it cannot metabolize further, thereby killing the organism (57). (Humans are able to metabolize xylitol-5-phosphate.) Consequently xylitol is an excellent sweetener for use in toothpaste as an anti-cariogenic ingredient.

Lipases. Some lipases, especially the bile-salt-stimulated lipases in human milk, destroy the protozoan *Giardia lamblia* (58). The free fatty acids are responsible for the action. This was shown by the requirement for incubation of the cream with the skim to develop the inhibitor. Studies with the addition of *Giardia* to milk (59) showed that preincubation was necessary to get a good kill of the added cells. There was substantially no change in viable count without cholate, whereas with cholate the viable count went to virtually zero over the two hours.

Salivary lipase, lingual lipase, phospholipase, and pregastric lipase all seem to have the same desirable hydrolytic effect.

Lactoperoxidase and myeloperoxidase. Lactoperoxidase and myeloperoxidase are halogenating and oxidative enzymes. Lactoperoxidase, coupled with a peroxide-generating oxidase, converts SCN^- to $SCNO^-$, a very reactive and lethal ion for microorganisms. The peroxide-generating oxidase is believed to go through a singlet oxygen (superoxide) stage, with the O_2^- being further oxidized to hydrogen peroxide; however the superoxide ion also can be quite lethal. Both the superoxide ion and hydrogen peroxide probably exert their antimicrobial effects through

oxidation of -SH groups of essential sulfhydryl enzymes to disulfides (-S-S-), sulfenyl thiocyanates (-S-SCN), or sulfenic acid (-S-OH). (60). Milk normally contains thiocyanate ions (SCN^-) and lactoperoxidase, which is why low levels of hydrogen peroxide added to milk serve as an effective deterrent to slow the growth of microorganisms. The effect is not long-lasting because of the high reactivity and transient nature of the hypothiocyanate ion ($SCNO^-$). In colostrum, the level of lactoperoxidase in milk produced by cows in the days following parturition is ten times that in milk at other times. This serves as an important protective factor for newborn calves. That is why the trend to putting calves on so-called calf milk replacers results in a slower growth rate and higher disease rate in those calves unless lactoperoxidase, thiocyanate, and a suitable oxidase are included in the calf milk replacer, to give "bottled milk" the same protective factors as "mother's milk" (61). This system applied to soft-serve ice cream mix stored at 5^0 results in the count remaining under about 20 CFU/mL for 60 days whereas the untreated control went from under 100 to 10,000 in 10 days. On pastry cream the count went from about 3,500 CFU/mL to almost 100,000,000 in 7 days, whereas with the lactoperoxidase system, the count dropped from the original 3,500 to about 750 (J-P. Perraudin, Oleofina, personal communication).

The myeloperoxidase-H_2O_2-Cl^- system has been suggested for food preservation (62) based on the production of hypochlorous acid (HOCl), and/or halogenation of microbial components to form chloramines. In addition to these mechanisms the phagocytes are reported to kill the engulfed invader-cells by a process catalyzed by myeloperoxidase to form cyanide. The MPO-H_2O_2-$Cl^{\bullet}$ can produce HCN, which can bind microbial heme, or inhibit catalase present in the organism under attack, to conserve the peroxide formed (63).

Physical disruption of the cell or change in permeability. The cell is a heterogeneous structure. Removal of any element in that wall, or of the binding material, results in a breaching of the wall. Total destruction of the cell wall isn't necessary. Crude trypsin, combined with lysozyme and lysolecithin, is reported to lyse *E. coli* and *Shigella flexner* (63).

Gram negative organisms have a peptidoglycan layer between the lipopolysaccharide outer membrane and the inner membrane. Disruption of the lipopolysaccharide layer changes permeability and results in death of the cell.

Lysozyme. Lysozymes (1,4-ß-acetylmuramidase) cleave peptidoglycans between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine. Some lysozymes also have chitinase and/or esterase activity. Lysozymes from different sources differ in specificity, microbial spectra, number of conformation-stabilizing disulfide groups and other ways. Lysozyme is effective by itself against a number of gram positive organisms, but not gram negatives, as the latter have a lipopolysaccharide shield over the peptidoglycan layer attacked by the lysozyme. The gram-positive cell wall peptidoglycan layer is covered substantially only with a very porous layer of capsular material, and some scattered protein. On the other hand the gram-negative organism has the peptidoglycan layer covered by a tight lipopolysaccharide-protein-divalent cation-phospholipid layer. Both gram-positive and gram-negative organisms are problems, but the gram-negative organisms

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are by far the greater problem from an enzymatic attack standpoint. Various agents that can disrupt the lipopolysaccharide layer can act as potentiators for lysozyme by giving it access to the peptidoglycan layer. These include EDTA, Tris buffer, Polymyxin B, polylysine (20mer), unsaturated fatty acids like arachidonic, and complement (64). Polycations (e.g. $lysine_{20}$) seem to disrupt the lipopolysaccharide layer, and make the lipopolysaccharide layer sensitive to the anionic detergent sodium dodecyl sulfate (65-66).

Lysozyme derived from hen egg albumen has been applied commercially to prevent late blowing of certain hard cheeses, such as grana, provalone, emmenthal, asiago, and montasio, where it effectively lyses the emerging bud from the germinating spore of *Clostridium tyrobutyricum*, a common contaminant in cows' milk where high moisture silage is used. Over 100 tons of hen egg lysozyme is used annually for that purpose (32). Lysozyme has been reported effective against *Listeria* and *Clostridium botulinum*, and said to "hold promise" as a replacement for nitrite or sulfite (67).

Mannanase. Mannanase, with and without proteases is important for yeast lysis, along with the β-glucanases. Yeast (*Saccharomyces*) cell walls consist primarily of β-glucans-proteins. A protein-mannan outer layer forms a smaller covering layer. These can be readily disrupted by a combination of lysozyme and an endo-β-1,3-glucanase (68-69). Mannanase and mannosidase are also important, and are now commercially available (70, Imai, T., Godo Shushei Co., personal communication).

Chitinase. Chitinase is a system involving at least three enzymes, an endo-chitinase, an exo-chitinase, and chitobiase (N-acetylglucosaminidase) (71). Roken (72) estimated chitin:glucan ratio of 1:1.4 in the cell wall of *Aspergillus niger*. Chitinase activity is an essential component of an anti-fungal system. However proteins and lipids have also been demonstrated as very important in the cell walls of fungi and appropriate activities should be included for these, as well, in an anti-fungal "cocktail." A very potent "chitinase" from *Aeromonas hydrophila* subsp. *anaerogenes* A52 has been marketed now for several years (73).

"Killer enzymes" or "anti-enzymes enzymes", enzymes that inactivate other enzymes. Of course proteases come immediately to mind. Also sulfhydryl oxidase for -SH enzymes. Other specific enzymes are possible.

CONCLUSION

The field is developing rapidly, though no new markets like detergent protease, or immobilized glucose isomerase yet appear to be close at hand. The next "$10,000,000" or more single enzyme market is likely to be in a "reverse reaction" such as using a hydrolytic enzyme in a water-limited environment to catalyze a condensation reaction. But enzymes with smaller but still very significant markets are emerging for the reasons given at the beginning of this presentation.

Add to this the present skills in genetic engineering to improve yields and lower costs, and protein engineering to alter the pH, temperature, or other characteristic of an enzyme, and the future looks very bright from the enzymologist's viewpoint, provided management is willing to speculate on the development costs!

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Chapter 13

Immobilized Plant Cell Reactor Systems

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Plant cell cultures provide an alternate means of producing valuable plant-derived biochemical compounds compared to conventional field cultivation. Commercially important products which can be generated in this manner include drugs, flavors, fragrances and pigments. However, productivity of these Systems is often low and thus a major impediment to the commercialization of such processes. Use of bioreactors containing plant cells immobilized on or inside membranes and beads is one approach to improve productivity. In this paper, several examples of immobilized plant cell cultures are presented. Data on growth characteristics and kinetics of product formation for suspended (free) and immobilized cells are shown. Different biological and process engineering approaches to enhance product synthesis have been demonstrated. Problems of scaling up these cultures are discussed.

Use of plant cells for production of biochemicals represents a new area of biotechnological exploration. Higher plants are valuable sources for a wide range of important chemicals, including pharmaceuticals, food colors, flavors and fragrances and insecticides. Production of these compounds from higher plants is currently carried out by cultivation of the plant and extraction of the material. In general, this is a labor-intensive process that has a number of associated disadvantages such as geographical restrictions, politics, economics and climate conditions. One attractive alternative has been to develop cultures of isolated plant cells that can be grown in suspension or immobilized on some support similar to the use of aseptic fermentors and other biochemical reactors for the culturing of microorganisms.

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Products synthesized by the parent plant, in a variety of cell types and throughout the development of the plant, are made in culture under a range of conditions and considerable scope exists for improving the productivity of such cultures. Development of stable plant cell lines of sufficiently high productive capacity on which to base commercial processes remains an important problem. Immobilization of plant cells within a support matrix appears to offer both bioengineering and biochemical advantages compared with free cells. These include ease of use in a continuous process with retention of biomass; reuse of biocatalyst (cells), cofactors or precursors; protection of cells from mechanical stresses; and superior productivity and longevity of cells.

Immobilization of cultured plant cells by entrapment in a gel matrix appears to provide conditions that are optimum to cell differentiation resulting in higher yields of secondary products. Compact or organized and slow-growing cultures often synthesize higher amounts of secondary products than growing cultures.

Immobilized cell preparations as artificial aggregates of cells have been employed to investigate the effects of cell aggregation on secondary metabolite formation and cell metabolism under defined conditions. The size of aggregates and cell concentration can be controlled within immobilized cell preparation to compare the effects of cell aggregation. Brodelius (1) reported that cell suspension cultures of Coffea arabica that contained large aggregates show a higher production of methylxanthine alkaloids than cultures with finely suspended cells. Thus, this work provides an appropriate model system to investigate the effect of aggregation on secondary metabolism for immobilized cell culture.

Biotransformation involves the conversion of an externally applied compound into another form, by hydroxylation, for example, and utilizes only a small number of intracellular enzymes, although it relies on the primary metabolism of the cells to regenerate any necessary cofactors. Biotransformation can probably be achieved at relatively high external substrate concentrations. Therefore, immobilization of plant cells shows promising potential for the production of secondary metabolites by synthesis from appropriate precursors. Two such processes that have been reported along these lines -- the bioconversion of codesinone to codeine (2) and the hydroxylation of digitorin to digoocin (3) have significant commercial importance.

Plant derived pharmaceuticals are estimated to have an annual value of $9 billion in the U.S. alone (4). Flavors and fragrances have a current worldwide market of about $1.5 billion. Market data for insecticides and other fine chemicals such as pigments are not readily available. The first example, and the only current commercial process based on plant cell culture, is for the production of shikonin in Japan. This compound is both used in medicine and as a pigment (5-7).

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CHALLENGES TO THE SCALE-UP OF PLANT CELL CULTURES

The process engineering requirements of plant cell, tissue or organ systems are quite different than those typically seen with bacteria or non-plant eukaryotic systems. The sizes of plant cells or cell aggregates, for example, are almost four orders of magnitude larger than a typical bacterial cell. Organized plant structures in culture are macroscopic and easily seen with the naked eye. The ability to handle effectively the variety of possible plant structures is a formidable task, but one which is necessary for successful commercial exploitation for chemicals production.

The greatest amount of work on scale-up of bioprocesses relevant to plants has been with cell suspension cultures. The potential for chemicals production and for biomass generation, from large scale plant suspension cultures has indeed been recognized for some time (8-10). Many factors remain to be resolved, however, as evidenced by the fact that only one process during the past 25 years has reached commercialization (5). Some of the barriers to successful commercialization are associated with:

- the growth rates and growth characteristics of the cell in suspension,
- the mixing and mass transfer in large scale reactor systems,
- the long term variation, i.e., instability, of cells in culture,
- product formation, release and associated downstream processing steps
- process modeling, synthesis and optimization.

Downstream processing steps are also important process components and have received only limited attention. In general, however, the types of downstream processes needed to extract chemicals from cell culture would be similar to the steps involved in their extraction from whole plants. But the extraction, separation and purification steps can generally use harsher conditions than those usually employed in the biotechnology industry for the recovery of protein products from recombinant microorganisms or animal cell culture. A major emphasis is needed, however, in the integration of these steps into an overall process system.

GROWTH CHARACTERISTICS OF PLANT CELLS IN CULTURE

Biological Perspective

Successful growth and propagation of plant cells freely suspended in liquid media was first demonstrated almost 50 years ago (11-13). Much attention has been focused since on elucidating the necessary media components that support growth and differentiation of the culture. The role of growth regulators was pointed out in 1957 (14) and the process of somatic embryogenesis was realized in

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1958 (15). Work on plant protoplasts (16) in culture, that allows for somatic hybridization of different plant species and work on anther cultures (17), that produces haploid cells and plants, opened up new ways for carrying out genetic modifications that greatly accelerated the pace at which plants can be screened for new and improved properties. Also in the late sixties, it was reported that genetic variability could be found in cell suspension cultures (18), usually after the cultures had been maintained for long periods of time. The regeneration of various plants from a somatic cell suspension culture quickly led to the realization that this technique, referred to as somaclonal variation, can be used for crop improvement (19) and for the selection of cells with altered biochemical pathways (20). Recently, the variation in plants regenerated from cell cultures of tomato has been used to ascertain the genetic basis of somoclonal variation (21). The variability arises both from the ex-plant source (intrinsic) and from spontaneous variation (extrinsic) during culture propagation.

Process Engineering Perspective

Growth of large amounts of plant cells or tissues in culture is hampered by the extremely slow growth rates. Doubling times for plant cells in cultures vary from 20 to 100 hours and this often necessitates the use of large reactors when one is concerned with chemicals production to obtain reasonable yields. Contaminating organisms are a problem under these conditions and must be dealt with by extreme attention to aseptic conditions. Cell suspensions also tend to grow as aggregates of varying sizes. Cells are also sensitive to fluid shear and the power input to the reactor is thus limited. This restriction works against the need for uniform conditions inside the reactor and often precludes the direct use of fermentors that have been designed for microbial fermentations. Nevertheless, some large scale systems have been successfully operated including a two-state 950 liter fermentor for the production of shikonin (5) and a 750 liter fermentor for the production of Catharanthus alkaloids (22). Fermentors as large as 20,000 liters have been reported as well for the production of tobacco cell mass (23).

The use of air lift fermentors has been advocated as a means of overcoming the sensitivity to shear while still maintaining adequate oxygen and mixing characteristics (24). Scale-up of such fermentors has shown mixed results in that the productivity has either decreased (22-25) or increased (24) in going to larger sizes. The hydrodynamics of such systems are extremely complex to model. Some work on modeling of air lift systems for microbial fermentations has been carried out (26-29); however, none of the models so far proposed has been tested with plant cell systems.

The aggregation of cells in suspension culture leads to a heterogeneous population that confound the analysis and operations of the reactor types mentioned above in many cases. For non-growth associated products, immobilization of cells provides a

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means to circumvent various problems including aggregation. Immobilization can be carried out to give a uniform (homogeneous) degree of aggregation or to eliminate aggregation as a confounding factor. Furthermore, in continuous systems, the slow growth rates limit the dilution rates for free cells in suspension whereas for immobilized systems the dilution rate is not similarly limited. Thus, there are a number of advantages to working with immobilized plant cell systems. Reviews of the techniques and reactors configurations for immobilization of plant cells have been presented by us and other groups as well (30-32).

Another important observation concerning growth characteristics of plant cells deals with the separation of growth and production phases, especially for a large number of secondary metabolities. Certain media components will favor growth while other components will favor secondary metabolite production. This has been extensively documented with pigment formation by Lithospermum erythrorhizon (33) and the phenolics production in Nicotiana tabacum (34).

Mixing and Mass Transfer

The aggregation of cells due to their natural growth characteristics in suspension culture or due to an imposed condition by various immobilization schemes leads to mass transfer and mixing constraints. An important parameter in non-photoautotrophic cultures is the oxygen transfer coefficient that is typically used as one of the scale up criteria for the air lift systems mentioned previously. Other factors can also be expected to be important such as the local carbon to nitrogen balance. These factors are made even more important in plant cell cultures since the relative balance of nutrients can lead to distinct morphological changes and even to differentiation of the plant cells. It can certainly be expected that the different cell types will possess different biochemical or regenerative abilities. In cell cultures that are selected to be photoautotrophic and can thus utilize photosynthetic pathways in culture, carbon dioxide transport is a major design factor.

Cell Stability

It is well known that plant cells in suspension undergo gradual but continuous changes in the genetic and, ultimately, biochemical characteristics. In operation of reactors for chemical production or in the cloning of elite cell lines, stability is to be encouraged. This is in contrast to purposeful variability induced deliberately in order to select for such elite cell lines. One method of maintaining stable cell lines would be to limit their growth as has been observed in immobilized plant cell systems. Apparently, the high density culture that is achieved by the immobilization process slows cell growth even further than its usual low value. If the cells can be reused and maintained in active nongrowth states, then this is a preferred method of operating plant cell reactors. In other cases where cell growth

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is required, such as in somatic embryogenesis, other factors for selecting against instability, or controlling stability specifically must be sought. These factors are currently unknown.

Product Formation, Release and Downstream Processing

Product formation rates have already been mentioned above as being influenced by environmental factors and by plant differentiation. Much previous research has approached the questions of environment and cell state empirically. Studies related to methods for optimizing product formation rates should be based, however, on a more fundamental understanding of cell biochemistry and physiology.

Products formed in plant cells are usually intracellular storage products and must therefore be released from the cells if the product is to be continuously collected in the medium. This is especially important in immobilized cell systems. There are several environmental factors that will allow for release of products such as modifications in the media pH (35) and addition of solvents that permeabilize the cells (1 , 36). It has also been demonstrated that operation in two-phase reactors, where the products are extracted into the nonaqueous phase, can be effective. Both liquid-liquid (37) and liquid-solid (38) systems have been used. The continuous release of products in this manner would also favor their continuous production since the storage capacity of the cells would not become limiting.

The integration of production and separation stages that is suggested by the above observations has not been pursued on a large scale. Lipophilic products, such as many fragrances (essential oils) and alkaloids, could be produced effectively by such methods. Downstream processing is also simplified by the preliminary extraction into a second phase. Subsequent purification of the product and general downstream processing for the secondary metabolites can use relatively harsh techniques that are quite familiar to the chemical processing industries.

Aggregated Cell Cultures

We have recently investigated the effects of aggregated states on plant cell culture productivity and morphogenic capacity. In one case, cultures of coffee cells were artificially aggregated by immobilization in alginate gels (39). In the other case, carrot cell cultures were observed to form somatic embryos from small, dense cell aggregates after growth on low hormone media (40). These experiments are described briefly below.

Coffee Cell Cultures

Suspension cultures of *Coffea arabica* cv *bourbon amarelho* derived from primary callus cultures have been maintained on media (14) supplemented with cysteine, thiamine and kinetin (39). The cells were sieved through nylon nets of 1 mm mesh size to obtain small aggregates and single cells. Large aggregates were not used. This selection was done during weekly subculturing. Alginate

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beads of 3-4 mm diameter were made by mixing 2% sodium alginate with a sieved cell suspension and dripping the alginate cell suspension (6/1) into medium fortified with 60 mM $CaCl_2$. The beads were allowed to form for thirty minutes and then collected by rapid filtration. They were washed with media containing 5 mM calcium salt.

After four days, seven flasks containing the growing, suspended cells were taken. The suspended cells were aggregated using the alginate bead procedure and reintroduced into the four day old media. In this way, only aggregation is being manipulated. The identical procedure was done for seven and twelve day old suspension cultures as well as for cells being subcultured (Zero day old).

Cell dry weight, glucose concentration and purine alkaloid levels were followed at two- to three-day intervals for all types of aggregated cells. Cells immobilized on day Zero grew slower and produced less total alkaloids than for cells maintained in suspension. The glucose uptake rate was also slower. Productivity, measured on a cell dry weight basis is much improved for aggregated cultures, however, compared to the freely suspended cells. The caffeine levels are almost twice as high. For cultures immobilized at day seven, the cell growth rate immediately decreased, with glucose uptake also slowing. Alkaloid levels are only slightly improved (theobromine) or even decreased (caffeine). Therefore, however, this effect is dependent on the initiation time of aggregation. For coffee cultures, aggregation effects are important at early stages of growth in fresh media.

Cultures of Daucus carola cv Nantes have been maintained in 125 mL shake flasks on primary media containing growth regulators. The cultures were switched to media containing no hormones or different levels of hormones and aggregated cells (clusters of differentiated tissue) were followed using an image analyzer. An analyzer has been developed to recognize embryos from morphologically distinct cell aggregates in suspension culture systems (40). The maxima in the curves occur with depletion of sugar from the media. These data can be collected on large numbers of embryos in a rapid, automated fashion.

Conclusions

Bioreactors employing plant cell cultures have use in chemicals production systems and in micropropagation (biomass) systems, as well. Factors related to the performance of these reactors from an engineering point of view have been addressed in this paper. Some preliminary data from our laboratory suggest how mass

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transfer in aggregated systems affects productivity in coffee cell cultures. Aggregated cells are also characteristic of developing somatic embryos and we have designed image analyzer based protocols for rapidly quantifying numbers of developing embryos. These tools and techniques are expected to be of use to the development of efficient bioreactor systems for plant cell culture.

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Chapter 14

High-Level Synthesis and Secretion of α-Amylase from Rice Callus

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Rice seed callus was found to synthesize and secrete high levels of α-amylase for up to 17 months after initiation. Approximately 43% of the total secreted protein is α-amylase. This level of expression was not increased by exogenous gibberellic acid, nor was expression reduced by the gibberellin biosynthesis inhibitor paclobutrazol. Alpha-amylase expression, however, was reduced 41-fold in seed callus derived from Tan-ginbozu, a mutant lacking gibberellins, suggesting that gibberellins are involved in the regulation of α-amylase biosynthesis in the callus. This low expression in Tan-ginbozu callus, however, was not increased by exogenous gibberellic acid. Surprisingly, shoot-derived callus also secreted α-amylase at levels similar to seed-derived callus, even though shoot tissue normally produces only low levels of intracellular α-amylase.

Alpha-amylase, particularly from bacterial and fungal sources, is a well known example of an important industrial enzyme, and is widely used in the production of paper products, bread, alcoholic beverages, and syrups. With the exception of barley α-amylase, which is used by the brewing industry, the cereal grain α-amylases are better known for their agricultural rather than industrial importance. This is due in part to the fact that α-amylase activity is critical to the seed germination process, and most likely affects several other important agronomic traits of the plant. There are a number of unique and interesting features about the rice α-amylase gene/enzyme system that makes it well suited for protein engineering and plant genetic engineering studies:

a. Rice has a small diploid genome of 2 x 10^8 base pairs (1) which makes it five times larger than the Drosophila genome but twenty times smaller than the corn, wheat, and barley genomes. The small size of the rice genome makes it possible to clone and characterize many rice genes, quickly and easily.
b. Rice was the first of the cereal grains to be regenerated into whole plants from single protoplasts (2-4). This opens the way for the creation of transgenic rice varieties carrying novel genes and gene rearrangements.
c. The biology and biochemistry of cereal grain α-amylase has been well-studied and considerable information is available on the structure and function of these enzymes in a wide variety of organisms (5,6).

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d. Several studies have shown significant and positive correlations between rice α-amylase activity and major agronomic traits such as germination rate (7), seedling vigor (8), cold tolerance (9) and seed weight (10).
e. Rice α-amylase is a thermally stable enzyme with a broad pH range (11). Only 1% of rice α-amylase activity is lost after 40 min of incubation at 60° C. Rice α-amylase retains nearly full enzymatic activity at pH values ranging from 4 to 10 (a slight pH optima is observed at 4.5).
f. Rice α-amylase is expressed and secreted in large amounts from seed- and shoot-derived callus tissue.

For these and other reasons, the rice α-amylase gene family has been chosen for genetic and protein engineering studies. The long-term objectives of these studies are to: (a) genetically manipulate the germination process using recombinant DNA technology; and (b) construct hybrid genes and overproduce their protein products in rice seeds or callus tissue.

During germination, α-amylase, in concert with other degradative enzymes, is primarily responsible for the α-(1-4)-endoglycolytic cleavage of amylose and amylopectin, the major components of the starchy endosperm of the seed. Although α-amylase is expressed in both the aleurone layer and scutellar epithelium during germination, the latter tissue appears to be the initial and primary site of α-amylase biosynthesis in rice (12-14). It has been shown that α-amylase accounts for about 15% of the protein secreted from the scutellum (15). Because barley aleurone layers lack endogenous gibberellic acid (GA_3) and can be easily isolated, they have served as a model experimental system for studying the hormonal regulation of the α-amylase genes. The results of these studies clearly indicate that the α-amylase genes are induced, at the level of transcription, by the phytohormone gibberellic acid (16-18). While similar studies on the scutellar epithelial tissue have not been performed, it is generally assumed that the α-amylase genes expressed in this tissue are regulated in a similar manner. This assumption is supported by the studies on another germination specific hydrolase, (1-3,1-4)-β-D-glucanase, which is GA_3-regulated in both the scutellum and aleurone of barley (19). Whether or not α-amylase expression in rice scutellar tissue is GA_3-regulated is not yet certain.

The technique of culturing plant cells, tissues, and organs has long served as a valuable research tool for investigating plant biochemistry, cell physiology, the processes of tissue differentiation (20), and has recently been used to study the molecular mechanisms regulating plant gene expression (21,22). In rice, callus tissue can be readily generated from the young germinating seed. This seed callus is predominantly derived from the scutellum of the seed (23; Ram, R., Sungene Tech. Corp., personal communication, 1987). Since the scutellum is a principle site of α-amylase synthesis, seed callus provides an opportunity to examine tissue-specific and perhaps hormonal regulation of α-amylase in both organized and unorganized tissues.

This study addresses whether or not the callus retains the mode of α-amylase expression indicative of the tissue from which the callus was derived, and whether the expression of α-amylase in seed callus is regulated by gibberellins.

Materials and Methods

Rice seeds (*Oryza sativa* L. cv. M202) were obtained from Dr. Neil Rutger, University of California, Davis. Tan-ginbozu rice seed were obtained from Dr. L. Rappaport, University of California, Davis. Seeds were dehulled and surface-sterilized in 95% ethanol for five min and 40% commercial bleach for 30 min, followed by five washes with sterile distilled water. For shoot production, M202 seeds were incubated for five days on 0.8% agar medium in the dark at 30°C. When

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the etiolated leaf pierced through the coleoptile, the shoot was cut at the base and cut into three segments. For callus formation, seeds or shoot segments were plated on an agar 1.5D Medium [Murashige-Skoog miminal organics medium (Gibco), 2% sucrose, 100 mg/L casamino acids, and 1.15 g/L L-proline, Yamada vitamins (4), 0.8% bacto-agar, and 1.5 mg/L (6.8 x 10^{-6} M) 2,4-D (2,4-dichlorophenoxyacetic acid)]. For the gibberellin biosynthesis inhibition experiment, paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl 4,4-dimethyl-2-1,2,4-triazol-l-yl)pentan-3-ol] (ICI Americas Inc.) was added at 0.5, 5.0, 20.0, and 50.0 mg/L, a range of 1.68 to 168 µM.

The seeds and shoot segments were cultured at 30°C in the dark. After two weeks of culture, the seed callus was dissected away from organized seedling tissue and subpropagated on fresh medium. Shoot callus required five weeks before subpropagation. After an additional two weeks, the callus was dissected into one to three millimeter diameter pieces and placed in eight mL of 1.5D medium lacking agar which, depending upon the experiment, may have contained 2.5 µM gibberellic acid (GA_3) or the aforementioned levels of paclobutrazol. For the pulse-label experiment, 20 mCi of ^{14}C-aminoacid mix (Amersham) were added directly to the media. After 48 h, the media were collected and filtered through a 0.2 mm Nalgene filter to remove debris, and the filtrate was collected for protein, enzymatic activity, and ^{14}C-incorporation determination. Embryogenic callus was defined as a hard nodular callus which produces organs or embryos, especially root-like tissues. Nonembyogenic callus was defined as a loose, friable callus which produces no embryos or organs.

Enzyme activity was determined by the starch-iodine method described previously (16), except that the reaction was carried out for ten min instead of five min. One unit of activity is defined as a 10% reduction in the OD_{620} absorbing starch-iodine complex. Samples were heated to 70°C for ten min before the assay to inactivate β-amylase (24). The amount of protein was determined by the Bradford dye-binding assay (25). In preparation for gel electrophoresis, samples were precipitated with four volumes of 0°C acetone and after one hour centrifuged at 15,000 g for one min. Protein samples (generally 20-30 mg) were resuspended in 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 0.0625 M Tris, pH 6.8, and 0.05% bromophenol blue, and then heated to 100°C for five min. Samples were loaded onto 4% stacking,12.5% resolving polyacrylamide gel (acrylamide:bis 30:1) as previously described (26). Electrophoresis was carried out at 18 mA for ten h. The gels were stained in 5% acetic acid, 10% methanol, 0.1% Coomassie brilliant blue R, and destained in 7% acetic acid and dried.

To determine α-amylase levels in germinating seedlings, rice M202 or Tan-ginbozu seeds were surface-sterilized and then incubated on 1.0% agar medium with 10 mM $CaCl_2$ plus or minus 2.5 mM GA_3 and/or 0.5, 5.0, 25.0 or 50.0 mg/L paclobutrazol for five days at 30°C. To generate shoot tissue, M202 seedlings were grown on 1.0% agar medium for seven days in the dark at 30°C. Seed or shoot tissues were homogenized in 44 mM KH_2PO_4 buffer, pH 5.4, centrifuged at 7000 g for five min, and the supernatants were assayed for α-amylase activity.

Rabbit antiserum to wheat α-amylase was obtained from Dr. Thomas Okita (University of Washingtion, Pullman), and rabbit anitserum to barley α-amylase was obtained from Dr. Russell Jones (University of California, Berkeley). The wheat and barley α-amylase antigens were purified by Sepharose 6B-cycloheptaamylose affinity chromatography (27). Rabbit antiserum to rice α-amylase was produced in our laboratory using rice callus α-amylase purified by band elution from SDS-PAGE. The α-amylase bands to be eluted were identified by strong cross-reactivity with wheat and barley α-amylase antiserum in the 43 to 44 kDa range. Western blots were conducted as described (28).

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Results

Callus was generated from M202 rice seeds, M202 shoot basal meristems, and Tan-ginbozu seeds. Although the M202 shoot and Tan-ginbozu seed calli were slower to initiate than M202 seed calli, all calli grew at similar rates once initiated. Callus tissue was rinsed and transferred to liquid 1.5D medium and incubated for 48 h at 30°C. The medium was assayed for α-amylase using three criteria: relative mobility of protein on SDS-PAGE, immunological crossreactivity, and enzyme activity.

On the basis of these criteria, it was determined that M202 seed callus expresses and secretes high levels of α-amylase into the culture media. As Figure 1 indicates, the predominant protein on SDS-PAGE migrates with a relative mobility of 43-44 kDa, a size which corresponds to that previously reported for rice α-amylase (29). This band strongly crosssreacts with α-amylase antisera in western blots (Fig. 2). Approximately 12,000 units/g dry weight callus of heat-stable amylolytic activity was detected in culture media after 48 h of incubation (Table I). Callus homogenates also contained α-amylase, but at most 4000 units/g dry weight callus.

Scanning densitometry of the gel shown in Figure 1 revealed that α-amylase corresponds to approximately 43% and 20% of the total protein in the media and homogenates, respectively. For the secreted α-amylase, this corresponds to 3.0 mg of α-amylase/g dry weight callus. These values varied less than 2% between comparable experiments. Radioactive pulse labeling with ^{14}C-amino acids demonstrated that most of the α-amylase is due to active synthesis and not due to preferential accumulation of the relatively stable α-amylase in the media. Callus samples pulse-labeled for 48 h demonstrated that α-amylase comprises 30% of the total actively synthesized and secreted protein (autoradiograph not shown). This high level of α-amylase expression has continued unabated for at least 17 months after initiation and through monthly subculturings. We have tested other rice varieties (Italica Livorno, Short Labelle, and L202), as well as ryegrass (*Lolium multiforum*), for expression of α-amylase in seed callus, all of which were found to secrete high levels of α-amylase.

Since gibberellins are known to induce cereal α-amylase expression during seed germination (16-18), we examined the effects of exogenous GA_3 at 2.5 μM on α-amylase expression in seed callus cultures. No significant change in α-amylase levels was detected after 48 h of treatment (Fig. 1). As a control, M202 seeds were germinated for five days with 10 mM $CaCl_2$ and 2.5 μM GA_3. Seedlings treated with GA_3 had four times as much α-amylase in whole seed homogenates, and had shoots of twice the height, compared to untreated seedlings, indicating that the GA_3 is biologically active and that the rice variety M202 is GA_3 responsive in the two classic gibberellin response systems of α-amylase expression and shoot elongation.

The lack of callus responsiveness to exogenous GA_3 suggested that α-amylase expression in callus tissue may be independent of gibberellin stimulation, or due to gibberellins produced endogenously by the callus. To test the latter possibility, the effects of the gibberellin biosynthesis inhibitor paclobutrazol on α-amylase expression was examined. Callus was cultured on solid medium containing paclobutrazol at levels bracketing its effective range (0.0 to 168 μM) (30). After one month of culture, segments of callus were transferred to liquid media containing the same concentrations of paclobutrazol and the media was collected for SDS-PAGE after 48 h. Alpha-amylase expression was found to be similar at all levels of paclobutrazol and comparable to the high level expression in untreated callus (Fig. 3). The untreated callus actually had somewhat less α-amylase than the paclobutrazol treatments, but the variation was nonetheless within a two-fold variation and was not considered highly significant. This experiment suggests that *de novo* synthesis of gibberellins is not required for the α-amylase expression by the callus. As a control, M202 seeds treated with these levels of paclobutrazol showed up to a 50% reduction in α-amylase activity

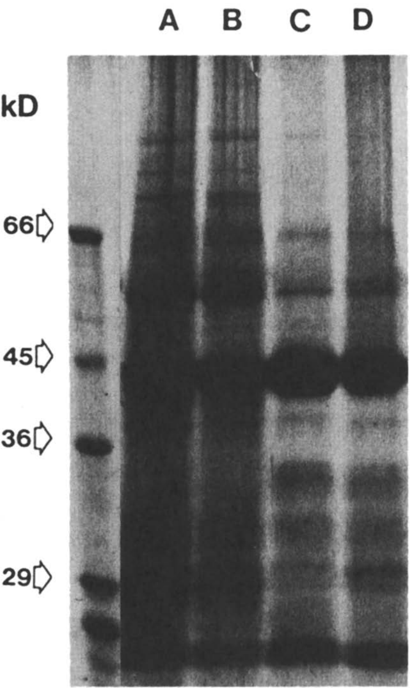

Figure 1. Crude extract of proteins from M202 seed callus. 12.5% SDS-PAGE stained with Coomassie blue. Lanes A and B contain proteins from callus homogenates treated without and with 2.5 µM GA_3. Lanes C and D contain proteins secreted from callus treated without and with 2.5 µM GA3. Alpha-amylase is the predominant protein with M_r 43-44 kDa.

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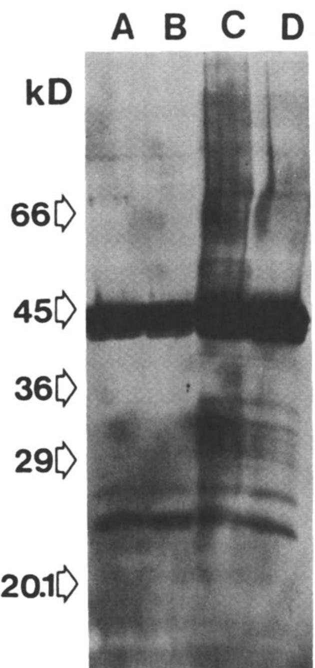

Figure 2. Western blot of samples in Figure 1. The bands at Mr 43-44 kDa cross-reacted strongly with α-amylase antiserum, indicating the presence of intra- and extracellular α-amylase.

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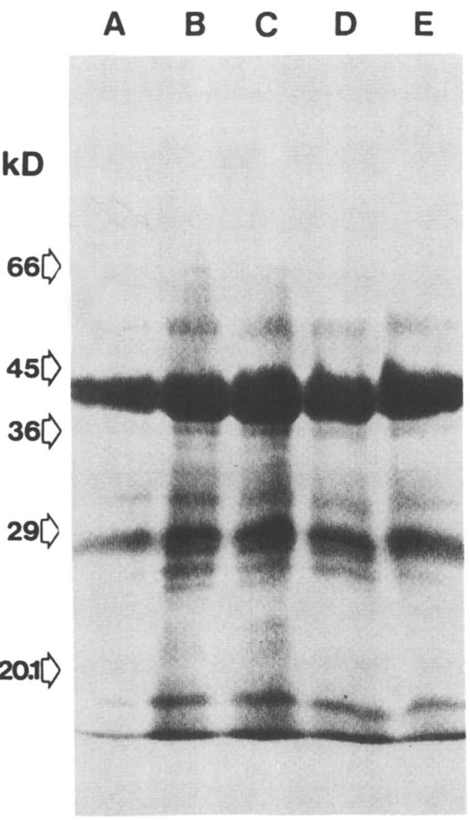

Figure 3. Total proteins secreted by callus which was treated with paclobutrazol. 12.5% SDS-PAGE stained with Coomassie blue. Lane A, 0.0 mg/L; Lane B, 0.5 mg/L; Lane C, 5.0 mg/L: Lane D, 20 mg/L; and Lane E, 50 mg/L paclobutrazol. Alpha-amylase is the predominant band at 44 kDa.

in whole seed extracts and a three-fold reduction in shoot height, indicating that the paclobutazol is biologically active. When 2.5 μM GA_3 is added in conjunction with paclobutrazol to M202 seedlings, α-amylase activity and shoot height were similar to untreated M202 seedlings.

To investigate the possiblility that α-amylase expression in seed-derived callus is independent of gibberellins, callus tissue was initiated and propagated from Tan-ginbozu seeds, a rice dwarf mutant which lacks gibberellins (31). Alpha-amylase expression, however, was found to be significantly lower than M202 seed callus, which, in contrast to the results of the paclobutrazol experiment, suggests that gibberellins greatly enhance α-amylase expression in callus (Fig. 4 and Table I). About 8% of the total protein secreted from Tan-ginbozu callus was α-amylase, corresponding to 0.075 mg/g dry weight callus. This represents a 41-fold decrease in α-amylase protein, and a 13-fold decrease in α-amylase activity, relative to that produced by M202 seed callus. The differences in α-amylase expression between M202 and Tan-ginbozu callus is mirrored in differences in germination rate and α-amylase activity of control seedlings. M202 showed a five-fold greater level of α-amylase activity per seed and a 4.5-fold greater shoot length than Tan-ginbozu. (The starch in the seed homogenates binds α–amylase and has a levelling effect on the differences between the two rice varieties not encountered when measuring α–amylase in the callus media and so the difference between the varieties may be underestimated).

Tan-ginbozu callus was treated with 2.5 μM GA_3 for five days, but the Tan-ginbozu callus did not respond as expected by increasing α-amylase expression. As a control, Tan-ginbozu seeds were germinated for five days with or without 2.5 μM GA_3. When treated with GA_3, seedling homogenates had twice the α-amylase activity and shoots were twice the height relative to untreated seedlings.

Table I. Comparison of α-Amylase Expression in M202 Seed, M202 Shoot, and Tan-ginbozu Seed Callus

	M202 Seed callus	M202 Shoot callus	Tan-ginbozu Seed callus
Micrograms α-amylase / g dry wt callus	3000	3300	74
Units α-amylase activity / g dry wt callus	11,600	19,700	930
α-Amylase as % of total secreted protein	43	41	8

Enzyme assays and immunodetection methods have been used to show that young etiolated rice shoots express moderate amounts of α-amylase protein and activity which is intracellular, not secreted. Shoot tissue yielded 560 units α-amylase/g dry weight tissue. To determine whether this level of expression persists in the callus state, α-amylase expression in M202 seed and shoot callus was compared. Interestingly, the shoot callus was also found to secrete high levels of α-amylase (19,600 units/g dry weight callus), comparable to those levels expressed by seed callus (Fig. 4 and Table I). Approximately 41% of the total secreted protein (corresponding to 3.3 mg/g dry weight) was found to be α-amylase. Thus, the total amount of α-amylase protein secreted by seed and shoot callus is similar, with the shoot callus α-amylase having a somewhat higher amylolytic activity. Five-day

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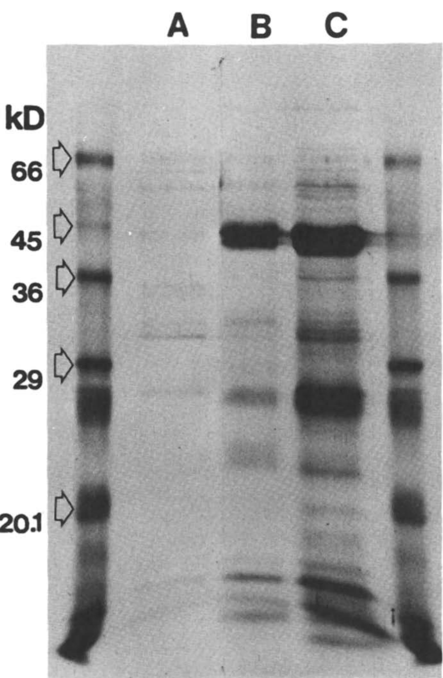

Figure 4. Total proteins secreted by callus from Tan-ginbozu rice seeds (Lane A), M202 rice seeds (Lane B), and M202 shoot (Lane C). 12.5% SDS-PAGE stained with Coomassie blue. Protein content reflects total protein secreted per g dry weight callus.

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germinated M202 rice seeds yield at most six isozymes or isoforms of α-amylase on two-dimensional western blots. In the M202 seed callus there are about 10 such forms. M202 shoot callus produces a similar, though slightly more complex, distribution of 12 such forms (data not shown). Clearly, the normal low-level intracellular expression of α-amylase in shoot has been converted once the tissue became callus. Rice anther callus (courtesy Shannon Pinson) was tested and also observed to produce high levels of secreted α-amylase. Evidently the high level expression of α-amylase in rice callus is independent of the tissue source. Nonembryogenic callus exhibited the same high level expression of α-amylase as did embryogenic and organogenic callus, indicating that organized tissues within the callus are not responsible for the α-amylase expression.

Discussion

We have observed that rice seed callus secretes high levels of α-amylase for long periods of time. This expression is not affected by exogenous GA_3 or the inhibition of gibberellin biosynthesis by paclobutrazol. A role for gibberellins is nevertheless implicated since the gibberellin-deficient dwarf Tan-ginbozu has a much reduced expression of α-amylase in seed callus. The α-amylase expression in callus is not dependent upon tissue source because seed, shoot, anther, nonembryogenic, and embryogenic callus all secrete high levels of α-amylase.

This high level production of α-amylase by callus is not restricted to the M202 variety, since we observe similar expression in three other rice varieties, there is a report of expression in a cell suspension culture of a japonica variety (Akazawa, T, Nagoya University, personal communication, 1987), and Saka and Maeda (32) also report expression in callus homogenates of several other varieties. Since many rice varieties, as well as ryegrass (*Lolium multiflorum*), all secrete high levels of α-amylase, the callus production of α-amylase appears to be a general phenomenon of rice callus and perhaps other cereal callus.

M202 is not isogenic with Tan-ginbozu, so the magnitude of the differences in α-amylase expression between them may not solely be an indication of the effect of gibberellins. Saka and Maeda (32) reported that Ginbozu, the progenitor to Tan-ginbozu, produced three-fold more α-amylase activity in callus homogenates than Tan-ginbozu, indicating that there is a definite reduction of α-amylase expression in Tan-ginbozu callus which is indeed attributable to the absence of gibberellins and not to varietal differences. The level of secretion of α-amylase between Ginbozu and Tan-ginbozu has not yet been compared.

The two-dimensional western blot analysis shows numerous isozymes or isoforms in both M202 seed and shoot callus, several of which don't appear in the germinating seed. Evidently in the callus there is a derepression or stimulation of many, if not most, of the α-amylase genes. Some of the spots on the western blot may be due to charge modifications of some isozymes, and therefore do not represent products of separate genes.

