Properties of cellulolytic enzyme systems

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Cellulolytic enzymes are synthesized by a large number of micro-organisms which include fungi, actinomycetes, gliding bacteria and true bacteria. However, only the fungi appear to excrete large amounts of cellulase enzymes in active form into culture media and not surprisingly these are the enzyme systems that have been most extensively studied. Bacterial cellulases are cell-wall bound, in the main.

While many cellulases are known to solubilize the amorphous and inter-crystalline phases of the cellulos, very few appear to have the capacity for solubilizing native cellulose to an extent that denotes that the most highly ordered crystalline areas are being degraded. Notable in this regard are the cellulases of the fungi *Trichoderma viride* (Ogawa & Toyama, 1972), *T. reesei* (Mandels & Reese, 1964), *T. koningii* (Halliwel, 1965; Wood, 1968), *Fusarium solani* (Wood, 1962), *Penicillium funiculosum* (Wood & McCrae, 1978), *Penicillium chrysogenum* (Eriksson, 1975) and *Talaromyces emersonii* (Folan & Coughlan, 1979). Of the cellulolytic bacteria studied, only the anaerobe *Clostridium thermocellum* appears to liberate an extracellular enzyme system that effects the extensive degradation of crystalline cellulose (Johnson et al., 1982).

Little is known about the properties of the cellulases of the bacteria as yet, but significant advances have been made recently in the understanding of the enzymes and their modes of operating in the fungi. It is well established, for example, that rapid dissolution of native cellulose requires the independent and co-operative action of a mixture of enzymes. The enzymes present in the mixture have been well characterized, but it is not known yet how they interact and what factors control the interactions on the face of the cellulose crystallite. These uncertainties have resulted in much speculation regarding the mechanism of cellulase action.

Fungal cellulases

Fractionation studies on culture filtrates of the fungi mentioned above have demonstrated that there are three major types of enzyme involved in hydrolysis of native cellulose (Wood & McCrae, 1979). These are: endo-1,4-\(\beta\)-glucanase (endo-1,4-\(\beta\)-D-glucan 4-glucanohydrolase, EC 3.2.1.4), cellobiohydrolase (1,4-\(\beta\)-D-glucan cellobiohydrolase, EC 3.2.1.91) and \(\beta\)-glucosidase (EC 3.2.1.21). Some cellulase systems also contain a glucohydrolase (1,4-\(\beta\)-D-glucan 4-glucanohydrolase, EC 3.2.1.74), but these are minor constituents. Cellobiohydrolase, which is present as a major constituent of the cellulase systems of fungal cellulases that can degrade highly ordered crystalline cellulose, is absent in other cellulase systems: thus, this exoglucanase would appear to be the distinguishing feature of cellulases that are able to degrade crystalline cellulose.

The specificities of these enzymes have been studied by using a range of substrates: cotton fibre and *Avicel* (microcrystalline \(\alpha\)-cellulose), which are highly hydrogen-bonded structures; CM-cellulose or \(\text{H}_2\text{PO}_4\)-swollen cellulose, which are, in essence, model substrates for measuring degradation of amorphous cellulose; and cellobiose, nitrophenyl-\(\beta\)-glucosidase or other arylglucosides, which are substrates for \(\beta\)-glucosidase activity. As a result of these studies the following generalizations can be made:

(i) Endoglucanases, often called CM-cellulases or \(C_\text{X}\) enzymes, attack CM-cellulose or \(\text{H}_2\text{PO}_4\)-swollen cellulose in a random fashion, resulting in a rapid decrease in chain length together with a slow increase in reducing groups (Wood & McCrae, 1979). Water soluble cello-oligosaccharides are the intermediate products of attack, but these are hydrolysed to glucose and cellobiose, the rate decreasing with decreasing degree of polymerization. Purified endoglucanases are highly polymer dependent and have little apparent capacity for hydrolysing crystalline cellulose. An ability for synthesizing longer chain oligomers from cellobiose and short chain cello-oligosaccharides has been reported.

