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Cellulase: A Perspective

D. E. Eveleigh

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Cellulase: a perspective

By D. E. Eveleigh

Department of Biochemistry Microbiology, Lipman Hall, Cook College, Rutgers University, New Brunswick, New Jersey 08903, *U.S.A.*

[Plate 1]

Cellulose, a polymer of β-1,4-linked D-glucose residues, is the World's most abundant natural polymer. It occurs predominantly in plants, forming their main structural component, but also occurs widely in other organisms, such as bacteria, algae, fungi and animals. With annual production of around 1.8×10^{12} tonnes, it has attracted considerable study encompassing its synthesis, biodegradation and utilization in several recent reviews (M. P. Coughlan *Biotechnol. Genetic Engng Rev.* **3**, 39–109 (1985); B. S. Montenecourt & D. E. Eveleigh in *Gene manipulations in fungi* (ed. J. M. Bennett & L. L. Lasure), pp. 491–512, New York: Academic Press (1985); J. N. Saddler *Microbiol. Sci.* **3**, 84–87 (1986)). With this wealth of data at hand, a perspective of fungal cellulase is presented with consideration of current models of action, nature of the enzyme complex, analytical methods and approaches for enhanced production.

The substrate

Cellulose, the major structural polysaccharide of plants, has high tensile strength and is recalcitrant to degradation, properties useful to the plant cell but not necessarily so to the biotechnologist. It is composed of insoluble, linear chains of β-(1→4)-linked glucose units with an average degree of polymerization *in situ* of 10000, yet can be as low as 15. Commercial celluloses are considerably reduced from the upper value. The polysaccharide chains associate to form insoluble elementary fibrils that further aggregate as larger microfibrils. Hydrogen bonding and van der Waals forces promote effective interchain binding with the resultant formation of the insoluble polymer. Although the interchain forces are strong, the association between the elementary fibrils is generally imperfect and celluloses of greater than 90% crystallinity are relatively rare. These include cotton and *Valonia* cell walls, although substrates, such as filter paper and commercial microcrystalline cellulosics commonly employed in cellulase assays, are not highly crystalline. The resistance of cellulose to hydrolysis is, in part, caused by the crystalline state, although crystallinity indices are not an absolute criterion and are not directly indicative of the susceptibility to enzyme hydrolysis. Indeed, Marchessault and co-workers, with both regenerated cellulose films (Marchessault & St-Pierre 1980) and steam-exploded woods (Marchessault *et al.* 1983; Puls *et al.* 1985), found that the degree of swelling and fibrillar structure were more useful as parameters that were indicative of susceptibility. Similarly Grethlein (1985), with pretreated soft and hard woods, showed that their susceptibility to cellulase was directly related to their pore volume, and that the crystallinity index is not an overriding factor. Thus for practical enzyme saccharification schemes, a principal focus of research has been to gain greater enzyme access to the inner microfibrils by increasing the pore volume of the substrate (Saddler 1986).

A further important consideration in cellulolysis is that the repeating unit of cellulose is cellobiose, the crystal unit cell being 10.5 Å × 8.35 Å × 7.9 Å†. Alternate glucose moieties are rotated 180° along the axis of the polymer chain, and thus a terminal residue can occur in either of two orientations. With the two stereodistinct cellobiose repeating units, two broad classes of stereospecific enzyme can be postulated to occur, as has been emphasized by Wood (1985). From a stereological perspective, it can be considered as two intimately associated polymers. In brief, cellulose is a recalcitrant polysaccharide whose susceptibility to enzyme hydrolysis is considerably enhanced by increasing its pore structure.

Cellulase

A diverse spectrum of cellulolytic fungi and bacteria are the major mineralizers in soil and aquatic ecosystems. Yet, because many of these microbes fail to release copious amounts of cellulase into culture broths, they have received scant study. In contrast, microbes that are prolific cellulase producers, including those from such fungal genera as *Trichoderma* and *Phanerochaete*, and bacteria such as *Cellulomonas*, *Pseudomonas* and the actinomycetes *Thermomonospora* and *Microbispora*, have received far greater attention. Microbes with surface-bound cellulases such as *Clostridium thermocellum* and *Ruminococcus*, which appear typical of many bacteria, are only recently receiving deserved attention.