The paclobutrazol and Tan-ginbozu experiments are in apparent contradiction. The paclobutrazol experiment suggests that gibberellins are not responsible for callus α-amylase expression, whereas the Tan-ginbozu experiment suggests that they are involved. The experiments do differ, however, in that the paclobutrazol experiment only rules out *de novo* synthesized gibberellins as being important, whereas the Tan-ginbozu experiment rules out both *de novo* synthesis and gibberellins which may have been in the mature seed. Perhaps gibberellins in the mature seed trigger the high level of α-amylase expression as the seed tissue becomes callus, and thereafter the expression is maintained without gibberellins. The preliminary results of an experiment with scarified Tan-ginbozu seeds germinated in the presence of GA_3, but during callus growth without GA_3, indicates that this hypothesis is incorrect. Callus

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is not responsive to exogenous GA_3 in either M202 or Tan-ginbozu. There may be a different GA_3 concentration or period of exposure which will reveal GA_3-responsiveness; however, the controls indicate that there is an expected enhancement of α-amylase activity by GA_3. Since the controls utilize whole seeds, they may be demonstrating mostly the aleurone response to GA_3. The scutellum may respond at a different GA_3 concentration or respond less vigorously than the aleurone. The Tan-ginbozu callus experiment nonetheless remains an indication that gibberellins are somehow involved in the callus α-amylase regulation, but the mechanism is aberrant and not yet defined.

The observation that seed, shoot, and anther callus all produce and secrete high levels of α-amylase raises intriguing questions about the tissue-specific control of α-amylase in rice. For example, callus is often thought to consist of a mass of undifferentiated cells exhibiting little if any tissue-specific gene expression. Any tissue-specific identity which may have existed in the precallus state is eventually lost during callus growth and subpropagation (33). Our findings indicate that seed callus seemingly retains the ability to express α-amylase in a mode normally restricted to the scutellum and aleurone layer of the germinating seed. Shoot and anther callus, on the other hand, lose their low-level intracellular expression and adopt the high level secreted mode of expression. Seed and shoot callus, however, are not identical in the production of all other proteins (Fig. 3), indicating that perhaps some level of tissue-specific identity remains imprinted on the callus. Callus expression of α-amylase may only superficially resemble seed-like expression. Perhaps it would be more apt to speak of a callus-specific α-amylase expression than of a tissue-specific expression carried over into the callus state.

High level expression of α-amylase by callus may be a type of stress response, or perhaps an artefact due to the 2,4-D. Because 2,4-D is the only factor in the media which triggers callus formation, 2,4-D is likely accountable in some way for the high level α-amylase expression. The auxin indole-3-acetic acid has been reported to influence the GA_3-responsiveness of dwarf wheat aleurone layers apparently by alterations in the composition of the membrane lipid bilayer (34). In a related way 2,4-D may be disrupting the normal gibberellin responsiveness and α-amylase expression of the tissue. There is a report of a 50-fold gene amplification that occurs when rice becomes callus and then reverts to the normal copy number when the rice regenerates (35). The possibility of such an amplificaton for α-amylase will be investigated using southern blots.

The purpose of this work was to develop a callus or cell suspension culture system suitable for studying the tissue-specific and hormonal regulation of α-amylase. Although for hormonal regulation, the aleurone is most desirable since it is the best characterized tissue to respond to gibberellic acid, aleurone cells are not amenable to cell culture since they rapidly vacuolate and die. Scutellum-derived callus, on the other hand, grows vigorously in culture, and so it was exploited, even though α-amylase regulation in the scutellum is less well characterized. The callus system has advantages over the scutella or aleurone systems in that (a) it can be propagated for indefinite periods of time, (b) it can be easily reduced to protoplasts which can be used for transient expression of transformed gene constructs, and (c) it produces large amounts of the enzyme which can be purified without the need for laborious dissections or extensive manipulations to remove whole seed debris. For these reasons we anticipate that this callus system, although apparently somewhat abnormal, will nonetheless be useful for studying the α-amylase regulatory system.

Perhaps the greatest potential application of this work lies in the development of genetically engineered plant calli or cell suspension cultures which produce proteins of commercial importance. The rice α-amylase gene promoters, now being characterized in our laboratory, could be linked to coding regions for a protein of interest, such as an industrial enzyme or therapeutic agent, and introduced into the rice genome by, for

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example, electroporation of protoplasts. Calli or cell suspension cultures expressing and secreting the protein could be derived from the transformed cells. The rice callus system satisfies two important criteria for commercial success, namely sustained and high-level expression. The callus system, however, is of additional interest because it has advantages over other expression systems, such as bacteria, particularly *E.coli*, because it secretes the protein, thus facilitating protein purification. As a eukaryotic system, plant callus is also capable of glycosylation, which may be necessary for protein stability and function.

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Chapter 15

Structure–Function Properties of the Sperm Enzyme Acrosin

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The sperm enzyme acrosin functions in sperm binding to, and penetration of, the egg zona pellucida. Acrosin is a three domain glycoenzyme, possessing a serine active site protease, a hydrophobic binding domain, and a recently described carbohydrate (fucose) binding domain; it is the first described lectin-protease. Its functional properties, including activation of a proenzyme as well as subsequent processing and separation of its three functional domains, are controlled via limited proteolysis (autolysis). Structure-function models for acrosin are presented. Functional membrane-bound forms of the lectin-protease are proposed which both hydrolyze and bind carbohydrate or just bind carbohydrate. Acrosin's properties provide for sperm binding and progressive penetration of the zona pellucida, essential cellular steps in the fertilization process.

All animal eggs are surrounded by egg envelopes which must be traversed by sperm on their journey to the egg surface. These egg envelopes are known by various terms in different organisms, being called the vitelline layer in sea urchins, the vitelline envelope in amphibians, and the zona pellucida in mammals. The fertilizing sperm must penetrate the egg envelope in order to fuse with the plasma membrane and transfer its haploid set of chromosomes into the cell cytoplasm.

Involvement of sperm proteolytic enzymes in fertilization processes has a long history. Perhaps the first definitive observation that sperm proteases affected egg envelopes was that of Yamane in 1935. He demonstrated that an extract of rabbit sperm dispersed the cumulus cells and solubilized the zona pellucida (ZP) or egg envelope of the rabbit egg (1). The presence of proteases in the sperm extract was presumed by analogy with the dispersing action of the trypsin activity in pancreatin. In 1939, Tyler obtained an extract from sperm of the giant keyhole limpet *Megathura crenulata* which dissolved the egg envelope without affecting the egg itself. He coined the term "egg membrane lysin", which has since been

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shortened to "lysin", to describe those enzymes and factors in sperm which assist the sperm in penetrating the egg envelopes. The observations from Dan's laboratory in 1956 associated sperm lysin with the acrosome in Mytilus edulis sperm and its release or exposure during the sperm acrosome reaction. After the description of the acrosome reaction in mammalian sperm by Austin's laboratory in the late 1950's, studies on acrosomal proteases from mammalian sperm were initiated by several groups, first perhaps in Hartree's laboratory in Cambridge (for review see 2), but also by Stambaugh's lab in Philadelphia and Williams' group in Georgia. After intensive debate during the late 60's, the name acrosin was assigned to the trypsin-like activity of the enzyme associated with the acrosome in mammalian sperm. The term lysin remains a generic term referring to the enzyme or factors which solubilize or specifically hydrolyze components of the egg envelopes, whereas acrosin is a specific or varietal name for one particular enzyme which proteolytically hydrolyzes selected glycoproteins of the ZP. Non-enzymatic lysins, discovered by Haino-Fukishima, bind stoichiometrically to envelope components thereby effecting their solubilization (for review see 3). However, this mechanism for sperm penetration of the egg envelope seems to be limited to marine mollusks.

Contemporary studies on the ZP lysin acrosin were perhaps initiated with the observation that synthetic protease substrates were hydrolyzed by sperm extracts (4) and that the enzyme could be effectively extracted from sperm at pH 3 (5). The enzyme has now been purified from several species, shown to be specific for Arg/Lys peptide bonds, and to be a serine active site endoprotease. In 1972, Meizel provided evidence that a zymogen form of acrosin existed, termed proacrosin (6).

Sperm hydrolases have often been suggested as being sperm lysins based largely on circumstantial evidence, such as egg envelope dissolution by heterogeneous sperm enzyme extracts. Envelope dissolution by itself is an insufficient criterion for a particular enzyme or complex of enzymes functioning as sperm lysins. Criteria which must be met by sperm enzymes to qualify as sperm lysins were suggested by Hoshi (3). We have modified the criteria in view of recent observations by a number of investigators. Criteria which must be met to establish that a sperm enzyme is a lysin are: a) The active enzyme must be spacially and temporally located on the sperm so that it is in the right place at the right time to function in gamete interaction. b) The enzyme must selectively hydrolyze peptide bonds present in the egg envelope glycoproteins. c) Inhibition of the activity of the enzyme on the sperm surface must inhibit egg envelope penetration. d) Appropriate chemical or immunological modification of the egg envelope glycoprotein, which is the substrate for the sperm lysin, will prevent penetration of the egg envelope.

No sperm enzymes have yet been described which meet all of these criteria. However, acrosin satisfactorily meets the first three criteria and, with the identification of the zona pellucida glycoprotein which is a substrate for acrosin, it is now possible to approach experimentally the fourth criterion (7).

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THE STRUCTURAL PROPERTIES OF ACROSIN

A variety of protocols have been used to purify acrosin from sperm of different species. Two approaches are commonly used: a) purification of the zymogen form, proacrosin, from sperm extracts followed by autoactivation of the purified zymogen and subsequent isolation of the active enzyme, and b) purification of the active enzyme from sperm extracts in which proacrosin activation has already occurred. Properties of proacrosin and acrosin which have been exploited for purposes of purification include: a) resistance to irreversible denaturation by acidic pH or by chaotropes, b) hydrophobicity, c) glycosylation, d) high pI, and e) chemical specificity of the enzyme's substrate binding site. Since neither proacrosin auto-activation nor proteolysis by acrosin occurs at acidic pH, nearly all acrosin and proacrosin purification methods use chromatography at a pH less than 4.5 for one or more steps (8-13), thereby avoiding premature autoactivation of proacrosin or loss of acrosin activity due to autolysis. Chaotropes such as urea and guanidine-HCl have been used to disrupt molecular interactions during chromatography (10,13). Hydrophobic interaction chromatography (11) and lectin affinity chromatography (8) are effective for acrosin and proacrosin purification, respectively. Due to their high pIs, acrosin and proacrosin elute at relatively high salt concentrations in cation exchange chromatography, enabling purification at most pH values using this method (8,11,13). Finally, chromatography on affinity columns conjugated with the inhibitory ligand p-aminobenzamidine is an effective purification step (11,12).

Proacrosin purified from rabbit testes is 68K Mr by SDS-PAGE (8), while that purified from porcine (14) or ovine (19) spermatozoa is 55K Mr. Guinea pig testicular proacrosin is 62K Mr, and two guinea pig sperm proacrosins are 55K Mr and 43K Mr (13). On the basis of these observations, Hardy et al. (13) proposed that testicular proacrosin is processed, presumably proteolytically, to form the lower molecular weight proacrosins found in spermatozoa. Direct evidence of such processing has not yet been reported.

During autoactivation of purified proacrosin, multiple acrosins differing in molecular weight are formed sequentially, the largest acrosin being formed first, with the smaller acrosins being formed by subsequent autolysis. Acrosins generated by autoactivation of purified 55K Mr porcine sperm proacrosin are 49K Mr (designated m_α-acrosin), 38K Mr (designated m_β-acrosin), and 25K Mr (designated m_γ-acrosin) by SDS-PAGE (14). m_α-Acrosin is present only transiently, being quickly degraded to m_β-acrosin, which is relatively more stable (14). m_γ-Acrosin is formed only after prolonged incubation at basic pH (14). Acrosins purified directly from extracts of porcine or caprine spermatozoa are 38K Mr (11) and 42K Mr (15), respectively, by SDS-PAGE. The structural relationship between acrosin generated from impure proacrosin and that generated from purified proacrosin has not been determined. Thus, it is unknown whether acrosins purified directly from sperm extracts are identical with acrosins generated by autoactivation of purified proacrosin.

Proacrosin and acrosin are glycosylated. Rabbit proacrosin and porcine acrosin are 8% carbohydrate by weight (8,16). Porcine acrosin has an N-linked oligosaccharide at Asn3 (17). Data for

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caprine acrosin are consistent with the results for the porcine enzyme (15). The amino acid composition of various acrosins indicate a relatively high proline content (18,19). Interestingly, when one compares the compositions of m_α- and m_β-acrosins, the peptide portion removed is remarkably high in proline content: 31 of the fragment's 85 residues are proline (18). Since the limited amino acid sequence data available indicate that all of the N-terminal amino acids present in 55K Mr proacrosin are also present in 42K Mr acrosin (discussed below), it is likely that the portion lost on conversion of m_α- to m_β-acrosin is at the C-terminal end. It is not yet known whether this unusual fragment is removed intact or in pieces.

Acrosin is inhibited by the serine active site reagents diisopropylfluorophosphate, phenylmethylsulfonylfluoride, and p-nitrophenyl-p'-guanidinobenzoate (9,12,15). Acrosin is also inhibited by tosyllysylchloromethylketone and tosylarginylchloromethylketone but not by tosylphenylalanylchloromethylketone (9,12,15), indicating specificity for hydrolysis on the carboxyl side of lysine and arginine, and providing evidence for an active site histidine. The catalytic triad of serine protease active site amino acid residues is present (22).

Initial attempts to determine the N-terminal amino acid sequence of porcine acrosin were complicated by the presence of a secondary sequence that was removable after treatment with reducing agent in the presence of 6 M guanidine-HCL (20). Further study showed that the enzyme is comprised of two polypeptide chains linked by disulfide bonds (17). A similar two-chain structure was subsequently demonstrated for caprine acrosin (15). The two chains, designated heavy and light, are 37K Mr and 4200 Mr, respectively, for porcine acrosin (17), and 38K Mr and 3700 Mr, respectively, for caprine acrosin (15). The similarity between the amino acid sequences of porcine acrosin light chain and the activation peptides of other serine proteases led Fock-Nuzel et al. (17) to propose that the mechanism of proacrosin activation is limited hydrolysis of the single proacrosin polypeptide chain to produce the two-chain active enzyme. Subsequent sequencing of porcine proacrosin showed that its N-terminal sequence is identical to that of porcine acrosin light chain, and the N-terminal sequence of the heavy chain is encountered in the proacrosin sequence immediately after the C-terminal arginine of the light chain (A. Henschen, personal communication), thus providing further support for the proposed activation mechanism. During initial autoactivation of guinea pig proacrosins, a 5000 $\pm$ 1000 Mr fragment (light chain) is generated which remains bound to the remainder of the acrosin molecule (heavy chain) by disulfide bonds, regardless of whether the proacrosin is the 62K Mr size variant isolated from testis or the 55K Mr or 43K Mr size variants isolated from spermatozoa (15). These latter results are presently the only direct evidence for a proacrosin activation mechanism involving limited proteolysis of a single polypeptide chain to produce a two-chain acrosin.

The sequences of porcine and caprine acrosin light chains and the N-terminal sequences of the acrosin heavy chains are shown aligned with the sequences of several serine proteases in Figure 1. Sequence similarity between porcine and caprine acrosins is readily apparent, as is the similarity of the acrosin sequences (range of

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	1–10	11–20	21–	Positional identity	Reference
Acrosin light chain (po)	RDNATCDGPC	GLRFRQKLES	GM-R		17
Acrosin light chain (ca)	RDNTTCDGPC	GIRFRQNR		78%	15
Chymotrypsinogen (bo)	C	GVPAIQPVLS	GLSR	40%	25
Hepsin (hu)	RFLAAICQDC	GRRKLP----	-VDR	26%	24
Plasminogen activator (hu)	YCDVPSCSTC	GLRQYSQPQF	---R	22%	26
Plasminogen (hu)	PQCAAPSFDC	GKPQMEPKKC	PG-R	17%	68
Trypsinogen (bo)		VD	DDDK	0%	27

	1–10	11–20	21–30	31–40	41–50	51–	Positional identity	Reference
Acrosin heavy chain (po)	VVGGMSAEPG	AWPWMVSLQI	PMYHNNRRYH	TCGGILLNSH	WVLTAAHCFK	NK		20
Acrosin heavy chain (ca)	IIGGQDAAHG	SWPWMVSLQI	FTYHNNRRYH	VCGGSLL			70%	15
Plasmin (hu)	VVGGCVAHPH	SWPWQVSLRT	RFG-----MH	FCGGTLISPE	WVLTAAHCL-	--	48%	68
Hepsin (hu)	IVGGRDTSLG	RWPWQVSL--	----RYDGAH	LCGGSLLSGD	WVLTAAHCF-	--	48%	24
Chymotrypsin (bo)	IVNGEEAVPG	SWPWQVSLWD	KTG-----FH	FCGGSLINEN	WVVTAAHCG-	--	46%	25
Elastase (po)	VVGGTEAQRN	SWPSQISLQY	RSG--SSWAH	TCGGTLIRQN	WVMTAAHCVD	--	44%	29
Trypsin (*S. griseus*)	VVGGTRAAQG	EFPFMVRL--	SMG-------	-CGGALYAQD	IVLTAAHCVS	--	42%	28
Trypsin (bo)	IVGGYTCGAN	TVPYQVSLN-	-SG-----YH	FCGGSLINSQ	WVVSAAHCY-	--	40%	27
Prothrombin (bo)	IVEGQDAEVG	LSPWQVMLF-	--RKSPQE-L	LCGASLISDR	WVLTAAHCLL	YP	38%	30
Cathepsin G (hu)	IIGGRESRPH	SRPYMAYLQI	---QSPAGQS	RCGGFLVRED	FVLTAAHCW-	--	35%	32
Plasminogen activator (hu)	IKGGLFADIA	SHPWQAAIFA	KHRRSPGERF	LCGGILISSC	WILSAAHCF-	--	35%	26
Clr b-chain (hu)	IIGGQKAKMG	NFPWQVFT--	------NIHG	RGGGALLGDR	WILTAAHTL-	YP	33%	31
Conserved	IVGG A G	S PWQVSL	SG H	FCGG LI	WVLTAAHC		77%	33
Invariable	G	P		CG	V AAHC		100%	33

Figure 1. Sequences of acrosin light chains and N-terminal sequences of acrosin heavy chains shown aligned with the sequences of corresponding portions of other serine proteases. Positions identical with the porcine acrosin sequence are underlined. Alignments for plasmin, prothrombin, Clr b-chain, cathepsin G, and plasminogen activator are from (24), except minor adjustments were made to maximize similarity with acrosin. Sequence similarities expressed as percent positional identity to the porcine sequences are indicated to the right of each sequence. Abbreviations: po = porcine, ca = caprine, bo = bovine, hu = human.

positional identities, 33% to 48%) with those of the other serine proteases shown. A majority (77%) of the amino acids that are conserved in serine proteases, and all of those that are invariable, are also conserved in the partial sequences of acrosin available (20,22); thus, acrosin is homologous (21) with the trypsin family of serine proteases. Similar comparisons using a longer acrosin sequence (124 residues) produced similar positional identities, leading Fock-Nutzel (22) to the remarkable proposal that acrosin diverged from the other serine proteases after Streptomyces griseus trypsin but before pancreatic trypsin and thrombin during the evolution of the serine proteases. Using the limited sequence comparisons shown in Figure 1 and a divergence time for the porcine and caprine lineages of 50 million years ago, Hardy et al. (15) calculated a unit evolutionary period (time required for a 1% change in amino acid sequence) of 1.8 million years for acrosin. This sequence divergence rate is more than three times as rapid as that of trypsin, and similar to those of enzymes such as carbonic anhydrase, ribonuclease, and lactalbumin (23). The portions of the acrosin sequence compared for this calculation are homologous with the other serine proteases. Since the C-terminal portion of m_α-acrosin, and therefore proacrosin necessarily, has an unusually high proline content, it seems unlikely that this region of the acrosin sequence is homologous to the other serine proteases. It will be very interesting to compare the amino acid sequence divergence rate of this portion of the acrosin molecule, as it might be expected to be involved in functions unique to acrosin.

THE FUNCTIONAL DOMAINS OF ACROSIN

PROTEOLYTIC ACTIVITY. In comparison with pancreatic trypsin, acrosin is 50% larger, contains carbohydrate, and is more hydrophobic (11). Although acrosin has often been referred to as trypsin-like, the above structural differences are reflected in kinetic and hydrolytic specificity differences, in different interactions with membranes, and in the newly described lectin function of acrosin.

Substrate specificity differences between boar acrosin and trypsin are not particularly manifest when using small substrates, but these enzymes show distinctly different kinetics of porcine ZP hydrolysis (34). The loss of 30% mass in the conversion from m_α- to m_β-acrosin has little effect on the kinetic analyses of inhibition and substrate preference with artificial substrates and small trypsin inhibitors, indicating that this excised portion of the enzyme contributes little to the topography of the active site (35). From Km_{app} analyses with amide and ester substrates of Arg and Lys, acrosin prefers the Arg substrates over Lys, and Km_{app} differences between amide and ester substrates indicates that acrosin proceeds kinetically through a classical double displacement mechanism as does trypsin (36).

Acrosin causes the limited and specific cleavage of only certain portions of the ZP (7,37,38). The ZP is composed of three families of glycoproteins, denoted 90K, $55K_\alpha$ and $55K_\beta$, and two derived components, 65K and 25K (39). The 65K and 25K components are produced from the 90K family by a proteolytic event in the follicle. The 90K family, and the 90K and 65K components, were specifically

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hydrolyzed by acrosin to small polypeptides not retained in SDS-PAGE (40). The 55K families were not affected by acrosin in the particulate or solid forms of the ZP. The ZP remained visually intact even after extended acrosin hydrolysis reduced the 90K family to small polypeptides (40), and the interacting hydrolyzed peptides could only be liberated with mercaptoethanol and denaturants.

ZONA PELLUCIDA BINDING. Several molecular mechanisms have been proposed for sperm-egg binding. Sperm binding to the ZP has been suggested to involve carbohydrate-protein interaction, protein-protein interaction, or both (41). Inhibition of mouse sperm binding to mouse eggs was observed with trypsin inhibitors (42,43), and anti-acrosin antibodies inhibited sperm binding in the rabbit (44). Two molecules that have trypsin binding specificity were observed on mouse sperm: a molecule that turned over active-site titrant inhibitors of trypsin, but that was not itself catalytically active (46), and a molecule that bound seminal plasma inhibitor (45). Catalytically active acrosin bound ZP preferentially over a control protein, as would be expected from a normal enzyme-substrate relationship (34). A possible explanation of the above data is that the proteolytic substrate binding site of acrosin, or a portion of the molecule that retains binding activity after autolysis, resides on the sperm surface and is involved in binding ZP.

When all acrosins (including small autolytic forms of 12-18K Mr) were Western blotted, all of the forms bound ^{125}I-ZP even when the enzyme was inhibited with DFP (47-49). This implied a second binding site on acrosin for ZP. Recently, a fucose binding protein with a molecular weight corresponding to boar proacrosin, 53K, and having the same N-terminal sequence as acrosin (50), was described on the surface of acrosome intact boar sperm (51). The carbohydrates that bound acrosin inhibited sperm binding (52), and these carbohydrates also inhibited the amidase activity of acrosin (53). In addition to acrosin, low molecular weight polypeptides involved in the species-specific binding of rabbit sperm to rabbit ZP were described as fucose binding lectins (54). These data are consistent with acrosin binding a fucose containing ligand on the ZP, and also suggest that the binding site for fucose resides in the small Mr forms of acrosin.

The inhibition of acrosin by polysulfated polysaccharides is interesting since different acrosins are inhibited differentially. The m_α- and subsequent hydrolytic products of m_β-acrosin are not as susceptible to inhibition with these carbohydrates as is m_β-acrosin (53). These data provide evidence for a model for the penetration of ZP by sperm by a sequential binding, hydrolysis, and subsequent rebinding that would be necessary for sperm penetration of the ZP.

HYDROPHOBIC BINDING. Acrosin is an unusual protease in that it has high affinity for lipids and detergents, and its amidolytic activity is modified by these agents. Boar acrosin aggregates with itself and with other proteins in the absence of detergents (10,55). Its hydrophobic nature (11) and lipophilic properties led to its description as an extrinsic membrane protein. Non-ionic detergents stimulated acrosin's amidase activity at critical micelle concentrations of those detergents (56), but the enzyme did not bind to a micelle of Triton X-100 (56).

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m_{β}-Acrosin has been reported to be inhibited by phospholipid vesicles (56), whereas anionic phospholipid dispersions stimulated the autolysis of proacrosin (57). With detergent couple electrophoresis (58), where amphiphilic proteins bind charged detergent-couples resulting in a mobility shift and hydrophilic proteins are unaffected, proacrosin, m_{α}- and m_{β}-acrosin, peptides released from acrosin autolysis, and a 28K binding protein, behaved as amphiphilic or intrinsic membrane proteins (Urch, unpublished observations).

Since the 28K-proacrosin interaction required either 8 M urea or 6 M guanidine HCl to disrupt it (10), this protein-protein interaction may act to anchor proacrosin to the acrosomal membranes or to other proacrosin molecules. While acrosin does not fit all the criteria for an intrinsic membrane protein, acrosin was associated with and extracted from preparations of sperm membrane vesicles with 1 mM HCl and Triton X-100 (59, Urch, unpublished observations). Salt or combinations of salt and detergent were ineffective. The interaction of acrosin with membranes, particularly sperm acrosomal membranes, is obviously important in ZP binding and penetration. Thus, while the exact definition of acrosin's interaction with membranes remains to be explained, it is apparent that acrosin hydrophobicity is important to its function in fertilization.

THE CELLULAR FUNCTION OF ACROSIN AS A SPERM LYSIN. Acrosin fulfills most of the above listed criteria for a sperm lysin. It has been localized onto both the inner and outer acrosomal membranes by histochemical and immunochemical localization procedures (60,61), and is at the correct place to function in fertilization. Complete ZP dissolution by homologous acrosins, the second lysin criterion, is not observed with all species. With boar acrosin, dissolution of the porcine ZP was never visually observed, as was true with sheep ZP and ram acrosin (62). To visualize the effect of acrosin on the ZP, electrophoretic or other separation procedures that employed denaturants and disulfide bond reduction were required (7,37,38). Complete dissolution of the ZP does not occur in normal fertilization, since the ZP is needed for the continued development of the embryo.

The third criterion of the inhibition of fertilization by active site inhibitors was observed both *in vitro* (63) and *in vivo* (64) fertilization. However, this inhibition was never total, and once the sperm had tightly bound the ZP, trypsin active site inhibitors did not prevent further penetration or hydrolysis of the ZP (65). Therefore, while acrosin does not strictly follow all of the criteria for a ZP lysin, it does cause limited hydrolysis of the ZP and it is in the correct place to act in penetration of the ZP. While acrosin has been localized to the acrosome, there are conflicting reports as to exactly where in the acrosomal compartment acrosin is localized before the acrosome reaction and the timing of its release in the fertilization process. From the description of acrosin as a ZP binding lectin, acrosin must be available to bind the ZP, and this would place the molecule on the surface of the intact sperm, or in some species, of the acrosome-reacted sperm. In the mouse, only acrosome intact sperm bind the ZP (66). Once the sperm has undergone the acrosome reaction and the outer membranes (limiting

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plasma membrane and outer acrosomal membrane) have vesiculated, the inner acrosomal membrane becomes the limiting surface of the sperm and of importance in gamete interactions. In the hamster, acrosome reacted sperm bind to the ZP, and the fertilizing sperm appears to remain bound to the ZP by a remnant of the vesiculated acrosomal membrane or acrosomal ghost (67). With boar sperm, acrosin has been localized to the acrosomal surface of live sperm with immunofluorescent techniques (Urch, unpublished observations). From these observations, acrosin is in the correct place to interact with the ZP.

STRUCTURE-FUNCTION MODELS OF ACROSIN

Any attempt at proposing a model for acrosin's structure-function relations must necessarily generate a working hypothesis, due to insufficient data and the multiple and dynamic molecular forms of acrosin that exist. Acrosin is much like the Greek god of dreams, Phantasos, in that it changes its forms via autolysis so quickly and readily that it seems almost phantom-like. This polymorphism, and presumably the multiple pathways leading to these multiple forms, provides a great experimental challenge to those of us who wish to understand the structure-function relations of acrosin.

PROCESSING OF ACROSIN'S FUNCTIONAL DOMAINS. In Figure 2, the processing via autolysis of acrosin is illustrated beginning with proacrosin. Figure 2 is highly schematic and does not include all of the steps in acrosin processing or all of its molecular forms. The purpose of the scheme is to provide a reasonable suggestion as to the relation of acrosin's functional domains and how these domains might be hydrolytically processed to generate molecules which have different catalytic and binding properties. The single glycosylated polypeptide chain proacrosin is converted from an inactive form to an active form by limited proteolysis in the N-terminal region of the polypeptide chain. In the case of boar sperm acrosin, this processing occurs between Arg22 and Val23 thereby producing a two-chained molecule. The light (L) and heavy (H) chains are crosslinked via disulfide bonds. Additional processing occurs in the C-terminal portion of the H chain eventually producing an enzymatically active m_{β}-acrosin. These two proacrosin processing events probably occur by more than one pathway and produce several intermediate forms. The proteolytically functional molecule is additionally processed such that the three functional domains of the enzyme are separated. Two pathways are suggested, one which separates the carbohydrate binding domain from the hydrophobic and proteolytic domains while the other pathway separates the proteolytic domain from the hydrophobic and carbohydrate binding domains. Thus, membrane bound acrosin forms are produced which can hydrolyze peptide bonds or bind to carbohydrate residues (m_{γ} - forms). Further processing separates all domains from each other producing a mixture of molecules with Mr's of 12-18K which individually possess hydrophobic binding and carbohydrate binding domains and a non-functional proteolytic domain. Presumably, completely processed acrosin molecules (12-18K) with separated domains would no longer be functionally important in the sperm binding/penetration process.

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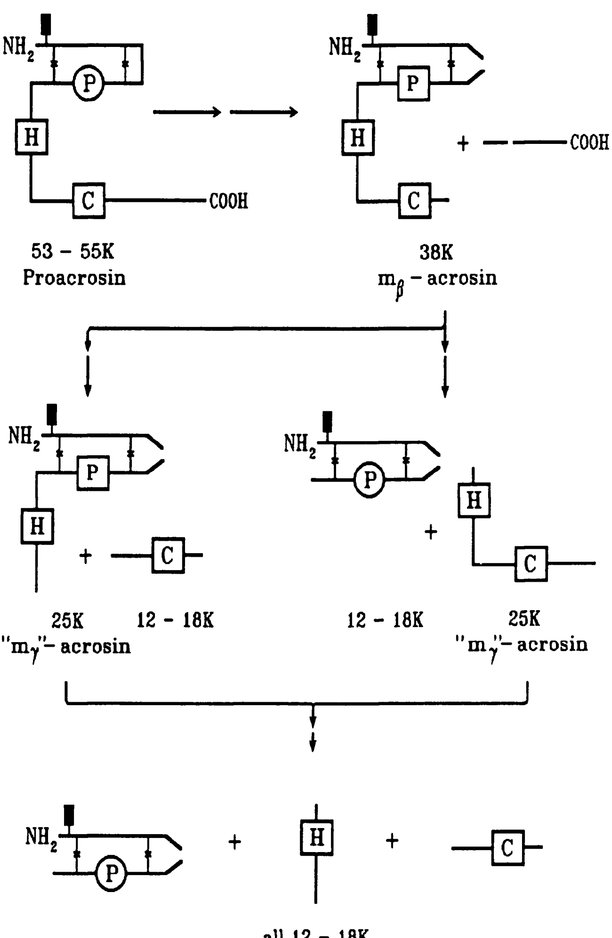

Figure 2. Processing of Acrosin's Functional Domains. The symbols used are defined in the legend of Figure 3.

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AN ACROSIN MECHANISM FOR ZP BINDING AND PENETRATION BY SPERM. Figure 3 is a scheme illustrating the way m_β-acrosin may assist the sperm to bind and penetrate the ZP. Acrosin is bound to the sperm membrane via its hydrophobic domain. The scheme suggests that acrosin may be an intrinsic membrane glycoprotein in that the hydrophobic domain is inserted into the sperm membrane. However, alternate schemes are possible including an extrinsic rather than intrinsic mode of binding or binding to the sperm membrane via an intermediate "acrosin binding protein" (10). Binding of the sperm to the ZP is via the carbohydrate binding site. While the acrosin is bound to the ZP, its proteolytic site activity is reduced. Thus, when acrosin is functioning in the binding mode it may not be fully functional in its proteolytic mode. Other acrosin molecules on the sperm membrane would interact with the ZP via the proteolytic site, thereby hydrolyzing peptide bonds and allowing the motile sperm to progressively penetrate the ZP matrix. Possession of both binding and hydrolyzing domains would permit acrosin to hydrolyze a penetration path while still binding the motile sperm to the ZP. Release from the ZP-acrosin binding mode to permit progressive sperm penetration could be via three mechanisms: a) the carbohydrate binding domain-ZP interaction is dynamic and could simply be chemically reversed, b) the acrosin carbohydrate binding domain could be processed (hydrolyzed) by another acrosin molecule, and c) the bound ZP oligosaccharide could be hydrolyzed from the ZP glycoprotein by an acrosin molecule and the ligand-oligosaccharide released as a glycopeptide. Mechanism "a" involves a cycling of binding-release steps between acrosin and the ZP. Mechanism "b" is a non-cycling process where the carbohydrate binding function is a singular event. Mechanism "c" is the least likely of the three mechanisms postulated as proteolysis by boar acrosin is limited to one (90K) of the three glycoprotein families of the pig egg ZP. One or both of the other ZP components ($55K_\alpha$ and $55K_\beta$) function as ligands for sperm binding. Thus, using the lectin-protease functions of acrosin, through a series of proteolytic and cycling/non-cycling carbohydrate binding events, acrosin could provide the necessary cellular binding and penetration functions for the motile sperm to traverse the ZP on its way to fusion with the egg plasma membrane, the subsequent step in the fertilization process.

FUTURE RESEARCH

A primary focus of future research should be determination of the primary (10) structure of acrosin. Protein sequencing by the classical Edman degradation method and recombinant DNA methods will be needed to elucidate completely the primary structure of acrosin. Knowledge of the primary structure is of fundamental importance for additional studies on the functional properties of the enzyme.

With the 1° structure known, the functional domains of the enzyme can be more readily defined. By comparison with the proteolytic site of other serine active site proteases, it should be possible to deduce binding and catalysis functions that are common to serine proteases where structural homology is high, and to pursue the discovery of unique functions where homology is low. The structure of the carbohydrate binding domain can be compared with other fucose

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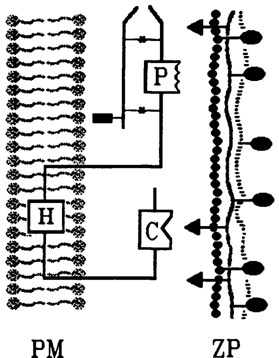

Figure 3. A Model for Acrosin's Role in Zona Pellucida Binding and Penetration of Sperm. The model uses the m_β-acrosin form depicted in Figure 2. Acrosin is bound to the sperm plasma membrane (PM) via its hydrophobic domain and interacts with the egg zona pellucida (ZP) via its protease and carbohydrate binding domains. The ZP is composed of three glycoproteins which have unique polypeptide chains and both shared and unique oligosaccharide moieties. Symbols used: ▮,⬬,◀ = oligosaccharide moieties, shared and unique; □ = functional domain; ○ = non-functional domain; P = protease active site; H = hydrophobic binding site; C = carbohydrate binding site; ⸸ = disulfide bond.

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lectins, again searching for homology/analogy and heterology/uniqueness in understanding its function. Knowledge of the 1° structure can assist in understanding the molecular nature of acrosin binding to membranes and perhaps also organization of proacrosin in the acrosomal matrix.

Understanding the activation process (proacrosin to acrosin) requires a knowledge of the 1° structure. Regulation of acrosin activity also includes limited proteolysis (autolysis) which modulates and separates the functional domains of the enzyme from one another, so that we can determine what forms of the enzyme are "where and when"; information essential for understanding the cellular function of acrosin.

Knowledge of acrosin's structure will facilitate the identification of the substrate(s) and ligand(s) sites of the ZP glycoproteins. The ZP glycoproteins are complex and difficult to study. Progress toward understanding the role of acrosin's "partner molecules" in sperm binding and penetration of the ZP will be greatly assisted by additional understanding of acrosin's structure.

From the understanding gained in the above studies will come the potential to create specific chemical reagents which can alter/inhibit acrosin's molecular interactions and the binding/penetration of the ZP. This will provide the needed unequivocal evidence of acrosin's role in fertilization and a basic understanding of gamete interactions, knowledge which is potentially useful for regulating the fertilization process.

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Chapter 16

Enzyme-Probe Conjugates as Analytical Tools in Diagnostics

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Applications of enzymatic detection systems in the context of diagnostics are presented in this article. An overview of detection systems, including those based on non-enzymatic markers such as radioactive, fluorescent and particulate labels, is discussed initially. Direct and indirect strategies for applying detection systems are also presented with respect to the identification of immunoglobulin and nucleic acid probe molecules. Enzymatic detection systems are discussed with an emphasis on chromogenic enzyme reactions. Methods of designing enzymatic assays often involve using molecular bridges between enzyme molecules and the probe species; immunodetection is discussed in this context as well as procedures for attaching immunodetectable moieties to probe molecules. Finally, current and potential developments which may improve the application of enzymatic detection systems are briefly outlined.

In this presentation we will discuss analytical detection systems composed of enzyme and probe molecular components working in tandem, with the enzymes serving the role of the signal-generating mechanism linked to ligand-specific biomolecular probes. In general, we will draw a distinction between the enzyme and the probe components. These two components may be applied simultaneously as a single conjugated reagent or in series with the enzymatic component following the application of the probe. In this design, the enzyme molecules offer the means for evaluating the result of an assay, and in large part affect the sensitivity of the assay in question by the degree of substrate metabolism and the signal to background ratio; however, the probe species, which will be more fully described later, directs the specificity of the assay by virtue of its particular binding relationship with the defined target ligands.

Since the broad topic of the agricultural application of enzymes is being addressed and since some of the recent work of the

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authors has been concentrated on diagnostic applications of molecular probes for analyzing biological specimens for the presence of infectious pathogens, chiefly of veterinary interest, the applications to be discussed will be in large part from the point of view of a diagnostic laboratory, rather than an analytical research laboratory. While maximum specificity and sensitivity are desirable in both laboratory situations, the potential hazards, expense, rapidity of execution, and labor requirements of an assay technique are factors which might require greater consideration when adapting an assay system for practical application in a diagnostic laboratory. The latter concerns, in general, support an interest in enzyme-based detection systems. Before limiting the discussion to the use of enzyme-based detection methods, we will present an overview of detection scheme, including non-enzymatic techniques.

The probe systems currently used for analyzing the macromolecular composition of biological specimens are typically composed of a specific probe molecule linked in some fashion to an, oftentimes, multipurpose detection system. The two major probe groups we will discuss will be antibodies and nucleic acid probes. Antibody probes, of either polyclonal or monoclonal origin, are used for the identification of specific antigenic determinants or epitopes; nucleic acid probes, commonly developed nowadays through recombinant cloning techniques, are used for identifying specific genetic sequences. Selection of the probe system is dependent on the target molecules available for detection. As an example, a polyclonal antibody reagent may prove superior to a more specific monoclonal antibody reagent in diagnosing the presence of a virus in an infected clinical specimen because of the ability of the former reagent to detect multiple target epitopes on the viral particles, whereas the latter reagent may detect only a solitary epitope subset within the sample, thereby limiting the sensitivity. The polyclonal reagent may sacrifice assay specificity, however, by cross-reacting with other related viruses that bear a few common epitopes. In the case of a latent viral infection though, both the polyclonal and monoclonal antibody reagents might prove useless because insufficient amounts of viral peptides are synthesized by the infected tissue. In this instance nucleic acid probes might detect the viral genome within the specimens more readily; this is the case with the provirus form of the human immunodeficiency virus and other retroviruses. Following selection of the probe system, the detection system is chosen to complement the needs of the user and to offer appropriate and acceptable levels of sensitivity.

Detection systems may be divided into radioactive/non-radioactive (Table I) and direct/indirect detection systems.

Radioactive/Non-radioactive Detection Systems. Radioisotopic detection methods frequently employ molecules containing radionuclides of hydrogen, sulfur and phosphorus, and less frequently iodine and chromium. Oftentimes, the probe molecules themselves are directly radiolabeled for immediate detection. Nucleic acid probes are commonly labeled by enzymatic incorporation of radiolabeled nucleotides or enzymatic addition of radiolabeled phosphate groups to the nucleic acid chain. Proteins, in particular immunoglobulins, are labeled commonly by direct

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radioiodination which achieves a high specific activity and may be accomplished by chemical means using chloramine T (1), N-succinimidyl 3-(4-hydroxy,-5-[^{125}I]iodophenyl) propionate (2), or 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (3). Radioiodination of proteins may also be accomplished by enzymatic means by lactoperoxidase-catalyzed iodination (4). Proteins may also be tritiated by reductive methylation (5) or radiolabeled with a variety of isotopes by biosynthetic incorporation of labeled amino acids. Radioisotopic labels may also be linked to probe molecules through a series of bridge molecules, which will be presented under indirect detection methods. The sensitivity of radioisotopic assays is frequently excellent; however, low signal to background noise ratios may impair assay interpretation. The limiting factors in the application of radioisotopic techniques in diagnostic laboratories are the biological hazard, potential contamination problems, and short half-life of labeled probes. Despite the drawbacks, radiolabeled probes are used frequently in nucleic acid blot and in situ hybridization assays, and in radioimmunoassays.

Current non-radioactive detection methods generally only approach the sensitivity of radioisotopic assays; however, reduced hazards and enhanced reagent stability are valid considerations in their defense. While enzymatic detection systems that yield colorimetric results, which we will discuss more fully later, are perhaps the most popular of the non-radioactive methods, a variety of other techniques for detecting probe molecules exist, as well as a variety of techniques for linking detection systems to probe molecules. Other common detection systems are based on (a) inert materials, typically particulate, such as latex, ferritin and colloidal gold (6-8), and (b) fluorescent compounds (9). The particulate markers are typically used by coating them with a material that can bind them to target-probe complexes. Latex particles are commonly used in agglutination assays. Ferritin and colloidal gold more recently have been used as markers in electron microscopic studies because of their electron dense properties (8). Colloidal gold is also being used in tandem with silver coating enhancement for light microscopic studies. Fluorescent compounds tagged onto immunoglobulins have long been used in fluorescent antibody techniques for in situ visualization of antigens and are now being incorporated into relatively new areas such as flow cytometry (10) and nucleic acid sequencing. Less common, and at this point esoteric from the standpoint of agricultural applications, are chemiluminescent (11-13) and electrochemical (14) detection methods. These latter detection systems because of purported enhanced sensitivity may find applications in clinical chemistry.

Direct/Indirect Detection Systems. Direct detection of molecular probes requires physical attachment of the detection system component to the probe, such that binding of the probe to its target molecule results in immediate attachment of the detection system to the target-probe complex. In contrast, indirect detection requires that further separate steps be performed to identify target-probe complexes after they are formed. Direct and indirect target-probe-

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enzyme interactions as they are used in immunoassays are briefly illustrated in Table II. Radioisotopes are an obvious example of a direct labeling system. Countless biomolecules have been synthesized and are commercially available for different radioisotopic assays. As noted above, there is a trend away from radioactive assays which has led to the design of different methods of directly linking detectable moieties, such as fluorescent compounds or enzymes, to molecular probes, like immunoglobulins and nucleic acid hybridization probes. The direct method has the obvious advantage of requiring fewer steps to identify target-probe complexes; however, this technique does require the specific synthesis of probe-detector reagents for each target assayed.