(ii) Cellobiohydrolases (Wood & McCrae, 1972; Berghem & Pettersson, 1973) degrade cellulose by splitting off cellobiose units from the non-reducing end of the chain. Notwithstanding these problems, a significant feature of all studies is the synergism observed between the various types of enzyme found in the cellulase systems that can solubilize crystalline cellulose (Wood & McCrae, 1979; McHale & Coughlan, 1980). Fractionation studies carried out in our laboratory on the cellulases of *T. koningii* and *F. solani* show that the activity with cotton fibre as substrate, under certain conditions, is completely dependent on the cooperative action of the enzymes (Wood & McCrae, 1979). Thus, the activity against cotton is lost when cellobiohydrodase, endoglucanase and \(\beta\)-glucosidase activities are separated, but it can be recovered when they are recombined. Mixtures of endoglucanase and cellobiohydrolase can account for most of the cellulase activity of the unfraccionated enzyme system. However, maximum efficiency in rate and extent of hydrolysis is obtained only when \(\beta\)-glucosidase is present in the mixture. The role of the \(\beta\)-glucosidase is to remove the inhibitory effects of cellobiose, which is one of the principal products of the other two enzymes.
Oxidases and oxidoreductases. Whereas in some fungi β-glucosidase is the only enzyme capable of hydrolysing cellbiose to glucose to reduce end-product inhibition, in others this process is aided by the enzymes cellbiose oxidase and/or cellbiose–quinone oxidoreductase. As yet only the white rot fungus *S. pulverulentum* (Eriksson, 1981) has been reported to synthesise cellbiose oxidase, but there is some evidence, albeit indirect, that *T. koningii* (Wood & McCray, 1978) and *T. reesei* (Eriksson et al., 1979) may also produce it. Cellbiose–quinone oxidoreductase has been reported to be produced by several types of fungi, namely, the white rot fungus *S. pulverulentum* (Eriksson, 1981), the thermophilic fungus *S. thermophile*, phylogenetically belonging to the Ascomycetes (Coudray et al., 1982), and a species of the imperfect fungus, *Monilia* (Dekker, 1980).

The cellbiose oxidase of *S. pulverulentum*, which is a halophilic thermophile, oxidizes cellbiose and higher cellodextrins to their corresponding organic acids by using molecular oxygen. Cellbiose–quinone oxidoreductase, which has been shown to be involved in both lignin and cellulose degradation, oxidizes cellbiose (but not cellobio-oligosaccharides) to cellobiono lactone in the presence of electron acceptors such as quinones or phenoxacyanides. The nature of the oxidation causes the production of superoxide anion, which is a product of the reaction. The cellbiose oxidase is localized in the superficial zone (Eriksson, 1981). The enzyme electron acceptor for the enzymes of *S. thermophile* and *Monila* sp. have been identified to be the study to be done *in vitro*, but the natural electron acceptors of these non-lignin-degraders are not known. It has been speculated, however, that as they inhabit the same environment as the lignin-degrading fungi they may be present in cellbiose oxidase.

An interesting aspect to the involvement of cellbiose oxidase in cellulose degradation in the *S. pulverulentum* cellulase system, but perhaps also in other cellulases, is the generation of superoxide anion as a product of the reaction. Superoxide anion may be involved in initial attack on cellulose (Eriksson, 1981). However, it is possible that the H₂O₂ which is produced by the dismutation of superoxide anion by the enzyme superoxide dismutase may also be involved in this way. Koenigs (1975) has suggested that initial attack on crystalline cellulose by brown-rot fungi is via a H₂O₂/Fe²⁺ system.

Multiple components. Fractionation studies have demonstrated that each of the types of enzyme comprising the cellulase system consists of a multiplicity of forms with apparent homology. The components of these isoenzymes have been the subject of much discussion in the literature. This discussion has addressed the possibility that the multiplicity of components could be genetically determined or be caused by partial proteolysis (Nakayama et al., 1976; Gong et al., 1979) or by differential glycosylation of a common polypeptide chain (Gum & Brown, 1977). All possibilities seem to operate.