The basic model of cellulase action is based especially on fungal systems. Cellulase is a multicomponent enzyme system generally considered to be composed of three major components: endo-1,4-β-glucanase (endo-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4), cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.21). A further component, glucohydrolase (1,4-β-D-glucan 4-glucohydrolase, EC 3.2.1.71) is sometimes present. These enzymes act cooperatively and sometimes synergistically in the hydrolysis of crystalline cellulosic substrates (figure 1). In the early 1970s, K.-E. Eriksson, K. Nisizawa and T. Wood independently proposed a basic model in which they envisaged endoglucanases to initiate attack at multiple internal sites in amorphous zones of the substrate, thereby creating new sites for subsequent attack by cellobiohydrolase. This latter enzyme initiates its attack at non-reducing termini and then sequentially cleaves cellobiose residues from the glucan chain. The action of these two enzyme species is synergistic and results in soluble oligomers, cellobiose and glucose, the former two products subsequently being cleaved to yield glucose by cellobiase. Each enzyme by itself is essentially inactive towards crystalline cellulose. The model has been a useful basis for study but now must be amended, because there are multiple forms of each type of enzyme that have differing substrate specificities; besides, it fails to define adequately the manner of cooperative interaction between the components, which is still poorly understood. Furthermore, two stereospecific classes of endoglucanases (A:A′) and cellobiohydrolases (B:B′) (figure 1) can be theoretically predicted (although not yet observed), based on the fact that the cellobiose repeating unit occurs in two stereospecific forms. Thus the model continues to be revised and some aspects of it are now considered.

The proposed modes of interaction have been confirmed, in part, by an elegant electron microscopic study with *Trichoderma reesei* cellulase (cellobiohydrolase CBH II and endoglucanase EG II) acting against highly crystalline *Valonia* cellulose fibrils (figure 2, plate 1,

† 1 Å = 10^{-10} m = 10^{-1} nm.

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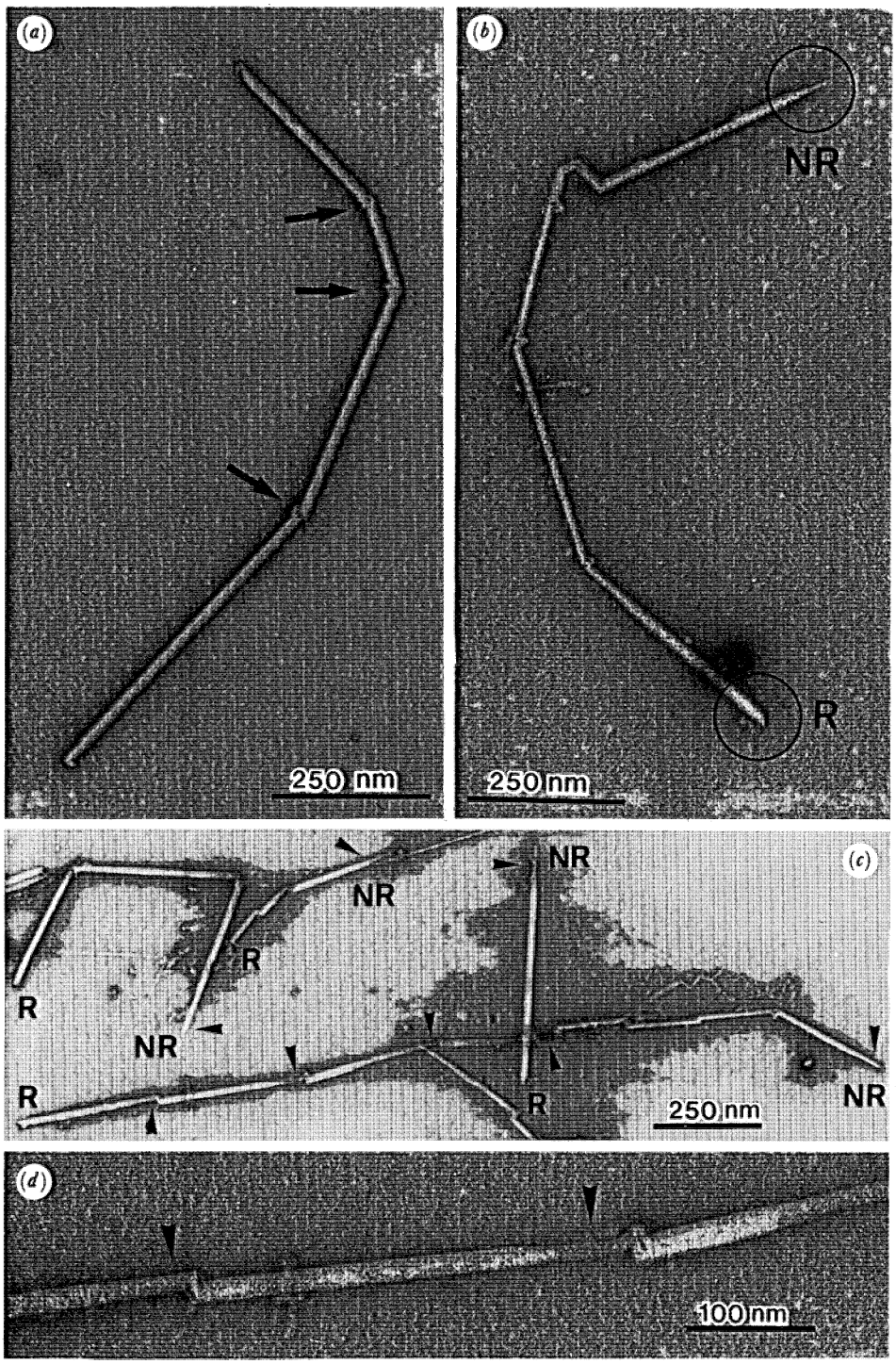