Indirect detection does require more steps, but oftentimes yields amplified signals relative to direct methods because layering of bridging molecules may increase the number of detector molecules per probe molecule. It is probably this bridging/amplification technique that has allowed current enzyme detection systems to approach the sensitivity of radiolabeled systems. The use of these indirect methods reduces steric problems that might arise from having enzyme molecules directly bound to probe molecules. Acceptable bridging molecule systems have been developed which have also simplified the utilization of different detection systems. To illustrate this point, a researcher who has developed a unique monoclonal antibody (a primary antibody) in the mouse may select from a variety of commercially available products consisting of different detection systems (e.g. fluorescein, alkaline phosphatase, colloidal gold) attached to an immunoglobulin that will specifically bind to mouse antibodies (a secondary antibody). In this way the researcher may readily obtain and test a number of detection methods for visualizing target-probe interactions without having to directly label the monoclonal antibody probe. For nucleic acid probes, which in themselves are not readily immunodetectable, it is useful to incorporate or attach detectable moieties to the nucleotides. Biotin has served this purpose well in both nucleic acid and antibody probe systems. As well as being easily detected with immunoglobulins specific for biotin, biotin may also be detected non-immunologically with avidin or streptavidin, two proteins which share a marked, highly specific affinity for biotin. The affinity constant for avidin-biotin interactions is approximately 10^{15} liters/mole, much higher than the range for antigen-antibody interactions which are commonly between 10^{3}-10^{9} liters/mole. Consequently, a vast number of detection complexes composed of avidin or streptavidin bound to a detection system are commercially available (e.g. streptavidin-alkaline phosphatase).

Two relatively new immunodetectable moieties have recently been developed for nucleic acid probes systems which allow us to illustrate two different methods of incorporating labels into nucleic acids. Both methods have been employed for biotin labeling. Originally nucleic acid probes were labeled by enzymatic incorporation of pre-labeled nucleotides, radiolabeled nucleotides, biotin-UTP/dUTP, 5-bromodeoxyuridine (15), or most recently a steroid hapten linked nucleotide analogue, digoxigenin-dUTP (16).

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Table I. Detection Systems

	Detection Label	
	Radioactive	Non-radioactive
Signal types	Radioisotopic	Particulate Fluorescent Enzymatic: Chromogenic Chemiluminescent Electrochemical
Advantages	High sensitivity Direct detection	Reduced hazard Stable shelf-life
Disadvantages	Biological hazard Potential contaminants Short shelf-life	Typically used by indirect detection-time consuming Less sensitive, although potential for enhancement exists

Table II. Immunoassays: Target-Probe-Enzyme Interactions

Direct Detection	Ag = target antigen
	Ab_1 = primary antibody
Ag - Ab_1*Enzyme	Ab_2 = secondary antibody
	B = biotin
Indirect Detection	SA = streptavidin
	- = non-covalent interaction
Ag - Ab_1 - Ab_2*Enzyme	
Ag - Ab_1*B - SA - B*Enzyme	* = covalent interaction
Ag - Ab_1 - Ab_2*B - SA - B*Enzyme	

The latter is detected using a proprietary enzyme-linked immunoassay. A new development appears to be post-synthesis labeling, in which subsequent to nucleic acid probe synthesis or purification, the unlabeled nucleic acid probe is subjected to a non-enzymatic, chemical process that modifies the original probe molecule. Chemical methods for attaching biotin to macromolecules using succinimide esters containing biotinylated moieties have been described (17,18). Biotinylation occurs under mild conditions by reaction of the succinimide esters with amino groups in the proteins or on transaminated nucleotides. A relatively new reagent, photobiotin, is a photoactivatable compound that apparently achieves significant labeling of nucleic acids with biotin upon brief exposure to an intense sunlamp source (19). Another method of generating chemically modified, immunodetectable nucleic acid probes employs a rather toxic chemical N-acetoxy-N-2-acetylaminofluorene (10,20). A newer system with reduced potential hazard is available which chemically modifies nucleic acids, specifically forming sulfonated cytidine residues (21) using bisulfite ions and O-methylhydroxylamine. DNA probes containing the sulfonated cytidine residues are detected by a proprietary system consisting of a mouse monoclonal antibody to sulfonated DNA, which in turn is detected by a secondary antibody-enzyme complex composed of anti-mouse antibodies covalently linked to alkaline phosphatase. This last case probably best exemplifies the rather furious manner in which new techniques may be piled upon one another to achieve a goal or a product.

Enzyme-Probe Conjugates

While enzymes may be covalently attached directly to primary probe molecules, as noted above for reasons of reagent versatility, steric factors, and potential signal amplification, indirect detection systems appear to be the more popular. Consequently, enzyme-probe conjugates are typically complexes of a desired enzyme marker and a secondary level probe; that is, a probe molecule that can specifically identify a primary level probe molecule, such as an alkaline phosphatase-streptavidin conjugate can identify a biotinylated nucleic acid probe by virtue of the binding affinity between streptavidin and biotin. Other examples of enzyme-probe systems are given in the preceding section on direct and indirect detection systems.

Enzyme markers and associated substrates are selected for several properties, including stability and a low detection limit. A low detection limit directly influences the sensitivity of the enzyme-based assay. The final enzyme-substrate interaction must yield an ample amount of some end product which can be accurately monitored and, hopefully, quantitated. The authors' experiences have been chiefly with enzymatic detection systems which culminate in a visible chromogenic reaction (e.g. alkaline phosphatase, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate). Results are assessed either by direct visual observation or spectrophotometrically. For the sake of completeness, it should be mentioned in this section that enzyme detection systems have been described which are monitored by alternative methods. These

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techniques, as noted earlier, seem esoteric with respect to most diagnostic assay procedures at this time. Rather than standard colorimetric monitoring, these other methods involve enzyme catalyzed generation of luminescent (11-13) or electrochemically (14) detectable end products which are subsequently monitored by specialized equipment.

A variety of enzymes have become popular for use in non-radioactive detection systems. The most popular enzymes, as judged by what is commercially marketed, are horseradish peroxidase, alkaline phosphatase, and β-galactosidase. As an aside, the gene for β-galactosidase is also commonly used in molecular biology as a marker for gene transfer into β-galactosidase deficient bacteria. Bacterial colonies grown in the presence of substrate-laden agar generate a blue pigment if the gene has been successfully transferred and translated. In general, these enzymes are similar in sensitivity, although some authors have indicated higher signals on solid phase systems with alkaline phosphatase relative to horseradish peroxidase. Selection of enzyme is often based on the samples being analyzed and the reaction environment. Some clinical specimens or other samples may have a high endogenous level of a given enzyme, which might yield a high background signal in enzyme-based assays. β-Galactosidase-based assays are subject to less interference for this reason because this enzyme is not present in mammalian systems. In the case of high endogenous activity, methods for selective inactivation of endogenous enzymes have been devised. The presence of interfering compounds, such as preservatives (e.g. sodium azide), might also influence the selection of enzyme. In the case of sodium azide, horseradish peroxidase would be inhibited and, therefore, of limited value.

Enzyme-linked Immunoassays. Enzyme-linked immunoassays (EIA) are, by the most general definition, systems which use an enzyme-immunoglobulin conjugate to detect the presence and amount of a given antigenic compound. Likely antigens may include infectious microorganisms, drugs, hormones, tumor antigens, and even other immunoglobulins. In veterinary medicine EIA have been developed to detect a variety of exogenous agents such as bacteria, viruses, metazoan parasites, and mycotoxins, and host factors such as progesterone. The technical subdivisions of EIA are covered elsewhere (22).

In general, immunodetection is usually accomplished by binding the antigen to a solid matrix prior to application of the enzyme-antibody conjugate. The interaction between antigen and solid matrix is a variable factor depending on the nature of the assay format and the original state of the antigen. If the antigen is in solution it may be applied directly to a solid matrix, such as plastic multi-well plates (enzyme-linked immunosorbent assay [ELISA] plates), and nitrocellulose or nylon sheets. Oftentimes, these media are specially modified or coated to enhance the binding of macromolecules, typically proteins. The interactions between the antigenic material and binding matrices in these cases are not specific or selective for the desired antigen. An antigen solution may also be subjected to electrophoresis prior to blotting to a nitrocellulose or nylon sheet (western blots), allowing some non-

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immunologic differentiation of the antigen from other materials in the solution. Specific antigen trapping to a solid matrix may be accomplished by first coating the matrix with antibodies that are specific for the desired antigen. In this fashion when the antigen solution is exposed to the specially prepared matrix, the antigen will be selectively bound to the matrix by virtue of the antibody coating.

Antigens not in solution, as in a piece of biopsied tissue, may obviously be liberated to form an antigen solution; however, frequently it is desirable to visualize the antigens in situ. In the case of infectious microorganisms the relationship of a pathogen's antigens with respect to infected host tissues may reveal critical information about disease processes. In the instance of in situ antigen detection the antigen-bearing material is usually preserved by methods that hopefully will not modify the antigen. The detection of antigens in situ often follows similar procedures to those used on antigens bound to artificial media.

Regardless of the different methods of preparing antigens, enzyme-linked immunodetection techniques are reasonably similar. As noted above, detection may proceed directly or indirectly. In the former case only a primary layer of antibodies are used. These antibodies are specific for the desired antigen and are directly conjugated to molecules of a selected enzyme. For indirect detection the primary antibody is not directly conjugated to an enzyme. Instead, the enzyme is bridged to the primary antibodies by a layer of enzyme-conjugated, secondary antibodies usually directed to the species-specific, xenospecific epitopes on the primary antibodies. Alternatively and commonly, biotin is employed for indirect detection. The primary or secondary layer of antibodies are chemically biotinylated before use and after application are followed by a biotin-specific immunoglobulin or avidin/streptavidin molecules conjugated to the detection enzyme, which identify the antigen-biotinylated antibody complexes.

After establishing a molecular bridge between antigen and the detection enzyme by means of specific antigen-antibody reactions, the antigen-antibody-enzyme complexes are visualized typically by chromogenic reactions. A variety of enzyme-substrate systems have been defined which yield soluble or insoluble, pigmented end products of different colors based on the needs of the researcher. It should be noted that while the enzymes and bridging molecules are relatively harmless, the substrate chemicals may vary from harmless, to unknown biohazard potential, or to recognized carcinogenic/mutagenic compounds.

An interesting twist has been added to the basic ELISA format using alkaline phosphatase as the detection enzyme. Rather than the usual system of measuring the attachment of enzyme molecules by following immediately with a chromogenic substrate mixture, a system has been reported which generally amplifies the enzyme signal 30-50-fold (23) by layering multiple enzyme systems. Application of the technique for assaying thyroid stimulating hormone resulted in a 70-fold increase in sensitivity (24). The amplification of the alkaline phosphatase signal is accomplished by incubating a $NADP^+$ solution in the presence of alkaline phosphatase specifically bound to the surface of an ELISA well and following this incubation with

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the addition of an amplifier mixture containing alcohol dehydrogenase, diaphorase, ethanol, and a chromogenic substrate, p-iodonitro-tetrazolium violet. NAD^+, initially formed by dephosphorylation, activates a redox cycle driven by alcohol dehydrogenase and diaphorase. The NAD^+ is reduced by the alcohol dehydrogenase, and the resultant NADH in turn reduces the tetrazolium salt substrate to form a formazan dye (25). The amount of dye formed is proportional to the original amount of NAD^+ formed by the bound alkaline phosphatase. The design of this system limits its application at this time to assays which are carried out in solution phase; the technique has not been adapted to blot or *in situ* assay formats.

Nucleic Acid Hybridization. The potential diagnostic uses of nucleic acid hybridization assays (NAHA) have been discussed (26,27), usually with respect to identifying pathogenic organisms by the presence of their genetic material. Some infectious agents of interest to the livestock industry for which nucleic acid probes have been reported include infectious bovine rhinotracheitis (28), bluetongue (29,30), pseudorabies (31), and *Anaplasma marginale* (32). Hybridization assays have also been developed for detecting the bacterial contaminants *Salmonella* and *Campylobacter* in food (33). In addition, nucleic acid probes have been applied for the identification of host genetic factors associated with the occurrence of hereditary diseases (34). Nucleic acid hybridization may also be applied with DNA fingerprinting techniques for animal identification and paternity testing (35). NAHA differ from EIA at the level of the specific target-probe interaction. In EIA the target-probe complex is formed by the union of antigen and specific antibody, whereas in NAHA the target-probe complex is formed by the hybridization between complementary target and probe nucleic acid strands with the intent of identifying specific genetic sequences rather than specific antigens. Despite this rather distinct fundamental difference, the methods of enzymatic detection of antigen-antibody or nucleic acid target-probe complexes are essentially the same, so a lengthy discussion on enzymatic detection in hybridization assays will be not be made here.

Nucleic acids are not particularly immunogenic under normal conditions and antibodies to nucleic acid strands generally do not distinguish between the nucleotide sequence of the probe strand or any other nucleic acid. By incorporating unique nucleotide analogues or chemically modifying the nucleic acid probe, it is possible to make nucleic acid probe molecules distinctly immunodetectable. In the case of biotin moieties, again these may be detected non-immunologically with avidin or streptavidin. Once the nucleic acid probe is made unique relative to the target or any other nucleic acids, it is possible to utilize any of the enzymatic detection sequences outlined above in the section on enzyme-linked immunoassays, subsequent to the specific hybridization reaction. As in the case of EIA, typically it is necessary to link nucleic acid target-probe complexes to a solid matrix for visualization. NAHA may be performed on samples applied directly to nitrocellulose or nylon sheets (dot blot assays) or samples may be electrophoretically separated prior to application to the sheets (northern [RNA] (36) or

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Southern [DNA] (37) blots). Recently, a technique has been described for treating nucleic acid target-probe complexes as antigens in an antigen capture system performed in plastic ELISA plates (38). The latter technique is of interest because it allows automated equipment designed for performing standard ELISA to be used for nucleic acid hybridization techniques.

A new enzymatic technique currently being explored enhances the sensitivity of nucleic acid assays by amplifying target sequences prior to reaction with probe molecules. This method is unique because virtually all prior attempts to enhance the sensitivity of NAHA have focused on amplifying the assay signal after formation of the target-probe complexes. The new technique, dubbed polymerase chain reaction (PCR), employs a DNA polymerase, originally the Klenow fragment of DNA polymerase I, and predetermined oligonucleotide primer sequences (39). The technique requires prior knowledge of a nucleotide sequence specific for the target being probed, for instance the human immunodeficiency virus (40) and human papilloma virus (41). Oligonucleotide primers sequences which flank the target sequence, generally between 15-30 nucleotides long, are synthesized. The DNA sample being assayed is heated to 95°C to heat denature the double-stranded DNA target, followed by a lower temperature incubation to allow the primer sequences to anneal to the complementary regions within the denatured target DNA sample. Addition of the DNA polymerase and nucleotide substrate generates new copies of the primed DNA sequences by primer extension. Repeated cycles of heat denaturation, primer annealing, and enzymatic synthesis specifically increase the amount of target sequences up to 10^6-fold after approximately 20-40 cycles. A recent modification of the technique has incorporated the DNA polymerase of the thermophilic bacterium, *Thermus aquaticus*, which resists heat inactivation during the denaturation cycles (42). The PCR technique, although quite simple in theory, will undoubtedly improve the potential sensitivity of NAHA for a variety of pathogens.

The Future

Non-radioactive detection methods, particularly enzymatic systems, will increase in popularity as the level of sensitivity of these methods are enhanced. Already some enzymatic detection techniques allow the identification of subpicogram amounts of nucleic acid target and nanogram levels of protein in NAHA blot and immunoblot assays, respectively. Applications employing multiple enzyme systems, such as the amplified ELISA technique, as well as the PCR technique for amplifying the amount of nucleic acid target molecules within samples, will greatly enhance the sensitivity of current assays in the near future. If the enzymatic marker systems become more potent to enhance sensitivity, with the aid of molecular linkers to reduce steric hindrance between enzyme and probe molecules, it may be possible to return to direct detection methods. The steps required to employ bridging molecules will be removed, resulting in time and labor reductions per assay. Automated systems will also reduce time and labor, while improving performance consistency. Design of less toxic substrates and more stable reagents would also enhance adoption of enzymatic detection systems.

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Finally, development of new systems which link chromogenic reactions to solid phase detection of target molecules, similar to those employed in dipsticks for clinical chemistry would help move assays from the laboratory into the field, and facilitate rapid diagnoses.

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Chapter 17

Immobilized Enzymes as Processing Aids or Analytical Tools

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The primary products of agriculture are biological materials; therefore, the use of enzymes as processing aids and analytical tools would seem to be a natural consequence. Nevertheless, the number of enzymes actually used commercially is quite small in comparison with the total number of enzymes which have been characterized. Essentially, only hydrolases have been extensively developed as processing catalysts and analytical reagents, although there are certain important exceptions including isomerases, lyases and oxidases. Enzymes can be used in either soluble or immobilized forms. In some cases, use of soluble enzymes requires incorporation of a subsequent inactivation step. Immobilized enzymes provide opportunities for process control and automation, and may stabilize the catalyst to conditions required in a given process. However, the operation of an immobilized enzyme bioreactor must be compatible with the entire process, and a bioreactor's half-life must be sufficiently long to compensate for its added cost. The recent advent of recombinant DNA technology and site-directed mutagenesis should increase the potential for using enzymes of greater stability and modified specificities.

Although the first immobilization of an enzyme was described over seventy years ago (1), wide-spread application of this technology to industrial processing has not occurred at the rate once predicted (2). Many factors are probably responsible for the slow growth rate of immobilized enzyme technology, but chief among them are: a) there have been sizeable cost reductions for soluble enzymes (2); b) with the exception of several of the successful applications, the technology has attempted to replace existing soluble enzyme processes; and c) most of the agricultural industry involves high volume, low value-added products and hence is slow in capitalizing high technology, capital-intensive processes. Nonetheless, this technology provides certain advantages, as listed in Table I, which may lead to adoption of immobilized enzymes for certain processes. The 3.4-fold

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reduction in labor costs reported by Tanabe-Seiyaku Co. (3) in their comparison of immobilized and soluble aminoacylase processes for production of L-amino acids illustrates the potential gain of an automated, continuous immobilized enzyme bioreactor process.

Table I. Advantages of Immobilized Enzyme Technology

* Processes can be readily adapted for automation and continuous processing
* The enzyme(s) can be potentially stabilized
* Processes offer easy and precise control of the extent of reaction
* The enzyme preparation does not contaminate the processed product
* Enzyme is reused; therefore, productivity of enzymes can be much greater with lower cost

During the late 1960's and early 1970's the immobilized aminoacylase and glucose isomerase technologies, respectively, were commercialized (4). Sugar conversions are well suited to the adaptation of immobilized enzymes because nonenzymatic methods are either unavailable or less desirable and the substrates' small size and lack of charge minimizes unwanted diffusional restrictions and partitioning effects. Although only about a half-dozen processes have been subsequently commercialized, the advent of genetic engineering and the possibility of tailoring enzymes for new applications will undoubtedly provide impetus for growth of commercial immobilized enzyme technology over the next 10-15 years.

An area of particularly attractive application is that of the new protein technologies. As relationships between protein structure and functionality become elucidated, selective proteolytic modification to yield a particular functionality should become feasible. Thus, rather than trial-and-error development of functionality, it should be possible to design functionality based on the principles of protein structure and function and the specificities of the enzymes used for modification. Use of immobilized exo- and endopeptidases in such technology could be especially attractive for the reasons listed in Table I, particularly since problems associated with autolysis would be eliminated and the extent of proteolytic reactions could be controlled with some precision.

Another promising area for adaptation of enzyme bioreactor technology is that of lipid modification. Several examples are: a) the interesterification of triacylglycerols to change their composition; b) limited lipolysis for production of flavors; and c) conversion of cholesterol to forms that are not absorbed. The potential stabilization of enzymes to the presence of organic solvents would provide a definite advantage to enzyme bioreactor technology for the modification of lipid molecules.

Finally, if enzymes can be stabilized in the presence of organic solvents by immobilization, an area that appears especially promising is the synthesis of organic flavor compounds. This is likely to require regeneration of expensive cosubstrates, such as the nicotinamide coenzymes, and such processes remain to be optimized. However, the stereospecificity of enzyme-catalyzed reactions and the high

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value-added nature of such flavor ingredients favors future developments in this area.

Methods of Immobilization

Methods for immobilization of enzymes on insoluble supports can be generally classified as using adsorption, entrapment or covalent attachment techniques (Table II). In a few cases, enzymes have also been rendered insoluble by covalent crosslinking; however, more often, enzymes are crosslinked within entrapped cells. These methods have been reviewed extensively in the past [for example, see refs. (5) and (6)]; consequently, this overview will focus only on a few salient features and briefly summarize the general aspects of immobilization technology.

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Table II. Methods of Immobilization

Method	Type
* Adsorption	
	Electrostatic
	Hydrophobic
	Biorecognition
* Entrapment	
	Cells
	Gels
	Fibers
	Membranes
* Covalent attachment	
	Organic polymers
	Biopolymers
	Inorganic polymers
* Crosslinking	

Basically, immobilization techniques can be divided into those involving noncovalent interactions or physical entrapment and those resulting from covalent bond formation. The procedure of choice depends largely on the intended application. Thus, if the bioreactor is to be used for bioprocessing on a large industrial scale, economy of preparation and use will be an overriding factor. In general, noncovalent methods such as adsorption are likely to be less expensive since the support is more readily reused and the bioreactor can be reloaded with active enzyme following losses in the initial levels of enzyme activity. However, it is also important to consider the characteristics of the substrate and product. For example, an endopeptidase or nuclease that was immobilized by entrapment would not function well due to limited access by the substrate. On the other hand, if the bioreactor is intended for an analytical use, covalent immobilization of the enzyme may be more desirable as a means to increase stability and to completely eliminate enzyme from the analysis stream. These generalities should not be interpreted as exclusive, however, as some methods of covalent immobilization and the supports used may be economical if the bioreactor stability is sufficient and, likewise, some noncovalent methods may not be subject to significant enzyme leaching.

Noncovalent Methods - Adsorption. Electrostatic adsorption of enzymes on ion-exchange materials was the first method used commercially for immobilization of enzymes: aminoacylase by Tanabe-Seiyaku (3) and glucose isomerase by Clinton Corn Processing (7). Obviously such methods are simple, the support can be used over a long period of time, and regeneration of the bioreactor can be performed in-place by simply desorbing the inactive enzyme (for example by changing pH) and adding back active enzyme under conditions which favor adsorption. Enzymes also can be adsorbed to hydrophobic matrices such as those used for hydrophobic interaction chromatography (8-10). The surface hydrophobicity of the enzyme, and thus the strength of binding, can be increased by chemical modification (9). Recently, enzymes also have been immobilized by adsorption on transition metal-activated supports (11).

All adsorption methods represent equilibria, albeit some of extremely high affinity; therefore, they are subject to leaching of enzyme from the support. Such leaching may become more serious when the adsorbed enzyme is subjected to complex mixtures, due to competitive displacement by molecules that interact more strongly with the matrix. Possibly, genetic engineering of the enzyme could be used to provide both a means for simple isolation and for increasing the thermodynamic strength of the interaction with a matrix. In this regard, Mosbach and co-workers (12) have designed proteins with affinity tails consisting of polycysteine to allow rapid purification on thiol matrices. Similarly, it may be possible to genetically attach polyaspartyl or polyvalyl peptides, for example, to either the N-terminus or the C-terminus of a protein, thereby greatly increasing its ionic or hydrophobic character.

Recently, immobilization techniques which utilize biorecognition have been developed. Thus, both carboxypeptidase A (13-15) and lactate dehydrogenase (15) have been adsorbed on support surfaces with covalently immobilized monoclonal antibodies. Use of a monoclonal antibody allows selection of the epitope, that is, the portion of the enzyme's surface that interacts with the antibody, and hence offers the potential of presenting the immobilized enzyme in a manner designed to achieve maximum stereoaccessibility. Consequently, such immobilized enzymes should have high specific activities. Furthermore, binding to the antibody may stabilize an enzyme as revealed by increased heat stability. One disadvantage of this method of immobilization lies in the fact that the monoclonal antibody is also a protein and thus can be subject to at least some of the factors that result in loss of enzyme activity. Hence, although inactive enzyme can be desorbed and replaced with active enzyme, if the covalently immobilized antibody should become irreversibly denatured, the support would have to be discarded. Furthermore, although the enzyme is tightly bound to the antibody under optimal conditions ($K_d \sim 10^{-8}$ M), suboptimal conditions could result in some dissociation.

Another biorecognition which we propose could be useful as an adsorption method depends on the interaction of biotin with avidin. The affinity in the biotin-avidin complex is extremely high ($K_d \sim 10^{-10}$ M), so that the stability to leaching would approach that of covalently immobilized enzymes. Development of a support surface with covalently immobilized biotin would allow specific adsorption of avidin/enzyme conjugates. Such conjugates could be prepared chemically, or, using genetic engineering, the structural gene for an

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enzyme could be fused to that for avidin in a manner similar to the fusion of the genes for galactokinase and β-galactosidase (12). Due to the specificity and affinity of the avidin-biotin interaction, loading of a bioreactor and isolation of enzyme-avidin could be developed as a single-step process. Since biotin is a robust small molecule, such enzyme supports could be unloaded and reloaded many times.

In our view, genetic engineering of future enzymes for industrial uses should consider not only their catalytic properties, but also their potential for isolation and immobilization. Designing enzymes to allow selective, high affinity immobilization by adsorption on a relatively inexpensive matrix should greatly increase the attractiveness of enzyme bioreactor processes.

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Noncovalent Methods - Entrapment. Entrapment of enzymes in crosslinked whole cells represents the most common method of preparation of commercial enzyme bioreactors at the present time. Methods currently used for preparation of immobilized glucose isomerase are listed in Table III. Using the Novo process to illustrate the entrapment method, a cell slurry is homogenized to disrupt the plasma membrane, thus improving access to the enzyme and exposing more surface protein for crosslinking (16). The disrupted cell mass is then crosslinked with glutaraldehyde and the wet crosslinked cell mass is extruded through a small orifice (<1 mm diameter) and cut into short cylindrical particles which are then dried and sized by screening. For some preparations, glucose (which dissolves during use to give a more porous matrix) and magnesium oxide are included prior to extrusion. Such bioreactors of immobilized glucose isomerase typically exhibit a half-life of 75 days (16).

Covalent Methods. Nearly all conceivable insoluble polymers have been used for enzyme support matrices. For example, organic polymers including polystyrene, nylon, phenol-formaldehyde resin, and acrylic copolymers, biopolymers including cellulose, Sephadex, agarose, collagen, and chitin, and inorganic polymers including glass, ceramics, stainless steel and sand all have been used (4). The variety of chemical activation procedures is, perhaps, even greater and has been reviewed extensively (5,6,10). Generally, the most successful coupling methods are those in which enzymes' amino or carboxyl groups react, since residues with these groups are usually more abundant on the surfaces of proteins.

The most commonly used biopolymers, such as agarose, contain alcoholic hydroxyl groups which can be activated with cyanogen bromide; however, better methods have recently been developed including activation with sulfonyl chlorides (17), 2-fluoro-1-methylpyridinium toluene sulfonate (FMP) (10), and chlorocarbonates (18). The first two are commercially available as activated supports: tresyl-activated Sepharose (Pharmacia) and FMP-Trisacryl (BioProbe International). The newer methods yield more stable bonds, which preclude leaching of the enzyme from the matrix. Most of these activated supports are too expensive for commercial use in a large process bioreactor; however, they may be extremely useful for preparing analytical bioreactors.

The functional groups of inorganic supports are usually derived from surface hydration of the metal oxides; for example, the silanol

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Table III. Commercial Methods Used for Immobilization of Glucose Isomerase

Company	Source	Immobilization Method	Reference
Novo Industri (Sweetzyme)	*Bacillus coagulans* *Streptomyces murinus*	Cells are homogenized, crosslinked with glutaraldehyde, extruded, dried, and screened	16,31
ICI Americas Inc.	Arthrobacter Sp.	Cells are flocculated, heated, and granulated	32
Gist Brocades (Maxazyme)	*Actinoplanes missouriensis*	Cells are trapped in gelatin, crosslinked with glutaraldehyde, and granulated	31,32
Miles (Takasweet)	*Flavobacterium arborescens*	Cells are flocculated with polyamine, crosslinked with glutaraldehyde, and extruded	31
Miles Kali-Chemie (Optisweet 22)	*Streptomyces rubiginosus*	Enzyme adsorbed on silica and crosslinked with glutaraldehyde	31
SNAM Progetti	Streptomyces Sp.	Cells are entrapped in cellulose acetate fibers	32
Finnsugar Co. (Spezyme IGI)	*Streptomyces rubiginosus*	Crystalline enzyme adsorbed on DEAE-cellulose in a granular base containing polystyrene for rigidity and TiO_2 density	31,33
Nagase (Sweetase)	*Streptomyces phaechromogenes*	Cells are heat treated, granulated, and adsorbed on an anion-exchange resin	31
Godo Shusei (Godo-Agi)	*Streptomyces griseofuscus*	Cells are treated with chitosan, crosslinked with glutaraldehyde, and granulated	31
CPC International	Streptomyces Sp.	Enzyme adsorbed on granular ceramic carriers	32

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groups of glass or silica. For covalent immobilization, these surfaces are usually treated with an organosilane which gives a functional group on a carbon chain attached through a stable siloxane bond. Frequently, an aminopropyl derivative of the surface is prepared and the enzyme is then immobilized using glutaraldehyde to activate the surface. This technique was used by Corning Glass for their commercial preparation of immobilized lactase (19). It represents a relatively simple and inexpensive method for covalent immobilization. Development of the tresyl chloride method for polyhydroxylic surfaces offers another efficient technique for immobilization to glycerylpropyl silica which is prepared by reaction with γ-glycidoxypropyltrimethoxysilane (17). Using small bead diameter, large pore size tresyl-silica (10 μm, 1000 Å pore), a loading for horse liver alcohol dehydrogenase of 21 mg/g dry support was obtained (17). Furthermore, due to the lack of pore diffusion limitations for these small beads, the immobilized enzyme exhibited 95% of the soluble enzyme's activity.

We have had very good success with succinamidopropyl derivatives of inorganic surfaces. These derivatives, prepared by succinylation of the aminopropyl surface, can be activated with carbodiimide (20). The resulting amide bond formed upon reaction with free amino groups is very stable; for example, we have not been able to detect leaching following extensive washing with 6 M guanidinium chloride. Although the procedure is too expensive for preparation of a process bioreactor, it should be good for production of analytical bioreactors. We have used this method for preparation of immobilized exo- and endopeptidases, oxidases, dehydrogenases, and various affinity matrices (20-24). For example, an optimized procedure using controlled-pore glass (120/200 mesh, 720 Å pore diameter CPG) yielded 37 mg active trypsin/g of dry support as determined by the active site titrant p-nitrophenyl-p'-guanidinobenzoate (25). Even at that high loading, the fraction of active/total trypsin fell only about 20% due to immobilization.

A characteristic of immobilized enzymes that is often ignored is the potential partitioning of ions and substrates and/or products due to electrostatic potentials or hydrophobic moments. This factor could be used to advantage, for example, if the optimal conditions for enzyme activity do not match those of the process stream. To use the example cited earlier, a succinamidopropyl surface was shown by electrostatic partitioning of ions and independent chemical analysis to have 96 μmol charged groups/g dry beads (25). Attachment of 2 μmol trypsin/g did not significantly alter this characteristic. The electrostatic partition coefficient was determined from the ratio of the partitioning of the ion probe N-benzoyl-L-arginine amide to that of the zero net charge analog N-benzoyl-L-arginine. Data in Figure 1 indicate that substantial electrostatic partitioning occurs below an ionic strength of 0.1. For example, at an ionic strength of 0.06 the concentration of anions would be 2.34 times less at the surface than in the bulk solution. Furthermore, the pH would be 0.4 units lower at the surface.

Another advantage of succinamidopropyl derivatives as compared to aminopropyl surfaces is the reduced tendency to adsorb proteins. Since most food proteins under physiological conditions have net negative charges, they would be repelled from a negatively charged surface. Data in Table IV indicate that succinamidopropyl glass is

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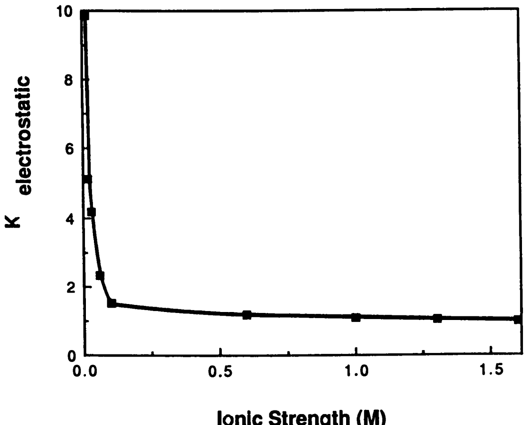

Figure 1. Electrostatic partition coefficient of succinamidopropyl-glass beads as a function of ionic strength.

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Table IV. Adsorption of Glutamate Dehydrogenase on Various Derivatized Glass Surfaces[a]

Matrix[b]	mg Protein/g Beads after Buffer Wash	mg Protein/g Beads after Denaturant Wash[c,d]
—OH, —OH Raw glass	6.9	5.1
$-CH_2CH_2CH_2NH_2$ Aminopropyl-glass	10.2	0.64
$-CH_2CH_2CH_2NHC(=O)CH_2CH_2C(=O)OH$ Succinamidopropyl-glass	0.36	0.15
$-CH_2CH_2CH_2NHC(=O)CH_3$ Acetamidopropyl-glass	0.17	0.15
$-CH_2CH_2CH_2OCH_2CH(OH)CH_2(OH)$ Glycerolpropyl-glass	0.15	0

[a]Reprinted from DuVal et al., J. Appl. Biochem. 6, 240-250 (1984) with permission.

[b]Prepared from 80/120 mesh, 73-nm-pore-diameter glass.

[c]Immobilized protein assayed by amino acid analysis of matrix acid hydrolysate.

[d]Twenty-milliliter portions of a 1.0-mg/ml glutamate dehydrogenase solution in 50 mM sodium phosphate, pH 7.0, containing 2 mM DTT and 1 mM EDTA were recycled through columns containing 0.6 g of the indicated matrices. After recirculation for 3 h using a fluidized-bed configuration at room temperature (24-26 C), the protein solutions were drained from the columns and each column was then washed with 500 ml of 50 mM phosphate buffer containing 2 mM DTT and 1 mM EDTA using a fixed-bed configuration at room temperature and a flow rate of 150 ml/h. Each matrix was then assayed for adsorbed protein.

almost as inert as glycerylpropyl glass with respect to adsorption of glutamate dehydrogenase (26). Also, we have observed less fouling using the succinamidopropyl matrix for immobilized enzymes exposed to milk.

An inexpensive method for activating succinamidopropyl matrices involves treatment with thionyl chloride (26). Although the chemistry of the resulting activation is not understood, it has been shown that the activated surface will react only with thiol or amino groups yielding a covalent bond. Furthermore, the activated matrix can be stored for long periods and immobilization of enzyme can be achieved simply by adding a solution of the enzyme to the activated matrix.

Characteristics of Bioreactors

The major factors affecting the economics of a bioreactor process are (4): a) the catalyst's specific activity, b) the catalyst's stability, and c) the regenerative capability. The catalyst's specific activity determines the size of bioreactor required for a particular production rate and thus affects the capital cost. Catalyst stability and regenerative capability determine the amount of product produced per unit of catalyst and the production per unit of support matrix. The productivity of a bioreactor, kg product/L of immobilized enzyme/half-life, is determined by both the catalyst's specific activity and stability. Obviously, increasing the productivity decreases the cost per unit of product. The regenerative capability includes both potential reactivation of the immobilized enzyme and the reusability of the support material.

If enzyme inactivation is not due to covalent changes in structure, the native active structure can be reformed in immobilized enzymes by refolding from a random coil state (22,27). In fact, if they are attached to the matrix by multiple points, even thermally inactivated multichain enzymes can be reactivated (27). Although such regeneration steps apparently have not been used commercially, their use should be considered in certain cases, especially since in many cases the inactivation may result from adsorption onto the enzyme matrix of components in the process stream. For example, we have found that immobilized sulfhydryl oxidase activity can be regenerated numerous times following treatment of UHT milk by washing with 4 M urea (28).

Obviously, the ease with which the enzyme support can be reclaimed and enzyme activity re-immobilized will make a significant contribution to the cost of bioreactor operation. The higher the initial cost of the support matrix, the more important this regenerative capability becomes. Thus, future development of well defined, stable enzyme supports may depend upon concomitant development of efficient, high affinity, and selective adsorption techniques to allow simultaneous isolation and immobilization.

Current and Potential Bioprocessing Technology

Descriptions of the current commercial use of immobilized enzyme technology have appeared over the past several years (3,4,29,30); consequently, this area will be summarized briefly to allow more time for discussion of developing technology.

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Technologies Applied Commercially

1. Glucose isomerase. Production of high fructose corn syrups (HFCS) is by far the most abundant commercial use of immobilized enzyme technology, with a production of 6.7 million tons of HFCS in 1984 (31). This technology has continued to advance with the development of continuous processes that allow operation at a higher pH which eliminates the requirement of Co^{2+} for stability (16). Typical production of a 42% fructose syrup from a feed stream containing 43-47% (w/w) concentration of 95% glucose uses a 1.5 x 5-m fixed-bed bioreactor operating at pH 7.5-7.8 and 60°C with Mg^{2+} added at a level of 20 x $[Ca^{2+}]$ (16,31). The half-life of such a bioreactor is 1200-1500 h, yielding a productivity of 3000-4000 kg syrup dry substance per kg of biocatalyst and a production cost of 10-20 cents per 100 pounds of isosyrup dry substance (31).

2. Aminoacylase. Production of L-amino acids by Tanabe Seiyaku Company using aminoacylase adsorbed on DEAE-Sephadex represents the first industrial use of an immobilized enzyme (3,29). The process uses the enzyme to resolve the racemic mixture of an amino acid, derived by chemical synthesis, by biospecific hydrolysis of the acylamino acid followed by separation of the L-amino acid from the acyl-D-amino acid by crystallization. The D-forms are then racemized and passed back through the reactor thus improving the yield. The entire process, including a fixed-bed bioreactor, is a continuous, automated operation.

3. α-Galactosidase. Production of sucrose from sugar beets is substantially improved by hydrolysis of the raffinose present to give sucrose and galactose (4,30,34). The major benefit is derived from elimination of the inhibition of sucrose crystallization by raffinose. The enzyme, produced in the mycelia of *Morteriella vinacea*, is crosslinked and pelletized for commercial use by Hokkaido Sugar Company and the Great Western Sugar Company (4).

4. β-Galactosidase (lactase). Two types of β-galactosidases have been used industrially depending upon the application; *viz.*, the fungal enzymes with low pH optima for treatment of cheese whey and the yeast enzymes with neutral pH optima for treatment of milk. Reasons for hydrolyzing lactose in milk or whey are manyfold (35), including a) lactose intolerance exhibited by many adults, b) increased stability of concentrated milks and ice cream to frozen storage, c) more rapid acid production in fermented dairy foods, d) production of fermentation stocks from whey, and e) production of a syrup. Most of the development has evolved around use of the enzyme from *A. niger* or *A. oryzae*, the latter being preferred because of a low pH optimum, 3.0-3.5 (35-37). The Corning process, which uses β-galactosidase immobilized on porous aminopropyl silica ceramic (19,36), has been used industrially in the U.S., England, and France (the Nutrisearch facility in the U.S. has ceased operation due to the economics of yeast production). This bioreactor process included a daily cleaning cycle to remove proteins, peptides, and cells adsorbed to the carrier surface, most likely due to the reasons given earlier. Valio in Finland also has produced lactose-hydrolyzed whey for a number of years using an immobilized enzyme process (35). Recently a bioreactor with excellent stability using enzyme immobilized on a macroporous Plexiglas type support has been described (37). Using this technique, Röhm GmbH observed 80% activity after 100 days of operation with a productivity of 12,000 gal/lb of immobilized enzyme.

The only reported commercial use of the yeast enzyme is the production of lactose-hydrolyzed milk in Italy using the Snam Progetti process for entrapment of enzyme in cellulose triacetate fibers (4).

5. Aspartase. Tanabe Seiyaku has used aspartase in lysed *E. coli* cells immobilized by entrapment in polyacrylamide (3) or κ-carrageenan (38) for production of aspartic acid since 1973. Using a substrate stream containing 1 M ammonium fumarate and 1 mM Mg^{2+} at pH 8.5 and 37°C, a continuous, automated bioreactor with a 120-day half-life will produce L-aspartate at 60% of the cost of a batch fermentation (3). Recently, a process for immobilization of the cells in polyurethane has also been described (37).

6. Fumarase. The development and use of this immobilized enzyme by Tanabe Seiyaku for production of L-malic acid is very similar to that of aspartase (3). Lysed *Brevibacterium ammoniagenes* or *B. flavin* cells are treated with bile acid to destroy enzymatic activity which converts fumarate to succinate. As with aspartase, the cells can be immobilized in polyacrylamide or κ-carrageenan gels. Using a substrate stream of 1 M sodium fumarate at pH 7.0 and 37°C, L-malic acid of high purity has been produced since 1974 by a continuous, automated process (3,39); for example, using a 1000-L fixed-bed bioreactor, 42.2 kg L-malic acid per hour was produced continuously for 6 months.

Enzyme Bioreactors Nearing Commercial Application

1. Glucoamylase. Although the technology for industrial use of immobilized glucoamylase has been available for some time (40), it has not gained acceptance. The major causes for the lack of industrialization are: a) the cost of the enzyme has decreased during the past several years (2); b) the immobilized enzyme lacks sufficient stability to operate at temperatures above 55°C (29,40); and c) glucose yields are less with the immobilized enzyme, presumably due to pore diffusion limitation and resulting formation of reversion products such as isomaltose (4,40). The latter two problems potentially could be solved by improvements in the technology. A bioreactor with increased thermal stability was developed and patented by Tate and Lyle, Ltd., London, and was reported to be operating at a pilot plant scale (4).

2. 5'-Phosphodiesterase. Several reports have appeared describing the semi-industrial scale application of immobilized 5'-phosphodiesterase for production of 5'-mononucleotides (IMP, AMP, GMP, UMP, CMP) to be used as flavor enhancers (30,41,42). In Japan, the enzyme is immobilized on a porous ceramic support and used (in combination with a similarly immobilized 5'-adenylate deaminase to convert AMP to IMP) to produce the 5'-mononucleotides from RNA (30,41). The deamination is desirable since IMP and GMP act synergistically with monosodium glutamate as a flavor enhancer.

A process was developed in Germany using the enzyme immobilized on 350 Å pore diameter Eupergit C for production of 5'-ribonucleotides from crude nucleic acid mixtures containing both RNA and DNA (42). Apparently the larger DNA is excluded from the pore volume while the smaller RNA has access to the enzyme, thus eliminating the requirement for a difficult separation of RNA from DNA. Using a substrate stream of crude nucleic acid adjusted to 0.1 mM $ZnSO_4$ at pH 5.0 and 60°C, a 10-inch x 25-inch fixed-bed bioreactor produced 10

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tons/year of 5'-ribonucleotides. No detectable loss of activity occurred during 500 days of continuous operation.

3. Aspartame synthesis. Peptidase-catalyzed synthesis of peptide bonds by establishing conditions favoring reversal of hydrolysis is well known. Synthesis of the sweetener α-L-aspartyl-L-phenylalanine methyl ester (Aspartame) can be performed most effectively with thermolysin since this endopeptidase does not have esterase activity (43). Advantages of enzymatic *vis-à-vis* chemical synthesis include: a) protection and deprotection of the β-carboxyl is not required to prevent formation of the bitter tasting β-aspartame; and b) less expensive DL-Phe-OMe can be used since the enzyme does not act on D-Phe-OMe (43). The D-Phe-OMe can be racemized, re-esterified, and passed back through the bioreactor. The bioreactor is operated in a two-phase system of ethyl acetate:water (7:3 w/w) which partitions reactants in the aqueous phase and products in the organic phase, thus driving the reaction towards synthesis. The reactants, carbobenzoxy-L-Asp and DL-Phe-OMe, are the least expensive available reactants for chemical or enzymatic synthesis (43). Enzyme covalently immobilized to a hydroxylic gel appears to provide the optimum biocatalyst at present.

Recently the possibility of total enzymatic synthesis of Aspartame has been suggested (44). It was shown that immobilized penicillinacylase would hydrolyze *N*-phenacetyl L-L-aspartame; thus, if thermolysin will accept phenacetyl L-Asp, use of the carbobenzoxyl protecting group and its subsequent removal by hydrogenation would not be required.