A typical cellulase found in cultures of *T. koningii* has been found to contain ten cellulase components. These included two cellobiohydrolases, two β-glucosidases and six endoglucanases, four of which were major endoglucanases (Wood & McCray, 1978). Cellulases of *S. pulverulentum* (Eriksson, 1981), *P. funiculosum* (Wood et al., 1980) and *T. emersonii* (McHale & Coughlan, 1981) have been reported to be equally heterogeneous.

Most of the enzyme components are glycoproteins with carbohydrate content ranging from 1 to 10% of the total (Enari, 1983). However, at least one has been reported to contain as much as 50% carbohydrate (McHale & Coughlan, 1981). The principal sugar appears to be mannose.

Mₘ values of the enzymes range from 12 000 to 75 000 (Enari, 1983), but some β-glucosidasises appear to be much larger. Thus, the five β-glucosidasises of *S. pulverulentum* range from 16 5000 to 18 2000 (Deshpande et al., 1978), while that found in cultures of *F. solani* is approx. 40 000 (Wood, 1971).

The cellulase of *T. reesei* has been the subject of intense study because of its commercial potential. Like the other cellulases, it has proved to be heterogeneous with respect to β-glucosidase, endoglucanase and cellobiohydrolase, the heterogeneity depending on the duration of culture (Gong et al., 1979) and the conditions of pH maintained during fermentation (Pettersson, 1981). The cellobiohydrolase isolated from a *T. viride* preparation differed only in the degree of glycosylation (Gum & Brown, 1977); they were similar with respect to amino acid composition, Mₘ, heat stability and C-terminal residues. The enzymes also exhibited immunological cross-reactivity, and this is significant now that two immunologically unrelated cellobiohydrolases (designated I and II) have been found in cut culture filtrates of *T. reesei* (Fagerstam & Pettersson, 1980; Nummi et al., 1983) and in the *P. funiculosum* cellulase system (Wood, 1984).

The nature of the multiplicity can be determined by amino acid sequence analysis. When this was carried out on cellobiohydrolases I and II of *T. reesei*, no apparent relationship was found between the two enzymes (Pettersson et al., 1979). This surprising observation, however, when the analysis was extended to the main endoglucanase component a clear homology between the enzyme and one of the cellobiohydrolases was found.

Clearly these results are of great interest in that they raise the possibility that (a) each cellobiohydrolase has a definite role to play in the degradation of cellulose and (b) that the possibility that the multiplicity can extend to the active site region that cellobiohydrolases and endo-cellulases can use the same catalytic mechanism for hydrolysis. The recent publication revealing a similarity between the active site region of an endoglucanase of the fungus *Schizophyllum commune* and a sequence in *T. reesei* cellobiohydrolase (Paice et al., 1984) gives some credence to the latter possibility.

An interesting property of the two cellobiohydrolases of *T. reesei* and *P. funiculosum* (Wood, 1984), giving support to the suggestion that the two enzymes have an important role to play, is that they appear to act synergistically with each other in solubilising both Avicel (Fig. 1) and cotton fibre. Synergism between two exoglucanases is difficult to explain, but a reasonable speculation might be that cellobiohydrolases I and II are two enzymes exhibiting different substrate specificity and the synergism is utilized to attack the different non-reducing end groups that can be found, in all probability, in the cellulose crystalite (Fig. 2). If this hypothesis is tenable, one could speculate that the observed synergism could be explained if one envisages that the action of one cellobiohydrolase, by removing cellbiose units successively from one type of non-reducing chain end, could expose a non-reducing end group on another chain with the correct configuration for attack by the other stereospecific cellobiohydrolase (Wood, 1984).

As already discussed, multiplicity in terms of endoglucanase is also the rule in most fungal cellulases. The various endoglucanases found in any one cellulase system are reported to differ in respect of their Mₘ values, isoelectric points and their association with carbohydrate (Enari, 1983). A characteristic property of the various endoglucanases is the slope of the line obtained by plotting the change in viscosity of a solution of CM-cellulose against the increase in reducing power (Wood, 1981). As viscosity is a parameter related to chain length the differences in slope have been interpreted to indicate the existence of enzymes varying in the randomness of their substrate attack. However, it is in fact still not clear whether these enzymes have been synthesized by the fungus in the forms in which they exist in the culture filtrates or if they are artefacts, resulting from proteolytic
Fig. 1. Synergism between cellobiohydrolases I and II of P. funiculosum in solubilizing filter paper.