FIGURE 2. (*a*) Electron micrograph of a typical microcrystal of cellulose from *Valonia macrophysa*. The arrows indicate the kinked defects of the microcrystal. (*b*) Identical to (*a*), but after 16 h of digestion with CBH II. Circled areas: R, reducing end of the microcrystal; NR, non-reducing end. (*c*) Identical to (*b*), but after 16 h of digestion with a 60:40 mixture of CBH II and EG II. Abbreviations: R, reducing ends of the microcrystals; NR, non-reducing ends. The arrowheads denote the areas of GBH II attack. (*d*) Enlargement of an area of (*c*) (reproduced from Chanzy & Henrissat 1985).

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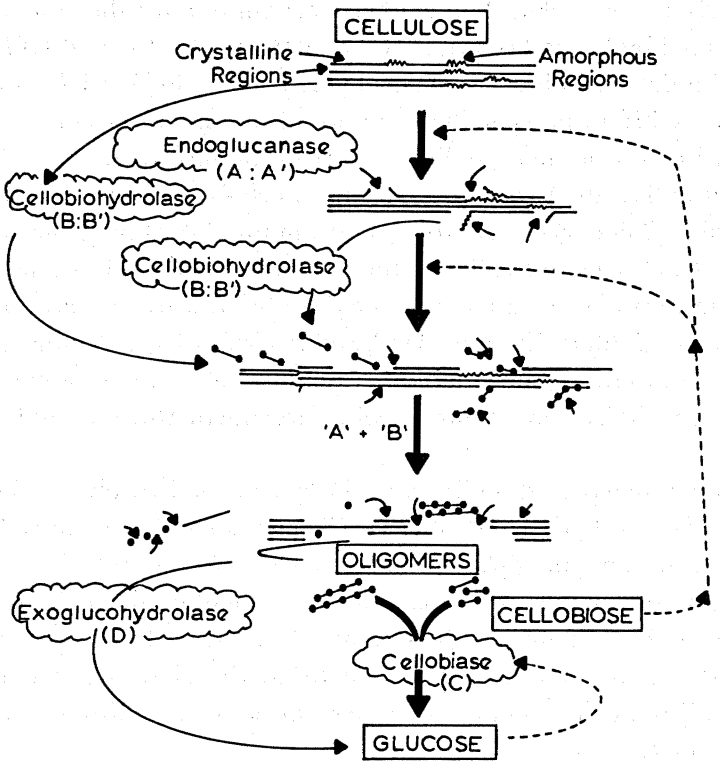

FIGURE 1. Generalized scheme for cellulolysis. Cellulose is initially attacked in amorphous zones by endoglucanases, (A:A′), thus generating multiple sites for attack by cellobiohydrolases (B:B′). The continued cooperative action between exo- and endo-splitting polysaccharides continues, combined with the terminal action of cellobiase (C) to yield glucose. The cooperative action of cellobiohydrolase and endoglucanase is synergistic. Most individual enzymes do not promote effective hydrolysis, although certain cellobiohydrolases will completely degrade crystalline cellulose. Exo- and endopolysaccharases can each occur in multiple forms and can exhibit different specificities in relation to the degree of polymerization of the substrate. Two forms of each endoglucanase (A:A′) and cellobiohydrolase (B:B′) are theoretically possible based on the two stereoconfigurations of the cellobiosyl unit in the chain (see text). In contrast, in the attack of soluble oligomers such as by exoglucohydrolase (D) (Klyosov *et al.* 1980), only one enzyme is necessary as the enzyme can initiate attack from either 'side' of the substrate.

Chanzy & Henrissat 1985). The fibrils were eroded only at one end by CBH II. The subsequent addition of endoglucanase EG II resulted in selective attack at amorphous zones along the length of the fibrils, followed by surface stripping by CBH II. This study confirmed visually the proposed synergistic and unidirectional combined degradative action of endo- and exoglucanases, at least with respect to EG II and CBH II. The study also confirmed the parallel (all non-reducing termini pointing in the same direction) rather than antiparallel conformation of cellulose I chains through the sequential angular thinning of the crystallites. From these results, a prediction arises that if such synergism between endo- (EG) and exoglucanases (CBH) is merely the result of additional sites being formed initially by endoglucanase for subsequent attack by exoglucanase, then optimal synergism will occur with a high ratio of CBH:EG. This was observed with CBH II and EG II (95:1) (Henrissat *et al.* 1985). However, the total *T. reesei* cellulase system is more complex, as the synergistic interaction between CBH I and EG I is quite different, with an optimal ratio of 1:1; this obviously cannot be explained merely on the basis of creation of new sites by endoglucanase followed by attack at the newly created sites

by the CBH (Henrissat *et al.* 1985). Some other mechanism must therefore also be operative. One concept, espoused by Wood (1985), is of the interaction of EG and CBH at the substrate surface, with the initial hydrolytic cleavage by EG being followed by instantaneous and combined action by CBH to prevent the reclosure of the initial scission; such closure would otherwise be likely in view of the crystalline nature of the substrate.