4. Isomaltulose production. The enzyme isomaltulose synthetase, located in the periplasmic space of *Erwinia rhapontici* cells, can be used for production of crystalline isomaltulose (6-*O*-α-D-glucopyranosyl-D-fructofuranose; palatinose; lylose) from sucrose syrups (45,46). Semi-industrial scale bioreactors have been developed by entrapment of the nongrowing cells in calcium alginate gel beads. The process, developed at Tate and Lyle, uses 1.6 *M* (55% w/w) sucrose as the substrate feed with a 99% conversion. The bioreactors, with a one-year half-life, are capable of producing 1500 times their weight in crystalline isomaltulose and the immobilized cells are 350 times more stable than free cells. Isomaltulose, also found in honey, has a number of uses including its function as a noncariogenic bulking agent and as a growth promoter for bifidobacteria (46).

Since the disclosure by Tate and Lyle, several other companies, including the South German Sugar Co., Mitsui Sugar Co., and Miles Laboratories, have reported isomaltulose production with bioreactors (46). For example, Mitsui has announced plans for a 600-ton/year plant using their bioreactor technology.

Potential Industrial Applications of Bioreactors

Discussion of potential applications of bioreactors is a highly subjective undertaking since the number is essentially limited only by the ingenuity of the authors and the known list of enzymes. Because of this fact and the limitation of space, we will illustrate the range of potential with a few selected examples.

1. Lipase. Interesterification of triacylglycerols in the presence of a regiospecific lipase or a nonspecific lipase and free fatty acids can be used to modify the physical or nutritional characteristics of fats. Lipases from *Aspergillus* sp. (47,48) and *Rhizopus*

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arrhizus (47,49) have been immobilized by adsorption on diatomaceous earth (Celite from Johns-Manville) and used to incorporate myristic acid into palm mid-fraction with hexane as the solvent. A successful pilot-scale interesterification of shea oleine and stearic acid, as well as shea oil and myristic acid, has been characterized (49). The adsorbed protein included a reservoir of bound water necessary for catalysis. The enzymes from Rhizopus delemar and Candida cylindracea have also been adsorbed onto Celite using a slightly different procedure (50). These immobilized enzymes were used to interesterify olive oil and stearic acid or oleic safflower oil and palmitic acid dissolved in hexane.

Immobilized lipases can also be used for triacylglycerol hydrolysis and hence the potential for production of cheese flavor concentrates.

2. Sulfhydryl Oxidase. The mammalian enzyme, particularly that from bovine milk, is the best characterized (51), although the enzyme from Aspergillus niger has received recent attention (52,53). Both enzymes catalyze oxidation of sulfhydryl or thiol groups to disulfides with dioxygen as the electron acceptor. However, they apparently differ somewhat in their specificity, with the bovine enzyme showing specificity for cysteine, peptides, or proteins (54), whereas the microbial enzyme displays greatest activity with dithiothreitol and other small thiols and little or no activity with proteins (52,53). Furthermore, the microbial enzyme is a soluble flavoprotein, while the mammalian enzyme is a membrane metalloglycoprotein. Cooked off-flavors in products such as UHT milk are caused by thermal degradation of protein disulfides resulting in the formation of thiol compounds. We have shown that treatment of UHT milk with bovine sulfhydryl oxidase effectively eliminates this off-flavor, yielding a product indistinguishable from pasteurized milk (28,55). Moreover, it is known that autoxidation of thiols leads to formation of active oxygen forms, and our preliminary studies have suggested that enzyme-treated UHT milk does not develop the typical oxidative-type flavors during long periods of storage. The bovine enzyme has been immobilized on porous glass and silica and used for continuous treatment of UHT milk (28,55). Since loss of bioreactor activity was largely caused by adsorption of milk components, a method for regeneration of activity was developed which utilized a periodic wash with sterile 4 M urea.

The activity of this enzyme suggests other potential applications including flavor modification of other food products and as a bioreactor in the downstream processing of recombinant proteins.

3. Endopeptidases. Our expanding understanding of the relationship between structure and functionality of food proteins presents the opportunity for designing functionality into proteins by selective, specific proteolytic modification. Control of reaction and prevention of autolysis offered by immobilization are essential to establish the conditions for a highly selective modification. Hydrolysis at specific positions in the primary structure of proteins could be coupled with resynthesis of peptide bonds by selection of conditions, for example, as in the plastein reaction. By careful choice of enzymes and conditions according to the characteristics of the substrate proteins, it may be possible to design new structures from known food proteins.

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Such applications appear to be more attractive for the use of bioreactors than traditional uses of endopeptidases for chillproofing beer, juice clarification, and curd formation in cheesemaking which currently use well established soluble enzyme processes. In the case of curd formation, hydrolysis of micellar κ-casein by immobilized chymosin is questionable (56).

4. Dehydrogenases. The necessity for recycling coenzymes is a major obstacle to the use of dehydrogenase bioreactors. For this reason immobilized cell bioreactors will likely be the method of choice for such conversions except in special cases such as for organic synthesis of high value-added products. Methods have been developed for recycling coenzymes by coupling enzyme reactions and retaining the coenzymes by covalent attachment to the enzyme, to the matrix, or to soluble polymers (57-59). For example, alcohol dehydrogenase bioreactors, which have broad substrate specificity, have been used for synthesis of various compounds in an immiscible organic phase, in which the coenzyme is retained with the enzyme in a low-volume aqueous phase where it can be recycled, for example, by using another enzyme with a coupled substrate regeneration.

Another potential application is the coupling of oxidases and reductases to convert cholesterol to coprostanol, which is not absorbed and is not atherogenic (61,62).

Analytical Bioreactors

This review would not be complete without mentioning some of the analytical uses of immobilized enzymes. Numerous enzyme electrodes compatible with various types of detectors have been developed by Guilbault and co-workers (63), and enzyme thermistors have been developed by Mosbach and co-workers (64). Commercial instruments are available from several companies, including Leeds & Northrup, Midwest Research Institute, Technicon, Universal Sensors, and Yellow Springs Instruments, for analyzing glucose, lactose, maltose, sucrose, galactose, cholesterol, urea, pesticides, and amino acids. For an excellent review of these systems the reader is referred to the book by Guilbault (63). Rather than repeating what has been presented previously, we will discuss two applications that may be of interest to the food scientist.

1. Preparation of enzymic hydrolysates of protein for amino acid analysis. Food chemists are well aware of the limitations of using an acid-hydrolysate of proteins for compositional analysis. Many of the nutritionally important residues such as Trp, Cys, and Met are not easily determined. Recently we developed an immobilized endo- and exopeptidase bioreactor that was capable of complete hydrolysis of substrate proteins (65). We have since modified the bioreactor to contain Pronase (a mixture containing four types of activities), intestinal mucosal peptidases, prolidase, and proline-specific peptidases from *E. coli* (66). An example of the preparation and analysis of a bioreactor hydrolysate of β-lactoglobulin is given in Table V. Inclusion of 2 M urea in the reaction mixture improves the recovery of amino acids, possibly by solubilizing hydrophobic peptides and by destabilizing protein structure. Data from analysis of the bioreactor hydrolysate are in excellent agreement with the known composition

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from the primary structure, thus yielding substantially more information than that from an acid hydrolysate. Similar results were obtained for a hydrolysate of lysozyme. Although prolidase and *E. coli* aminopeptidase P were less stable and had to be replaced every third day, the other enzymes were stable through numerous digestions.

Table V. Compositional Analyses of Various Hydrolysates of β-Lactoglobulin[a]

Acid	Acid Hydrolysate (mol/mol)	Enzymic Hydrolysate of CM-Protein in 2 *M* Urea (mol/mol) ± S.D.	Sequence (mol/mol)
Asp	15.9	11.1 ± 0.6	11
Asn	<0.1	4.28 ± 1.04	5
Thr	7.13	7.74 ± 0.37	8
Ser	5.17	7.23 ± 0.33	7
Glu	25.3	15.7 ± 0.18	16
Gln	<0.1	4.60 ± 0.45	9
Pro	7.68	7.74 ± 0.36	8
Gly	3.05	3.26 ± 0.03	3
Ala	14.1	14.7 ± 0.96	14
Cys	1.60	<0.3	5
Val	9.72	10.5 ± 0.41	10
Met	3.83	3.9 ± 0.40	4
Ile	9.54	10.7 ± 0.21	10
Leu	22.5	21.9 ± 0.34	22
Tyr	3.63	4.19 ± 0.14	4
Phe	3.82	4.03 ± 0.05	4
His	2.03	1.92 ± 0.03	2
Trp	0	2.17 ± 0.06	2
Lys	14.6	14.9 ± 0.45	15
Arg	2.77	2.78 ± 0.11	3
CM-Cys	0	5.05 ± 0.97	0

[a]Each value is the average of triplicate hydrolysates.

2. Determination of protein digestibility. A number of *in vitro* methods for assaying protein digestibility have been developed in an effort to replace expensive, time-consuming animal bioassays. The availability of such an assay for more frequent monitoring of protein nutritional quality is desirable as proteins are increasingly handled as ingredients. Our effort to develop an *in vitro* digestion system included an initial digestion with pepsin under acidic conditions followed by neutralization and digestion with pancreatic and intestinal mucosal peptidases, all using immobilized enzymes so the two bioreactors can be used for many assays. By measuring the extent of hydrolysis rather than the rate, this method does not underestimate the digestibility of proteins that are very stable and yet are completely digestible. The immobilized digestive enzyme assay (IDEA) method has been used to monitor changes in digestibility resulting from racemization, crosslinking, and Maillard reactions and results have been correlated with *in vivo* digestibilities. Recently we have

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used this method in a fluidized-bed bioreactor that allows assay of samples containing particulate matter. An example of analyses obtained with this method and a comparison to digestibilities measured *in vivo* is given in Table VI. The correlation between *in vitro* and *in vivo* values is quite good (r = 0.8) taking into consideration the typical variability experienced for independent measurements *in vivo*.

Table VI. Comparison of Digestibilities of Proteins in Various Foods Determined *in Vivo* and by the IDEA Method[a]

	Digestibilities		
	IDEA		
Food Products	D[b] (*In Vitro*)	Predicted[c] *In Vivo*	*In Vivo*[d]
Animal:			
ANRC casein	0.471 ± 0.049	92.5	97.9
Nonfat dry milk	0.338 ± 0.036	89.3	93.8
NFDM (heated)	0.105 ± 0.012	83.7	90.1
Sausage	0.446 ± 0.012	91.9	94.0
Animal-vegetable mixture:			
Macaroni & cheese	0.352 ± 0.090	89.7	94.8
Vegetable:			
Soy isolate	0.453	92.1	95.0
Rolled oats	0.357 ± 0.041	89.8	92.4
Chick peas	0.386 ± 0.043	90.5	88.5
Pea protein concentrate	0.448	92.0	92.8
Wheat cereal	0.361 ± 0.023	89.9	91.2
Pinto beans	0.027 ± 0.035	81.9	75.9
Rice-wheat gluten	0.509 ± 0.048	93.5	93.9

[a]W. C. Thresher *et al.* (N. C. State University, unpublished data).
[b]Defined as the fraction of the total peptide bonds hydrolyzed (triplicate analyses).
[c]Calculated from the *in vitro* values using the regression: Y = 81.14 + 24.19D (69).
[d]Average of *in vivo* values determined by independent laboratories in a cooperative study. Y = 80.94 + 30.33D; R = 0.80.

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Chapter 18

Aryloxyphenoxypropanoate and Cyclohexanedione Herbicides

Inhibition of Acetyl Coenzyme A Carboxylase

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Haloxyfop, an aryloxyphenoxypropanoate, and tralkoxydim, a cyclohexanedione, inhibit acetyl coenzyme A carboxylase (ACCase) from moncotyledoneous and dicotyledoneous species in a manner indicating that this enzyme is the target site for these classes of herbicides. The concentration of haloxyfop and tralkoxydim required for 50% inhibition (I_{50}) of ACCase activity in extracts from susceptible species (maize, wheat, and tall fescue) ranged from 0.5 μM to 1.2 μM. The I_{50} values for a resistant monocot, red fescue, were 10 μM and 30 μM, and for a resistant dicot, soybean, were 140 μM and 520 μM for haloxyfop and tralkoxydim, respectively. The I_{50} values correlate very well with the herbicidal activity of theses compounds except for wheat, which is resistant to tralkoxydim. The herbicidally inactive S(-) enantiomer of haloxyfop did not inhibit maize ACCase.

Aryloxyphenoxypropanoates and cyclohexanediones are two classes of herbicides that control many monocotyledoneous species. Although these herbicides are structurally very different (Fig. 1), there has been some conjecture that they have a similar mode of action because of their similarity in selectivity and symptomology. This paper describes the experiments that led to the discovery that aryloxyphenoxypropanoate and cyclohexanedione herbicides inhibit acetyl coenzyme A carboxylase (acetyl-coenzyme A: bicarbonate ligase [ATP], EC 6.4.1.2) activity in susceptible species (1). In addition, evidence is presented indicating that the inhibition of acetyl coenzyme A carboxylase (ACCase) is well correlated to observed herbicidal activity. Similar, independent findings have recently been reported by two other research groups (2,3).

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Mode of Action

Since the discovery of the herbicidal properties of the aryloxyphenoxypropanoates, there have been many studies aimed at determining their mode of action. Fewer reports have been published regarding the mode of action of cyclohexanediones. Neither class of compounds interferes with photosynthesis, respiratory O_2 uptake, protein biosynthesis, or nucleic acid biosynthesis (4-6). Several physiological processes are disrupted by both the cyclohexanediones and aryloxyphenoxypropanoates, namely growth and development, maintenance of membrane integrity, auxin induced growth, and lipid metabolism (4,5,7,8). In addition, the aryloxyphenoxypropanoates have been reported to depolarize membrane potentials (9,10). However, no specific target site had been identified for either class of compounds.

Of the postulated modes of action of these compounds, the inhibition of lipid biosynthesis has been most thoroughly investigated, principally by Hoppe and colleagues (4,5,11,12) for the aryloxyphenoxypropanoates and Lichtenthaler and colleagues (13,14) for the cyclohexanediones. Hoppe's group demonstrated first that the aryloxyphenoxypropanoate diclofop-methyl (methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate) altered lipid metabolism (4). They went on to show that acetate incorporation into fatty acids was inhibited after a 60 min incubation in diclofop methyl in chloroplasts of susceptible but not tolerant species (11). The cyclohexanedione sethoxydim (2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one) also alters lipid metabolism by inhibiting acetate incorporation into fatty acids (14). These studies strongly suggested that *de novo* fatty acid synthesis was inhibited but did not identify the specific site of inhibition.

Experiments Leading to ACCase

Our goal was to determine the specific target site(s) of action of the aryloxyphenoxypropanoate and cyclohexanedione herbicides. We set two simple criteria as prerequisites for identifying physiological processes as possible target sites. The site of action must be affected (1) rapidly (within minutes) and (2) by low concentrations (<μM) of the herbicides. Among the several proposed modes of action postulated by other researchers, we chose to focus our attention on the disruption of lipid metabolism because it seemed most sensitive to these herbicides (11,12).

Experiments were conducted to determine how quickly a low concentration (1.4 μM) of an aryloxyphenoxypropanoate, haloxyfop (2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]-propanoic acid) (Fig.1) could affect lipid metabolism. We used leaf discs as experimental material because it would mitigate effects of herbicide uptake and translocation. Maize (*Zea mays*) leaf discs were prepared as previously described (15) and incubated in ^{14}C-acetate. At various times, discs were removed and cell constituents were separated into organic and aqueous soluble fractions (16). Within 30 min of application, 1.4 μM of either the free acid or the methyl ester of haloxyfop inhibited lipid

biosynthesis by about 50% (Fig. 2) but had little or no effect on the amount of acetate incorporated into the aqueous fraction (data not shown). Further studies in our laboratory indicated that these effects were discernible within 15 min of herbicide application. To our knowledge, this was the fastest physiological response reported for aryloxyphenoxypropanoates *in situ*. To facilitate uptake into plant tissue, these compounds are often applied as esters, which are believed to be rapidly deesterified to the herbicidally active free acid. That both the free acid and the methyl ester were equally effective suggested that either there was a very active esterase or that the ester itself was effective at the active site. A cyclohexanedione, tralkoxydim (2-[1-ethoxyimino)propyl]-3-hydroxy-5-mesitylcyclohex-2-enone) (Fig.1) had a similar effect as did haloxyfop in reducing acetate incorporation into lipids (Fig. 3).

Aryloxyphenoxypropanoates and cyclohexanediones are herbicidal to most monocotyledons but not to dicotyledons. A comparison between a susceptible species, barley (*Hordeum vulgare*), and a tolerant species, soybean (*Glycine max*) showed that haloxyfop was effective in reducing lipid biosynthesis in barley but not in soybean leaf discs (Table I).

Table I. Effect of Haloxyfop on ^{14}C-Acetate Incorporation into Lipids in Soybean and Barley Leaf Discs After a 40 Minute Incubation Period

	Acetate Incorporation (nmol/cm2)	
Treatment	Barley	Soybean
Control	3.10	1.41
1.4 μM Haloxyfop	2.04	1.63

We decided to focus our attention on *de novo* fatty acid biosynthesis based upon our observations and those reported in the literature (12). To determine whether fatty acid biosynthesis was inhibited, we determined the effect of the herbicides on ^{14}C incorporation into palmitic (16:0) acid, the first product of fatty acid biosynthesis. Maize leaf discs were incubated for 40 min in ^{14}C-acetate, after which fatty acids were extracted. As shown in Table II, both tralkoxydim and haloxyfop significantly reduced the amount of acetate incorporated into palmitic acid. Thus, these compounds affected a step early in lipid biosynthesis.

The pathway from acetate to palmitic acid (actually a palmitic acid-acyl carrier protein complex) involves at least nine enzymes: acetyl CoA synthetase, acetyl CoA carboxylase, and the seven enzyme fatty acid synthetase complex. We chose first to test the effect of these compounds on acetyl CoA carboxylase (ACCase) activity. There were several reasons to select ACCase as the

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Haloxyfop

Tralkoxydim

Figure 1. The structures of haloxyfop and tralkoxydim.

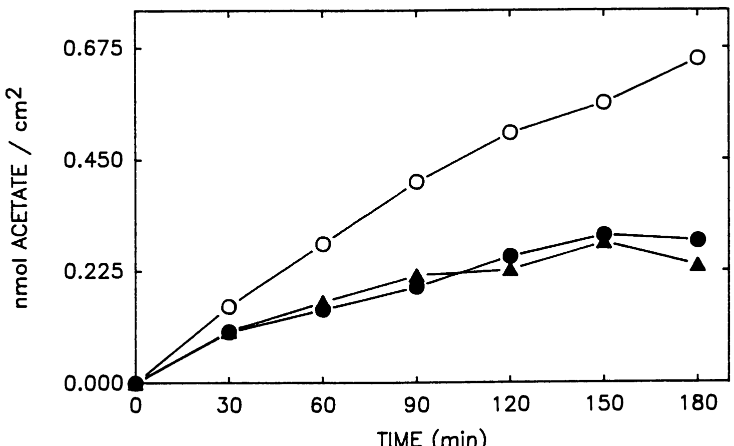

Figure 2. Comparison of control (○), 1.4 μM haloxyfop methyl ester (●), or 1.4 μM haloxyfop free acid (▲) on ^{14}C-acetate incorporation into lipids in maize leaf discs.

target. It has been reported that an aryloxyphenoxypropanoate herbicide can act as a hypolipidemic drug by reducing serum cholesterol and triacylglyerol levels in animals (17). Some hypolipidemic drugs act by inhibiting ACCase activity (18). Thus, it was possible that aryloxyphenoxypropanoates acted in a similar mannner by inhibiting ACCase activity in plants. This is in contrast to a conclusion made by Hoppe and Zacher (19), who had suggested that ACCase was not the site of action of diclofop. Their reasoning was based on the observation that diclofop inhibited malonate incorporation into polar lipids in maize root tips. They assumed that malonate was esterified to malonyl CoA, which entered the fatty acid biosynthetic pathway. This reaction requires a thioesterase which, in many species, is less active than is a malonate decarboxylase, which converts malonic acid to acetic acid (20). Even if a malonate decarboxylase was not active, malonic acid would not necessarily be converted directly into malonyl CoA nor would malonyl CoA obligatorily be converted into fatty acids because there are several biosynthetic pathways utilizing malonyl CoA (20,21). There is even a question whether malonate is used at all as a precursor for fatty acid biosynthesis (22). For these reasons, we decided to test aryloxyphenoxypropanoates and cyclohexanediones on ACCase activity.

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Table II. The Effect of Tralkoxydim and Haloxyfop on ^{14}C-Acetate Incorporation into Palmitic Acid in Maize Leaf Discs

Treatment	^{14}C Incorporation (dpm ± sem)
Control	619 ± 95
1.4 μM Tralkoxydim	144 ± 13
1.4 μM Haloxyfop	297 ± 49

Inhibition of ACCase Activity

The activity of ACCase extracted from maize was inhibited by both tralkoxydim and haloxyfop acid in a concentration dependant manner (Fig. 4). The concentration that inhibited activity by 50% (I_{50}) was about 1 μM for both compounds. These values are similar to those recently reported by others (2,3). The methyl ester of haloxyfop was more than 100 fold less inhibitory than the free acid (Fig. 4), which is consistent with the methyl ester being deesterified in the plant. The data presented in Fig. 2 suggest that the deesterification occurs rapidly in leaf tissue.

Aryloxyphenoxypropanoates have a chiral carbon at the 2 position of the propanoate moiety of the molecule (Fig. 1). The

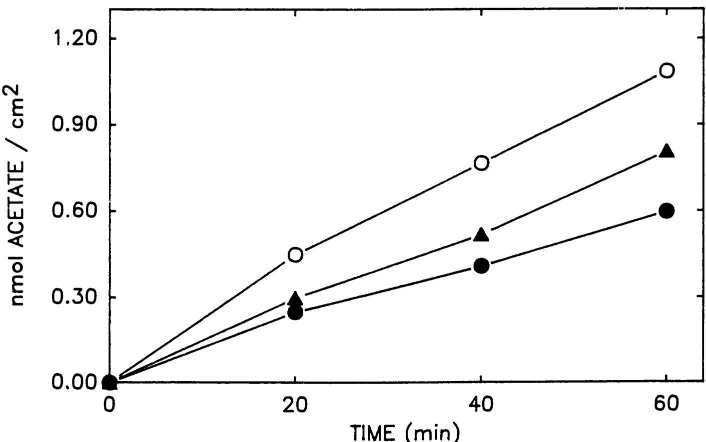

Figure 3. Comparison of control (○), 1.4 μM tralkoxydim (●), or 1.4 μM haloxyfop free acid (▲) on ^{14}C-acetate incorporation into lipids in maize leaf discs.

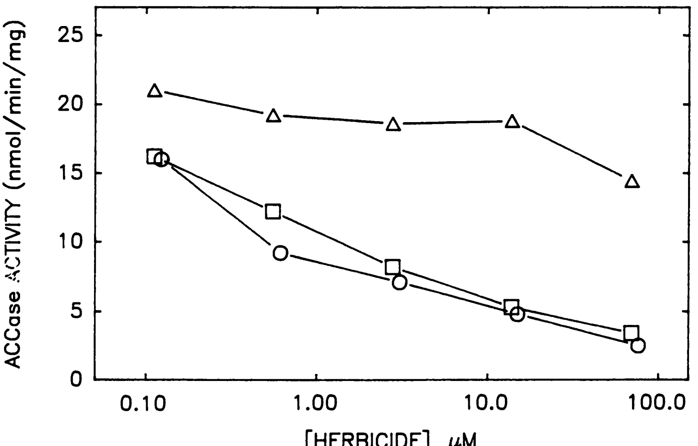

Figure 4. Effect of haloxyfop methyl ester (Δ), haloxyfop free acid (○), and tralkoxydim (□) on acetyl CoA carboxylase activity from maize.

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R(+) enantiomer is herbicidally active (23,24). Hoppe and Zacher (12) showed that the R(+) enantiomer of diclofop was more effective than the S(-) enantiomer in reducing acetate incorporation into free fatty acids in isolated maize chloroplasts. ACCase activity is inhibited by R(+) (98% enantiomeric excess) haloxyfop acid but not by the S(-) (94% enantiomeric excess) enantiomer (Fig. 5). The inhibition caused by the S(-) enantiomer could be accounted for by the 3% contamination in the S(-) preparation by the R(+) enantiomer.

A comparision of five aryloxyphenoxypropanoate herbicides shows that they are very active inhibitors of maize ACCase (Table III). The I_{50} values range from less than 25 nM for quizalofop to about 3 μM for fluazifop. The I_{50} values are not necessarily well correlated with whole plant activity. For example, diclofop has a higher recommended use rate than does haloxyfop, yet diclofop is about three times more active at the enzyme level. A reason for this discrepancy is that the outer benzene ring of diclofop can be readily hydroxylated in plant tissue to form a herbicidally less active molecule (25).

Table III. Effect of Five Aryloxyphenoxypropanoate Herbicides on Maize ACCase Activity

R-O-(phenylene)-O-CH(COOH)-CH_3

Herbicide	R	I_{50} (nM)
Fluazifop	CF_3-pyridyl (N)	2820
Haloxyfop	CF_3, Cl-pyridyl (N)	309
Diclofop	Cl, Cl-phenyl	130
Quizalofop	Cl-quinoxalinyl (N, N)	24
Fenoxaprop	Cl-benzoxazolyl (N, O)	108

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Resistant and Susceptible Species

Further evidence supporting our hypothesis that ACCase is the target site of aryloxyphenoxypropanoate and cyclohexanedione herbicides can be obtained by studying susceptible and tolerant species. Plants may be tolerant to herbicides due to several factors including reduced translocation, metabolic detoxification, and reduced active site sensitivity to the herbicide. We were interested in learning whether species tolerant to haloxyfop and tralkoxydim had ACCases that were less sensitive to inhibition than those from susceptible species. The effects of haloxyfop and tralkoxydim were tested on five species: one dicotyledoneous species, soybean, which is tolerant to both herbicides, and four monocotyledoneous species (1) red fescue (*Festuca rubra*), which is tolerant to both herbicides, (2) tall fescue (*Festuca arundi nacea*), which is susceptible to both herbicides, (3) wheat (*Triticum aestivum*), which is tolerant to tralkoxydim but susceptible to haloxyfop, and (4) maize, which is susceptible to both herbicides.

Whole plants were sprayed with a range of concentrations of the herbicides to determine the concentration needed to reduce growth by 50% (GR_{50}) two weeks after application. As expected, soybean and red fescue plants were tolerant to both herbicides, wheat was resistant to tralkoxydim but not to haloxyfop, and the other species were susceptible (Table IV). The I_{50} data for ACCase inhibition reflected well the whole plant data. Soybean was most tolerant to the herbicides at both the whole plant and enzyme levels whereas maize was the most susceptible at both levels. The only notable exception was for wheat which, as expected, was tolerant to tralkoxydim but its ACCase was sensitive to inhibition. Thus, wheat tolerance is not due to insensitivity of its ACCase.

Table IV. Effect of Haloxyfop and Tralkoxydim on Whole Plant Growth and ACCase Inhibition in Five Species

	GR_{50} (μM)		I_{50} (μM)	
Species	Haloxyfop	Tralkoxydim	Haloxyfop	Tralkoxydim
Maize	19	18	0.50	0.52
Wheat	83	>760	1.22	0.91
Tall fescue	133	225	0.94	0.40
Red fescue	1250	>6000	23.32	13.83
Soybean	>6000	>6000	138.50	516.72

Reversibility of Binding of ACCase and the Herbicides

Once that it was established that ACCase was a target site of the aryloxyphenoxypropanoates and cyclohexanediones, we began to investigate the relationship between the herbicides and the enzyme. Using a protein extract prepared as previously described (1) and further purified through a Sephacryl S-300 gel filtration column, we determined whether the inhibitors were covalently bound

to the enzyme. ACCase was incubated in a 7 μM herbicide solution and then assayed before and after passing through a desalting column (Sephadex G-25). As expected, the herbicides inhibited ACCase activity (Table V). After passage through the desalting column, ACCase was no longer inhibited by the herbicides. These results indicate that the herbicides are not covalently bound to the enzyme.

Table V. Effect of Desalting an ACCase-Herbicide Mixture on Enzyme Activity

	Enzyme Activity (nmol/min/mg)		
Treatment	Control	Haloxyfop[1]	Tralkoxydim[1]
15 min incubation at 35 C	254	72	64
Desalting	168	185	169
Haloxyfop[1]	43	-	-
Tralkoxydim[1]	31	-	-

[1]Herbicide concentration in assays was 1.4 μM.

There are many other questions that need to be addressed. For example: What are the kinetics of the inhibition? Do the different inhibitors bind at the same site? What are the molecular requirements for inhibition? What are the differences between susceptible and tolerant ACCases? and so on. ACCase purified 40 to 100 fold may not be sufficiently pure to answer many of these questions. For example, an extract purified on a Sephacryl S-300 column can have a specific activity up to 400 nmol/min/mg. We have observed that this preparation can catalyze the carboxylation of other short chained acyl CoA's in addition to acetyl CoA (Table VI). Both haloxyfop and tralkoxydim inhibit the carboxylation reaction regardless of whether n-propionyl CoA or acetyl CoA are substrates either individually or together (Table VII). At present, we are unsure whether n-propionyl CoA can be used as a substrate for ACCase or whether a n-propionyl CoA carboxylase is present in the preparation and the herbicides also inhibit that enzyme.

Table VI. Carboxylation of Different Acyl CoA Molecules

Substrate (0.3 mM)	Reaction Rate (nmol/min/mg)
Acetyl CoA	192
n-Propionyl CoA	59
n-Butyl CoA	32
Isobutyl CoA	0
Valeryl CoA	1

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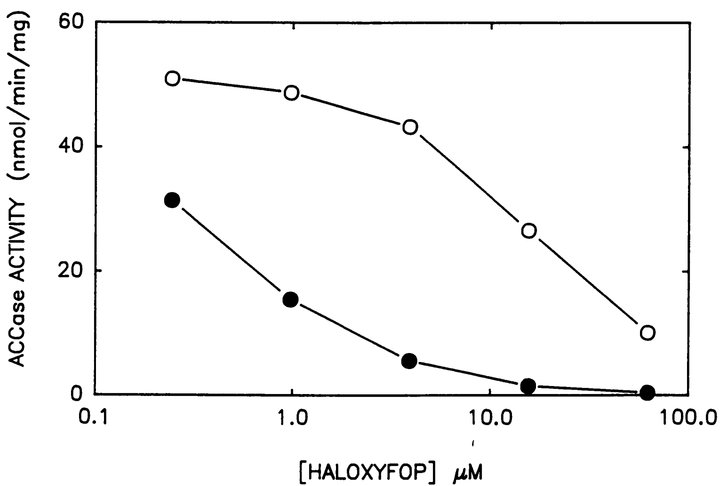

Figure 5. Effect of the S(-) enantiomer (○) and the R(+) enantiomer (●) on acetyl CoA carboxylase activity from maize.

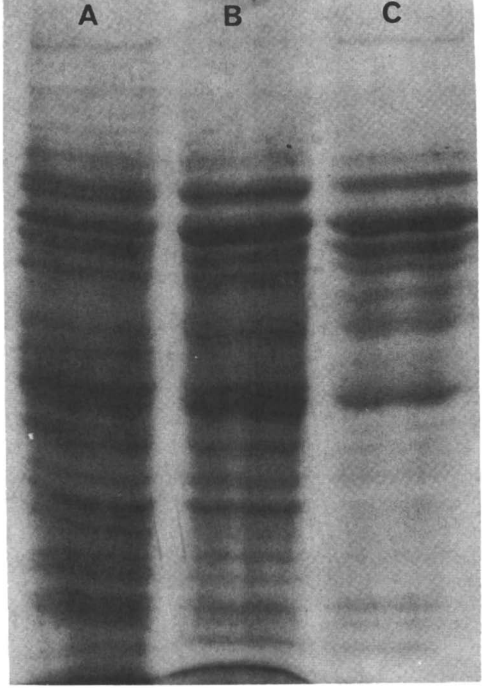

Figure 6. Results of SDS polyacrylamide gel electrophoresis on the supernatant of a crude maize extract (A), a 6 to 14 % polyethylene glycol precipitate (B), and pooled fractions having ACCase activity from a Sephacryl S-300 column (C).

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Table VII. Effect of Haloxyfop and Tralkoxydim on the Carboxylation of Acetyl CoA and n-Propionyl CoA

	Reaction Rate (nmol/min/mg)		
Substrate	Control	Haloxyfop[1]	Tralkoxydim[1]
Acetyl CoA[2]	86	30	24
n-Propionyl CoA	28	16	7
Acetyl CoA + n-Propionyl CoA	88	35	23

[1]Herbicide concentrations were 1.4 μM
[2]Acyl CoA concentrations were 0.25 mM

To examine the purity of the protein that was eluted from a Sephacryl S-300 column, we subjected the protein to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then performed a Western blot (26) using avidin linked to phosphatase as a probe. The SDS-PAGE indicated that there were many polypeptides in this partly purified preparation (Fig. 6). Among these were two major and several minor biotin-containing bands (Fig. 7). This preparation may contain propionyl CoA carboxylase or a partly degraded form of ACCase that can use propionyl CoA as well as acetyl CoA as a substrate. Therefore, the enzyme data presented here are quali-

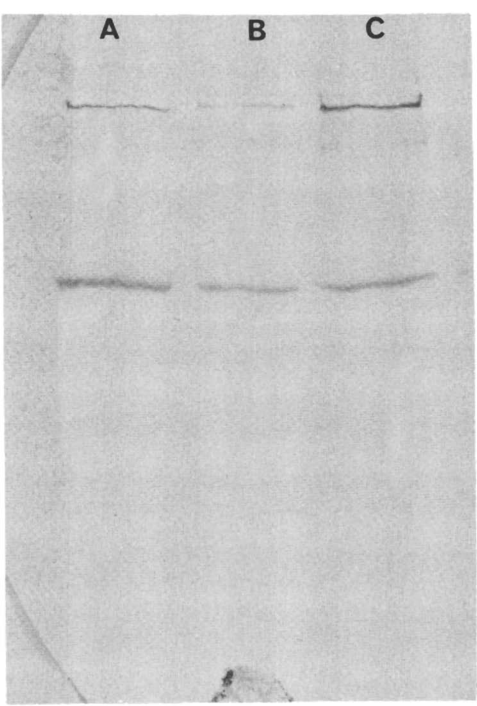

Figure 7. Western blot showing proteins containing biotin. The lanes correspond to the lanes in Figure 6.

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tative and further purification of the enzyme is needed to describe quantitatively the interaction of these herbicides with ACCase.

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Chapter 19

Inhibition of Acetolactate Synthase by Triazolopyrimidines

A Review of Recent Developments

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ALS is the first common enzyme in the biosynthetic route to valine, leucine and isoleucine. It is the site of action for the triazolopyrimidine (TP) herbicides as well as the sulfonylureas (SU) and imidazolinones (IM). These compounds act on the meristem and are slow to bring about plant death. Hence the opportunity to use metabolism of these herbicides to impart crop selectivity has been exploited successfully.

Additions of valine, leucine, and isoleucine were found to relieve the growth inhibitory effects of TP on Bacillus cell cultures, soybean cell cultures, and Arabidopsis seedlings. ALS isolated from a number of sources was found to be sensitive to TP at nM levels. The barley enzyme has been amenable to purification. A purification procedure that gives >60 % recovery and 235-fold purification is described. The interaction of TP with ALS is non-covalent and the inhibition is of a mixed type with respect to pyruvate. Like IM and SU, TP is a slow, tight binding inhibitor with a much greater affinity for the steady state complex than the free enzyme. Mutants of tobacco and soybean resistant to TP have been isolated in tissue culture. Initial analysis suggests ALS in these mutants is desensitized to TP.

Acetolactate synthase (ALS, EC 4.1.3.18) is the first common enzyme in the biosynthetic route to the branched chain amino acids, valine, leucine and isoleucine. It is the primary target site of action for at least three structurally distinct classes of herbicides, the imidazolinones (IM), sulfonylureas (SU), and triazolopyrimidines (TP) (Figure 1). SU and IM were discovered in greenhouse screening programs whereas TP was subsequently targeted as a herbicide. Numerous substitution patterns can be incorporated into the basic structure of all three classes of herbicides to provide crop selectivity as well as broad spectrum weed control. This is amply demonstrated in the seven products based on SU and four based on IM already in the market. A number of others are in various stages of development. The rapid success of ALS inhibitors as herbicidal products has attracted a world-wide research commitment. Not since the photosystem II

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inhibitors (1), has a single mode of herbicide action had such potential to reshape weed control chemistry and technology.

SU and IM (Figure 1), are proprietary chemistry of DuPont (2,3) and American Cyanamid (4), respectively. The substituted 1,2,4-triazolo[1,5-a]pyrimidines (TP, Figure 1) are a new class of herbicides under development at Dow Chemical Co. (5). A number of reviews have already appeared on the biology and biochemistry of SU (6-10) and IM (11,12). Hence, the focus of this article has been on the work done at our premises on TP. Wherever appropriate, our results have been compared with those of SU and IM.

Herbicidal Characteristics of TP

The inhibition of the primary target, ALS, results in a number of distinctive whole plant symptoms. Hence, the herbicidal biology of TP is strikingly similar to that exhibited by SU and IM. Growth of sensitive species is retarded within a matter of hours of application although visible effects may not be observed for several days (for an account of observations with IM and SU see references 12 and 13, respectively). Symptoms appear first in the upper meristematic regions of the plants as chlorosis and necrosis. The upper new leaves frequently take on a wilted appearance. The effects then spread to the remaining parts of the plant. In many species including most legumes and the Panacoid grasses, there is a clear reddening of the midrib and leaf veins. Complete desiccation of the plant may occur in 7-10 days under ideal growing conditions but may take up to 6-8 weeks under lesser conditions.

The actual lethal event resulting from inhibition of ALS by TP, SU, or IM has been the subject of debate. Although the plant is deprived of valine, leucine and isoleucine as a consequence of enzyme inhibition, protein synthesis continues at normal rates even after plant growth has begun to slow (12,13). Accumulation of one of the substrates of ALS, 2-ketobutyrate, has been implicated in the cause of death in microbes (9,14). This substrate has also been shown to undergo transamination to the corresponding amino acid in *Lemna minor* (15). However, the toxicity due to this keto acid has not been clearly demonstrated in higher plants. One of the earliest biological responses of SU treatment of plants is the inhibition of cell division (13). It is not clear how this is related to ALS inhibition. While there is consensus among workers that ALS is the primary target site for these herbicides, the actual biochemical cascades leading to death remain equivocal.

Since TP, SU and IM are slow to bring about plant death, there are significant opportunities to exploit metabolism of the herbicide to influence crop tolerance. Metabolism has indeed been the overriding parameter determining crop selectivity (5c,16,17). ALS inhibiting herbicides in development and/or full commercialization are known to have selectivities to all the major crops including corn, soybeans, wheat, barley, rice, cotton and canola.

Mode of Action of TP

Several analogs of TP at 1500 ppm cause a growth lag of 25-50 hrs of *E. coli* (K-12) growing in minimal media. The growth lag was completely abolished with the addition of casamino acids or a mixture of

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valine, leucine and isoleucine to the media. None of the other amino acids were effective in alleviating the growth lag. The growth of Bacillus subtilis was also completely inhibited by TP but at concentrations as low as 80 ppm. Again, the growth inhibition was nullified upon addition of all the three branched chain amino acids (Figure 2). Both chlorsulfuron (CS), a SU and imazaquin, an IM, produced identical effects to TP on the above two bacteria. The difference in the effect of these herbicides on the two bacteria can be attributed to the genetic regulation and isozyme patterns of ALS in the two organisms. It is well documented that E. coli has an isozyme (ALS I) that has very low sensitivity to SU (18).

Concentrations as low as 6 ppb of TP completely inhibited soybean suspension cultures and Arabidopsis thaliana. Consistent with the observations on bacterial cultures, the presence of valine, leucine and isoleucine in the media completely reversed the growth inhibition (Figures 3 & 4). In the case of Arabidopsis, the seedling growth in the presence of TP was proportional to the concentration of the three amino acids in the medium. A growth level equal to the control was achieved at an amino acid concentration of 0.35 mM (Figure 4). Collectively, the growth studies support that the primary mode of action of TP in microorganisms and plants is the disruption of branched chain amino acid biosynthesis.

The verification of the mode of action of TP followed the logic established in studying SU (18-21) and IM (22-24). Although originally thought to inhibit plant growth by arresting cell division (13,25), subsequent experiments using bacteria (18), excised pea root tips (19) and pea seedlings (19) clearly proved that SU blocked the biosynthesis of the branched chain amino acids. Likewise, growth studies with corn cultures and corn plants revealed the mode of action of IM (22-24).

Inhibition of ALS by TP

The requirement for all three branched chain amino acids to nullify the inhibitory effects of TP suggested that the target enzyme is ALS. ALS was isolated from barley seedlings as a 0-33% Ammonium Sulfate precipitate and examined for inhibition by TP. It is apparent from Figure 5 that the enzyme is very sensitive to the compound. The I(50) value (concentration required for 50% inhibition) was calculated to be 0.047 uM. This value is within the range reported for CS tested against ALS from different species (19). Imidazolinones are less potent with I(50) values in the range 2-12 uM (26). ALS isolated from several species and their I(50) values for TP is shown in Table I.

Table I: Inhibition of ALS from different species by TP

Source of ALS	I(50) uM
Barley	0.046
Arabidopsis	0.038
Tobacco Cultures	0.032
Cotton Cultures	0.036
Soybean Cultures	0.040

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Figure 1. Three chemical families known to exhibit herbicidal activity through the inhibition of acetolactate synthase. A. sulfonylurea (sulfometuron) B. imidazolinone (imazapyr) and C. A representative triazolopyrimidine.

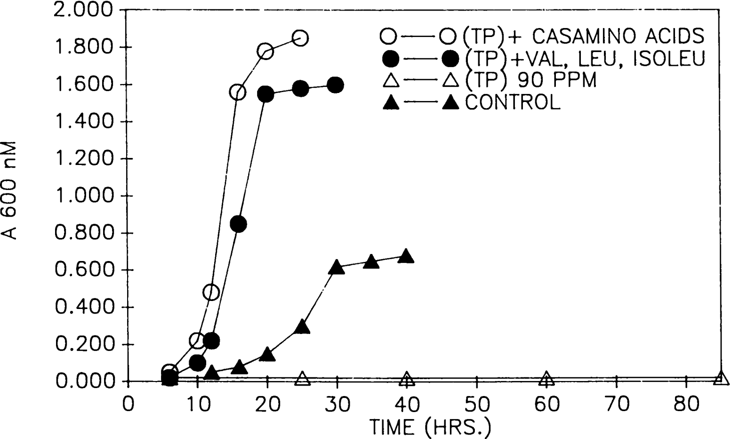

Figure 2. Inhibition of growth of <u>Bacillus</u> <u>subtilis</u> by triazolopyrimidine; reversal by valine, leucine and isoleucine.

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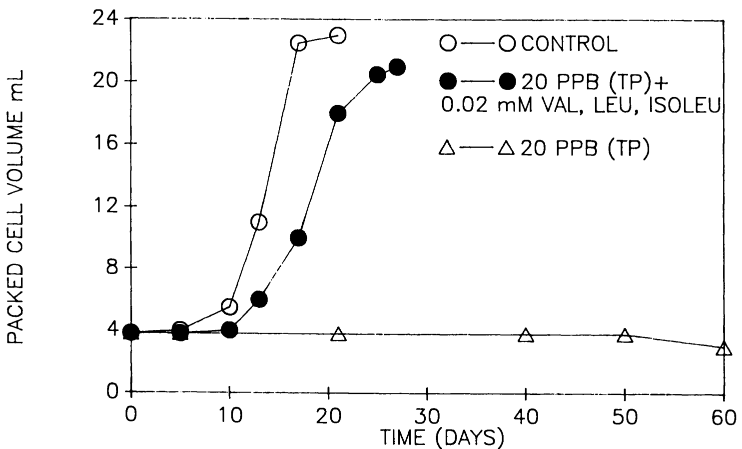

Figure 3. Effect of triazolopyrimidine on soybean suspension cultures.