It is also now clear that the two *T. reesei* forms of cellobiohydrolase (CBH I and CBH II) are both functionally (Tilbeurgh *et al.* 1985) and immunologically (Fägerstam & Pettersson 1980) distinct. CBH I clearly binds all over the cellulose surface and can independently break down *Valonia* cellulose crystals (Chanzy *et al.* 1983). Other researchers have similarly noted the independent action of cellobiohydrolases (Halliwell & Griffin 1973; Nummi *et al.* 1983, Sasaki *et al.* 1979). In these instances, the situation is reminiscent of the original Reese suggestion that cellulolysis is initiated by some factor C_1, with subsequent attack by endoglucanase (Reese 1977).

A further and quite unexpected finding was the synergistic hydrolysis exhibited by the two *T. reesei* cellobiohydrolases (CBH I and CBH II) (Fägerstam & Pettersson 1980). One rationale for this cooperative exo–exo synergistic action is that each enzyme exhibits a different substrate stereospecificity that corresponds to the two possible orientations of the terminal cellobiose moiety of the glucan chain (figure 3). The removal of one surface polymer chain results in exposure of buried chains, some of which will be in the alternate stereo-orientation; the combined action will therefore facilitate hydrolysis of both stereodistinct polymer chains (for review see Wood (1985)).

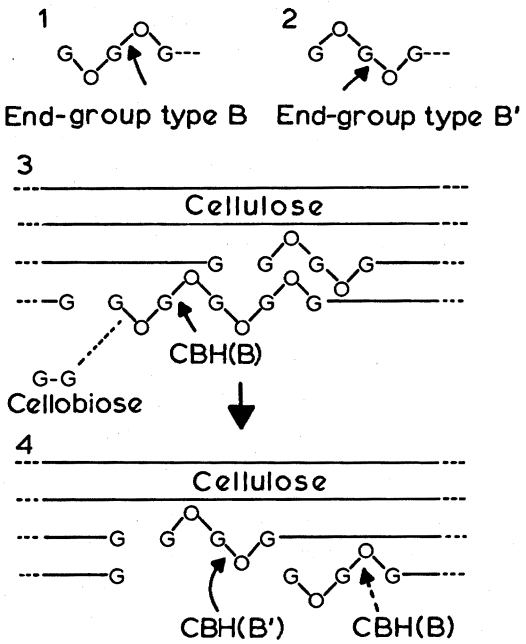

FIGURE 3. Theoretical synergistic attack by two distinct stereospecific cellobiohydrolases [B, B'] in the degradation of crystalline cellulose (after Wood 1985).

In contrast to the well-studied fungal systems, bacterial cellulases have only recently been studied in detail. Analogous bacterial systems with exo- and endo-splitting glucanases have been described in *Acetivibrio cellulolyticus* (Saddler & Khan 1980), *Clostridium* (Creuzet *et al.* 1983) and

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Microbospora bispora (Bartley 1986). However, in spite of considerable study with *Clostridium thermocellum* (Duong *et al.* 1983; Johnson *et al.* 1985; Ljungdahl & Eriksson 1985; Millet *et al.* 1985) and *Pseudomonas* sp. (Ramasamy *et al.* 1980), no cellobiohydrolase has yet been demonstrated in these bacteria. This raises the intriguing possibility that cellulase systems exist lacking exoglucanases, and perhaps based on synergism between endoglucanases or other components.

Multiplicity of enzyme forms

The occurrence of multiple forms of cellulase components has complicated the study of cellulases. This multiplicity is due to several factors including (i) multiple genes; (ii) macroheterogeneity based on the formation of multienzyme aggregates; (iii) microheterogeneity due to complexing of cellulases with proteins, glycoproteins or polysaccharides; (iv) synthesis of variants of a single gene product via infidelity of translation, proteolysis, variable glycosylation or interaction with components of the culture broth.

All such processes occur to some extent and it is difficult to assign significance to each process with regard to its effect on cellulolysis. However, (i) the demonstration of ten genes associated with cellulolysis in *Clostridium thermocellum* adds a new dimension to the complexity of this bacterial cellulase (Millet *et al.* 1985). The recently found (ii) macroheterogeneity (Sprey & Lambert 1984) and (iii) microheterogeneity (Alurralde & Ellenrieder 1984; Sprey & Lambert 1983) of 'purified' cellulase complexes add cautionary notes on the criteria needed to assess enzyme purity. In addition, they have implications for the actual *in vivo* mechanism of action of cellulases. (iv) The modifications that can arise through a variety of physiological processes range from the question of fidelity of translation to poorly characterized changes that occur to proteins in culture broths, and raise numerous questions with regard to their significance in cellulolysis.