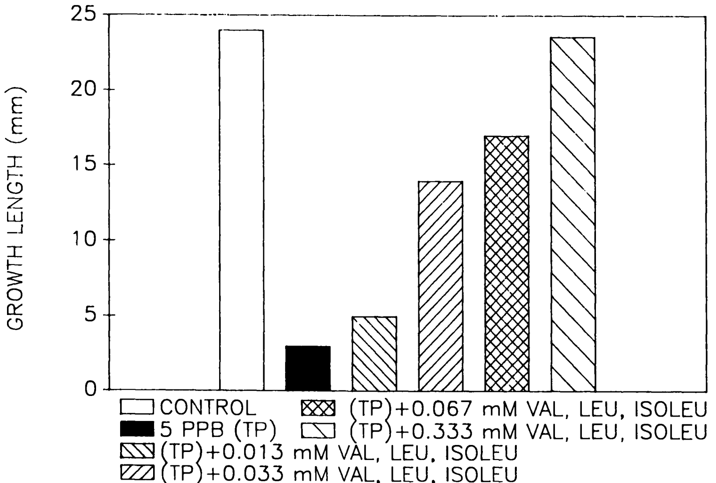

Figure 4. Effect of triazolopyrimidine on the growth of Arabidopsis thaliana seedlings.

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ALS was assayed by quantifying the amount of acetoin formed from acetolactate (27). The enzyme from seedlings were assayed at pH 7.15 and that from cultures at 8.3. ALS from a number of higher plants has been readily extracted and assayed (19,22). However due to the instability of the enzyme, there is no efficient procedure for its purification. Recently, Muhitch et al (28) described a four step purification procedure for ALS from maize suspension cultures. This method yielded enzyme preparation with high specific activity, but the recovery was low. We have examined ALS from different species in order to identify the best source for purification and the results are given in Table II.

Table II: ALS Activities from Various Plant Sources

ALS Source	Specific Activity (SA)		
	Crude Extract	Ammonium Sulfate ppt	
		0-35%	35-50%
Soybean Cultures	87.8	N.D	865
Tobacco Cultures	420	1121	1004
Morningglory Cultures	10	-----N.D-----	
Alfalfa Sprouts	50	289	50
Mungbean Seedlings	60	420	87
Barley Seedlings	8	1200	20
Cotton Cultures	N.D	460	880
Arabidopsis Seedlings	N.D	800	22

SA in Table II = nmoles/hr/mg protein and N.D = not detectable. Tobacco, soy cultures and barley seedlings were the best source of ALS, both in terms of specific activity and total units. The enzyme preparations from all sources were unstable in buffer solutions in spite of protective thiol agents. The inactivation of ALS in the crude extract of tobacco showed a distinct biphasic kinetics, implying the presence of at least two isozymes (unpublished observations). The presence of two ALS genes in tobacco (29) and at least three in microorganisms (18) has also been noted by other workers. ALS from barley was most amenable to purification. Table III gives a profile for the rapid purification of this enzyme with high recovery.

Table III: Purification of ALS from Barley Seedlings

Step	Volume (ml)	Protein (mg/ml)	Total Units	S.A
Crude Extract	620	2.1	91.1	0.07
0-33% AS ppt.	10	8.8	106.8	1.213
P2 Column (desalting)	14	6.0	117.6	1.40
Mono-Q-HPLC	2.2	5.5	82.32	6.8
Phenyl Agarose	2.1	1.69	58.8	16.6

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In Table III, units have been defined as the amount of protein required for the formation of 1 umole product/min. The activity of the enzyme in the crude extract was rather low accounting for an anomalous (>100 %) recovery in the early stages of purification. The overall recovery of the enzyme was >60 % with a 235-fold purification. The final preparation, however, was not homogenous. Barley has two isoforms of ALS which can be separated on a phenyl agarose column immediately after the ammonium sulfate precipitation. One of the forms does not bind to the column and was too unstable to attempt purification. The details of purification in Table III pertain to the fraction with affinity for phenyl agarose.

The ALS isolated as described in Table III displayed typical Michaelis-Menten kinetics with respect to pyruvate with a Km of 2.44 mM. Substrate concentrations as high as 50x Km had no effect on the rate of the reaction. Thiamine pyrophosphate, FAD and Mg(2+) were an absolute requirement for catalysis by the purified enzyme. These properties are consistent with observations made by others (30). Optimum activity was obtained at pH 7.1 and 37C, which were also the best conditions for inhibition by TP. There was no significant difference in the I(50) value of TP whether ALS was taken after step 2 or 5, indicating low potential for non-specific binding of the herbicide to other proteins.

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Interaction of TP with ALS

The interaction of all of the herbicides shown in Figure 1 with ALS is non-covalent (5b,28,30). Radiolabelled TP can be quantitatively separated from barley ALS in a gel filtration column, with complete restoration of activity (data not shown). None of the three classes of herbicides bear any structural similarity to the substrate or cofactors of ALS. This is also reflected in the kinetics of TP inhibition with respect to varying pyruvate, shown in Figure 6. The pattern is suggestive of a linear mixed type of inhibition. We have not examined the mode of inhibition with respect to varying thiamine pyrophosphate or FAD. SU have been reported to be a competitive inhibitor of ALS from Salmonella typhimurium (with respect to pyruvate, 30), and an uncompetitive inhibitor of the enzyme from Methanococcus (31). IM is also an uncompetitive inhibitor of corn ALS (26). The time course of ALS inhibition by the three classes of compounds (Figure 1) is distinctly biphasic (5b,28,30) with progressively decreasing enzyme activity. There is at least a 5-10 fold difference between the initial and the final steady-state I(50) value (5b,18,28,30). TP, like the SU and IM, are slow, tight binding inhibitors with a greater affinity for the steady state complex of ALS than for the free enzyme (30). The interaction of SU and ALS II from S. typhimurium has been examined in detail. Based on kinetics and spectroscopic evidence, Schloss et al (30) have proposed that the inhibitor binds tightly to ALS-FAD-TPP (decarboxylated)-Mg(2+)-pyruvate complex, at the site of the second keto acid.

Relationship between the binding sites of TP, SU, and IM

The similarity in the herbicide biology and biochemistry of the above structurally distinct compounds is interesting as well as intriguing. One of the key questions is whether these inhibitors compete for the

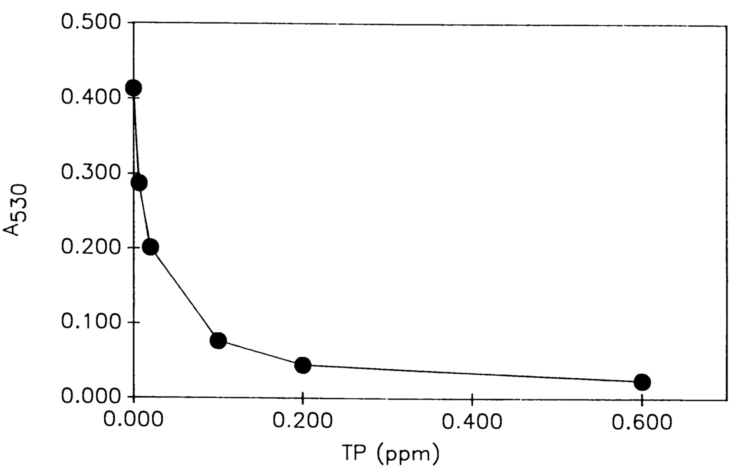

Figure 5. Inhibition of barley acetolactate synthase by triazolopyrimidine.

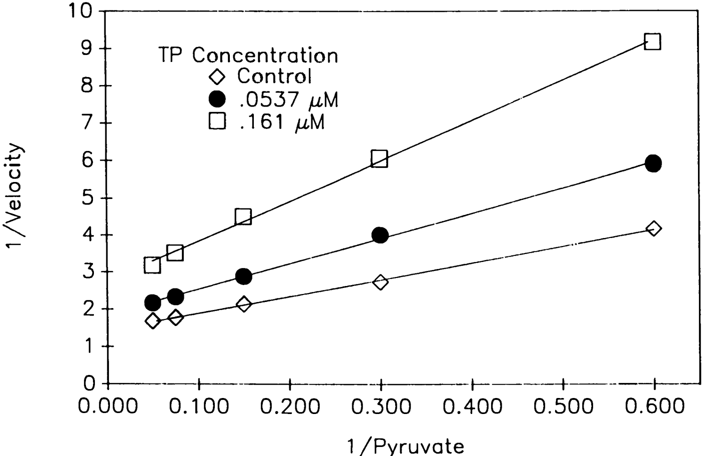

Figure 6. Kinetics of inhibition of barley ALS by triazolopyrimidine; varied substrate = pyruvate.

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same site on ALS. In order to address this question, we isolated several mutants of tobacco, 10-100 fold resistant to growth on TP compared to the wild type (which succumbed at 6.5 ppb). Growth of a number of mutants was comparable to the control. Initial analyses of the mechanism of resistance revealed that most mutants had ALS desensitized to TP (new data, manuscript in preparation). A few of them had susceptible enzyme but clearly tolerated higher concentrations of the compound. These are suspected to have enhanced metabolism of the herbicide. Among the 15 mutants examined, all were cross resistant to SU (chlorsulfuron) as well as IM (imazaquin). Many of these mutants remain to be characterized. However, our preliminary results suggest that all three classes of herbicides have highly overlapping binding (inhibitory) sites on ALS. Cross resistance between SU and IM has already been documented by others (32). Recently, Schloss et al (33) showed that IM and TP were able to quantitatively displace a radiolabelled SU herbicide from ALS, indicating competitive binding. Curiously, the SU ligand was also displaced by the quinone, Qo. It was proposed that SU, TP, and IM bind to ALS in a vestigial quinone binding site associated with the evolution of ALS from pyruvate oxidase. This enzyme is an FAD-protein that catalyzes the oxidation of pyruvate to acetate. The reduced FAD is regenerated via electron transfer to a quinone. Similarly, ALS is a flavoprotein but its reaction (condensation of two keto acids) does not involve a net redox change. Schloss et al (33) have suggested that these two enzymes share a common evolutionary heritage and that the quinone (herbicide) binding site on ALS has been conserved due to the constraints of the catalytic mechanism. While this is an attractive theory, the water soluble quinone Qo is a relatively weak inhibitor of barley ALS (I(50) = 30 uM in our studies) compared to SU and TP. Further, the quinone, Q6, does not inhibit barley ALS even at 200 uM. Finally, it is not known if the three classes of herbicides inhibit pyruvate oxidase.

Recently, Shaner et al (34) reported a mutant maize line resistant only to IM. Similarly, McDevitt et al (35) have also shown that site directed substitution of different amino acids at five different loci on the ALS sequence resulted in specific resistance to SU herbicides. These studies suggest that IM and SU herbicides have non-overlapping binding domains on ALS. We have not examined the several TP resistant mutants for specificity of resistance.

The genetics of SU resistance has been extensively studied. Mutants that are tolerant to SU have been isolated in bacteria (36), yeast (36), algae (37), haploid cultures of tobacco (38,39) as well as *Arabidopsis* (40). In all of the cases examined, ALS was found to be 100-fold less susceptible to the herbicide (36-40), compared to the wild type enzyme. A similar change in the sensitivity of ALS has been reported for IM resistant corn cultures (11). Plants regenerated from resistant tobacco cultures fully retained the trait, and transmitted it as as a dominant or a semi-dominant nuclear gene (39). The molecular basis of SU resistance has been characterized in microorganisms (36) and plants (41,42), by sequencing both the wild type and mutant ALS genes. Comparisons showed a single nucleotide difference that translated into one amino acid substitution at the enzyme level. This change however, was not the same in all resistant organisms. For example, in *E. coli*, valine was substituted for alanine (36) and in yeast, serine for proline (36). In tobacco,

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mutations in both the ALS genes conferred resistance to SU (41). Introduction of resistant ALS gene into wild type resulted in SU resistant transgenic plants (43). Collectively, the genetic studies offer unequivocal evidence that ALS is the sole primary target for the three classes of herbicides.

Other applications for herbicide resistant mutants are imminent. SU resistant tobacco plants are expected in field trials. The most advanced in this new approach to crop selectivity are IM resistant corn lines. These plants are reportedly in breeding trials with Pioneer Seed Co., to transfer the trait into high yielding hybrid lines. Success in this task will not only expand market opportunities for commercial imidazolinones, but also allow corn production in areas where imidazolinone carry-over may be a concern. A number of other crops engineered for herbicide resistance (either due to altered target site (32,44), or as a result of rapid herbicide metabolism (45), are at or near field trials (44,46). Engineering resistant crops is quickly emerging as an alternative approach to achieving crop selectivity.

Another major application of herbicide resistance is its utility as a selectable marker. Like antibiotic resistance in bacterial transformation, herbicide resistance should prove extremely useful for selecting transformants that are insect resistant, disease resistant, or engineered for other non-selectable traits. Many of these herbicide resistant markers have already been integrated into plant cloning vectors (32,43).

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Chapter 20

Plant Enzymes in Resistance to Insects

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During the last several decades, traditional approaches to host-plant resistance have shown that a number of plant natural products act as constitutive bases of resistance against insects. More recently, it has been realized that plant resistance against insects also consists of inducible and dynamic elements triggered by insect-feeding damage. These inducible elements of resistance have been found to be both plant enzymes and their end-products. The potential use of such enzymes and their products as bases of resistance against insects is discussed.

The field of plant resistance to insects has been dominated for the last few decades by the idea that specific constitutive characters confer resistance (1-5). The greatest attention focused on organic molecules (MW < 2000) which directly interfere with the insect's behavioral, physiological, and/or biochemical processes. The breeding of resistant plants is usually accomplished by selecting for plants with enhanced constitutive levels of the chemical agent (3,4). The plant response to insect feeding was generally considered passive or static because of the assumed constitutive nature of defense. Concern for the dynamics of production of these characters was mainly limited to ensuring a plant phenology consistent with the stage(s) of the insect to be controlled. Also, the plant's enzymatic processes responsible for the formation of chemical defenses were of secondary importance, for inbreeding, acquisition of phenotype supposes genotype. Hence, the role of plant enzymes in insect resistance is comparatively less defined.

More recently it has become apparent that plants respond dynamically at a metabolic level to insect feeding, and that these induced responses can be of sufficient diversity and intensity to confer resistance (6-11). Many of these responses in the plant's chemical composition are identical to those induced as a result of attack by microorganisms (9,12-14). For example, insect feeding

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can induce the de novo production of isoflavonoids, furanoterpenoids, and proteinase inhibitors and can elevate constitutive defenses such as phenolics, curcurbitacins, quinones, and terpenes (6,9-11).

Obviously, these induced changes involve activation and/or alteration of enzymatic activity. Although such changes are often a first line of defense against invasive organisms, their impact on these organisms is not predictable. Entomologists are just beginning to understand the relationships among these enzymatic changes, the subsequent metabolic end-products, and how to utilize these inductive processes to confer insect resistance. Although the field of plant pathology has provided a detailed biochemical understanding of these inductive processes (12-16), entomologists must still a) verify that insect feeding simulates the physical stress caused by invading microorganisms, b) determine if the insect induces changes unique to the specific plant/insect interaction, and c) understand the impact of the changes upon the specific insect. Thus, a detailed knowledge of the behavioral, physiological, and metabolic susceptibilities of insects to plant natural products is essential in order to effectively employ inducible or activatible plant resistance.

We will discuss selected current knowledge on the use of plant enzymes as bases of insect resistance; the review is not comprehensive. Particular attention will be given to induced *de novo* synthesis of proteinase inhibitors in the tomato plant *Lycopersicon esculentum*, and on the simultaneous use of plant polyphenol oxidases as a means of conferring resistance to insect pests.

The relevance of such use of enzymes to agriculture will be examined in terms of: a) feasibility in crop resistance, b) compatibility with the concurrent use of biological control agents in insect pest management (IPM) systems, c) compatibility with products of gene transfers, and d) compatibility with human health.

Rationale for Employment of Plant Enzymes

For simplicity, we only consider those enzymatic processes in annual plants that immediately follow insect feeding and whose consequences affect the insect within seconds up to 24 hours after inflicting feeding-damage upon the plant. A discussion of long-term inductive processes in perennial or biennial plants is germane to a comprehensive understanding of resistance (8,10) but is beyond the scope of this paper.

Plant enzymes can confer resistance by perturbing the utilization by an insect of chemicals (agonists = kairomones) that are essential in utilizing its host plant. The perturbation arises from plant enzymes creating chemicals (antagonists = allomones) that are inhibitory to insects, or removing chemicals (agonists) that are beneficial. These perturbations can be caused by several general mechanisms that proceed when plant cells are broken by the feeding insect and during ensuing masticative and digestive processes. Thus, enzymes may act in the following manners:

Direct production of antagonists wherein an enzyme converts a chemical to a more biologically active form.

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Indirect production of antagonists wherein an enzyme acting on a substrate liberates a chemical messenger that triggers the *de novo* synthesis of a new and biologically more active chemical.

Direct removal of agonists wherein an enzyme converts a chemical to a less biologically active form.

Indirect removal of agonists wherein an enzyme liberates a product which reacts with a second chemical rendering the last less biologically active.

Direct action of enzyme wherein an enzyme acts directly against the insect as a substrate.

Direct Production of Antagonists

Many plant products are stored within the plant in bound form, e.g., as glycosides, esters, or peptides, presumably to avoid autotoxication and to enhance solubility and storage (2,17). These compounds may affect herbivores, but very often their biological activity is magnified by conversion to unbound forms via enzymes of plant or herbivore origin. The plant enzyme responsible for conversion is usually separated from its substrate by compartmentalization, and it is only when plant tissue is damaged by an invading organism that the substrate and enzyme meet and the activation ensues. Examples of such binary chemical systems are given below.

Glycosidases and Thioglycosidases. Of the glycosides produced by plants (e.g., iridoids, saponins, phenolics, cardenolides, and alkaloids), perhaps the best studied in terms of influence upon insects and other animals are the cyanogenic glycosides and the glucosinolates (2,17-19).

Over 2000 species of plants are known to be cyanogenic (20). The production of HCN and an aldehyde or ketone is mediated by two enzymes, a β-glucosidase and a nitrile lyase (Figure 1a) which mix with cyanogenic glycosides during cell disruption. It is usually assumed that the mode of action of HCN against insects is via inhibition of oxidative respiration (21-23). Unfortunately, this assumption must be checked, for some species are known to be insensitive to cyanide as a result of detoxicative processes or target site insensitivity (18,22-25). It is equally reasonable to suppose that the aldehyde or ketone (benzaldehyde in the case of prunasin) rather than HCN is the toxic agent (26,27). The cyanogenic glycosides themselves are not toxic to most organisms; the presence of a plant or animal β-glucosidase is the minimal prerequisite for release of active agents. Many plant species are known to be polymorphic in their ability to produce HCN (18), because even though they may synthesize cyanogenic glycosides certain polymorphs are acyanogenic because they lack β-glucosidase activity. Hence, any attempt to breed for cyanogenesis as a basis of resistance should consider the well-described genetic systems regulating the many enzymes involved in the biosynthesis and degradation of cyanogenic glycosides (18,28).

Cyanogenesis is thought to be a plant defense against unadapted, chewing insects (26,27,29-32). The mechanisms of cyanide-

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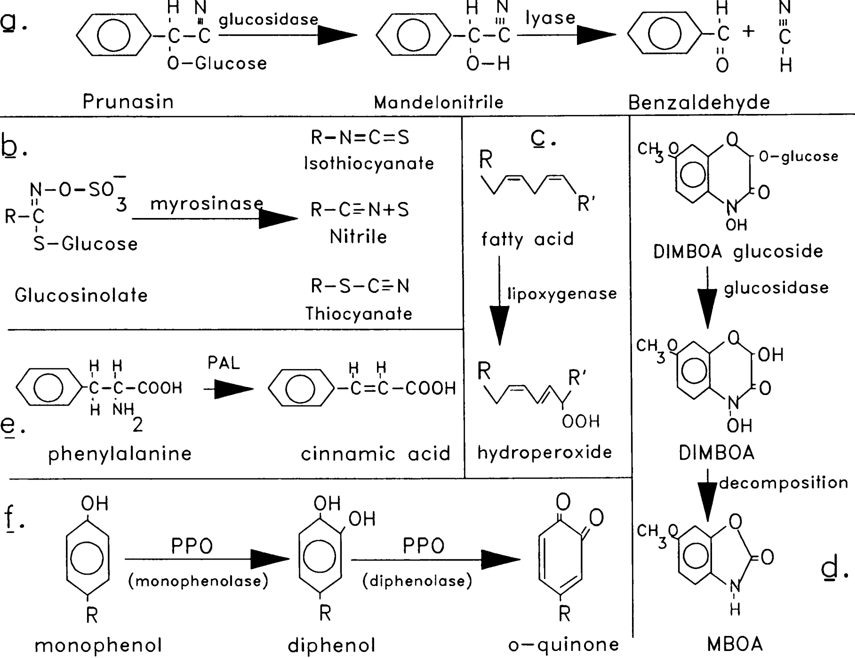

Figure 1. Major Products of Some Enzymatic Reactions. PPO - polyphenol oxidase; PAL - phenylalanine ammonia lyase.

based resistance against them are poorly known, although interference with feeding behavior and toxicity has been implicated. However, cyanogens are usually ineffective against many insects that utilize cyanogenic plants as hosts because they are metabolically counter-adapted to circumvent toxification (18,22,24), and against sucking insects that feed on vascular fluids where cyanogens are absent (33). Based on current knowledge, our ability to direct cyanogenesis as a force of resistance against key insect pests and disease-causing organisms is limited (17,34).

A parallel situation is found in plants, particularly in crucifers, that store mustard oil glucosides. There the action of myrosinase (= thioglycosidase; Figure 1b) on the glucoside, followed by spontaneous rearrangement of the aglycone, liberates several products, nitriles, thiocyanates, and isothiocyanates (35,36). Such products (e.g., allylisothiocyanate) are often considerably more toxic to non-adapted insects than to insects that are evolutionarily adapted to crucifers as host plants (17,37). In fact, many insects that are adapted to crucifers utilize mustard oils as ovipositional and/or feeding stimulants, but often show a preference to oviposit on plants with low glucosinolate levels (38). Hence, it is theoretically possible to use high levels of glucosinolates accompanied by high myrosinase activity to restrict attack of crops by both adapted and non-adapted insects.

The use of glucosinolates as bases of resistance against insects seems consistent with their role in disease resistance (39). However, with crops possessing high levels of cyanogens or glucosinolates to effect resistance, the value of these crop plants as food for humans, livestock, and/or poultry is often greatly depreciated because of the toxicity of the end-products (35,36,40).

Another example of direct production of antagonists is the presence of the glucoside of 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one (DIMBOA) in graminaceous plants, confers resistance to corn borers and aphids (41,42). As is true for cyanogenic glucosides and mustard oil glycosides, DIMBOA glucoside is not the active agent, but following exposure to glycosidase activity it is hydrolyzed (as shown in Figure 1d) to form DIMBOA and MBOA (6-methoxy-1,3-benzoxazolinone), of which DIMBOA is thought to be the primary active agent (42,43). The role of DIMBOA in plant resistance to fungal disease is not well established, but the study of natural benzoxazinone compounds has proven invaluable in the development of synthetic fungicides such as benomyl (16).

Little else is known about how glycosidases can be used to enhance resistance to insects. Several potential uses come to mind: the activation of certain coumarin glycosides to more active photodynamic agents, for insects are known to be sensitive to such chemicals (44,45); the rapid hydrolysis of hydrolyzable tannins to their aglycones (e.g., gallic acid), which are toxic to a variety of organisms including insects (46-48); and the hydrolysis of phenolic glycosidases to enhance their toxicity (17).

However, the activity of other phytochemicals such as saponins against insects and other organisms depends upon the integrity of the 3-*B*-glycosidic linkage (49). Rapid hydrolysis of this linkage by plant enzymes during insect feeding and digestion could be counter-productive.

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Ureases and Liberation of Ammonia. The release of ammonia from urea by urease provides abundant substrate for the efficient assimilation of nitrogen in many plant species (50). In addition, foliage naturally releases ammonia via urease activity. The amount of ammonia released can be substantial (e.g., > 100 umoles/hr/gm fr. wt. in bean species of *Canavalia*; 50), and seems sufficient in some plants to compromise insects. However, we are unaware of any information showing that urease activity in plant tissues contributes to resistance against insects. The possibility of utilizing this as a mechanism of resistance seems promising because ammonia is acutely toxic to certain insects (25). Furthermore, optimal urease activity occurs near or slightly above the midgut pH of many insect herbivores (50), thereby facilitating release of ammonia. However, some insect species have adapted metabolically to handle a high level of dietary ammonia and even use it to advantage (51-53). Hence, the value for resistance may be primarily against non-adapted insects.

Polyphenol Oxidases. Plant trichomes and their exudates confer resistance to a variety of insects (54-56). In solanaceous plants, such as the tomato and potato, trichomes contain polyphenol oxidases and catecholic phenolics (e.g., caffeic and chlorogenic acids), which contribute to resistance to a variety of insect pests. In the potato plant, the polyphenol oxidases and phenolics are separated in different trichomes. When insects, such as aphids or leaf hoppers, walk across the surface of the plant they break the two types of trichomes. Trichomal fluids are liberated and, upon mixing, polymerize as a result of polyphenol oxidase activity on catechols, forming an often lethal adhesive trap for the insects (57,58). In tomato plants, the polyphenol oxidase and chlorogenic acid are separated by intracellular compartments, but upon breakage of trichomes by insects, polymerization and physical entrapment occurs (54).

Many other plant enzymes (e.g., lipoxygenases, peroxidases, and oxidases) are able to convert their substrates to more biologically active molecules (e.g., aldehydes, peroxides, epoxides; 59,60), which may disrupt insect sensory perception and/or metabolism. However, these enzymes can also depreciate the quality of food so that it is toxic or undesirable to animal and human consumers (19,61), so the application of these enzymes to insect and/or disease resistance is not straightforward.

Indirect Production of Antagonists

We are concerned here with the action of enzymes acting on substrates to liberate chemical messengers that trigger the *de novo* synthesis of new and more biologically active chemicals. Such a train of biochemical events inherently has a longer inductive and biosynthetic time-frame than does, say, the near instantaneous liberation of HCN following damage.

Phenylpropanoid Metabolism and Phytoalexins. The induced synthesis of phytoalexins in many species of plants due to attack by

microorganisms or other physical factors is a well-described phenomenon (16,62-64). Among the best studied of the phytoalexins are the isoflavonoids. The localized de novo synthesis of these post-infectional defenses at the site of damage is initiated by the activation of phenylpropanoid metabolism at the level of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), which provide cinnamic acid and o-coumaric acid for the synthesis of a variety of phenolics, including isoflavonoids. The value of these phenolics as a defense against disease caused by viruses, bacteria, and fungi is well documented (16,30,62-64). Phenolics have also been widely recognized as defenses against insect herbivores (2,30,65) but the direct involvement of PAL and TAL as an inducible defense against insects is poorly understood (5). Recent evidence shows that PAL activity can be induced in soybean by damage caused by the feeding of mites (66) or insects (67). Both constitutive and inducible resistance in soybean cultivars to Mexican bean beetle was shown to be positively correlated with PAL and TAL activities (67,68). The causal factor(s) regulating resistance were not determined but were suggested to be phenylpropanoid derivatives. Considering the importance of plant phenolics as inducible and constitutive defenses against insect herbivores (69,70), and their known action against many pathogens, it is surprising that the roles of PAL or TAL in the production of these defenses against insects have not received more attention.

Chitinase, Polygalacturonase, and Proteinase Inhibitors. An array of polypeptides are produced de novo in plants when they become infected with various types of pathogens. Several of these "pathogenesis-related proteins", induced by fungal elicitors, have been identified as chitinases (71,72). Chitinases are, however, not restricted to infected plant tissues but may occur constitutively in healthy tissues (73-75) as well as in mechanically wounded plant tissues (74,76). The role of chitinase in plants is hypothesized to be a defense against fungal and bacterial pathogens (77,78) because of its ability to degrade or lyse fungal and bacterial walls. The oligosaccharide fragments released from fungal walls by chitinase induces the de novo synthesis of certain phytoalexins and proteinase inhibitors (11,79-81). Similarly, damage inflicted upon plants by invasive organisms releases endogenous pectinases which liberate oligosaccharides from plant and/or fungal cell walls that are elicitors of phytoalexin and proteinase inhibitor induction (11,80-83). Elicitors of fungal cell wall origin cause the massive accumulation of at least nine major proteins in Solanum tuberosum including chitinases and B-glucanases (71). These elicitors also induce lipoxygenases (84) and PAL (85). Such elicitor-induced changes may represent a generalized defensive response of the plant to damage by a variety of potential pests.

An understanding of the role of these induced responses in resistance against insects is in the inceptive stages (5,9,11). Phytophagous insects contain a variety of digestive enzymes capable of releasing oligosaccharide elicitors from plant cell walls (86). Also, insect exoskeleton and gut lining (peritrophic membrane) are chitinous (87); hence, fragments of insect chitin

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released into the plant by abrasion, salivation, and/or fecal contamination may, upon enzymatic hydrolysis, elicit a defensive response in the plant. It has been shown that the saliva of aphids is capable of releasing oligosaccharides from vascular tissue cell walls which may then prime the defensive responses of sorghum (86). We are unaware of any studies demonstrating that chitinase activity is induced by insects' feeding. However, it is well documented that feeding of noctuid larvae on tomato foliage is capable of inducing the tomato plant to systemically produce levels of proteinase inhibitors detrimental to larvae (11,88,89). The induction of proteinase inhibitors offers a promising form of host plant resistance; it will be discussed in more detail below.

Direct Removal of Agonists

We are concerned here with enzymes that convert chemicals from forms that are facilitative or essential to the insect's use of the plant to forms that are less active or utilizable. The use of "negative resistance" against insects is rare, despite the fact that it may strike directly against the insect's most essential needs.

Phenylalanine Ammonia Lyase (PAL). The standard approach for utilizing PAL and TAL in resistance is as part of a chain of events leading to the creation of toxic phenolic products (Figure 1e). However, PAL may be used in a different manner, that is by depriving the insects of essential nutrients. Insects generally have similar nutritional requirements as mammals (90,91) but have a particular requirement for high levels of aromatic amino acids (phenylalanine and tyrosine) for cuticle biosynthesis. The potential for PAL or TAL to deprive an insect of these essential amino acids has been overlooked. Phenylalanine, critical for the development of the locust *Schistocerca gregaria*, may be in short supply in free and protein-bound form in plants that are otherwise nutritive (92,93). Such a shortage can become a rate-limiting factor for the insect, thus we propose that PAL may be used in such a manner to enhance resistance. We have shown that free [^{14}C]-phenylalanine applied to tomato foliage is substantially converted to cinnamic and *o*-coumaric acids (Table I) during feeding and digestion by the larval noctuid *Trichoplusia ni*. This transformation does not occur in larvae feeding on artificial diet, indicating that the loss of phenylalanine results from the action of PAL and associated enzymes.

The impact of this loss upon insect growth has not been assessed, although considering Bernays' findings (92,93), this approach may be useful for resistance. Double indemnity may accrue if the insect is toxified by cinnamic acid and coumaric acid. Unfortunately, these phenolics were not toxic to the noctuid *Heliothis zea* (M.B. Isman, S.S. Duffey and G.W. Felton, unpublished data).

Lipoxygenases. Lipoxygenases catalyze the peroxidation of polyunsaturated fatty acids with a *cis,cis* 1-4-pentadiene moiety to form conjugated diene hydroperoxides (Figure 1c). Free fatty acids are formed following wounding from the deacylation of membrane

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Table I. Metabolism of Phenylalanine by Plant Enzymes in the Digestive Tract of *Trichoplusia ni*

Compound	% Total Excreted ^{14}-C
phenylalanine	13.5
cinnamic acid	34.3
coumaric acid	27.4
unidentified phenolic	24.0

phospholipids and galactolipids by hydrolytic acyl hydrolases contained in vacuoles. Free fatty acids normally occur at very low levels in healthy, intact plant tissues but are rapidly released from membranes by acyl hydrolases following tissue disruption (59). Fatty acids are essential nutrients for many insects (87,91). Lipoxygenases may deplete levels of linoleic acid and related unsaturated acids necessary for growth and reproduction in insects. Lipoxygenases occur in a variety of plant species and tissues (60) but the enzyme's role in resistance via this mechanism is unknown.

Indirect Removal of Agonists

We are concerned here with the enzymatic liberation of products that convert chemicals to less utilizable forms. The idea is to employ plant enzymes to strike directly at the insect's most critical needs, essential nutrients. In general, plant tissues are low in total nitrogen and pose a "nutritional hurdle" for insect herbivores (94). Plant proteins normally have low sulfhydryl and lysine content (95), which are nutritional requirements for insects (90,91). The nucleophilic properties of these limiting amino acids make them particularly susceptible to covalent binding by strongly electrophilic molecules such as *o*-quinones, hydroperoxides, benzoxazinones, and isothiocyanates formed in damaged plant tissues.

Lipoxygenases and Production of Hydroperoxides. As noted, lipoxygenases catalyze the oxidation of fatty acids to produce hydroperoxides. Hydroperoxides are very reactive electrophiles that bind covalently to the -SH, $-NH_2$, and -NH- functional groups of amino acids causing substantial reductions in protein nutritional quality or enzymatic activity (96). Further degradation of hydroperoxides may occur releasing volatile C_6 or C_9 aldehydes (59,97). These aldehydes may also react covalently with the nucleophilic moieties, further reducing nutritional quality (96). The use of this system as a defense against insects has not been extensively investigated; however, the potential does exist. In an artificial system, lipoxygenase and linoleic acid were used as dietary additives to significantly reduce the larval growth of the tobacco hornworm *Manduca sexta* (98). It was concluded that the adverse effects on larval growth resulted from lipoxygenase acting on linoleic acid to produce toxic levels of linoleic acid hydro-

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peroxide, rather than depletion of linoleic acid. This view was partially corroborated by the fact the addition of linoleic acid hydroperoxide to diet also reduced larval growth (98).

Lipoxygenases may offer a means of resistance via several mechanisms that impair the ability of the insect to utilize its food. Such mechanisms include: depletion of essential fatty acids (90), reduced digestibility of protein, reduced availability of essential amino acids (e.g., lysine, cysteine, methionine) and/or reduced activity of digestive and other critical enzymes. But such approaches are not without their detractions. Volatile aldehydes released from wounded foliage following the oxidation of free fatty acids by lipoxygenases (e.g., hexanal, *cis*-3-hexenal,and *trans*-2-hexenal) are highly attractive to certain insects such as the Colorado potato beetle (99) or *M. sexta* (100). The release of these volatiles may have the undesirable effect of attracting additional pests to the plant. Moreover, many aldehydes impart objectionable odors or flavors to certain foods such as soybean protein, and thus, some plant breeders have sought to genetically remove lipoxygenases from crops (97).

Thioglycosidase and Production of Isothiocyanates. Alkyl isothiocyanates also bind to -SH and -NH_2 functions of free amino acids and proteins, reducing protein digestibility (101). Although mustard oils are toxic to certain insects (37), the biochemical mechanisms by which they operate are unknown. The role of alkyl isothiocyanates in reducing the nutritional quality of plant nitrogen for insect herbivores is worthy of investigation. Similarly, the toxicity of the benzoxazine derivative DIMBOA to aphids may result from its reaction with thiols causing depletion of sulfur amino acids and/or enzyme inactivation (42).

Polyphenol Oxidases and Production of Quinones. Polyphenol oxidases are compartmentalized enzymes that occur in virtually all green plants and catalyze the oxidation of monophenols and/or diphenols (Figure 1f) (102-04). Polyphenol oxidases (PPO) can be induced by insect damage (105,106) or by microorganism attack (63,64,103). PPO's rapidly oxidize *o*-dihydroxyphenols (e.g., catechol, dihydroxyphenylalanine, and chlorogenic acid) to the corresponding reactive, electrophilic *o*-quinones which are known to be potent alkylators of nucleophiles such as -SH,-NH_2, and -NH- functional groups of amino acids and proteins (107-11). This covalent binding of *o*-quinones to proteins may reduce protein digestibility (107,112,113) and inhibit the activity of a wide range of enzymes (114). Surprisingly, the role of PPO in resistance to insects (54,57,113) is virtually uninvestigated (65,115), and to pathogens controversial (17,64,103).

We are investigating the potential role of PPO's in the tomato plant as a basis of resistance to two fruit- and leaf-feeding larval noctuid pests, the tomato fruitworm *Heliothis zea* and the beet armyworm *Spodoptera exigua*. Some varieties and species of the tomato plant contain high foliar levels of PPO and the substrate chlorogenic acid (54). Chlorogenic acid can also be oxidized by peroxidase in tomato foliage but the endogenous levels of the cofactor, hydrogen peroxide, are insufficient for

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peroxidase activity to contribute significantly to quinone formation. Laccase may also oxidize diphenols but is absent from tomato foliage.

The growth of larvae fed alkylated protein in artificial diet was substantially impaired compared to untreated protein (control) as shown in Figure 2. The growth rate of larval H. zea on foliage from field-grown plants was significantly negatively correlated ($n = 200$; $r = -0.65$; $P < 0.001$) with foliar PPO levels. Ingested PPO is highly active in the gut fluid of the insect, and as a result, nearly 50% of the ingested chlorogenic acid (CHA) was found to be covalently bound to protein in the faeces. Chemical oxidation of chlorogenic acid in the absence of PPO did not occur appreciably.

The adverse effects of PPO on noctuid larvae result from reduced bioavailability of amino acids caused by the covalent binding of oxidized chlorogenic acid to protein. When dietary protein (casein) was treated with PPO and chlorogenic acid, a substantial reduction in in vitro protein digestibility occurred. This reduction in digestibility may result from alkylation of lysine and/or protein cross-linking, which may impair the ability of the insect's trypsin to hydrolyze the protein.

The use of PPO/chlorogenic acid-based resistance seems promising, but it is not without limitations. As mentioned above, the implementation of PPO/chlorogenic acid as an antinutritive basis of resistance is directed at the insect's utilization of dietary protein. If one makes an analogy of dietary protein as an alkylatable sink, then it is apparent that the magnitude of the antibiotic effect (decreased digestibility and utilizability of protein) is a function of the size of the sink. Accordingly, we have found that the antibiotic effect is dependent on the level of dietary protein. As can be seen in Table II, when larval S. exigua is fed an artificial diet containing 0.5% casein, the impact of alkylation (PPO + CHA) upon growth is significant, a 38% reduction in relative growth rate compared to the controls. If one raises the level of dietary protein to 1.0% or 4.0%, one observes corresponding reductions in relative growth of only 15% and 7%. Thus the adverse effects of PPO/CHA on growth are alleviated by increasing levels of dietary protein. Control experiments have been carried out which eliminate the possibility that melanin-like polymers (formed solely by covalent bonding of o-quinones) are the toxic agents.

Our results have been recapitulated with other proteins of varying nutritional value to S. exigua and H. zea; they include soy protein, tomato foliar protein, corn gluten and zein. In all cases, more than 2.5% dietary protein was required to alleviate antinutritional effects, because these proteins are less nutritious than casein (Table III). The ability of a protein to alleviate the toxicity of o-quinones is proportional to its nutritional value to the insect (Table III). The proteins' ability to function as an alkylatable sink (alleviation of antinutritive effects) is correlated with the relative amounts of alkylatable amino acids (e.g., lysine, cysteine, histidine, methionine; Felton and Duffey, unpublished data).

Tomato foliage contains on the average ca. 1.0% protein fresh weight. However, these levels vary phenologically and

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Table II. Effect of Dietary Protein on the Toxicity of PPO and Chlorogenic Acid (CHA) to *Spodoptera exigua*

Dietary Treatment	Relative Growth Rate (mg/day/mg larva)
0.5% casein	0.266[a]
0.5% casein + CHA	0.261[a]
0.5% casein + PPO + CHA	0.164[b]
1.0% casein	0.277[a]
1.0% casein + CHA	0.277[a]
1.0% casein + PPO + CHA	0.236[b]
4.0% casein	0.377[a]
4.0% casein + CHA	0.255[b]
4.0% casein + PPO + CHA	0.351[a]

Means within each protein concentration not followed by the same letter are significantly different at $p < 0.05$. PPO (mushroom tyrosinase) was added at 0.100 change in OD_{470}/min/gm diet as chlorogenic acid oxidase activity and chlorogenic acid at 3.5 umoles/gm wet weight.

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Table III. The Relationship Between the Nutritive Value of Various Proteins to *Spodoptera exigua* and the Ability of Protein to Alleviate Toxicity Resulting from the Formation of *o*-Quinones

Dietary Protein	Relative Nutritional Value	%Protein Required to Alleviate 90%
Casein	1	2.2
Soy	2	3.0
Tomato	3	4.5
Glutein (corn)	4	>6.0
Zein	5	>6.0

Chlorogenic acid at 3.5 um/gm wet wt diet with PPO (mushroom tyrosinase; 0.100 change in OD_{470}/min/gm diet chlorogenic acid oxidase activity).

spatially between leaflets of the same plant and range from 0.05 to 6.0%. Foliar chlorogenic acid averages about 3.5 umoles/gm wet wt, and varies phenologically; variation is not as extreme as is seen for protein. Foliar polyphenol oxidase levels average about 45.0 change in OD_{470}/gm wet wt during the growing season but often increase tenfold during the growing season (Figure 2). Hence, the ability to predict the antinutritive effect is dependent upon a knowledge of the age and physiological state of the plant. Therefore, the use of *o*-quinone-based resistance against these noctuids

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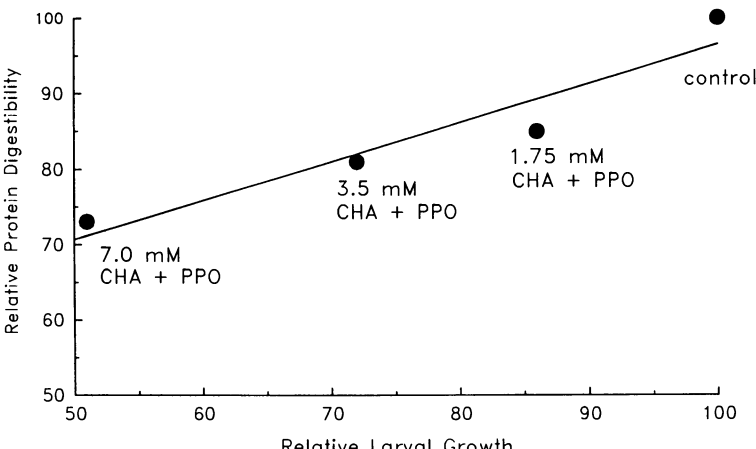

Figure 2. Effect of Alkylation of Casein By Chlorogenoquinone on _in vitro_ Protein Digestibility and Relative Growth of larval _Spodoptera exigua_. 1.0% casein was treated CHA concentrations shown and with 0.100 change in OD_{470}/min/gm diet PPO. Larval growth and protein digestibility reported as percent control where control equals 100%.

may involve manipulating multiple plant factors. This mechanism of resistance, aimed at the nutritive needs of the insect, theoretically provides a powerful antibiotic form of pest control free of certain problems associated with the use of synthetic insecticides (114).

Direct Action of Enzyme

We are concerned here with the direct action of the enzyme upon the insect as a substrate.

Chitinase. Chitin is the major structural component of the peritrophic membrane of insects which is thus theoretically susceptible to attack by plant chitinases. One of several functions of the peritrophic membrane of insects is to provide a physical barrier against the entry of pathogens or macromolecular toxins across the gut wall (117). Shapiro et al. (118) found that ingestion of fungal chitinases by larvae of the gypsy moth markedly increased larval susceptibility to baculoviruses. Earlier, Smirnoff (119) reported that chitinase enhanced larval susceptibility to the bacterium *Bacillus thuringiensis*. Although we are unaware of any reports of the direct action of chitinases on insects, the use of plants with damage-inducible chitinase may provide a means of a) increasing defense against a broad range of pests (fungi, nematodes, and arthropods) that use chitinous materials in their membranes or exoskeletons, and b) enhancing the susceptibility of insects to their own pathogens.

Polyphenol Oxidases. Some plant polyphenol oxidases possess both mono- and diphenolase activity (102,103), and thus are able to hydroxylate tyrosinyl residues in protein leading to the formation of the *o*-quinone of dihyroxyphenylalanine (108). This may lead to cross-linking between dietary proteins, digestive enzymes and/or proteins of the gut wall with ensuing reduction of digestive capabilties.