Proteases are clearly implicated in the release of specific enzymes from fungal cell walls; for instance, endoglucanase released by trypsin (Kubicek 1981). However, whether they have a function in the generation of multiple cellulase components is unknown. Modification of cellulases via proteolysis in the culture broth is well known (Mandels *et al.* 1961), and in *Schizophyllum commune* a second endoglucanase arises through extracellular proteolytic cleavage of the primary enzyme (Paice *et al.* 1984). Proteolytic modification of cellulases can also result in slight but significant changes in their substrate specificity (Nakayama *et al.* 1976), while proteolytic activation of cellulases has been observed in *Phanerochaete chrysosporium* (Eriksson & Pettersson 1982), and also for *Penicillium janthinellum* pre-endoglucanases with activation mediated by a protease from *P. funiculosum* (Deshpande *et al.* 1984). In contrast, Dunne (1982) failed to find major proteolytic modification of *T. reesei* Rut-C 30 cellulase, and Kammel & Kubicek (1985) also failed to demonstrate modification of proteins or glycoproteins after they entered the culture broth with *T. reesei* QM 9414. The multiplicity of cellulase components in these latter instances must arise before their release. Similarly, multiple endoglucanases and cellobiases are synthesized by young *T. reesei* protoplasts, again implying that their origin is before release from the membrane (Kolar *et al.* 1985; I. Labudova, personal communication).

Heterogeneous glycosylation is a further process by which multiple forms can be generated, as shown for *S. commune* exo- and endoglucanases during the secretory process (Willick & Seligy 1985). However, the significance of glycosylation of cellulases is unclear. Although glycosylation sometimes inhibits secretion in certain eukaryotic cells, *Schizophyllum commune* treated with

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tunicamycin (an inhibitor of asparagine-linked N-glycosylation) continues to secrete both endoglucanase and cellobiase (Willick & Seligy 1985). Similarly, tunicamycin-treated *T. reesei* QM 6a and Rut-C 30 secrete endoglucanases and cellobiases with reduced carbohydrate content, but these partly deglycosylated enzymes (and also those prepared *in vitro* via cleavage with endo H-glycosidase), show no major changes with respect to activity or susceptibility to thermal or proteolytic inactivation (Murphy-Holland & Eveleigh 1985). In contrast, Merivuori *et al.* (1985) claim that glycosylation is important for stability of cellulases towards proteases and heat. *T. reesei* cellobiohydrolase, when cloned in yeast, becomes highly glycosylated yet is still active (Shoemaker *et al.* 1984). From these fragmentary data, the role of glycosylation remains nebulous.

A further complexity with regard to the origin of the multiple endoglucanases has been raised by Niku-Paavola *et al.* (1985), who note that the original and most active *T. reesei* endoglucanase (M_r 43000; pI 4.0) becomes modified following release in the culture medium to yield a glucanase (M_r 56000–67000; pI 5.0) exhibiting one fifth the activity of the original enzyme.

Enzyme characterization is thus complex. However, when analysis is made with young cultures via molecular and immunological approaches, the problems associated with 'multiple enzyme variants' are largely circumvented. This will permit study of the roles of glycosylation and proteolysis in secretion (Willick *et al.* 1984). It also facilitates study of a practical goal of cellulase research, namely to define the optimal proportions of the enzymes necessary to gain efficient hydrolysis.

Modified substrates

Gaining an understanding of the mechanism of action of cellulases is complicated by the substrate being insoluble, while the homologous character of its repeating units yields few distinctive analytical features. To circumvent this problem, modified substrates have been employed. Dyed substrates, especially soluble forms such as Ostazin Brilliant Red H-3B-hydroxyethyl cellulose, can be used for a variety of detection and assay protocols (Farkas *et al.* 1985). These types of substituted cellulose, including carboxymethyl cellulose (CMC), appear as specific substrates for all endoglucanases that have been described to date and have been widely used in the characterization of these enzymes. Their application also includes the direct detection of recombinant DNA-derived endoglucanase clones when used in combination with staining by Congo Red (Lejeune *et al.* 1986; Millet *et al.* 1985).