The Tomato Plant and Interaction of Plant Defenses

The tomato plant contains a number of enzymes that we have already discussed and that may be important mediators of resistance to insects (e.g., polyphenol oxidases, peroxidases, lipoxygenases, ureases, chitinases, phenylalanine and tyrosine ammonia lyases, endopolygalacturonases). We discuss here the potential interaction of these enzymes.

A general scheme for the response of tomato foliage to wounding by insects or microorganisms is depicted in Figure 3. The responses of the plant are complex involving three general modes: a) the release of constitutive, non-activated defenses, b) the enzymatic activation of constitutive defenses, and/or c) the induction of multiple enzyme systems leading to the *de novo* synthesis of many chemical defenses (polyacetylenes, 120; phenolics, 121,122; proteinase inhibitors, 11). The action of the tomato plant's non-activated constitutive defenses has been discussed elsewhere (49,54,55,116,123). The impact and utility of these three general lines of defense upon insect pests are unclear.

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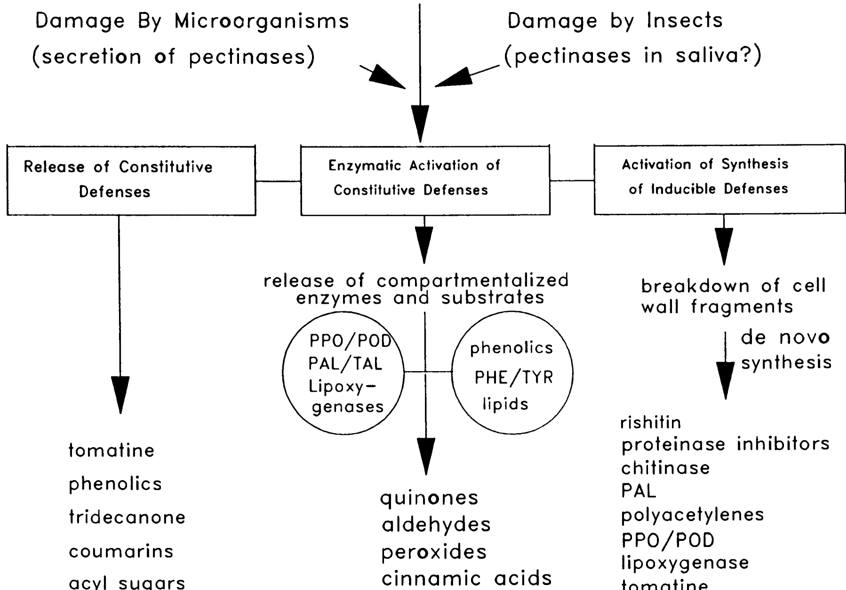

Figure 3. Diagram of Multicomponent Defenses in the Tomato Plant. PHE = phenylalanine; TYR = tyrosine; POD = peroxidase; PPO = polyphenol oxidase; PAL = phenylalanine ammonia lyase; TAL = tyrosine ammonia lyase.

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We have been using L. esculentum to understand the role of systemically inducible proteinase inhibitors (PI's I and II) as a potential mechanism of resistance against the beet armyworm S. exigua and H. zea (11,88). Damage to foliage as a result of larval feeding leads to the rapid induction of antibiotic levels of proteinase inhibitors, presumably via the mechanisms depicted in Figure 3. However, the use of proteinase inhibitors as a basis of resistance, although promising, has some constraints that relate a) to the intensity or degree of insect-induced wounding, and b) to their inactivation by other defensive chemicals in the tomato plant.

Wounding by Insects. If 3-4 fifth-instar S. exigua larvae are confined to feed on a single intact leaf for 24 hours, antibiotic levels of proteinase inhibitors are induced (88). However, in recent experiments conducted with Dr. C. A. Ryan (Washington State University), damage to tomato foliage as a result of feeding by larval H. zea did not always induce synthesis of proteinase inhibitors. When leaves are plentiful (more than 8 per plant) and larvae are given access to the whole plant, they may not inflict excessive damage upon any single leaflet, but rather feed on many leaflets. Also, the larvae often avoid damaging the midrib or cross-veins of leaves. This feeding behavior leads to no or very limited induction of proteinase inhibitors I and II (Table IV). Indeed, in control experiments similar to those of Ryan (124); where tomato leaves were mechanically damaged with a file, damage to cross-veins or the midrib caused greater induction of PI's than when damage was entirely interveinal (Table IV).

These results show the need to account for insect feeding behavior (site and intensity) in assessing the ability of plant inducible systems to function as factors in resistance. The fortuitous avoidance of PI induction by the insect's feeding behavior may be a consequence of reduced transport of proteinase inhibitor-inducing factor (PIIF) and/or release of PIIF by endopolygalacturonases (EPG), because of the site and degree of damage. It is well known that selective feeding on leaves by insects is a tactic that prevents mobilizing plant defenses (125,126). Whether such feeding behavior occurs in the field and whether plant defenses in field conditions are naturally induced because of damage by other factors is unknown.

There is considerable interest in utilizing proteinase inhibitors for host-plant resistance because of their adverse effects on insects (88,89,98) and pathogens (127). Current research is directed toward amplification of PI genes or the genetic transfer of PI genes into different crop species (128, 129). An alternative strategy may be the amplification or transfer of enzymes responsible for the degradation of cell wall polysaccharides. Because the induction of many potential plant defenses is dependent upon the release of cell wall fragments or cell contents, such a strategy may effect resistance. Higher levels of endogenous cell wall-degrading enzymes such as endopolygalacturonases may enable the plant to respond more rapidly to pest attack through a diverse array of inducible enzymatic defenses (e.g., proteinase in-hibitors, PAL, chitinases, polyphenol oxidases, and lipoxygenases).

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Table IV. Effect of Damage on PI Induction in Tomato Foliage

Damage and Treatment	Damage Area (mm^2)/Plant	% Relative Increase PI-I	% Relative Increase PI-II
Mechanical			
cross-vein	81.30	100	100
cross-vein	0.72	64	65
interveinal	0.72	47	59
Insect damage			
1st instar (20/plant)	1.11	0	8
4th instar (1/plant)	6.41	0	0
6th instar (1/plant)	20.52	0	0

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Inactivation of Proteinase Inhibitors by Other Enzymatically Based Plant Defenses. Unfortunately, the biological activity of tomato PI's against insects is not merely a function of PI level in foliage and/or fruit. A constraint upon the activity of PI's *in situ* may arise from the fact that they are susceptible to inactivation by alkylating agents such as *o*-chlorogenoquinone formed during the oxidation of chlorogenic acid by PPO and peroxidases (POD). Proteinase inhibitors are often rich in -SH and $-NH_2$ moieties, some near the active site (130). In experiments with tomato foliage damaged by feeding of larval *S. exigua*, the action of polyphenol oxidase after foliage is crushed reduces both the biological activity of proteinase inhibitors against *S. exigua* and the levels of proteinase inhibitors detectable by immunoassay (Table V). In one experiment, foliage was homogenized without PPO activity, and the proteinase inhibitor fraction was extracted and placed in artificial diet to assess its ability to support larval growth of *S. exigua*. Also, foliage was homogenized in buffer with PPO activity, allowed to sit 2 hours to simulate the time required for digestion in the insect, and then otherwise treated as above (G. W. Felton, R. M. Broadway and S. S. Duffey, unpublished data). The results clearly show that such oxidative processes reduce both the toxicity of proteinase inhibitors to the insect and the detectable foliar levels of PI-I and PI-II (Table V). In addition, the alkylation of PI's impairs their ability to inhibit trypsin (Table VI).

Extrapolation of these results to the real world suggests that the simultaneous use of PPO/phenolics and proteinase inhibitors as bases of resistance against certain insects may be mutually incompatible. If one were to rely on proteinase inhibitors as a basis, the elimination of high levels of polyphenol oxidase in the plant would not guarantee antibiotic activity because high levels of dietary protein can abolish PI toxicity (89). Hence, the activity of PI, PPO and phenolics *in situ* may require the manipulation of multiple plant factors.

Furthermore, in light of the ability of oxidized phenolics to inhibit a wide range of plant enzymes (114), the presence of high levels of PPO/phenolics may interfere with other potential

Table V. The Effect of Tomato Plant Foliar Polyphenol Oxidase Activity on Proteinase Inhibitors in *Spodoptera exigua*

Treatment	% Relative Growth	Proteinase Inhibitors		
		Total	PI-I	PI-II
Unwounded Plants				
w/oxidation	81.5	3.7	1.1	2.6
w/out oxidation	42.8	7.9	2.8	5.1
Wounded Plants				
w/oxidation	61.9	7.6	3.5	4.1
w/out oxidation	26.4	19.0	8.3	10.7

Table VI. The Effect of PPO and CHA on Soybean Proteinase Inhibitor Activity

Treatment	Tryptic Activity[1]	Relative Growth
Control	100	100
Soy PI	24	75
Soy PI + CHA	40	59
Soy PI + CHA + PPO	55	91

Tryptic activity measured *in vitro* with bovine trypsin. Tryptic activity and larval growth reported as percent of control where control equals 100%. Soybean inhibitor was Kunitz inhibitor. Percent relative growth determined by weight gain of *S. exigua* for 11 days on artificial diet containing the specified phytochemicals.

plant defensive enzymes (e.g., chitinases, lipoxygenases). Abundant literature exists showing that oxidized phenolics can inhibit pectinases (e.g., 114). This inhibition may interfere with the ability of the plant to respond to infection or wounding via induction of other defenses such as PI and/or phytoalexins.

Compatibility with Human Health. Many biochemical factors of resistance against insects and pathogens can constitute a health hazard for human consumers (19,61,109,131). But in the tomato plant, the use of proteinase inhibitors, phenolics, and/or PPO as bases of resistance appears compatible with human health because a) tomato fruit is not consumed in quantities sufficient to lead to acute or chronic poisoning, and b) at the time of ripening these substances are substantially or completely absent from the fruit (132; Felton and Duffey, unpublished data).

Compatibility of Plant Defenses with Biological Control. The compatibility of biochemical bases of resistance with the

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simultaneous use of biological control agents requires investigation (116). An additional constraint on the application of PPO/phenolic-based resistance is its incompatibility with microbial biological control agents currently used for insect control (133). We have found that phenolics, particularly in conjunction with PPO, detract from the action of entomopathogenic viruses. When the two major phenolics from the tomato plant, chlorogenic acid or rutin, were incorporated into the artificial diet of H. zea, the susceptibility of larvae to a nuclear polyhedrosis virus (HzSNPV) was significantly reduced (133). Because substantial amounts of ingested chlorogenic acid are converted to quinones in the insect gut, we have recently examined the effect of this oxidation on the ability of HzSNPV to cause disease in H. zea larvae. When HzSNPV was treated in vitro with levels of chlorogenic acid and PPO found in the plant, viral infectivity was reduced by as much as 90%. Binding of oxidized CHA to the viral occlusion body protein reduces the alkaline solubility and digestibility of the viral occlusion body (Felton and Duffey, unpublished data). This is critical because the virus must be solubilized and/or digested in the insect gut in order to release the infective virions (134). Processes that interfere with the course of infection can have a profound effect upon the occurrence of epizootics (135), and as a consequence may render the simultaneous use of viral insecticides with PPO-based resistance incompatible. Similarly, certain o-quinones may inactivate plant viruses (136,137).

Because chlorogenoquinone covalently binds to proteins, it is possible that other entomopathogens may be affected. For example, the bacterium Bacillus thuringiensis (BT) is a commercially available pathogen effective against a wide range of lepidopterous pests. The activity of this pathogen is derived from a proteinaceous endotoxin. Our laboratory has recently shown that oxidized chlorogenic acid increases the toxicity of BT by nearly 30% (C. T. Ludlum and S. Duffey, unpublished data). Thus, the adverse effects of phenolics and PPO on microbial control agents are not generalizable but must be considered for each individual organism.

Conclusions

Despite the limited research on the role of plant enzymes as mediators of resistance, opportunity knocks. Researchers in host-plant resistance have previously focused on constitutive factors of resistance rather than on activatible or inducible enzymes. Many plant enzymes, either by creating more reactive products or by removing essential substrates, have the potential to confer resistance against insects. Although plants have elaborate constitutive and inducible systems of defense against invasive organisms, their ability to function simultaneously against a diversity of pests remains to be fully determined. Our discussion of proteinase inhibitors and polyphenol oxidases demonstrates their use may be constrained by their mutual interaction, by their interaction with other plant enzymes, and by their interaction with other biological control agents.

Modern molecular techniques now make the interspecific transfer of genes feasible, and offer exciting, unique opportunities for developing crop resistance against a diversity of pests.

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The differing aims of scientists in genetically improving crop plants for increased resistance to pests, increased resistance to herbicides, or improved yield and nutritional quality, may, however, be at odds. For example, eliminating lipoxygenases or PPO to improve nutritional quality or marketability has been the aim of many scientists, but undesirable consequences to pest resistance may ensue. Resistance conferred by the transfer of genes (i.e., for proteinase inhibitors), may be adversely affected by inbuilt defenses of the receiving plant. Gene transfers for herbicide resistance, aimed at altering the oxidative potential of foliage to enhance plant resistance to certain herbicides (138), may interfere with the defensive responses of the plant that are mediated by oxidative enzymes.

Because the role of plant enzymes in host-plant resistance to insects is poorly understood at both an ecological and mechanistic level, the enhancement and/or transfer of genes for crop improvement should proceed with caution. The combined input of molecular biologists, agronomists, food scientists, plant pathologists, weed scientists, and entomologists is needed to achieve optimal success in crop improvements.

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Chapter 21

Elongation Reactions Involved in Hydrocarbon Biosynthesis in Insects

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The metabolic pathways by which insects produce hydrocarbons have been documented in several species. The methyl-branched hydrocarbons arise from the substitution of methylmalonyl-CoA for malonyl-CoA at specific points during chain elongation. The methylmalonyl-CoA arises from the carbon skeleton of valine and isoleucine in a cockroach and the housefly and from succinate in a termite. The microsomal elongation reactions have been characterized in the American cockroach and the common housefly, and show a high degree of specificity according to the degree of unsaturation of the fatty acyl-CoA substrates and the carbon chain length of the products.

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The cuticular lipids are essential components to the fitness of insects. The large surface-to-volume ratio of insects make insects susceptible to desiccation, and the cuticular lipids play an essential role in preventing water loss. They also affect the absorption of agricultural chemicals, may play a role in deterring microorganisms from attaching to and penetrating the cuticle, and in some species, play important roles in chemical communication, particularly as sex pheromones. The function, chemistry, physiology and biochemistry of insect cuticular lipids have been recently reviewed (1-7).

Insect cuticular lipids consist of aliphatic material which is present on the outer layer of the integument. In most species, they consist of complex mixtures of hydrophobic compounds including straight-chain saturated, unsaturated, and methyl-branched hydrocarbons, wax esters, sterol esters, ketones, alcohols, aldehydes, and fatty acids (1-4). In many insects, including the American cockroach (8) and the housefly (9), hydrocarbons are the predominant cuticular lipid component.

The hydrocarbons present on the surface of insects range in chain length from 21 to 55 carbons (1). *n*-Alkanes have been described in almost every insect examined. In some insects, such as the American cockroach, a limited number of hydrocarbons are present.

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About 70 % of the cuticular hydrocarbon in *P. americana* is (*Z*,*Z*)-6,9-heptacosadiene (27:2 Hy) (8), with the other two major components being *n*-pentacosane (25:0 Hy) and 3-methylpentacosane. This cockroach is unusual among insects in that there is essentially only one chain length for each of the major types of hydrocarbon present, i.e.straight chain saturated, unsaturated and methyl branched. In contrast, the housefly, *M. domestica*, has well over 100 hydrocarbon components in its cuticular lipids (9). In the housefly, the cuticular lipid composition is modified when the female becomes reproductively mature so that some of the components serve as the sex pheromone (10). Newly emerged females and males of all ages produce (Z)-9-alkenes of 27 carbons and longer. However, as the female becomes reproductively mature, an ecdysteroid, produced by the maturing ovary, initiates a change in the chain length of the alkenes so that (*Z*)-9-tricosene (23:1 Hy) becomes a major product (11). This alkene is then oxidized by a cytochrome P-450 polysubstrate mono-oxygenase to produce the corresponding 9,10-epoxytricosane and through a second oxidation, (Z)-14-tricosen-10-one (12). It has been shown that 20-hydroxyecdysone (20-HE) induces the change in the chain length specificity of the hydrocarbon-producing system (10).

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Biosynthesis of Insect Hydrocarbons: Methyl-branched Components.

Studies with widely diverse insect species, including both the American cockroach and the housefly, have established that the major site of cuticular hydrocarbon biosynthesis is the cells associated with epidermal tissue or the peripheral fat body, particularly the oenocytes (1).

There now exists overwhelming evidence that insects synthesize hydrocarbons from fatty acids by elongation reactions which produce very long-chain acyl moieties that are then converted to hydrocarbons one carbon shorter (1,2). The methyl-branched components, which are abundant in insects but rare in plants, are produced by the substitution of a methylmalonyl-CoA for a malonyl-CoA at specific points during chain elongation (Figure 1). Carbon-13 NMR studies (13-15) have shown that for methyl-branched components with a methyl group near the end of the chain, the branching group is added during the early stages of chain synthesis. The precursors of the methylmalonyl-CoA for a number of insects, particularly those lacking vitamin B_{12}, are the amino acids valine, isoleucine and perhaps methionine (Figure 1) (14,15). Recently it has been shown that the amino acids valine and isoleucine are also the precursors for the propionyl group used in ethyl branched juvenile hormone synthesis (16). In termites, which have high vitamin B_{12} levels, succinate is converted to methylmalonyl-CoA and serves as the methyl-branch donor group (Figure 1) (17).

Biosynthesis of (Z,Z)-6,9-Heptacosadiene.

The biosynthesis of 27:2 Hy has been studied *in vivo* with both radiotracer and ^{13}C-NMR techniques. Labeled acetate was incorporated *in vivo* about equally into the saturated and diene hydrocarbons, whereas [1-^{14}C]linoleic acid was preferentially incorporated into the diene fraction (18). Furthermore, [9,10-^{3}H]oleate was incorporated at low levels but almost exclusively into the 27:2 Hy, with less than

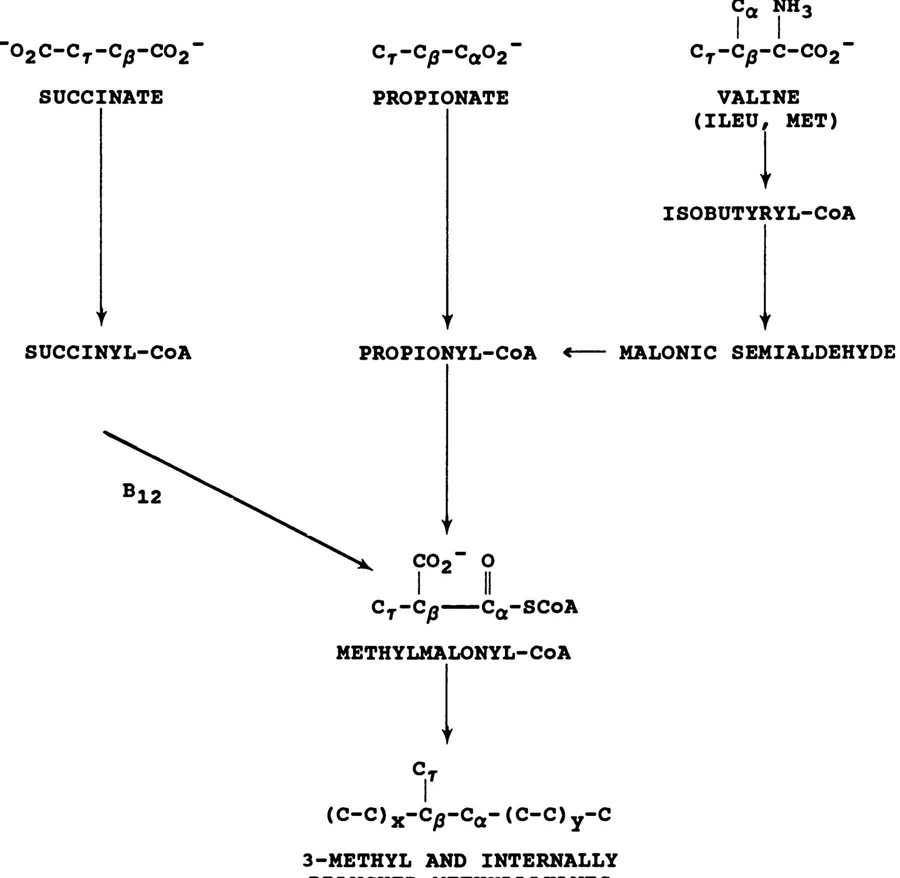

Figure 1. Origin of the methylmalonyl-CoA used for the biosynthesis of methyl-branched hydrocarbons in insects.

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6 % of the radiolabel being recovered in the saturated components (19). ^{13}C-NMR experiments showed that [2-^{13}C]acetate labeled carbon 25 but not carbon 3 of the 27:2 Hy (14). In addition, ozonolysis of the diene labeled from [1-^{14}C]acetate followed by radio-gas-liquid chromatographic (radio-GLC) analysis of the ozonolysis products showed that carbons 1-6 were not labeled. These data indicate that linoleate is elongated by the addition of acetate units, presumably via malonyl-CoA, and is then converted to 27:2 Hy (Figure 2).

Recent *in vivo* data have demonstrated that the American cockroach synthesizes not only linoleic acid (18:2 FA) (20), but also 20:2 FA and 22:2 FA (21). The view that animals are unable to biosynthesize linoleic acid (18:2, $\Delta^{9,12}$) has been overturned; some insect species can in fact synthesize linoleic acid *de novo*. Because labeled acetate is only detected in the carbons that result from the elongation of 18:2 FA in the 27:2 Hy, the insect does not directly elongate the newly synthesized 18:2 FA, but rather draws from a storage pool of linoleate. The fact that plants synthesize 18:2 FA from an oleoyl group esterified in the form of phosphatidylcholine (22) led to the suggestion that the same substrate for the Δ^{12} desaturase occurs in the cockroach. The 18:2 FA thus formed could subsequently be hydrolyzed from the phospholipid, converted to the CoA form, and then elongated. However, the form of the substrate for the Δ^{12} desaturase in the cricket, *Acheta domesticus*, is the CoA derivative rather than a phospholipid (Cripps, Borgeson, de Renobales and Blomquist, unpublished data) and may be the same for *P. americana*. Other factors, such as tissue specificity or the specificity of transacylase reactions may account for the observation that recently synthesized linoleate is not used for elongation reactions.

Elongation Reaction: In Vitro Studies.

To date, the elongation reactions involved in hydrocarbon biosynthesis have only been examined *in vitro* in the housefly (23, 24) and the American cockroach (25). Incubation of epidermal enriched microsomal preparations from both species with [1-^{14}C]stearoyl-CoA, malonyl-CoA, and NADPH resulted in the production of 20-30 carbon acyl moieties. The major *n*-alkane of the cockroach is 25:0 Hy, and it was, therefore, interesting to observe that very little radioactivity was incorporated into fatty acyl groups longer than 26 carbons by microsomal preparations from this insect. The 18:2-CoA was efficiently elongated to 26 and 28 carbon acyl moieties by microsomal preparations. In contrast, the 18:1-CoA and 18:3-CoA($\Delta^{9,12,15}$) were not elongated beyond 22 carbons (25). This shows that there is a high specificity according to the degree of unsaturation of the acyl-CoA elongation reactions. It is not known if the same enzymes elongate both the saturated and diunsaturated acyl-CoAs or if individual systems are used for each.

Microsomes prepared from epidermal enriched tissue readily elongated fatty acyl-CoAs in the cockroach, but microsomes prepared from fat body tissue elongated 18:2-CoA only to 20:2 FA and a trace of 22:2 FA (25). This demonstrates tissue specificity of the enzymes involved in fatty acyl-CoA elongation in the American cockroach.

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Hydrocarbon Formation.

Microsomes prepared from cockroach epidermal tissue also converted [1-^{14}C]18:2-CoA to hydrocarbon (25), in addition to labeling the very long-chain fatty acids. Radio-GLC analyses of the hydrocarbons formed showed that all the radioactivity in the hydrocarbon fraction (5.4 % of starting material, 54 nmoles/mg protein) was in the 27:2 Hy. Likewise, radio-HPLC analysis of the hydrocarbon formed from the elongation of 18:0-CoA showed that the major product was 25:0 Hy, with a small amount of radioactivity also recovered in 27:2 Hy. The latter probably arises from the desaturation of 18:0-CoA to 18:2-CoA. Thus, it appears that in cockroach microsomes the elongation enzymes are in close proximity to the enzyme system that converts the elongated products to hydrocarbon. This is the first demonstration in any organism that elongation reactions are coupled to the decarboxylation (decarbonylation) reactions, and presents an excellent opportunity to probe the system to further examine the nature of the step that converts the elongated fatty acid to hydrocarbon.

Conversion of Very Long Chain Fatty Acids to Hydrocarbons.

The conversion of high specific activity tritium-labeled fatty acids to *n*-alkanes one carbon unit shorter has been demonstrated in the American cockroach (26) and in the termite *Zootermopsis angusticollis* (27) *in vivo*, and in microsomal preparations from the termite (27). Most of the activity in the termite was located in the microsomal fraction and was stimulated two-fold by the addition of ascorbate.

The nature of the reaction that converts the long chain acyl moieties to hydrocarbon one carbon shorter is not well understood. It has been referred to as a "reductive decarboxylation" in both plants (28) and insects (2). However, Kolattukudy and co-workers have presented evidence that in plant (29,30) and vertebrate tissues (31) the reaction is a decarbonylation and that the carboxyl carbon is reduced to the aldehyde before being removed in the form of carbon monoxide. Clearly more work is needed to determine the nature of this reaction in insects.

Regulation of the Chain Length of Hydrocarbons.

The synthesis of 23:1 Hy in the female housefly occurs via the elongation of oleoyl-CoA to a 24 carbon acyl moiety which is then converted to an alkene one carbon shorter (10). Its production is hormonally regulated. Pre-vitellogenic females and males of all ages produce alkenes of 27 carbons and longer (Figure 3) (11). When the female becomes about two days old and begins producing vitellogenin, the ovaries initiate production of an ecdysteroid which triggers the production of the C_{23} pheromone components. Removal of the ovaries within 12 hrs of emergence prevents sex pheromone production. Implantation of the ovaries into ovariectomized females or treatment with 20-hydroxyecdysone (20-HE) restores pheromone production (11). Furthermore, implantation of ovaries or injection of 20-HE induces pheromone production in males (32). Thus, the presence of 20-HE induces a shift in the chain length of the alkenes produced from 27:1 Hy and longer to 23:1 Hy.

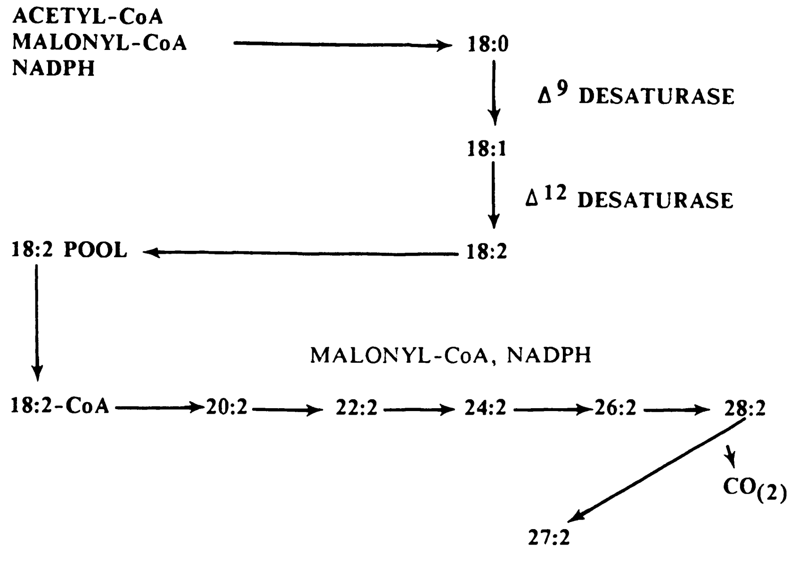

Figure 2. Proposed scheme for the biosynthesis of (Z,Z)-6,9-heptacosadiene in the American cockroach.

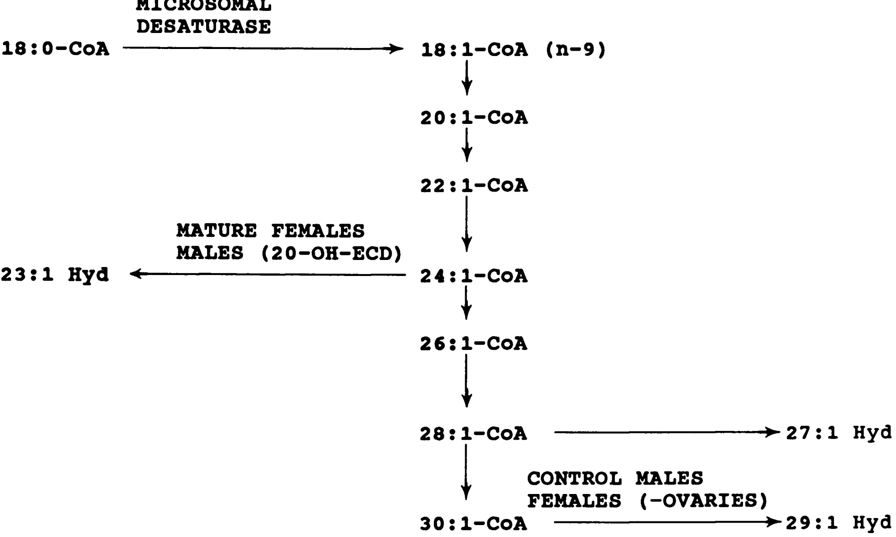

Figure 3. Biosynthesis of alkenes by houseflies. Mature females and males treated with 20-hydroxyecdysone (20-OH-ECD) produce 23:1 Hy whereas newly emerged females, control males and ovariectomized females produce alkenes of 27 carbons and longer.

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An examination of the elongation reactions in pre-vitellogenic females, in mature females, in control males, and in males treated with 20-HE suggests that it is the elongation reactions that are affected by 20-HE. Microsomes from males elongate 18:1-CoA to all even numbered fatty acids to 30:1 FA, whereas microsomes from mature females elongate 18:1-CoA to 24:1 FA, with very little product longer than 24 carbons produced (Vaz, Blomquist, and Reitz, unpublished data). Furthermore, microsomes from males elongate both 22:1-CoA and 24:1-CoA to fatty acyl moieties up to 30 carbons. In contrast, microsomes from females elongate 22:1-CoA only to 24:1 FA and traces of 26:1 FA. The 24:1-CoA is not efficiently elongated by microsomes from females, and only small amounts of 26:1 FA were observed. Thus, the elongation activity correlates with the expected pattern assuming that the elongation reactions were involved in regulating the chain length of the alkenes. It is not known whether it is the elongation activity itself, a hydrolase, or an acyl transferase that removes the fatty acyl moiety from the elongation pool once the appropriate chain length is reached.

Summary Statement

The metabolic pathways by which insects produce hydrocarbons are now documented in a few species, but only recently have studies been initiated towards characterizing the processes involved. Most of the methyl-branched hydrocarbons arise via the substitution of a methylmalonyl-CoA in place of a malonyl-CoA during chain elongation. The methylmalonyl group has been shown to be derived from either succinate or the amino acids valine, isoleucine and methionine. The elongation reactions have been described for the common housefly and the American cockroach, and preliminary data indicates that they play a key role in regulating the chain length of hydrocarbons. The ability to manipulate the chain length of the hydrocarbon products by ecdysteroids in the housefly should allow us to gain a better understanding of the process which controls hydrocarbon chain length. The process by which the very long chain acyl group is converted to hydrocarbon is not known for insects, although evidence has been presented in plants and vertebrates that the fatty acyl-CoA is reduced to the aldehyde and then decarbonylated. The development of an active microsomal preparation from the American cockroach that both elongates and converts the elongated product to hydrocarbon should allow rapid progress to be made toward gaining a better understanding of this process in insects.

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Chapter 22

Enzymes Involved in the Biosynthesis of Sex Pheromones in Moths

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A large number of moth sex pheromones are biosynthesized from common fatty acids by pathways involving three reactions: chain shortening, desaturation, and reduction. The chain shortening occurs in units of two carbons, while the desaturation may take place at the 11-12, 10-11 or 9-10 positions. The acid group is reduced to the aldehyde or alcohol, and the alcohol may be acetylated. Control of the E/Z ratio of the product can occur at the desaturation and/or reduction step. The various pathways and the enzymes involved in them are presented with emphasis on the Δ11 desaturase.

In the approximately thirty years since the first identification of an insect sex pheromone (1), interest in the chemistry of these compounds has increased dramatically. This is due both to academic interest and to the use of these compounds for insect monitoring and control. More recently, the biochemistry of these compounds has been studied (2-3). In this paper, we discuss the biosynthesis of a class of these compounds and consider some of the key enzymes involved in the biosynthetic pathway.

Initial Studies

Many Lepidopteran pheromones share a number of common features; a long, unbranched chain with an even number of carbons, a terminal oxygen-containing functional group (alcohol, acetate or aldehyde), and one or more sites of unsaturation, usually far from the oxygenated end of the chain. Species specificity is achieved by varying these characters and the stereochemistry of the double bond(s), as well as by mixing two or more compounds in specific ratios. The structures of these compounds suggest that their biosynthesis may be similar to that of the fatty acids. Initial studies attempted to establish this similarity.

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The fatty acid content of the ovipositor tips of a variety of Lepidopteran species were determined by acid methanolysis followed by gas-liquid chromatography (GLC) (4). In each case, unusual fatty acids corresponding to pheromone components were identified. For example, the tufted apple bud moth, *Platynota idaeusalis*, uses (*E*)-11-tetradecenyl acetate and alcohol as pheromone components (5) and also contains (*E*)-11-tetradecenoic acid in its ovipositor tip. In several species, the fatty acid content of the rest of the insect also was determined. In each instance, the unusual fatty acid components were found only in the ovipositor tip or in the pheromone-producing gland located in the tip.

One insect proved to be of special interest and was studied in greater detail (5-6). The redbanded leafroller moth (RBLR), *Argyrotaenia velutinana*, uses (*Z*)- and (*E*)-11-tetradecenyl acetates as pheromone components in the ratio of 9:1. The pheromone glands of these insects also contained the corresponding acids, Δ11-tetradecenoic acids, but the Z to E ratio was about 4:5. If these acids serve as precursors to the acetates, there must be strict stereochemical control exercised in the reactions that produce the final product in the 9:1 ratio.

Key Reactions

The above data suggest that pheromone component biosynthesis is related to fatty acid biosynthesis, but that some unusual reactions need to be added. Consideration of a large number of pheromone components (7) suggests that these reactions include chain-shortening by two carbons and desaturation at the 11-12 position. Fig. 1 shows the variety of potential intermediates that can be produced from palmitic or stearic acids employing these reactions.

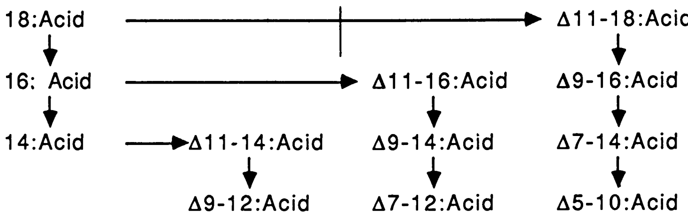

Figure 1. Δ11-18:Acid = (*Z* or *E*)-11-octadecenoic acid.

Evidence for the occurrence of a controlled, 2-carbon chain shortening reaction was obtained using the orange tortrix moth,

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Argyrotaenia citrana (8). Pheromone glands were removed and ground in buffer, and the resulting crude preparation assayed for activity. Addition of palmitic acid uniformly labelled with ^{14}C or labelled with ^{3}H at the 9 and 10 positions resulted in significant incorporation of radioactivity into tetradecanoic acid, whereas addition of palmitic acid containing ^{14}C at the 1 position led to little incorporation. A dual labelling experiment, using both carbon-14 and tritium in the same assay, gave a product of similar isotopic ratio as the starting material. This indicates that the conversion was by chain shortening rather than by degradation to acetate followed by resynthesis as the latter pathway would lose tritium by isotope exchange (9), which should alter the ratio.

A more explicit demonstration of chain-shortening was achieved by performing assays using 3-^{14}C-hexadecanoic acid as substrate (8). Over 80% of the radioactivity in the isolated chain-shortened tetradecanoic acid was in the 1-position, showing that most of the reaction was direct, though a small amount may have undergone degradation/resynthesis.

Performing the reaction with uniformly labeled hexadecanoic acid and separating the isolated tetradecanoic acids also demonstrated the introduction of the 11-12 double bond. Conversion of the unsaturated products into epoxides followed by GLC separation showed radioactivity in both the saturated and unsaturated acids. Similar to the results discussed above with RBLR, a closely related insect, the Z:E ratio of unsaturated acyl compounds was not the same as that in the pheromone. With the orange tortrix moth, the Z:E ratio of unsaturated acids was ca. 2:1, whereas the pheromone was found to be all Z.

These *in vitro* studies were consistent with *in vivo* experiments previously performed. The latter consisted of applying various labelled substrates dissolved in DMSO to the pheromone gland of RBLR and then excising and extracting the glands to obtain products (10). One particularly interesting result was obtained by using either E or Z-11-tetradecenoic acid labelled with ^{14}C in the 1 position as starting material. In both cases, the product obtained directly from the starting material was predominant, indicating that an E/Z isomerase was not active in the gland.

Another important result was obtained from *in vivo* experiments using the cabbage looper moth (CL), *Trichoplusia ni* (11). The biosynthetic pathway postulated for this insect is given in Fig 2. Substrates were applied to the gland and the (*Z*)-7-dodecenyl acetate isolated, cleaved at the double bond by ozonolysis, and the products isolated as benzyloximes. When acetate was used as substrate, radioactivity appeared in both products, but when hexadecanoic acid tritiated at the 16 position was used, radioactivity only appeared in the fragment derived from the terminal carbons. This demonstrated that incorporation took place as proposed in Fig 2, not by some scheme involving degradation and resynthesis.

16:Acid ⟶ (Z)-11-16:Acid ⟶ (Z)-9-14:Acid ⟶

(Z)-7-12:Acid —reduction⟶ —acetylation⟶ (Z)-7-12:Acetate

Figure 2. Biosynthetic pathway for *Trichoplusia ni.*

Other Reactions

While two reactions, chain shortening and 11-desaturation, are key steps in the biosynthesis of many pheromones, these pathways also require a reductive step where the acid group is converted to an alcohol, acetate or aldehyde. Studies on the spruce budworm moth (12), *Choristoneura fumiferana*, and various *Heliothis* species (13) indicate that there is a series of reactions, such as those in Fig. 3, to account for the various functionalities. Alcohols and acetates may be stored or released as pheromone; aldehydes are too reactive (and potentially dangerous to the insect) to store and so are only produced immediately before release.

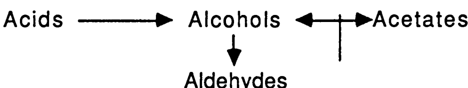

Figure 3. Reduction pathways for pheromone biosynthesis.

The reactions mentioned above account for many, but by no means all, pheromones whose biosynthesis is related to fatty acids. For example, the silkworm moth, *Bombyx mori*, has as its main pheromone component (*E,Z*)-10,12-hexadecadien-1-ol (1). The corresponding diunsaturated fatty acid also is present in the gland (14), as is a large quantity of (*Z*)-11-hexadecenoic acid (15). Recent studies have demonstrated that the monounsaturated acid is formed first, followed by what appears to be a 1,4-desaturation yielding the diunsaturated acid (16).

Other desaturases also may play a part in these pathways. The greenheaded leafroller moth, *Planotortrix excessana*, a New Zealand species, uses (*Z*)-8-tetradecenyl acetate as its main pheromone component. *In vivo* studies using deuterated precursors showed that the path given in Fig 4 was operative, indicating a desaturase that introduced a double bond at the 10-11 position was operative (17). Another New Zealand insect, the brownheaded leaf-

16:Acid ⟶ (Z)-10-16:Acid ⟶ (Z)-8-14:Acid

Figure 4. Desaturase and chain-shortening activity in *Planotortrix excessana.*

roller moth, *Ctenopseustis obliquana*, uses the same Z8 compound as well as (*Z*)-5-tetradecenyl acetate as major components. Studies with deuterated precursors showed the path indicated in Fig. 5 was

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operative, implicating oleic acid, (*Z*)-9-octadecenoic acid, and the common 9-10 desaturase in the biosynthesis of these pheromones (18).

18:Acid → (Z)-9-18:Acid → (Z)-7-16:Acid → (Z)-5-14:Acid

Figure 5. Desaturase and chain-shortening activity in *Ctenopseustis obliquana*.

An unusual 9-10 desaturase is present in the codling moth, *Cydia pomunella*. It forms (*E*)-9-dodecenoic acid, which then undergoes 1,4-desaturation and reduction to give the pheromone (*E,E*)-8,10-dodecadien-1-ol (19).

Some insects use hydrocarbons as sex pheromones. For example, the housefly, *Musca domestica*, uses a mixture including (*Z*)-9-tricosene, the corresponding epoxide and ketone, and several methyl alkanes (20). The tricosene is derived by chain elongation of oleic acid, and the epoxide and ketone are made from it. The methyl alkanes are made *de novo* from acetate and propionate, with one propionate unit per molecule supplying the branch carbon. Propionate can arise from the degradation of valine or *iso*-leucine, but not from succinate, although succinate may serve as an acetate precursor.

Several species of tiger moths, *Holomelina* spp., use 2-methylheptadecane as a main pheromone component. Studies involving the injection of radiolabeled compounds into whole insects have demonstrated that the path given in Fig 6 is most likely functional (21). This is similar to a known path in crickets (22).

$(CH_3)_2CHCH(NH_2)COOH \longrightarrow (CH_3)_2CHCH_2COOH$
leucine

$(CH_3)_2CH(CH_2)_{15}COOH \xrightarrow[-CO_2]{} (CH_3)_2CH(CH_2)_{14}CH_3$

Figure 6. Biosynthetic pathway for *Holomelina* spp.

Another step in the biosynthesis worthy of mention is that of the synthesis of the fatty acids themselves. Although we often consider the pathways to start with the acids (palmitic, oleic), fatty acid synthetase could be considered part of them, though, of course, not a unique part (23).

Enzymatic Properties

The study of the enzymes involved in these pathways is still in its early stages. However, enough has been done to give some insight into the characteristics and organization of these systems.

a) Chain Shortening. The first step in the sex pheromone biosynthesis in the redbanded leafroller involves chain shortening from hexadecanoic acid to tetradecanoic acid (3). Crude cell-free preparations from pheromone glands exhibit this reaction when

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provided with uniformly ^{14}C labeled hexadecanoic acid, NADH, NADPH, Coenzyme A, ATP, magnesium chloride and BSA (24). Assumedly, the Co A, ATP and $MgCl_2$ allow the crude preparation to synthesize the Co A derivative of the acid. Omission of the Coenzyme A causes the reaction to stop. Also, failure to provide NADP causes chain-shortening to no longer be exhibited. A small amount of dodecanoic acid and no shorter-chain acids were observed, showing the reaction to be limited, unlike the normal mitochondrial degradation sequence.

b) Δ11 desaturase. The desaturase from the cabbage looper moth has been partially purified and its properties determined (25). The pheromone glands were excised, ground in phosphate buffer containing sucrose and dithiothreitol and the microsomal fraction isolated by centrifugation at 100,000 g followed by resuspension of the pellet. The product was determined to be solely (*Z*)-11-hexadecenoic acid, using 1-^{14}C hexadecanoyl Co A as substrate. The pH optimum was broad and centered around 7.8. Production of product was linear for one hour, and then it quickly ceased. Activity was maximum in two-day-old insects and was almost gone by five days.