It is noteworthy that, in these latter studies, the *E. coli* clones principally synthesize periplasmically bound cellulase, and only a minor amount of enzyme (perhaps a maximum of 5%) is released in some manner into the surrounding milieu. However, Congo Red requires sequences of at least six unsubstituted glucose units for binding to the polysaccharide (Wood 1980). As CMC with a degree of substitution 0.79 contains only 0.036% of such unsubstituted sequences (Wirick 1968), the result is an extremely sensitive detection system, which allows for direct screening of clones that release very little cellulase into the medium. Modified CMC can also be employed for assay of exoglucohydrolase (figure 1, D). In this instance, CMC limit-dextrins are produced by the action of EG plus β-glucosidase on CMC, and as they contain several terminal unsubstituted glucose units at their non-reducing ends, they are a potential substrate for exoglucohydrolase (Rabinovich *et al.* 1982) and perhaps cellobiohydrolase.

Modified oligomeric substrates have been exploited by Claeyssens and his colleagues. For

instance, immobilized *p*-amino-benzyl-1-thio-β-D-cellobioside allows differential binding of *T. reesei* CBH I and CBH II and thus facilitates a single-step affinity purification of these two enzymes with selective elution by using lactose and cellobiose respectively (Tilbeurgh *et al.* 1984). Endoglucanases are not bound by this substrate. Methylumbelliferylglycosides (MU-X) have been employed with varying success. MU-cellobiose has proved useful in the demonstration of a putative binding site of four subsites for *T. reesei* CBH II by kinetic analysis of fluorescent substitution titrations in combination with other substrates (Tilbeurgh *et al.* 1985). The Belgian group have also shown that MU-lactoside (4-methylumbelliferyl-β-Galp-(1→4)-Glcp-) is a useful substrate for *T. reesei* CBH I, which specifically cleaves it to release only the fluorescent aglycone (Tilbeurgh *et al.* 1982). Analogously, *p*-nitrophenyl-β-D-lactoside has been used as a substrate for the CBHs of *T. reesei* and *Phanerochaete chrysosporium* although unfortunately it is also hydrolyzed by endoglucanase (Deshpande *et al.* 1984).

However, modified oligomeric substrates are not always as useful as predicted. 4-MU-cellobioside, theoretically predicted as a substrate for CBH, is not an absolute indicative substrate, because *T. reesei* CBH I cleaves it at both the hologlucosidic and aglycone bonds (Tilbeurgh *et al.* 1982), while in contrast, CBH II binds to this substrate but fails to cleave it (Tilbeurgh *et al.* 1985). *T. reesei* endoglucanases also hydrolyse this substrate. Furthermore, *T. reesei* CBH I fails to show specificity for the terminal cellobiosyl bond of methylumbelliferyl-higher-oligosaccharides (Tilbeurgh *et al.* 1982). In spite of these limitations, MU-cellobiose has been used sucessfully in the screening for *Clostridium thermocellum* glucanase clones, although no cellobiohydrolase clones have been found to date (Millet *et al.* 1985). Manifestly, these modified substrates have many useful attributes, but must be used with caution.

Production of cellulase

A major hindrance to the industrial cellulolysis of biomass is the high cost of cellulase, which has been estimated to be up to 60% of the total operating costs. To reduce production costs, hypercellulolytic mutant strains have been employed and fermentation conditions optimized with resultant high yields (15 filter paper units (fpu) ml^{-1}; 20 mg ml^{-1} extracellular protein) (Mandels 1985). With fed-batch fermentations as advocated by Hendy *et al.* (1984), spectacular yields of 6% extracellular protein (51 fpu ml^{-1}) have been obtained and also with cell mass optimized (35.9 g l^{-1}) productivities of 427 fpu l^{-1} h^{-1} were realized (Watson *et al.* 1984). These results are impressive. Can they be improved?

An apparently facile approach to increasing productivity is to use fast-growing organisms such as thermophiles, or employ rapidly utilizable substrates. To date, thermophilic bacteria although growing rapidly, have not yielded high productivities because of their inherent lower cellulase yields. Thermophilic fungi give higher cellulase yields, but have not been subject to intensive studies such as the selection of hypercellulolytic mutants. The recalcitrant nature of insoluble cellulosic substrates makes them particularly restrictive to gaining rapid growth. Soluble substrates are much preferred. Lactose, an inducer of cellulase synthesis in *T. reesei*, permits productivities of 200 fpu l^{-1} h^{-1} with a Celite immobilized Rut-C30 culture (250 ml), at low enzyme concentrations (2 fpu ml^{-1}; 15 CMCase ml^{-1}) but with enhanced specific activity (2 fpu mg^{-1} protein) (Frein 1986). On a larger scale (3 m^3 fermentor), a *T. reesei* CL-847 mutant selected for high cellulase yields when grown on lactose, gave yields ten fold greater than those of the wild strain when cultured on 6% lactose plus cellulosic supplements

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(Warzywoda *et al.* 1983). These data are simply illustrative of enhancing growth rate by using a soluble substrate to increase productivity. Although encouraging this is not an absolute solution because high growth rates *per se* are not synonymous with high enzyme yields. For gaining increased productivity a need exists for the selection of further mutants that are more effectively induced by lactose, or that are truly catabolite repression-resistant strains (Kawamori *et al.* 1985; Labudova & Farkas 1983); these would then produce high yields of cellulase when grown on glucose. Indeed, glucose-transport defective mutants of *T. reesei* have been obtained that 'behave as if they were derepressed in glucose repression of cellulase induction', although such strains do not transport glucose efficiently (Manczinger 1985).

What productivity goals can be set? Perhaps 1000 fpu l^{-1} h^{-1}; this figure is derived somewhat simplistically by assuming that dense cell cultures (70 g cells (dry mass) l^{-1}) composed of 60% protein can potentially direct half of their protein synthesis into production of cellulase enzymes,

i.e. enzyme per litre per hour from an 'overnight' culture

$$= 70 \times 0.6/20 \times 0.5 \text{ g } h^{-1} = 1.1 \text{ g } l^{-1} h^{-1}.$$

Assuming a specific activity of 1.0, this is equivalent to roughly 1100 fpu l^{-1} h^{-1}. Perhaps, with fungal cultures, only half this yield can be obtained, as maximal cell yields are routinely 35 g cells l^{-1}. Time will tell how practical such projections are on a large scale, or whether such high productivities can be obtained with immobilized cells.

A further major hindrance to the commercial application of cellulases is their very low specific activities, which are roughly 100-fold less than those of amylases. This low activity is probably a reflection of the refractory nature of the crystalline cellulose substrate. On the one hand, as endoglucanases exhibit a wide range of specific activities (*T. reesei* EG I, 27 (Shoemaker *et al.* 1983), *C. thermocellum* EG D, 430 (Millet *et al.* 1986)), there appears to be potential for developing cellulases of greater specific activity; through mutation and selection, via modification of the active site through site specific mutagenesis, or even by isolation of novel cellulolytic microbes with more effective cellulases. On the other hand, after a decade of worldwide general screening for hypercellulolytic mutants, few strains with increased specific activities have been detected, probably for the reasons stated above (Cuskey *et al.* 1983). With *T. reesei*, the Cetus strains showed small increases (Shoemaker *et al.* 1981), while a Kyowa Hakko Kogyo strain KY 746 shows nearly a 50% increase in filter paper activity over QM 9414, although with only a 25% increase in endoglucanase activity (Morikawa *et al.* 1985). One of the Rutgers mutants, RUT-P 37, shows a doubling of specific activity in both filter paper and endoglucanase units over the wild type QM 6a (Eveleigh & Montenecourt 1981; Montenecourt *et al.* 1983), and certain of its endoglucanases have more recently been shown to have over threefold greater CMCase specific activities (Sheir-Neiss & Montenecourt 1984). The above screening approaches all focused on selection of strains with increased cellulase yields. There is obviously a major need to devise a facile methodology for selection for cellulase mutants with increased specific activities. This will test the ingenuity of the researcher. One potential approach is the use of the 'plaque à trous' (Durand & Tiraby 1981).

A more fundamental approach to increasing specific activity is via modification of the binding site or the active site of the enzyme, but this requires detailed understanding of these sites. It is significant that the well-defined active site of lysozyme shares some homology with that of *Schizophyllum commune* endoglucanase (Yaguchi *et al.* 1983). Paice *et al.* (1984) have extended these findings to include the *T. reesei* cellobiohydrolase active site and also raised the

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possibility that endo- and exocellulases act by essentially similar mechanisms. The binding site is equally important. This is well illustrated by the enzymatic cleavage of lichenan, a glucan with β-(1→4)- and β-(1→3)-bonds (ratio 2:1) (Reese 1977). A *Streptomycete* β-(1→4)-endoglucanase binds to either of the β-(1→4)-bonds, and hydrolyses the adjacent β-(1→4) or β-(1→3)-bond. One product is *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose. Analogously, a *Rhizopus* β-(1→3)-glucanase binds through the β-(1→3)-bond of lichenan but cleaves the adjacent bond, namely one of the β-(1→4)-bonds, to yield *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose. In this instance, the binding site takes precedence over the site of hydrolysis. As knowledge of glycosidic hydrolysis at the molecular level is developed (see, for example, J. Knowles, this symposium), directed modification of the binding and active sites will present a logical approach to gaining increased specific activities.