The normal assay included, in addition to enzyme solution and substrate, BSA, NADH and NADPH. Omitting the BSA or NADH resulted in marked reduction in activity, whereas omission of NADPH gave only slight reduction in activity. The enzyme activity was highest when octadecanoyl Co A was used as substrate, almost as high when hexadecanoyl Co A was used, and very small when tetradecanoyl Co A was used as substrate.

The most common desaturase in most organisms, including insects, is stearoyl Co A desaturase, which introduces a double bond in the 9-10 position of long-chain fatty acids (26). Similarities between this enzyme and the Δ11 desaturase from cabbage looper include: location in the microsomal fraction, lack of sensitivity to carbon monoxide, inhibition by cyanide, use of a reduced nicotine-adenine nucleotide cofactor as an electron source and use of 16 and 18 carbon acids as preferred substrates.

There also are significant differences between the enzymes. First, there is the different site of introduction of the double bond. Then there is the preferred cofactor; both will accept NADPH or NADH, but the Δ9 enzyme prefers the former and the Δ11 enzyme the latter. Both are sensitive to cyanide, but the Δ11 is about 50 times less sensitive. The pH optimum of the Δ9 enzyme is somewhat more acidic (7.8-7.2). Finally, there is the tissue location. The Δ9 enzyme is generally present throughout the insect, while the Δ11 enzyme is localized in the pheromone gland (25).

The small amounts of the Δ11 desaturase available make solubilization and stabilization necessary before purification can be attempted. A series of detergents previously reported useful in desaturase purifications proved not to be useful in this case - indeed, most of them actually lowered the activity. However, octanoyl-N-methyl glucamide (27) did appear to stabilize and at least partially solubilize the enzyme. Treatment of enzyme solutions with Blue Sepharose, an affinity chromatography

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substrate, gave a degree of purification, but the activity was so low that further studies have proved difficult (24).

c) Reduction. The last step in the proposed biosynthetic paths involves reduction of the acids formed by chain shortening and desaturation. This can give product directly or can produce an intermediate that then gives the final product. For example, in the corn ear worm, *Heliothis zea*, which uses aldehydes as pheromone components, the immediate product of the reduction of 11-hexadecenoic acid could well be the corresponding alcohol. There exists in the insect cuticle a primary alcohol oxidase that converts alcohols to aldehydes (13). This oxidase shows little specificity for geometry or number of double bonds, has a broad pH profile (5-9) and was functional in both dichloromethane and hexane, an unusual and potentially quite interesting property.

More extensive studies have been done on the spruce budworm, *Choristoneura fumiferana*, (28) which uses (*E*)- and (*Z*)-11-tetradecenal as pheromone components. Once again, the first step appears to be reduction of the acid to an alcohol. Then an acetyl Co A: fatty alcohol acetyltransferase converts the alcohol to the acetate ester (12). The enzyme is specific for alcohols with chain lengths of 12-15 carbons, and prefers monounsaturated alcohols to saturated ones. It is located specifically in the pheromone gland, and appears to be microsomal.

Two other enzymes are involved in aldehyde production. The first is an acetate esterase, which is soluble, located in the gland, and hydrolyzes tetradecanyl acetate and its Δ11 unsaturated derivatives (*Z* or *E*) at the same rate. The second is an alcohol oxidase, which also is soluble and located in the gland. The oxidase requires oxygen, but does not require reducing cofactors (12). The saturated and unsaturated 14-carbon acids all react at similar rates in the reaction catalyzed by this enzyme.

The redbanded leafroller moth uses a mixture of (*E*) and (*Z*)-11-tetradecenyl acetates as pheromone components. The pheromone gland of this insect also contains an acetyl Co A: fatty alcohol acyl transferase (29). This enzyme will acetylate 12, 14 and 16 carbon alcohols, and prefers the saturated alcohol to the Z monounsaturated derivative, and the Z to the E, in the approximate ratios of 5:3:1 for saturated, Z and E, respectively.

d) Fatty Acid Synthatase (FAS). FAS is a commonly occurring enzyme complex whose occurrence and properties in insects has been recently reviewed (23). FAS activity in the pheromone gland of spruce budworm has been observed both *in vivo* and *in vitro* (12, 30). In both cases, the ratio of incorporation of malonate to acetate was 8:1 though *in vitro* this ratio decreased to 7:1 after two hours. Isolated acids ranged in chain length from 12 to 18 carbons and appeared to be primarily saturated.

In the redbanded leafroller moth, treatment of intact glands with DMSO containing radioactive acetate led to the formation of radiolabeled 14, 16 and 18 carbon saturated acids as well as Δ11-tetradecenoic acids and pheromone components (10). Cell-free preparations from redbanded leafroller pheromone glands also showed FAS activity when radiolabeled acetyl Co A, NADPH and malonyl Co A are supplied (31). The major products are the 16 and 18 carbon saturated acids, with the 18 carbon acid predominating.

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The activity does not sediment in a 100,000 g fraction, indicating it is soluble. A similar activity may be observed in preparations from the pheromone gland of the cabbage looper moth (31).

Areas for further study

The study of the enzymes involved in sex pheromone biosynthesis in insects has barely begun. There are far too many fruitful research projects in this field to even list them here. We will mention a few just to introduce the variety possible.

First, the use of two specific reactions -- Δ11 desaturation and controlled 2 carbon chain shortening of fatty acid precursors to account for the biosynthesis of a large number of pheromones -- has been an extremely fruitful approach. Even in a case where it seemed uncertain if this approach was appropriate (32), it turned out that it was (33). Other reactions should now be added to increase the range of products accounted for. Examples already mentioned include the Δ10 desaturase and the chain elongation of branched-chain starting materials. Other functional groups that appear in sex pheromones should also be accounted for, such as epoxides.

Another interesting problem involves the control of the synthesis. Hormonal control of the biosynthetic pathway is currently being investigated (33), and the existence of a brain hormone has been demonstrated. Other aspects of control, such as the ratio of compounds and when they are actually released, also require extensive further study.

Finally, although the Δ11 desaturase from cabbage looper is probably the most studied enzyme involved in these pathways, much more work should be done with it and related enzymes. For example, it only produces Z products and does not function efficiently with 14 carbon substrates, indicating a fundamental difference with the enzyme from redbanded leafroller moths, which desaturates 14-carbon acids to give a mixture of Z and E product (33). Projects such as this may well prove technically difficult, but will provide great insight into the enzymatic mechanisms.

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Chapter 23

Enzyme-Catalyzed Pheromone Synthesis by *Heliothis* Moths

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The specificity of blends of compounds used for pheromone communication by Lepidoptera species is the result of essentially two distinct sets of biosynthetic enzymes which regulate the production of specific olefinic bonds and synthesis of the oxygenated functional moiety, respectively. In Heliothis moths the regulatory systems that are responsible for production of the functional group during the final stages of pheromone biosynthesis consist of cellular acetate esterases and extracellular alcohol oxidases. Evidence indicates that the relative activities of these enzymes differ for each species of Heliothis. Thus, pheromone mediated reproductive isolation between closely related species of Heliothis is probably the result, in large measure, of the fact that some species require only aldehydes for communication while others use acetates, alcohols and aldehydes.

Knowledge of the chemistry, associated behavior and physiology of chemical communication systems of Lepidoptera has increased dramatically over the past 3 decades. However, studies on the biosynthesis of pheromone components are relatively new. Biochemical investigations are critical for accurately defining pheromone communication systems because biosynthesis determines the types of compounds that will be emitted as pheromones and establishes the ratio of components released.

The importance of elucidation of pathways of biosynthesis which regulate pheromone production is exemplified by the identification of additional pheromone components for the cabbage looper moth. Until the above research had been conducted, (Z)-7-dodecenyl acetate was the sole identified pheromone component. This compound was an effective attractant for males, but insects did not perform the complete range of behaviors that were exhibited in response to females. The additional components identified by Bjostad et al. (1) were found only after studies on biochemical precursors were

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conducted and these compounds were subsequently found to be critical for maximizing the pheromone mediated response of males (2).

Similarly, knowledge of the biochemical mechanisms which regulate the release of actual pheromone components is of value in explaining why analogues of behaviorally relevant pheromone components are present within the pheromone gland but are either biologically inactive, or inhibit the response of conspecific insects. For example, ethyl ether extracts of the pheromone glands obtained from females of Heliothis zea contain large amounts of (Z)-11-hexadecen-1-ol, the alcohol analogue of the aldehydic pheromone component present in greatest amount (3). However, the addition of as little as 0.1% of this alcohol to the four aldehydes that are released in the volatile pheromone blend (4) resulted in a significant reduction in the number of males captured in traps in field studies (3). The presence of the alcohol in the pheromone gland was subsequently explained (5-7) by studies on the terminal step of pheromone biosynthesis which showed that a primary alcohol oxidase present in the cuticle overlying the pheromone gland converts the alcohol to the behaviorally active aldehyde very rapidly, and apparently completely, as the alcohol passes through the cuticle.

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Biosynthesis of the Hydrocarbon Skeleton.

Initial studies on biosynthesis of pheromones of Lepidoptera were begun in the early 1970's when Inoue and Hamamura (8) showed that radiolabelled (E,Z)-10,12-hexadecadien-1-ol was produced when the pheromone glands of Bombyx mori were incubated with radiolabelled palmitic acid, and Kasang and Schneider (9) induced female gypsy moths to synthesize tritiated disparlure by injecting the ^{3}H labelled hydrocarbon analog. Subsequently, Jones and Berger (10) succeeded in inducing female cabbage loopers to produce ^{14}C labelled (Z)-7-dodecenyl acetate by injecting (1-^{14}C) acetate.

These initial studies were performed on isolated species from families which have distinct types of components. Systematic studies on biosynthesis by phylogenically related insects were not undertaken until the works by Roelofs and Brown (11) and Steck et al. (12) outlined the analogies in pheromones used by Tortricidae and Noctuidae respectively. The significant feature of these pheromones is that most of the Tortricinae use 14-carbon compounds with a double bond at the 11 position whereas the majority of identified compounds from noctuid moths have a double bond between the 5th and 6th carbons from the terminal methyl group (ie. Δ7-dodecenyl, Δ9-tetradecenyl, Δ11-hexadecenyl).

Studies by Roelofs, Bjostad and Wolf have been instrumental in elucidating the mechanisms by which these compounds are synthesized. Their studies on the cabbage looper (2, 13, 14), orange tortrix (15) and red banded leafroller moth (16) have demonstrated that a great number of insects utilize common saturated fatty acyl compounds as precursors of unsaturated pheromone components. In this biosynthetic scheme the common acid, palmitate, is the initial precursive compound. Palmitate can be acted upon directly by a Δ11-desaturase to give (Z)-11-hexadecenoate, which can then be subjected to partial ß-oxidation to yield (Z)-9-tetradecenoate and (Z)-7-dodecenoate, or can be chain elongated to provide

(Z)-13-octadecenoate. Alternatively, palmitate can undergo chain shortening to yield myristate which, when acted upon by the ▲11-desaturase, results in the production of (E)- or (Z)-11-tetradecenoate (17). The ▲-11-desaturase also appears to explain the production of a number of different types of compounds when associated with other enzymatic reactions. For example, the 2,13- or 3,13-octadecadienyl compounds found in moths of the family Aegeriidae can be explained by the action of the ▲-11 desaturase on palmitate, followed by chain elongation coupled with the action of a ß-ketoacyl reductase. Subsequent desaturation would yield either the ▲2, ▲13- or ▲3, ▲13-octadecadienoate (17). The biosynthesis of the conjugated diene, (E,Z)-10,12-hexadecadien-1-ol (Bombykol) also appears to result from incorporation of a double bond at the 11 position. In this case the (Z)-11-hexadecenoate formed is apparently acted upon by enzymes capable of isomerizing the double bond to yield the 10,12 conjugated system (17, 18, 19). A more complete discussion of the biosynthesis of the hydrocarbon portion of pheromone molecules is given by Wolf and Roelofs (this volume).

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Biosynthesis of Oxygenated Functional Groups.

While studies on biosynthesis of the hydrocarbon portion of pheromone molecules are of considerable importance, studies on the functional group are also critical. Often species use blends of compounds having the same functional group. However, numerous cases exist in which pheromone blends are comprised of compounds which have the same basic structure but differ in their functional group. Also many instances have been documented in which extracts of pheromone glands contain compounds that have different functional groups from the behaviorally significant compounds (3, 20, 21, 22, 23, 24, 25). In many of the above cases clear biosynthetic relationships exist between the classes of compounds.

The obvious first step in biosynthesis of a functional group is the reduction of the fatty acid or fatty acyl-CoA analogues. Among most organisms this is accomplished by microsomal acyl-CoA reductases which result in the production of the fatty aldehyde (26, 27, 28). This simple enzymatic system would seem a logical method of production of aldehydic pheromone components. However, as indicated by Morse and Meighen (29) and Teal and Tumlinson (5, 6, 7), aldehydes are highly toxic and reactive compounds. Consequently, as occurs in the pheromone gland of the spruce budworm moth the aldehydes are almost instantaneously converted to the alcohol analogues via the action of an aldehyde reductase. Evidence that acid reductases function in pheromone biosynthesis comes from *in vitro* studies on the orange tortrix moth (15) as well as *in vivo* studies on the redbanded leafroller (30). Aldehyde reductase activity occurs in the pheromone gland of the spruce budworm moth (29). Alcohols formed in the above reactions can be released as pheromone components directly, subjected to further enzymatic reactions, or both.

Two enzyme systems that act on alcohols formed in the above fashion have been documented to date. By far the most common is the action of an acetyl transferase which yields acetates. Acetyl transferase activity can be invoked to explain the production of acetate pheromone components by most tortricid moths (17), and we

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have demonstrated that this enzyme converts alcohols to the acetate components in a noctuid moth species (25). Acetyl transferases also produce acetate intermediates which are further metabolized to alcohol and aldehyde pheromone components. This occurs in the spruce budworm moth (31) and has been implicated in pheromone biosynthesis by Heliothis subflexa (25) as well as H. virescens (6, 25). The action of this enzyme, in these cases, appears to be somewhat of an anomaly inasmuch as the acetates are further metabolized back to the alcohols prior to oxidation to the aldehyde analogues (31-33). However, as discussed by Morse and Meighen (33) the acetates are stable high energy compounds which are relatively non toxic and thus make good storage precursors.

As indicated above, acetates formed in the pheromone gland of the above species are subsequently converted back to the alcohol via the action of an acetate esterase. Evidence to date suggests that the esterase resides in the soluble fraction of pheromone gland homogenates while the acetyl transferase is found in the microsomal fraction of homogenates (33, 34).

Aldehydes are produced via the action of alcohol oxidizing enzymes. In those species studied, a primary alcohol oxidase which appears to require only molecular oxygen and the alcohol substrate is involved (5, 29, 33, 34). However, the action of alcohol dehydrogenases which do not require molecular oxygen to function can not be discounted where aldehydes or ketones are produced as pheromones by other insects.

We have recently shown that the oxidase employed to convert the alcohols to aldehydes by three species of Heliothis moths is present in the cuticle which covers the cells of the pheromone gland (37), and evidence suggests that the oxidase used by the spruce budworm also resides in the cuticle (29). An important feature of the enzymatic system in Heliothis moths is that the cuticle oxidase converts all primary alcohols with equal efficiency. Consequently there is an excellent correlation between the ratios of alcohols produced in the gland and the ratios of aldehydes released (3, 22, 24,).

Comparison of Oxidase and Esterase Activities in Heliothis

Members of the Heliothis genus of noctuid moths have been the subjects of considerable study in our laboratory over the past 10 years not only because of their economic importance, but also because of the ability to hybridize two of the species, H. subflexa and H. virescens. Since members of this genus use variations of the same types of compounds for pheromone communication a commonality in the biosynthetic capability of these insects seems indicated (Table I).

As discussed earlier, the sixteen carbon aldehydes, used as pheromone components by all Heliothis species studied to date, are formed by the action of a primary alcohol oxidase in H. virescens, H. zea and H. subflexa. Interestingly, although acetate analogues of these aldehydes have only been found in extracts of the pheromone glands of H. subflexa (22, 35, 36), all three species possess esterases in the pheromone gland that are capable of converting the acetates to primary alcohols (25). Females of H. zea and H. virescens are capable of converting substantially more topically

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Table I: Compounds Identified from Extracts of Pheromone Glands of North American *Heliothis* Species*

	Species			
Compound	*H. zea*	*H. virescens*	*H. subflexa*	*H. phlaxiphaga*
Tetradecanal	-	+	+	-
(Z)-9-Tetradecenal	-	+	+	-
Hexadecanal	+	+	+	+
(Z)-7-Hexadecenal	+	+	+	-
(Z)-9-Hexadecenal	+	+	+	+
(Z)-11-Hexadecenal	+	+	+	+
Tetradecan-1-ol	-	+	-	-
(Z)-9-Tetradecen-1-ol	-	+	-	-
Hexadecan-1-ol	+	+	-	-
(Z)-9-Hexadecen-1-ol	-	-	+	-
(Z)-11-Hexadecen-1-ol	+	+	+	+
Hexadecan-1-ol acetate	-	-	+	-
(Z)-9-Hexadecen-1-ol acetate	-	-	+	-
(Z)-11-Hexadecen-1-ol acetate	-	-	+	-

*The presence of "+" indicates that a compound has been identified as being present in extracts of pheromone glands, a "-" indicates the absence of the compound. Data for *H. phlaxiphaga* are from Raina et al (47). Other data are from references 3, 5, 22, 35 and 36.

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applied acetate to alcohol than are females of H. subflexa (Figure 1), indicating that the relative activity of the esterase in glands is higher for the species which do not use acetates for pheromone communication. Consequently, acetates produced in the pheromone glands of H. virescens and H. zea are probably transitory intermediates in pheromone biosynthesis, and are converted to the corresponding alcohols as rapidly as they are produced. However, in H. subflexa this is not the case and acetates are released as pheromone components (22,35,46).

Studies of esterase activity in cell-free cuticle preparations and homogenates of pheromone gland cells have yielded interesting results. Over 96% of the esterase activity was associated with the cellular fraction of glands in H. zea and H. subflexa. However, the cellular fraction of the glands of H. virescens had less activity (85%) (figure 2). The small amount of esterase activity associated with the cuticle of both H. subflexa and H. zea may be an artifact of our experimental approach since microvillar extensions of the apical cell membranes go into the cuticle overlying the gland (37). These membranes would remain associated with the cuticle fragments unless the cuticle was finely ground thus solubilizing the enzyme. Therefore esterases bound to the apical cell membrane would be present in the cuticle used in our experiments. This is supported, in part, by preliminary studies conducted in our laboratory in which finely ground cuticle of the glands of H. zea had high oxidase activity but no esterase activity. Esterase activity in the cuticle of H. virescens is twice that found in the cuticular fractions of H. subflexa and H. zea. This suggests that females of this species maintain esterases in the cuticle, as has been hypothesized for the spruce budworm moth (33), and esterase activity has been found in cuticular pore canals of other insects (38). H. virescens and H. subflexa are very closely related species that are capable of hybridizing (39). However, in nature there is no cross attraction between the two species (40) and studies have shown that the sixteen carbon acetates are required for communication by H. subflexa (46). Consequently, the presence of the esterase in the cuticle of H. virescens may act as a fail-safe mechanism which converts any acetate which might enter the cuticle to the alcohol analogue prior to conversion to the aldehyde, thus maintaining the species specificity of the pheromone blend of H. virescens.

The oxidase that subsequently converts alcohol metabolites to aldehydic pheromone components is essentially limited to the cuticle which covers the cells of the pheromone gland (7). This enzyme becomes active after the adult cuticle has become fully developed just prior to eclosion from the pupal stage in H. zea and is active when females of H. virescens and H. subflexa emerge from the pupal case (Figure 3). This stage of development correlates well with structural changes in the pheromone gland that include the development of numerous lipophilic vesicles in the cells (41). In other species, notably the spruce budworm moth (42) which also appears to use a cuticular alcohol oxidase in pheromone biosynthesis, cellular changes that occur at this time include a switch from protein to lipid synthesis, as is indicated by the degradation of rough endoplasmic reticulum and evolution of numerous Golgi complexes, microbodies and extensive smooth tubular endoplasmic reticulum. Pore canals have been implicated as

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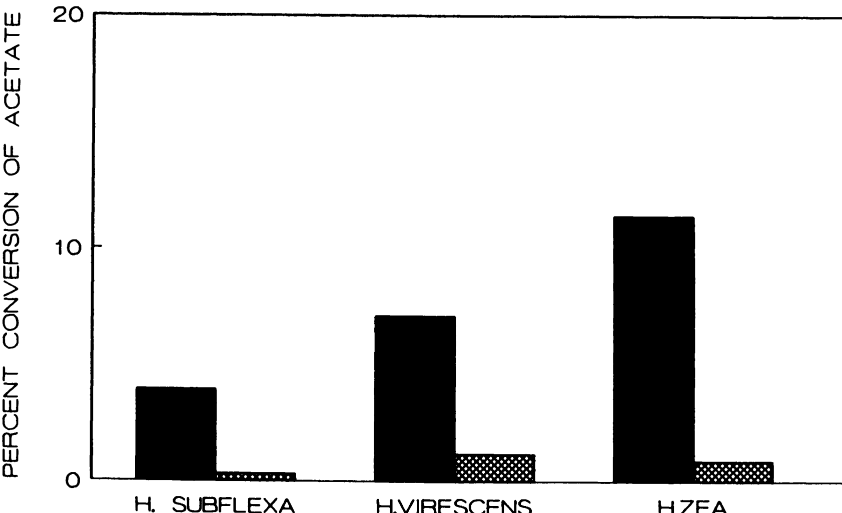

Figure 1. Percentage of (Z)-11-tetradecen-1-ol acetate (500 ng) converted to alcohol by the action of acetate esterase (solid bars) and subsequently to aldehyde (checked bars) via the oxidase in the intact pheromone glands of H. subflexa, H. virescens and H. zea (n = 10, each species).

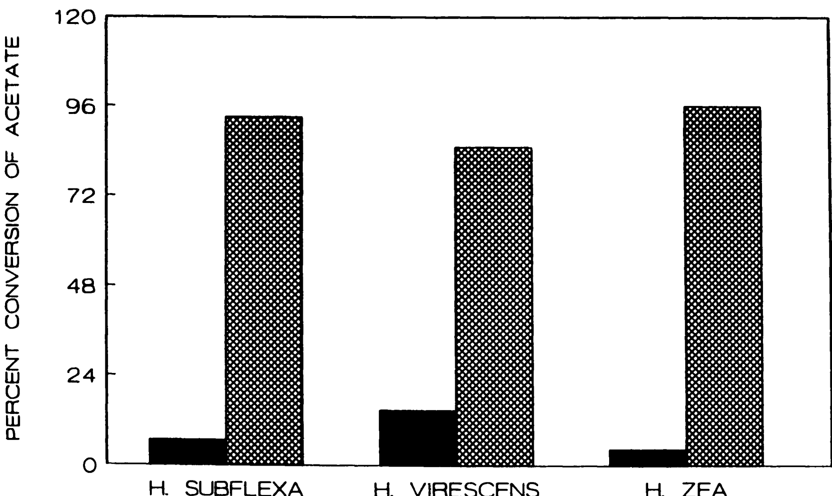

Figure 2. Distribution of esterase activity in the pheromone glands of H. subflexa, H. virescens and H. zea as indicated by relative conversion (percent) of (Z)-11-tetradecen-1-ol acetate to the alcohol analog when using the cell homogenate (checked bars) or cell free cuticle (solid bars) (N = 7 replications of 3 female equivalents per species).

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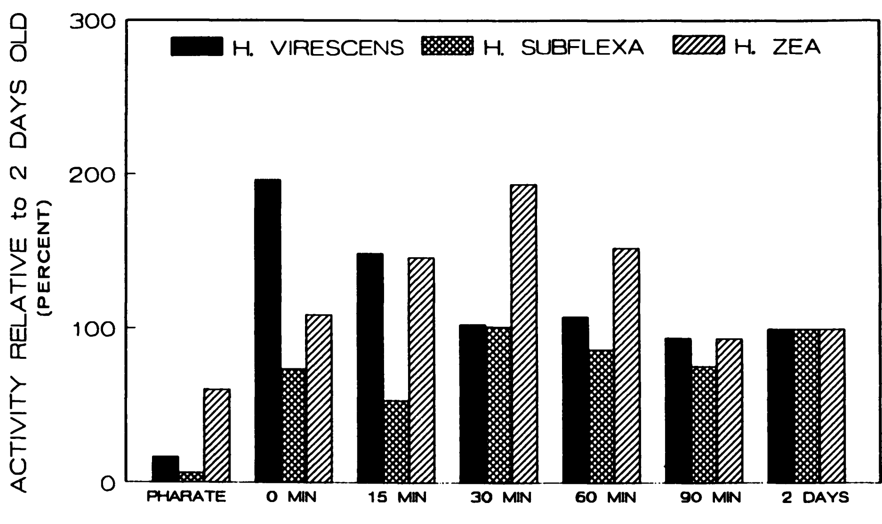

Figure 3. Dependence of oxidase activity on insect development as indicated by conversion of (Z)-11-tetradecen-1-ol to (Z)-11-tetradecenal in 30 min. by excised glands of H. subflexa, H. virescens and H. zea. Bars indicate the activity in intact glands at each time relative to that in glands of insects of the same species 48 h after adult emergence (100%) (n = 10, each species).

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structures by which pheromone can move through the cuticle (37), and are known to have oxidase activity (43). Since few changes have been found in the cuticle of the gland after it is formed, but prior to oxidase activation (42), the oxidase may be secreted by the cells of the pheromone gland into the extracellular pore canals of the cuticle just prior to the conversion of the cells from protein to lipid biosynthesis.

The presence of the oxidase in the extracellular cuticle of the pheromone glands of these species is associated with several unique features. Although activity in the cuticle is destroyed by soaking in 1% SDS or 7M urea, non-ionic detergents including Tween 80, NP-40, and Triton X100 have little or no effect. Similarly several organic solvents have little or no effect on oxidase activity in any of these species. In fact, the use of hexane as a carrier for the alcohol substrates is much more efficient than water (7). Solvents having large dipole moments appear to interact with the enzyme - cuticle matrix resulting in a loss of activity (Figure 4).

As indicated in Figure 5 there is a linear relationship between the amount of aldehyde produced with respect to time for the three species. However, the slopes of the lines are different which suggests that the activities of the oxidases present in the glands of the three species are different. This feature is also indicated by our study on conversion of acetate to alcohol by intact glands, because the alcohol products were converted to aldehyde. In this study we found that the glands of H. virescens converted approximately 20% of the alcohol that was produced by the action of the esterase, to aldehyde, whereas the glands of H. subflexa converted 9%. Taken collectively these data can be used to further explain the enzymatic regulation of blend specificity for the sibling species, H. virescens and H. subflexa. Both chemical and behavioral studies have shown that only aldehydes are required for pheromone communication by H. virescens (24, 44, 45). However, in addition to the aldehydes, H. subflexa uses acetates and (Z)-11-hexadecen-1-ol, the alcohol analogue of the major aldehyde component, for pheromone communication (46). It is probable that the lower activity of the oxidase in the cuticle of H. subflexa compared to H. virescens, ensures that a small proportion of (Z)-11-hexadecen-1-ol is released as a pheromone component by females of H. subflexa thus adding another important dimension to chemically mediated reproductive isolation between H. virescens and H. subflexa.

In summary, the pheromone blends produced by Heliothis species are generated by a common biosynthetic scheme which permits modifications that result in the production of species specific blends of pheromone components. Pheromone mediated reproductive isolation between H. zea and the other two species results from the fact that H. zea does not produce fourteen carbon aldehydes. However, the sixteen carbon compounds are present in a ratio that closely approximates that of H. virescens. Thus, it appears that females of H. zea do not have the enzyme system required for partial ß-oxidation of the sixteen carbon precursors. Females of both H. virescens and H. subflexa are capable of performing this chain shortening step. However, substantially less of the 16-carbon compounds are chain shortened to 14-carbon molecules in the glands of H. subflexa than H. virescens (see 5, 35). Pheromone blend

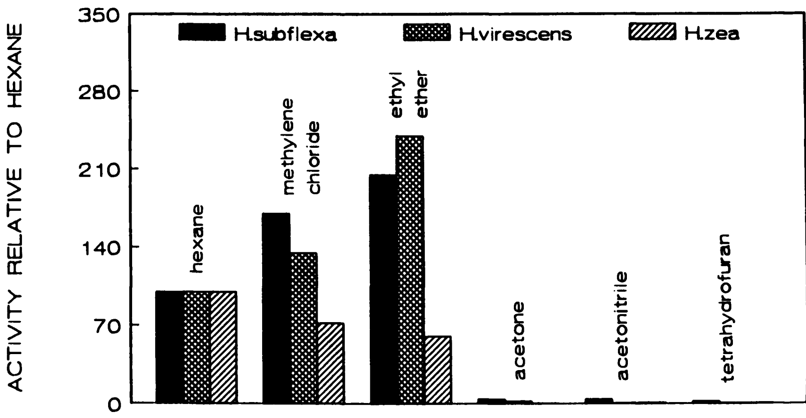

Figure 4. Effect on oxidase activity of soaking cell-free cuticle of H. subflexa, H. virescens, and H. zea in organic solvents for 10 min prior to incubation in (Z)-11-tetradecen-1-ol (500 ng/2µl hexane for 30 min). All values are relative to that found when using hexane (100%) (n = 8 replications of 5 female equivalents for each species).

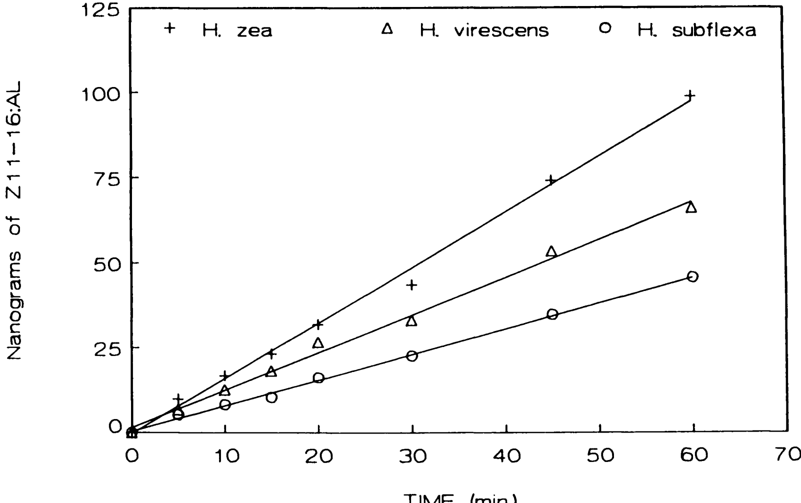

Figure 5. Effect of duration of incubation on oxidase activity in excised glands of H. subflexa, H. virescens, and H. zea as indicated by conversion of (Z)-11-tetradecen-1-ol (26.5 µg in 100 µl of hexane) to (Z)-11-tetradecenal as described elsewhere (7) (n = 10 replications, 1 female equivalent per replication for each species).

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specificity for H. virescens and H. subflexa can be accounted for, to a large extent, by the species specific activities of enzymes involved in production of oxygenated functional groups. This enables H. subflexa to release aldehydes, acetates, and alcohols while H. virescens employs only aldehydes for pheromone communication.

Acknowledgment

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Chapter 24

Preparation of Chiral Building Blocks by Biochemical Methods

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Microbial or enzymatic preparations of the chiral building blocks useful in organic synthesis are reviewed. Conversions of these chiral building blocks to important bioactive natural products are also illustrated.

In spite of the remarkable progress in the field of chemical asymmetric synthesis (1), microbial or enzymatic processes are still attractive to preparative chemists who want to synthesize enantiomerically pure bioactive natural products in amounts sufficient for extensive biological evaluation.

Since the publication in 1976 of a standard reference book by Jones, Sih and Perlman (2), an increasing number of organic chemists has become involved in studies on the application of biochemical systems in organic chemistry. Their approach is often different from that by biochemists, because organic chemists are much more aware of the general utility of a chiral building block obtainable by a particular biochemical reaction.

Our team at the University of Tokyo has pursued, over a decade, biochemical methods leading to versatile chiral building blocks for organic synthesis (3). This paper summarizes our recent work on the subject.

α-Amino Acids as Chiral Building Blocks

Enantiomerically pure α-amino acids can readily be pre-pared by resolving (±)-N-acetyl (or chloroacetyl)-α-amino acid with amino acylase of *Aspergillus* origin. This microbial enzyme is unexpensive, stable, and of broad substrate specificity allowing the resolution of α-amino acids with a branched- or a long-chain alkyl group.

α-Amino Acid Itself as a Building Block - Synthesis of Gizzerosine.

A disease named "black vomit" is a big problem in poultry industry. The disease is accompanied by gizzard erosion or ulceration in chicks, and known to be caused by brown fish meal in the diet. In 1983 Okazaki et al. isolated 2 mg of a toxic compound from 10 kg of heated mackerel meal. The toxin caused severe gizzard erosion in chicks within a week when fed to them at the level of ca. 50 μg/day

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(4). This toxin was named gizzerosine and assigned structure **1** with unknown absolute configuration (4). Our synthesis of (±)-**1** by the reductive coupling of histamine dihydrochloride (**2**) and an aldehyde **3** confirmed the proposed structure (5).

To clarify the aboslute configuration of gizzerosine, its enantiomers were synthesized as shown in Figure 1. Resolution of (±)-**4a** with amino acylase smoothly yielded (S)-**4b** and (R)-**4a**. Only (S)-**1** was found to be toxic when fed to chicks (6).

α-Hydroxy Acid and Epoxide as Building Blocks. Deamination of an α-amino acid with nitrous acid is known to give the corresponding α-hydroxy acid with retention of configuration. An α-hydroxy acid can be converted to an epoxide. Both α-hydroxy acids and epoxides are versatile chiral building blocks in natural products syntheses (3).

Alcohol as a Chiral Building Block

Various lipases and esterases are used for the preparation of optically active alcohols, which can serve as chiral building blocks.

(1S,4R)-4-t-Butyldimethylsilyloxy-3-chloro-2-cyclopenten-1-ol as a Building Block for Punaglandin 4. Punaglandin 4 (PUG 4, Figure 2) is one of the chlorinated marine prostanoids isolated from the Hawaiian octocoral *Telesto rusei* by Scheuer et al.(7). In our synthesis of PUG 4, the key chiral building block **9** was prepared by treating a stereoisomeric mixture of **8** with pig pancreatic lipase (PPL)(8). Fortuitously, the desired stereoisomer **9** of 100% e.e. was the only product (25% yield) of PPL-catalyzed hydrolysis. Oxidation of **9** gave **10**, which was subsequently converted to PUG 4(8).

(R)-2-Acetoxymethyl-3-phenyl-1-propanol as a Building Block for A-factor. A-factor (**14**, Figure 3) is the inducer of the biosynthesis of streptomycin in inactive mutants of *Streptomyces griseus* (9). It also induces the formation of spores in asporophological modifications of *S. griseus* (9). Our first chiral synthesis of **14** employed (S)-paraconic acid (**13**) as the key intermediate, which was obtained by the conventional optical resolution (10,11).

We recently developed a route in which (S)-**13** was prepared by an enzymatic process (Mori, K.; Chiba, N., The University of Tokyo, unpublished data). Treatment of diacetate **11** with PPL gave (R)-**12** of 86% e.e. Conversion of (R)-**12** to (S)-**13** was followed by its purification as an amine salt to give pure (S)-**13**, which was converted in several steps to A-factor (**14**).

β-Hydroxy Esters as Chiral Building Blocks

Due to their bifunctional nature, β-hydroxy esters are versatile building blocks in organic synthesis as detailed below.

Preparaton of the Enantiomers of Ethyl 3-Hydroxybutanoate. Reduction of ethyl acetoacetate with yeast yields ethyl (S)-3-hydroxybutanoate (**15**) as shown in Figure 4 (12-14). Purification of crude **15** as its crystalline 3,5-dinitrobenzoate gives (S)-**15** of 100% e.e. (14,15).

Ethyl (R)-3-hydroxybutanoate (**15**) of 100% e.e. is prepared by ethanolysis of poly-3-hydroxybutanoate (PHB)(16,17). Seebach et al. used PHB generated by *Alcaligenes eutrophus* (16), while we employed *Zoogloea ramigera*(15,17).

Ethyl 3-Hydroxybutanoate as a Building Block for Sulcatol and Pityol. (S)-Sulcatol (**16**, Figure 5) is the aggregation pheromone of *Gnathotrichus retusus*. This was synthesized in 73% overall yield from (S)-**15** as shown in Figure 5 (13,18). Sulcatol (**16**) was converted to pityol (**17**) by treatment with thallium (III) triacetate. Pityol (**17**) is a male-specific attractant of the bark beetle *Pityophthorus pityographus*(18).

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Figure 1. Synthesis of gizzerosine. Reagents: a) Aspergillus amino acylase, 37°C, 48 h; b) CbzCl/toluene, NaOH aq-An (92%); c) Cs_2CO_3/DMF; d) BnBr/DMF (45%); e) BH_3 THF (83%); f) $(COCl)_2$/DMSO-CH_2Cl_2, Et_3N (quant.); g) $NaBH_3CN$, MS 3Å /MeOH (69%); h) H_2/Pd-C/EtOH; i) dil HCl, Recrystallization from MeOH (33%).

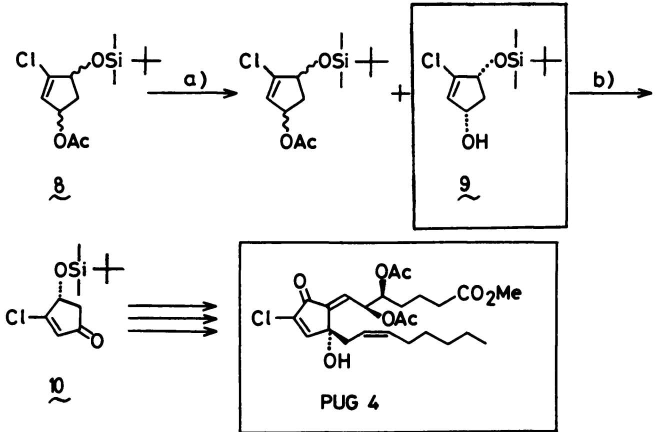

Figure 2. Synthesis of (1S,4R)-4-t-butyldimethylsilyloxy-3-chloro-2-cyclopenten-1-ol. Reagents: a) PPL (25%); b) PDC/DMF (91%).

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Figure 3. Synthesis of (3R)-(-)-A-factor. Reagents: a) $CH_2(CO_2Et)_2$, NaOEt/EtOH (58%); b) LAH/ether (78%); c) Ac_2O/C_5H_5N (66%); d) PPL/An-H_2O (56%); e) CrO_3 (98%); f) O_3; g) H_2O_2; h) H^+ (83%); i) purification via (R)-α- phenethylamine salt; j) BH_3 THF (92%); k) Me_3SiCl, $(Me_3Si)_2NH$ (52%); l) LDA, $Me_2CH(CH_2)_4COCl$; m) aq EtOH, Δ (30%).

Figure 4. Preparation of the enantiomers of ethyl 3-hydroxy butanoate. Reagents: a) Saccharomyces bailii KI 0116 (80%); b) 3,5-$(O_2N)_2C_6H_3CO_2H$, DMAP, DCC/CH_2Cl_2; Recrystallization; c) KOH/THF-EtOH-H_2O (45%); d) Zoogloea ramigera; e) EtOH-H_2SO_4/CH_2Cl_2 [33 g of (R)-15 from 50 g of Z. ramigera cells].

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Preparation of the Enantiomers of Methyl 3-Hydroxypentanoate. A mutant of *Candida rugosa* oxidizes pentanoic acid to give (*R*)-3-hydroxypentanoic acid of 93% e.e. (19). The corresponding methyl ester **18** can be purified via its crystalline 3,5-dinitrobenzoate to give (*R*)-**18** of 100% e.e. as shown in Figure 6 (20).

Reduction of octyl 3-oxopentanoate with baker's yeast furnishes octyl (*S*)-3-hydroxypentanoate of 97% e.e. After transesterification to the corresponding methyl ester **18**, it can be purified as its 3,5-dinitrobenzoate to give (*S*)-**18** of 100% e.e. (21).

Methyl 3-Hydroxypentanoate as a Building Block for Serricornin. Serricornin (**19**) is the female-produced sex pheromone of the cigarette beetle, *Lasioderma serricorne*, which is a serious pest of cured tobacco leaves (22). As shown in Figure 7, serricornin [(4*S*,6*S*,7*S*)-**19**] and its 4*R*-isomer were synthesized from methyl (*R*)-3-hydroxypentanoate **18** (20). Purification of crystalline **20** by recrystallization gave diastereomerically and enantiomerically pure **20**. The desired (4*S*,6*S*,7*S*)-**19** was readily separable from the by-product (4*R*,6*S*,7*S*)-**19** by silica gel chromatography (20).

A Cyclic β-Hydroxy Ester as a Building Block for Sporogen-AO1. Reduction of ethyl 2-oxocyclohexane-1-carboxylate with baker's yeast was first studied by Ridley in 1976 (12). Reduction of an analogous β-keto ester **21** with baker's yeast yielded ethyl (1*R*,2*S*)-5,5-ethylenedioxy-2-hydroxycyclohexane-1-carboxylate (**22**) of 98.4% e.e. (23).

The hydroxy ester **22** was employed as the starting material for the synthesis of sporogen-AO 1 (Figure 8, **23**). Sporogen-AO 1 (**23**) is a sporogenic sesquiterpene isolated from the culture broth of *Aspergillus oryzae*(24). The synthesis and bioassay of both the enantiomers of **23** revealed only the natural enantiomer (+)-**23** to be bioactive (25). The second synthesis as shown in Figure 8 was enantioselective to give only the desired enantiomer of sporogen-AO 1 (26).

β-Hydroxy Ketones as Chiral Building Blocks

Reduction of symmetrical and prochiral 1,3-diketones with yeast gives optically active β-hydroxy ketones, which are useful chiral building blocks in organic synthesis.

(S)-3-Hydroxy-2,2-dimethylcyclohexanone as a Building Block. Reduction of 2,2-dimethylcyclohexane-1,3-dione (**24**) with baker's yeast gives (*S*)-3-hydroxy-2,2-dimethylcyclohexanone (**25**) of 98-99% e.e. (27). This hydroxy ketone **25** was proved to be a versatile chiral building block in terpene synthesis (3).

Figure 9 illustrates the synthesis of the enantiomers of polygodial (**29**) starting from **25**(28). (-)-Polygodial (**29**) is a hot-tasting sesquiterpene first isolated from *Polygonum hydropiper* (29). It was also isolated from *Warburgia stuhlmanni*, and shown to possess antifeedant activity against some pest insects (30). The Diels-Alder reaction between **26** and dimethyl acetylenedicarboxylate yielded a mixture of **27** and **28**. These were separated, and further manipulated to give (-)-**29** and (+)-**29**, respectively (**28**). Both the enantiomers of **29** showed identical biological properties such as hot taste to human tongue, antifeedant activity against insects, and piscicidal activity.

Juvenile hormone III, an acyclic sesquiterpene epoxide, was also synthesized from (*S*)-**25** by submitting the acetate of (*S*)-**25** to the

Figure 5. Synthesis of (S)-sulcatol and (2R,5S)-pityol. Reagents: a) DHP, TsOH (quant.); b) LAH/ether (89%); c) $TsCl/C_5H_5N$ (quant.); d) $Me_2C{=}CHMgBr$, CuI/THF (quant.); e) $AcOH-THF-H_2O$, Δ (82%); f) $Tl(OAc)_3/HBF_4-An-H_2O$ (99%; 12% after MPLC purification).

Figure 6. Preparation of the enantiomers of methyl 3-hydroxypentanoate. Reagents: a) *Candida rugosa*; b) $MeOH-H_2SO_4$ (80%); c) $3,5-(O_2N)_2C_6H_3CO_2H$, DMAP, DCC/CH_2Cl_2; recrystallization; d) $KOH/THF-MeOH-H_2O$; e) *Saccharomyces cerevisiae* (70%); f) $K_2CO_3/MeOH$.