A further approach is to screen for further organisms that possess more efficient cellulases. A new bevy of cellulases are being reported, e.g. from plants (Ladisch *et al.* 1983), protozoa (Odelson & Breznak 1985) and rumen fungi (Wood *et al.* 1986). The relative metabolic cost of protein synthesis in a cell is extremely high (Stouthamer 1973). This is especially true for anaerobes, and presumably there has been evolutionary pressure for selection of cellulases of high specific activity in these organisms. This is perhaps illustrated by the cellulase from anaerobe *Neocallimastix frontalis*, isolated by Wood *et al.* (1986) who note that its cellulase is of biotechnological potential as it is several times more active (based on specific activity units per mg protein) in solubilizing cotton fibre than *T. reesei* Rut-C 30 cellulase. The simple expedient of screening for new anaerobic cellulase degraders could well result in the discovery of more efficient cellulases. In this vein, following the discovery of cellulolytic chytrids in ruminants, we have readily gained anaerobic cellulolytic fungi from muds (G. Compeau, personal communication), but have yet to evaluate their cellulases. Perhaps a further example of an energetically stressed system is the nitrogen-fixing cellulolytic bacteria associated with the glands of Deshayes of shipworms, which must synthesize cellulase and also fix nitrogen, both activities being metabolically expensive (Waterbury *et al.* 1983).

In brief, the enhanced cellulase productivities achieved through the cooperation between microbiologist and engineer have been remarkable. Even so, a major need continues for cellulases of markedly increased specific activities, and this should be attainable via judicious selection of highly cellulolytic organisms, combined with molecular approaches for maximizing efficiency of binding and active sites.

Conclusion

The energy crisis of the mid-1970s indirectly contributed to increased study of cellulases and, as briefly surveyed, has led to a considerably greater understanding of their mechanism of action, of factors regulating their synthesis and the bases for their heterogeneity, besides the practical achievements of remarkably high productivity.

In contrast to these detailed studies of the enzyme, there has been a slow realization of the complexities of the substrate whether it is 'practical' lignocellulose, modified cellulose (filter paper, microcrystalline cellulose), or naturally occurring 'pure' cellulose (cotton fibres, bacterial extracellular fibrils (*Acetobacter xylinum*) and algal cell walls (*Valonia*)). Fluorescent and colorimetric substrates have proved most useful in the analysis of cellulase action (Tilbeurgh

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et al. 1985). But it is abundantly clear that there is a need to apply physical analytical techniques to define such parameters as the crystalline–paracrystalline state (Kulshreshtha & Dweltz 1973) and pore structure (Grethlein 1985; Marchessault *et al.* 1983; Puls *et al.* 1985) to comprehend cellulolysis. It has been by employing well-characterized substrates that further understanding of the mechanism of action of cellulase was gained (figure 2; Henrissat *et al.* 1985). Unfortunately, in general terms there is a woeful lack of characterization of the initial stages of cellulolysis and reaction products, and the important roles of adsorption and readsorption (Gusakov *et al.* 1985) are only recently being more fully addressed.

A practical goal of cellulase research is to define both the optimal types and proportions of the enzymes necessary to gain efficient hydrolysis. The latter is especially difficult to define, in that the ratio depends on the changing status of the substrate throughout cellulolysis. However, with recombinant-DNA techniques at hand, one can envisage constructing the perfect cellulolytic microbe such as by incorporating effective fungal cellobiohydrolases, the high specific activity endoglucanases of *C. thermocellum*, and the cellobiase of *Microbispora bispora* (which is remarkably resistant to end-product inhibition (Waldron *et al.* 1986)), into a single organism. In this connection, the studies of bacterial cellulases have been remarkably productive. With *C. thermocellum* alone, there has been the concept (Bayer *et al.* 1985) and definition (Coughlan *et al.* 1985) of the cellulosome, the characterization of further specific cell–substrate binding factors (Ljungdahl *et al.* 1983), and cloning, which has permitted purification of several of its cellulase components that previously had proved recalcitrant to purification by routine biochemical techniques. The recombinant-DNA approaches now permit detailed comparison of cellulases at the molecular level (Mackay *et al.* 1985; J. Knowles, this symposium), and the first crystallization of a cellulase (*C. thermocellum* endoglucanase D) (Millet *et al.* 1986), as well as providing surprises such as a potential role of actin in cellulolysis (Klyosov *et al.* 1985). This is a far cry from the original C_1:Cx concept of Reese (1977) in which some factor (C_1) appeared to convert crystalline cellulose into a form accessible to hydrolytic endoglucanases (Cx). Such non-enzymatic microbial products are still with us (Griffin *et al.* 1984).

This paper is dedicated to Elwyn T. Reese on the occasion of his 75th birthday.

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FIGURE 2. (*a*) Electron micrograph of a typical microcrystal of cellulose from *Valonia macrophysa*. The arrows indicate the kinked defects of the microcrystal. (*b*) Identical to (*a*), but after 16 h of digestion with CBH II. Circled areas: R, reducing end of the microcrystal; NR, non-reducing end. (*c*) Identical to (*b*), but after 16 h of digestion with a 60:40 mixture of CBH II and EG II. Abbreviations: R, reducing ends of the microcrystals; NR, non-reducing ends. The arrowheads denote the areas of GBH II attack. (*d*) Enlargement of an area of (*c*) (reproduced from Chanzy & Henrissat 1985).