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Figure 7. Synthesis of (4S,6S,7S)-serricornin. Reagents: a) LDA, MeI; b) DHP, PPTS (61% from **18**); c) LAH (99%); d) NaH, $PhCH_2Cl$ (94%); e) TsOH, MeOH (quant.); f), 3,5-$(O_2N)_2C_6H_3CO_2H$, Ph_3P, $EtO_2CN{=}NCO_2Et$, recrystallization (40%); g) KOH (95%); h) t-$BuMe_2SiCl$, imidazole (quant.); i) H_2/Pd-C (97%), j) $TsCl/C_5H_5N$; k) NaI (99.6%, 2 steps); l) Et_2CO, LDA (80%); m) AcOH-THF-H_2O (43%).

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Figure 8. Synthesis of (+)-sporogen-AO 1. Reagents: a) baker's yeast (74%); b) DHP, PPTS (quant.); c) LAH/ether (98%); d) $TsCl/C_5H_5N$ (95%); e) PPTS/MeOH; f) aq $HClO_4$-ether (85%); g) t-BuOK/t-BuOH (78.5%); h) Ph_3P, $EtO_2CN{=}NCO_2Et$, $PhCO_2H$/THF; LiOH/MeOH (80%); i) t-$BuMe_2SiCl$, imidazole-DMF (quant.); j) Li/NH_3-t-BuOH, MeI (83%); k) Me_3SiI, $(Me_3Si)_2NH$; $CH_2{=}CHCOMe$, BF_3 OEt_2/i-PrOH-$MeNO_2$ (68%); l) pyrrolidine/C_6H_6 (78.6%); m) LDA, MeCHO (92%); n) $(COCl)_2$, DMSO, Et_3N (78.5%); o) DDQ/ether (83%); p) t-BuOOH-Triton B (70%); q) Me_3SiCH_2MgCl; H_2SO_4-THF; HF-MeCN (88%).

Figure 9. Synthesis of the enantiomers of polygodial. Reagents: a) baker's yeast (63-79%); b) t-$BuMe_2SiCl$ (81%); c) LDA/THF-HMPA, MeI (89%); d) NaC≡CH/liq NH_3 (99%); e) $CuSO_4$/xylene, Δ (51%); f) H_2/Pd-$CaCO_3$, quinoline in n-pentane (quant.); g) $MeO_2CC{\equiv}CCO_2Me$, 110°C, 30 h (97%); h) aq HF-MeCN (27% of **27** and 27% of **28** after MPLC); i) DBU/THF, Δ; H_2/Pd-C (80% from **27**); j) TfCl-DMAP/CH_2Cl_2 (86%); k) H_2/Pd (89%); l) LAH/ether (82%); m) $(COCl)_2$, DMSO, Et_3N/CH_2Cl_2 (63%); n) DBU/THF

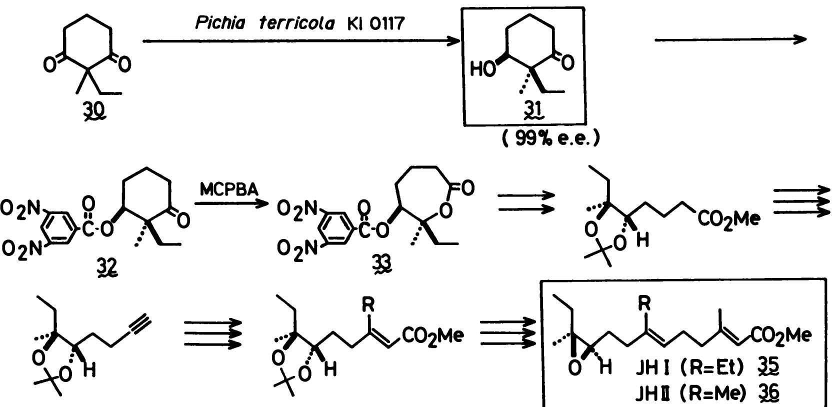

Figure 10. Synthesis of juvenile hormones I and II.

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Baeyer-Villiger oxidation reaction to cleave the cyclohexane ring (31).

(2S,3S)-2-Ethyl-3-hydroxy-2-methylcyclohexanone as a Building Block for Juvenile Hormones I and II. Reduction of a prochiral 1,3-diketone **30** with Pichia terricola KI 0117 furnishes (2S,3S)-**31** of 99% e.e. (32). As shown in Figure 10, **31** was employed as the starting material for the synthesis of enantiomerically pure (+)-juvenile hormones I and II (32). Purification of **31** was achieved by recrystallization of **32**, whose Baeyer-Villiger oxidation gave a crystalline lactone **33**. Methanolysis of the lactone **33** was follwed by protection of the glycol system to give **34**. This was converted to juvenile hormones I (**35**) and II (**36**).

(1S,4R,5S)-5-Hydroxy-1-methylbicyclo[2.2.1]heptan-3-one as a Building Block for Glycinoeclepin A. Reduction of 1-methylbicyclo[2.2.1]-heptane-3,5-dione (**37**) with baker's yeast gives (1S,4R,5S)-5-hydroxy-1-methylbicyclo[2.2.1]heptan-3-one (**38**) of 80-87% e.e. as shown in Figure 11 (Mori, K.; Watanabe, H., The University of Tokyo, unpublished data). By employing two β-hydroxy ketones **25** and **38** as the building blocks, glycinoeclepin A (**40**) was synthesized via **39** (Mori, K.; Watanabe, H., the University of Tokyo, unpublished data). Glycinoeclepin A is a degraded triterpenoid with significant hatch-stimulating activity for the soybean cyst nematode (33, 34).

Figure 11. Synthesis of glycinoeclepin A.

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Chapter 25

Baker's Yeast-Mediated Synthesis of Natural Products

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Baker's yeast, in a manner that is dependent upon the fermentation conditions and the nature of the α and γ substituents, converts α,β-unsaturated aldehydes into three sets of chiral adducts. These materials have been used for the synthesis of optically active forms of several insect pheromones.

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The ability of baker's yeast to transform unconventional substrates stereoselectively is well known, as one can judge from the considerable amount of work in this area reported in the early literature (1).

However, organic chemists only recently recognized the synthetic potential of baker's yeast, in the more general context of a synthetic approach to enantiomerically pure forms of biologically active natural products such as insect pheromones. These materials are of considerable importance in agriculture, and have been synthesized in some cases using readily available optically active natural products (2), (3). The natural materials employed, however, usually are available in only one enantiomeric form, a circumstance which represents a major drawback when both enantiomers of the target molecule are needed, or when the absolute configuration of the final product cannot be obtained from the starting material. Furthermore, the types of chirality present amongst this set of compounds are rather limited; they are particularly abundant in the R,R'CHX structure (X=oxygen or nitrogen functions) and sparse in the R,R',R"CH and R,R',R"C(OR"') moieties.

These observations stimulated a search for new, relatively small, highly functionalized chiral materials complementary to those already produced by nature, and it was thought that this need could be satisfied partially by the compounds available by enzymatic transformations of unconventional substrates using either isolated enzymes or microorganisms (4).

Organic chemists, who are not generally keen on growing cultures, began using baker's yeast which is readily available and cheap, using it like a common shelf reagent in reactions very similar to the natural transformations that it catalyzes. Most of the current applications of baker's yeast involve stereospecific

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reduction of compounds containing carbonyl groups and/or (carbonyl activated) double bonds, and wide substrate acceptance is shown. However, in some instances, the synthetic significance of these transformations is diminished by the limited reaction stereoselectivity perhaps, because several enzymatically active principles are acting on the same substrate with opposing stereobiases (5), (6). We became interested in the baker's yeast transformations of unconventional substrates in a study of the steric course of the known (7) conversion of cinnamaldehyde into 3-phenylpropanol. By means of deuteriated substrates it has been shown that saturation of the double bond occurs with formal trans addition of hydrogen; [2-^{2}H] cinnamaldehyde being transformed into (2S) [2-^{2}H] 3-phenylpropanol (8).

The latter material has been used in the synthesis of asymmetrically labelled L-homoserine in a study on the mechanism of the enzymatic formation of L-threonine (9). However, we soon realized that under the experimental conditions used, the reduction of the carbonyl carbon and the saturation of the double bond are only two of the synthetic manifestations that are possible using an α-β-unsaturated aldehyde (10). Since then, we have been exploring this area and it now appears that α-β-unsaturated aldehydes can be reduced by baker's yeast to yield the synthetically useful chiral products indicated in Scheme 1 in a manner that depends upon the fermentation conditions and the nature of the α and γ substituents.

The formation from cinnamaldehyde **(1)** of the (2S,3R) diol **(3)** (Scheme 2) that contained two additional carbon atoms to those of cinnamaldehyde and two adjacent chiral centres was new.

They occurred as the consequence of two distinct chemical operations. Addition of a C_2 equivalent of acetaldehyde onto the si face of the carbonyl carbon formed the (R) α-hydroxy ketone **(2)**, that was subsequently reduced on the re face to give the (2S, 3R) diol **(3)** (11). The C-C bond forming process is identical to the one reported by Neuberg (12) wherein benzaldehyde was transformed to (R)-acetylphenylcarbinol, a key intermediate in the manufacture of (-) ephedrine, in fermenting baker's yeast.

The identity of the enzyme(s) involved in the latter reaction has been debated (13). However, the formation of the above hydroxyketone, in analogy with acetoin, has been conceptualized as the consequence of the condensation of the "active" form of acetaldehyde, that is formed by decarboxylative addition of pyruvate to thiamine pyrophospate, with benzaldehyde. The role of pyruvate, in fact has been established. The same mechanism can be invoked for the reaction of cinnamaldehyde. It is known that the pyruvate decarboxylase (E.C. 4.1.1.1) accepts as substrates α-oxoacids higher than pyruvate. When we incubated cinnamaldehyde **(1)** with baker's yeast that had been washed with water at pH 5.5-6, and with α-oxobutanoate, we obtained (4R) **5**, that was subsequently reduced by yeast in the presence of D-glucose to the (3S,4R) diol **6** (14). It should be noted that the final product **6** is the result of coupling two reagents (aldehyde and α-oxoacid) that had been added to the reaction mixture.

This reaction, which would be often desirable, is seldom observed in the enzymatic catalysis (15).

There is some tolerance by the enzyme(s) involved in the above

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baker's yeast

R= C_6H_5, 2-furyl; EtOOC; C_6H_5COO
R^1= H, Me, Br, Cl

Scheme 1

1 R= H,Me

2 R= H,Me

3 R= H

4 R= Me

Scheme 2

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process with respect to the structure of the α,β-unsaturated aldehyde. Indeed, α-methyl and α-bromocinnamaldehydes afforded the corresponding diols **7** and **8** (Scheme 3), but α-ethylcinnamaldehyde is not a substrate for this reaction. Furthermore, α-methylcrotonaldehyde was not accepted, but when the position was oxidized, as in ethyl 3-methyl-4-oxo crotonate, up to 35% yield of the diol **9** was obtained in baker's yeast fermenting on **D**-glucose.Using washed yeast, the same aldehydoester in the presence of ethyl α-oxobutanoate afforded the corresponding (R) α-hydroxy ketone, that was subsequently reduced to the diol (**10**), although in lower overall yield.

The diols **4** and **6-10** are particularly suited for the preparation of small molecules, like insect pheromones, that contain relatively few chiral carbon atoms in their framework and whose chirality is due to oxygen substitution.From related isopropylidene structures it is possible to synthesize the chiral C_4,C_5 and C_6 carbonyl compounds **(11)-(14)** by ozonolysis, that are convertible into the α-epimers **(15)-(18)** by base treatment. Furthermore, the number of synthons that are potentially available is increased by the adducts accessible from **(11)-(18)** by reaction with suitable carbon nucleophiles under conditions allowing control of the stereochemistry by the two oxygen functions.

Synthesis of Pheromones from the Products of Acyloin-Type Condensation

Thus, (-)-frontalin **19** (Scheme 4), pheromone of Dentroctonus Frontalis bark beetle, was obtained from the methyl ketone **12** through the intermediacy of the adduct **20**, that was obtained as the sole transformation product by reaction with 4-methyl-pent-4-enylmagnesium bromide in THF. The latter material, once O-benzylated, with acid hydrolysis yielded **22**, and was then converted into **23** by periodate oxidation, followed by $NaBH_4$ reduction.

This sequence destroyed the two chiral centers of **12** that had assisted the formation of the quaternary chiral center required in **26**.

The conversion of **23** into **26** simply required ozonolysis to **24** and hydrogenolysis to the dihydroxyketone **25**, which spontaneously cyclizes to **26** (27% overall yield from **20**) (16).

The aldehyde **11** and its α-epimer **15**, on reaction with saturated Grignard reagents, afforded separable mixtures of diastereoisomers in which the syn adducts prevailed.

The two sets of adducts **26-31** so obtained (Scheme 5) were O-benzylated, hydrolyzed under acidic conditions, and oxidized with periodate, to yield the enantiomeric aldehydes **32-37**.

The aldehyde **32** was treated with a suitable C_5 Grignard reagent to produce the adducts **38** and **39**, (Scheme 6) that were then converted in unexceptional steps, into (+)-exobrevicomin **40** and into the (-)-endo diastereoisomer **41**, that were separated by preparative gas chromatography.

Similarly, from **35** the (-) and (+)- enantiomers, respectively, were prepared (17).

Compound **33**, was allowed to react with BrMg $(CH_2)_3CH{=}CH_2$, and the syn and anti diastereoisomers so obtained were separated to ultimately yield, (5S,6R) 6-acetoxy-5-hexadecanolide **42** and the

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5

6 R= Me; R^1= H
7 R= H; R^1= Me
8 R= H; R^1= Br

9 R= H
10 R= Me

11 R= R^1= H
12 R= H; R^1= Me
13 R= Me; R^1= H
14 R= R^1= Me

15 R= R^1= H
16 R= H; R^1= Me
17 R= Me; R^1= H
18 R= R^1= Me

Scheme 3

In Biocatalysis in Agricultural Biotechnology; Whitaker, J., el al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1989.

20 R= H

21 R= $CH_2C_6H_5$

22 R= $CH_2C_6H_5$

23 R= $CH_2C_6H_5$; X= CH_2

24 R= $CH_2C_6H_5$; X= O

25 R= H; X= O

19

Scheme 4

26 R= Me

27 R= $C_9H_{19}n$

28 R= $(CH_2)_2CH{=}CH_2$

29 R= Me

30 R= $C_9H_{19}n$

31 R= $(CH_2)_2CH{=}CH_2$

32 R= Me; R^1= $CH_2C_6H_5$

33 R= $C_9H_{19}n$; R^1= $CH_2C_6H_5$

34 R= $(CH_2)_2CH{=}CH_2$

35 R= Me; R^1= $CH_2C_6H_5$

36 R= $C_9H_{19}n$; R^1= $CH_2C_6H_5$

37 R= $(CH_2)_2CH{=}CH_2$

Scheme 5

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(5R,6R) diastereoisomer (Scheme 7). Again, from **34** the (5R,6R) product **43** was obtained. Products **42** and **43** represent the two enantiomers of the proposed major component of a mosquito oviposition attractant pheromone (18). The enantiomeric forms of 5-hexadecanolide, **44** and **45**, the pheromone of Vespa orientalis, were obtained from the enantiomeric aldehydes **34** and **37** (19). All the compounds obtained up to this point have been prepared in both enantiomeric forms, starting from a single chiral material.

Contrastingly, the chiral C_4 framework of the threo aldehyde **15** was incorporated into (2S,3S) octane-2,3-diol **46**, and into the derived ketone **47**, by a Wittig condensation with a C_4 reagent, followed by hydrogenation and hydrolysis. These materials are pheromones of Xylotrechus pyrrhorecus. From the same intermediate, by unexceptional steps, **47** was obtained (20). Incorporation of the carbon framework of the aldehyde **11** into (3S,4S) 4-methyl-3-heptanol **49** (Scheme 7) was accomplished via the epoxide **48**, but with extended manipulation of the chirality present in the precursor (21). Product **49** is a pheromone of Scolytus multistriatus. In a study of the mechanism of the acyloin-type condensation reported in Scheme 2, we obtained the (3S,4R) diol **50** by yeast reduction of the corresponding hydroxyketone. This served as the starting material for the synthesis of (4S,5R) sitophilure **53** (Scheme 8), a pheromone of the genus Sito philus. The key intermediate was the allylic epoxide **51**, that yielded the chiral homoallylic alcohol **52** on AlH_3 opening, and contained the chirality and the masked functionality for conversion into **53**. Indeed, O-protection by silylation, ozonolysis, addition of ethylmagnesium bromide, oxidation of the carbinol and deprotection yield the required product **53** (22).

Synthesis of Pheromones from the Products of Saturation of the Double Bond.

With the exception of the diol **9**, that was obtained from the corresponding aldehyde in up to 35% yield, most of the chiral diols mentioned above were isolated in yields of only 20-25%. The formation of the acyloin-type condensation products is in competition with the much more efficient reduction of the carbonyl carbon and saturation of the double bond of the unsaturated aldehydes that were used as substrates. We became interested in the mode of reduction of particular aldehydes such as **54-56** (Scheme 8) in a study of the total synthesis of natural α-tocopherol (vitamin E) (23). We expected to obtain chiral alcohols that would be useful for conversion into natural isoprenoids from the reduction of the α-double bond of the above aldehydes. Indeed, **54-56** afforded up to 75% yield of the saturated carbinols **57-59** by treatment with yeast. Whereas the ee of **57** and **58** was ca 85%-90%, that of **59** is 99%, as shown by NMR experiments on the (-)-MTPA derivative (24). The synthetic significance of carbinol **59** was based on the C_5 structural unit present in natural isoprenoids (see brackets in structural formulas).This protected synthon can be unmasked by ozonolysis, as indicated by the high yield conversion of **59** into (S)-(-)-3-methyl-γ-butyrolactone **60** (Scheme 9). Product **59** is a bifunctional chiral intermediate which does not need protective manipulation in that

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OH

OR

R

38 R= $\overline{O(CH_2)_2O}$; R^1= $CH_2C_6H_5$

OH

OR

R

39 R= $\overline{O(CH_2)_2O}$; R^1= $CH_2C_6H_5$

O

O

40

O

O

41

Scheme 6

OAc

O O R

42 R= $C_9H_{19}n$

OAc

O O R

43 R= $C_9H_{19}n$

O O R

44 R= $C_{10}H_{21}n$

O O R

45 R= $C_{10}H_{21}n$

OH

OH

46

OH

O

47

O

OR

48 R= $CH_2C_6H_5$

OH

49

Scheme 7

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Scheme 8

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In Biocatalysis in Agricultural Biotechnology; Whitaker, J., el al.;
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60 61 62

63 64

65 R= COC_6H_5; R^1= Me

66 R= COC_6H_5; R^1= H

67 R= COC_6H_5

68 R= COC_6H_5; R^1= different substituents

Scheme 9.

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the furan ring can be seen as a carbonyl protecting group and can be selectively removed permitting elongation at both ends of the molecule.The functionalities of **59** can be used in alternating manner. The direct ozonization with oxidative work up led to **60**. Alternatively, the alcohol functional group could be used to modify the side chain and the furan ring ozonized at a subsequent stage. Taking advantage of the above mentioned properties, product **59** has been used for the preparation of (4R,8R)-4,8-dimethyldecanal **64**, a pheromone component of Tribolium castaneum (24). The synthesis of **64** involved the use of two equivalents of **59** and proceeded through the intermediates **61-63** that were assembled according to the sequences: $C_5 + C_1 \rightarrow C_6$; $C_6 + C_5 \rightarrow C_{11}$; $C_{11} + C_1 \rightarrow C_{12}$. In addition to the two sets of chiral products mentioned above, another type of optically active material is available in fermenting baker's yeast from α,β-unsaturated aldehydes. During studies on the substrate specificity of the acyloin-type condensation indicated in Scheme 2 we submitted γ-oxygen substituted crotonaldehydes to the action of fermenting baker's yeast at pH 5.Whereas **65** afforded 35 % yield of the diol **9**, **66** gave rise to the diol **61** in ca 20% yield as sole chiral transformation product. Compound **67** is believed to arise by formal 1,4-addition of water across the double bond of the α,β-unsaturated aldehyde **66**, followed by reduction of the intermediate β-hydroxy aldehyde. The synthetic significance of **67** is that it has the (R) configuration. This is opposite to the identical material formally accesible from natural (S) malic acid, which has been already used as a starting material in pheromone synthesis. We expected to observe an extension of the latter mode of action of fermenting baker's yeast to more functionalized γ-oxygen substituted α,β-unsaturated aldehydes, hopefully to obtain stereospecific hydration/reduction and kinetic resolution with substrates like **68**. In such an event, we should have access to new chiral intermediates that could be useful in pheromone synthesis.

Many other substances belonging to quite different structural classes, in addition to those mentioned above, have been synthetised from the chiral materials obtained in fermenting baker's yeast and α,β-unsaturated aldehydes, thus lending further support to the significance for organic synthesis of the products obtained by biotransformations.

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Chapter 26

Preparation of Optically Active Pyrethroids via Enzymatic Resolution

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Efficient biochemical processes were developed for the preparation of the two optically active pyrethroid insecticides by a combination of enzyme-catalyzed reactions and chemical transformations. These are based on the findings that a lipase from Arthrobacter species hydrolyzes the acetates of the two important secondary alcohols of synthetic pyrethroids with high enantioselectivity and reaction rate. The two alcohols are 4-hydroxy-3-methyl-2-(2'-propynyl)-2-cyclopentenone (HMPC) and α-cyano-3-phenoxybenzyl alcohol (CPBA). The enzyme gave optically pure (R)-HMPC or (S)-CPBA and the unhydrolyzed esters of their respective antipodes. Kinetic studies suggested that the high enantioselectivity resulted from very selective binding of the substrates to the enzyme.

Synthetic pyrethroids are a group of ester compounds having excellent insecticidal activities. After the discovery of allethrin (1), a variety of useful synthetic pyrethroids have been produced mainly by structural modification of an alcohol having an asymmetric center. The insecticidal activities greatly depend upon the stereoisomers. Therefore, much effort has been expended to develop technologies for obtaining optically active isomers. However, contrary to the case of chrysanthemic acid, chemical methods of optical resolution were not very effective for these alcohols.

Because of the specificity and the enantioselectivity of some enzyme-catalyzed reactions, the application of enzymes is increasingly important in asymmetric induction and kinetic resolution in organic synthesis. A large number of publications were recently reviewed, focusing on utilization of enzymes and microorganisms to stereospecific hydrolysis and other reactions to produce pure stereoisomers (2,3). However, the use of an enzyme as a catalyst has usually been limited to small-scale experiments in the laboratory.

Lipases are a class of enzymes having high stability in the presence of large amounts of water-insoluble oily substrates, and catalyze hydrolysis at a water-oil interface. Such enzymes,

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requiring no cofactors, are now commercially available from various sources, and might be one of the most promising enzymes for large-scale application in organic synthesis.

This article describes our results on lipase-catalyzed enantioselective hydrolysis of carboxylic acid esters of two industrially important alcohols related to synthetic pyrethroids in two-liquid phase systems. The two alcohols are 4-hydroxy-3-methyl-2-(2'-propynyl)-2-cyclopentenone (HMPC) and the cyanohydrin, α-cyano-3-phenoxybenzyl alcohol (CPBA). The configurations, 1 and 2, are given for the liberated stereoisomers of the two alcohols in our lipase-catalyzed reactions.

H HO O

1

H C HO CN O

2

It is known that the (S)-forms are the essential stereoisomers for the insecticidal activities of both alcohols (4,5). Chemico-enzymatic processes are also reported in this article on the preparation of the optically active pyrethroid insecticides having the (S)-isomers of the two alcohols. Processes were developed that use enantioselective hydrolysis with a lipase.

4-Hydroxy-3-methyl-2-(2'-propynyl)-2-cyclopentenone (HMPC)

HMPC is an alcohol moiety of a new promising synthetic pyrethroid for household use, prallethrin. This ester has several times greater knockdown and killing effects than allethrin against various insect pests (4).

Hydrolysis by Lipase. The screening of our stock cultures revealed that a large number of microorganisms including bacteria, actinomycetes, yeasts and molds enantioselectively hydrolyzed the acetate of racemic HMPC to give (R)-HMPC preferentially in most cases (6). A study on the addition of an oil to the culture media suggested that the resposible enzyme might be a lipase rather than a carboxylic esterase. Thus, commercially available lipases were tested for the hydrolysis of the racemic ester. Ten out of twelve lipases tested were found to hydrolyze the ester with various rates of hydrolysis, yielding varying optical purities of the alcohol. Examples of microbial enzymes that gave the (R)-alcohol and the (S)-ester with relatively high optical purities are shown in Table I. The experiments were conducted at a substrate concentration of 8.8w/v%, pH 7.0 and 40°C.

A lipase from Arthrobacter species yielded pure (R)-HMPC at 50% hydrolysis with the smallest amount of the enzyme. Lipases from Pseudomonas fluorescens, Chromobacterium viscosum and Alcaligenes species were of less interest to us than the Arthrobacter lipase among others, judging from the optical purity of the product, degree

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of hydrolysis, and amount of the enzyme used. The three enzymes also gave high optical purities at around 50% hydrolysis.

Table I. Enantioselective Hydrolysis of Acetate of HMPC with Microbial Lipases

Origin of Lipase	Amount of Lipase Used (mg)	Degree of Hydrolysis (%)	Isomer Ratio of Liberated HMPC (S)/(R)	Enantiomeric Ratio*) E
Arthrobacter sp.	3	49.9	0.4/99.6	1280
Pseudomonas fluorescens	6	47.1	2.6/97.4	101
Chromobacterium viscosum	6	58.6	4.5/95.5	-
Alcaligenes sp.	20	47.4	2.2/97.8	124
Achromobacter sp.	100	32.9	2.4/97.6	64
Humicola sp.	100	36.8	9.3/90.7	15
Candida cylindracea	100	10.8	8.7/91.3	12

*) see Literature Cited (7).

All of the lipases used here are crude preparations, and hydrolyzed the acetate much slower than olive oil (Table II).

Table II. Activity of Microbial Lipase on Acetate of HMPC and Olive Oil

Origin of lipase	Activity on*) acetate of HMPC (µmol/min·mg-protein)	olive oil (µmol/min·mg-protein)	olive oil (µmol/min·mg-powder)
Arthrobacter sp.	17.4	230	190
Chromobacterium viscosum	6.7	790	330
Pseudomonas fluorescens	3.9	133	70
Alcaligenes sp.	2.0	59	22
Achromobacter sp.	0.8	82	10
Humicola sp.	0.2	400	120
Candida cylindracea	0.1.	110	21

*) pH 7.0 and 40° C

It should be noted that in the lipase-catalyzed hydrolysis the reaction mixture is biphasic consisting of water and water-insoluble oily substrate, and that the reaction is easily controlled by

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starting and terminating vigorous stirring. Furthermore, product recovery is simplified in the absence of organic solvents. Thus, the use of lipases that can be utilized as "an organic reagent" is much more advantageous than that of microorganisms for the enantio-selective hydrolysis of alkyl esters of secondary alcohols. This is especially true for the industrial preparation of chiral alcohols with biocatalysts (8).

Enantioselective Hydrolysis with *Arthrobacter* Lipase. Reaction performance with the *Arthrobacter* lipase was studied in detail. The pH profile curve of the zero-order reaction exhibited a pH-optimum around 7.0, and spontaneous hydrolysis was not significant at pH lower than 8.0 (Figure 1). The enzyme was sufficiently stable as long as the reaction was carried out at 40° to 50°C. The optical purity of the (R)-HMPC liberated was not influenced by the chain length from acetyl to capryl moiety at all ((S)/(R)=0.4/99.6~0.1/99.9).

Time-course of the hydrolysis clearly showed in Figure 2 that the reaction proceeded without difficulty even at a substrate concentration as high as 80%, and stopped spontaneously at 50% hydrolysis at which point the acetate of (R)-HMPC was completely hydrolyzed. The HMPC liberated was optically pure at each experimental point regardless of percent hydrolysis. Therefore, the *Arthrobacter* lipase distinguishes the (R)-isomer from its antipode quite completely.

Kinetic Study of Hydrolysis. It is of great interest to know the nature of the high enantioselectivity of the *Arthrobacter* lipase. The initial velocity measurements were conducted for the purpose of knowing which is the main factor of the enantioselectivity, the apparent Michaelis constant K'm or the catalytic constant k'cat.

In a lipase-catalyzed reaction of a water-insoluble oily substrate, the enzyme reversibly adsorbs at the substrate-water interface, and the enzymatic reaction takes place at that interface. The interfacial area strongly depends upon some physical parameters of emulsion, such as the size of the emulsion droplets as well as the amount of the substrate present. However, the importance of the interfacial area should not be overestimated in some cases (9). If the experiments were carried out whereby one makes very fine droplets of the emulsion and has a sufficiently high concentration of the substrate, all of the enzyme molecules are adsorbed at the interface and a "maximal velocity" is observed. In such a case, the usual kinetic treatment of an aqueous homogeneous reaction can be applied for the estimation of K'm and k'cat in a two-liquid phase reaction system. Two experiments were conducted to confirm that the conditions mentioned above are fulfilled when the substrate concentration is much greater than that of the enzyme.

A study of the influence of stirring cycles on the specific activity of the *Arthrobacter* lipase revealed that the activity increased with the stirring cycles and leveled off at 800 rpm. This might imply that the maximal interfacial area was obtained at more than 800 rpm of the agitation. This was supported by the effect of detergents on the activity at 1,000 rpm. Addition of various detergents to the reaction mixture decreased the activity. This is attributed to the decrease of the interfacial area, a part of which

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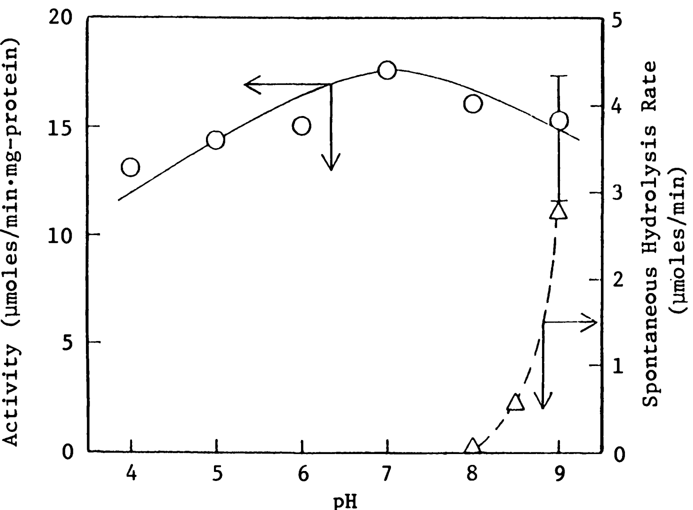

Figure 1. pH Profile for Arthrobacter Lipase-catalyzed Hydrolysis of Acetate of Racemic HMPC.

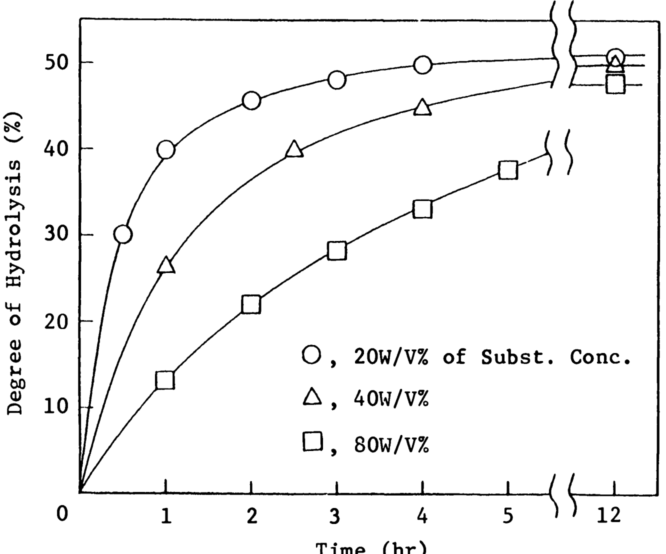

Figure 2. Reaction Performance of Acetate of Racemic HMPC with Arthrobacter Lipase.

is occupied by the detergent added. Thus, all of the experiments were performed under the conditions of 1,000 rpm and the substrate concentration greater than that of the enzyme.

According to the kinetic treatment of Lavayre et al (10) on two insoluble enantiomeric isomers, the relative initial velocities, V/VR were plotted against various ratios in the (S)-enantiomer. All of the initial velocity measurements were made at 50°C instead of 40°C to avoid crystallization of the acetate of pure (R)-HMPC, and at pH 6.0 to prevent spontaneous hydrolysis of the substrate at 50°C. The result gives a concave type of curve as illustrated in Figure 3. This implies that K'mS < K'mR and that the (S)-enantiomer is a strong competitive inhibitor. Thus, it is concluded that a very high optical purity of (R)-HMPC liberated with Arthrobacter lipase is entirely the result of the great catalytic constant of the (R)-HMPC ester.

The Lineweaver-Burk plots of the (R)-ester, in the presence and absence of the (S)-enantiomer, exhibited a typical pattern of a competitive inhibitor with two straight lines crossing on the vertical axis. Apparent kinetic constants were evaluated from the plots and summarized in Table III.

Table III. Apparent Kinetic Constants of Hydrolysis of Acetate of HMPC with Arthrobacter Lipase

Enantiomeric Isomer	K' (mM)	k'cat (μmole/min·mg-protein)
(R)-HMPC	100 (K'm)	66
(S)-HMPC	52 (K'i)	0

From the results, the enantioselectivity may be explained by the binding situation of the two enantiomers to the Arthrobacter lipase as illustrated schematically (Figure 4). This particular enzyme may have the ability to recognize the steric bulk of the two substituents on the asymmetric carbon of the alcohol so that the larger hydrophobic substituent L, is tightly bound to the larger hydrophobic subsite ρ_1, within the active center and the smaller substituents S, to the smaller subsite ρ_2. The carbonyl group of the (R)-ester is positioned very near to the catalytic site of the enzyme so that the (S)-enantiomer is hydrolyzed with the high velocity whereas that of the (S)-ester is placed too far from the catalytic site to be attached.

Preparation of Optically Active (S)-Prallethrin

To obtain the insecticidally active (S)-HMPC **6**, the enzymatically hydrolyzed (S)-acetate **4** must be separated from inactive (R)-HMPC **1** and hydrolyzed chemically to the desired (S)-HMPC **6**. This might not be very attractive from the industrial viewpoints, even if the

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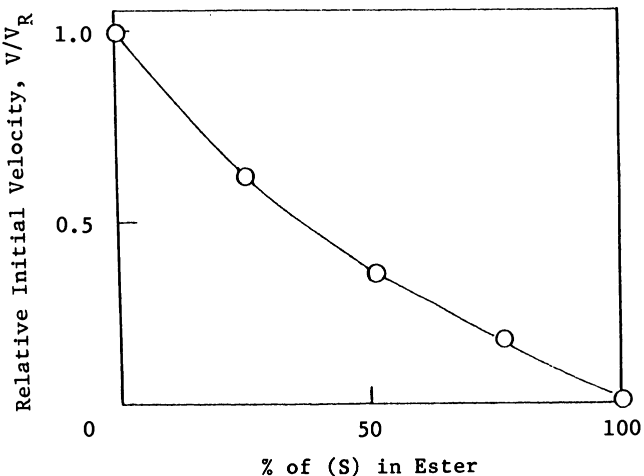

Figure 3. Initial Velocity of Hydrolysis of Mixtures of (R)- and (S)-HMPC Acetate with Arthrobacter Lipase.

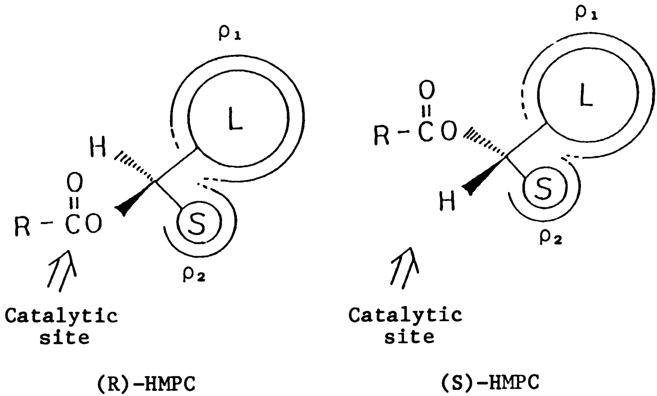

Figure 4. Binding Mode for High Enantioselectivity of Arthrobacter Lipase.

enzymatic optical resolution was quite efficient as was mentioned above. However, this problem was usefully solved by means of the chemical transformation of the (R)-HMPC 1 to the desired (S)-HMPC 6, as shown in Figure 5.

The mixture of the (R)-HMPC 1 and the (S)-acetate 4 from the enzymatic hydrolysis was esterified with methanesulfonyl choride and triethylamine to afford a mixture of the corresponding (R)-sulfonate 5a and the (S)-acetate 4. The (S)-acetate 4 was unaffected by the sulfonation conditions. The resultant mixture of two esters, 4 and 5a, was hydrolyzed in the presence of a small amount of calcium carbonate. The (R)-sulfonate 5a was converted into the (S)-HMPC 6 as a result of inversion of configuration. On the other hand, the (S)-acetate 4 was hydrolyzed with retention of configuration. Consequently, all of the racemic acetate 3 was converted with maximum efficiency to the desired (S)-HMPC 6 by the sequence: enzymatic hydrolysis and sulfonation followed by inversion of the chiral center in (R)-HMPC 1 without separation of the (S)-acetate 4. Similar transformations could also be carried out via nitrate ester intermediate 5b obtained from the reaction of the (R)-HMPC 1 with nitric acid and acetic anhydride.

Finally, the derived (S)-HMPC 6 was esterified with (1R)-chrysanthemoyl chloride 7 to give the most potent optically active form 8 of a new synthetic pyrethroid, prallethrin, which has significant knock-down and killing effects against various household insect pests. The insecticidal activities of some stereoisomers of prallethin are summarized in Table IV. Optically active (S)-prallethrin showed several times the lethal activities of the racemic mixture against housefly and cockroach.

Table IV. Insecticidal Activities of Stereoisomers of Prallethrin against Housefly and Cockroach (Topical Application)

Stereoisomers of Prallethrin		Relative Potencies	
Alcohol	Acid	Housefly (*M. domestica*)	Cockroach (*B. germanica*)
S	(1R)-trans	419	611
S	(1R)-cis	164	185
R	(1R)-trans	72	56
R	(1R)-cis	57	9
RS	(1RS)-cis,trans	100	100

α-Cyano-3-phenoxybenzyl Alcohol (CPBA)

CPBA is an alcohol moiety of highly potent synthetic pyrethroids such as fenvalerate (α-cyano-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methylbutyrate) or cyphenothrin (α-cyano-3-phenoxybenzyl chrysanthmate).

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Figure 5. Chemico-enzymatic Preparation of Optically Active (S)-Prallethrin from Racemic HMPC.

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The former is a photostable and efficient agricultural insecticide having no cyclopropane ring that is employed for cotton, fruits and vegetables.

Enantioselective Hydrolysis with Arthrobacter Lipase. The results of the enantioselective hydrolysis of the acetate of racemic CPBA are summarized in Table V for several commercial lipases that liberate very optically pure CPBA. The experimental conditions were chosen to give approximately 50% hydrolysis for each enzyme. It is noticed that all of the lipases in Table V hydrolyzed the ester of (S)-CPBA preferentially to give the insecticidally active (S)-isomer. This is apparently different from the case of HMPC. The highest activity and optical purity were again given by the *Arthrobacter* lipase. Spontaneous termination of the reaction at 50% hydrolysis was observed with this enzyme as was the case of HMPC.

The reaction rate of the CPBA ester was the same order of magnitude with that of the HMPC ester. This is astonishing considering the presence of two bulky substituents, 3-phenoxybenzyl and cyano groups, on the carbon atom in the α-position of the ester bond. Brockerhoff's generalization (11) predicts strong inhibition in such a case.

The pH profile of the hydrolysis of the acetate of CPBA showed that the activity of the enzyme was not greatly influenced in the range of pH 4 to pH 7. An undesirable conversion from the liberated (S)-CPBA to 3-phenoxybenzaldehyde was sharply increased when the pH was higher than pH 5. The reaction was therefore carried out at pH 4 to pH 5 as shown in Table V. Other reaction behavior was similar to that for the HMPC ester.

Table V. Enantioselective Hydrolysis of Acetate of CPBA with Microbial Lipases

Origin of Lipase	pH	Temp. (°C)	Conc. of Substrate (w/v%)	Degree of Hydrolysis (%)	Isomer Ratio of CPBA (S)/(R)
Arthrobacter sp.	4.0	35	20	49.0	99.9/0.1
Pseudomonas fluoresens	5.0	30	10	49.8	96.4/3.6
Chromobacterium viscosum	4.0	40	20	49.6	97.8/2.2
Alcaligenes sp.	5.0	30	10	49.8	96.7/3.3
Achromobacter sp.	5.0	30	10	47.5	96.7/3.3

Discussion on Binding Mode. The initial velocity measurements of the acetate of CPBA with various ratios of the (R)-enantiomer were observed to give almost the same line having a concave curve as illustrated for the acetate of HMPC in Figure 3. This result

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indicates that K'mR < K'mS in the case of the CPBA ester and that the (R)-enantiomer is a strong competitive inhibitor. The high optical purity of the liberated (S)-CPBA we conclude is due to the great catalytic constant of the (S)-enantiomer.

Why was the (S)-CPBA ester enantioselectively hydrolyzed when the same enzyme catalyzes the hydrolysis of the (R)-HMPC ester ? The answer to this question may be given by the comparison of the configuration of (R)-HMPC and (S)-CPBA (1 and 2) as well as the R and S definitions by the conventions of the sequence rule (12).

The relative sizes of the groups attached to the asymmetric carbon atom in HMPC and CPBA are, respectively:

$-C(CH_3)=C(-)-CH_2C\equiv CH \; > \; -CH_2-C(-)=O$

$3\text{-phenoxyphenyl} \; > \; -C\equiv N$

The stereochemistry of the (S)-CPBA is in good accordance with that of the (R)-HMPC as illustrated in 1 and 2. However, the definition of (R) and (S) in the two compounds are the results of nomenclature. Thus, the binding situation of the L and S substituents to the subsites ρ_1 and ρ_2 in the active center of the enzyme and the positioning of the carbonyl group of the (S)- and (R)-CPBA esters to the catalytic site are considered to be very similar to those in the case of the (R)- and (S)-HMPC esters.

Preparation of Esfenvalerate ((S)-(S)-Fenvalerate)

In contrast to the case of HMPC, most lipases hydrolyze the racemic acetate of CPBA 9 to give a mixture of the insecticidally active (S)-CPBA 2 and the (R)-acetate 10. Thus, the desired (S)-CPBA 2 could be separated from the (R)-acetate 10 by means of a continuous counter-current extraction using n-heptane solvent at 80°C. However, it is important to utilize the recovered (R)-acetate 10 for an efficient process. Fortunately, since the proton of the asymmetric carbon of the cyanohydrin acetate is labile, the antipodal (R)-acetate 10 is easily racemized by treatment with weak organic base such as triethylamine without any side reactions. The racemized acetate 9 thus obtained was recycled as shown in Figure 6. Therefore, all of the racemic acetate 9 was converted to the desired (S)-CPBA 2 in this recycling process. The (S)-CPBA 2 obtained was esterified with (S)-2-(4-chlorophenyl)-3-methylbutyryl chloride 11 to produce the most insecticidally active stereoisomer 12 of fenvalerate, namely esfenvalerate. The relative biocidal activities between

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Figure 6. Preparation of Esfenvalerate from Racemic CPBA.

esfenvalerate 12 and fenvalerate are shown in Table VI. Esfenvalerate has 3.5-4.0 times the insecticidal activities of fenvalerate and is not at all phytotoxic.

Table VI. Relative Biocidal Activities between Esfenvalerate and Fenvalerate

compound	absolute configuration		biocidal activities	
	alcohol	acid	insecticidal *)	chlorotic **)
esfenvalerate	S	S	3.5-4.0	1/16
fenvalerate	RS	RS	1	1

*) relative potency to housefly and cabbage army worm
**) relative chlorotic efficacy in tomato or Chinese cabbage

In conclusion, the combination of an enzymatic optical resolution and subsequent chemical transformations of epimerization or racemization of the asymmetric center of the unwanted antipodes have led to the successful development of processes for preparation of the two optically active pyrethroid insecticides. This work will provide a novel feature in the application of enzymes, especially lipases for the industrial production of chiral compounds.

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