Cellobiohydrolases (CBH) I and II were incubated alone and together with filter paper (20 mg) for 2 h at 37°C in 0.1 M sodium acetate buffer (pH 5.0). O-O, CBH I; Δ-Δ, CBH II; ▲-▲, CBH I and CBH II (sum). CBH I and CBH II (actual).

Fig. 2. Possible explanation of the synergism between cellobiohydrolases (CBH) I and II of P. funiculosum in solubilizing crystalline cellulose

Because cellobiose is the repeating unit in cellulose, theoretically there will be two types of non-reducing end groups (a and b) in the cellulose crystal. These end groups will be held in position by non-covalent bonds and will require two different stereospecific enzymes for hydrolysis. (c) shows only cellobiohydrolase I attacking; (d) shows cellobiohydrolase II attacking the new chain end exposed by cellobiohydrolase I action.

modification during culture, of a single endoglucanase. Nakayama et al. (1976), for example, have observed that partial proteolysis of endoglucanases yielded enzymes with altered substrate specificities, while Eriksson & Pettersson (1982) have reported increased specific activity of *S. pulverulentum* endoglucanase after treatment with two proteases from the fungus.

Obviously, proteolysis and differential glycosylation may account to some extent for the heterogeneous nature of the cellulase system, but just how many components are genetically determined and how many are artefacts still has to be decided. Of great interest in this regard, however, is the observation that a simple enzyme system which was induced in the fungus *T. reesei* by using the disaccharide sophorose as the sole carbon source, and which was found to contain only two immunologically unrelated cellobiohydrolases, one endoglucanase and a β-glucosidase, could hydrolyse Avicel at the same initial rate as an enzyme system containing multiple forms of the enzymes (Gritzali & Pettersson, 1982). Less speculative are results which demonstrate the importance of differences in adsorption on cellulose of the various enzymes (Berezin & Brown, 1979). On the basis of this evidence only a very simple enzyme system indeed is required to solubilize crystalline cellulose; but this may not be conclusive (Wood, 1983).

Mode of action. The currently accepted model for the enzymic hydrolysis of cellulose envisages a mechanism in which endoglucanase initiates the attack and the newly formed non-reducing chain ends are then acted on by the endwise-acting cellobiohydrolase. This mechanism, however, while being a satisfactory explanation of enzymic events resulting in the solubilization of amorphous areas of the cellulose, is really an oversimplification of the process that occurs on the face of the cellulose crystallite. It takes no account of the steric problems that exist for the enzymes when attacking highly ordered cellulose chains held rigidly in position by hydrogen bonds. Enzymes attacking such structures must exhibit a high degree of stereospecificity. The nature and extent of this stereospecificity was demonstrated recently by Sagar (1985), who concluded that
II) of *P. fuscans* can co-operate with the endoglucanases of the same fungus to solubilize cotton cellulose, only cellubiohydrolase I can co-operate with the endoglucanases of *T. koningii* and *F. solani*. Clearly, adsorption is not the only factor involved.

Thus the mechanism of cellulase action as it relates to solubilization of crystalline cellulose by endoglucanase and cellubiohydrolase is still controversial. The possibility that a non-hydrolytic chain-separating enzyme (so-called C3) might be involved has been suggested (Reese et al., 1951), but has been discounted by most. Recent studies with cellulase preparations from *T. reesei* may, however, support this hypothesis (Griffin et al., 1984).

**Bacterial cellulases**

Most cell-free and cell-wall-bound preparations are unable to solubilize highly ordered cellulose to a significant extent although they can hydrolyze CM-cellulose readily and 

\[ \text{H}_2\text{PO}_4^- \text{-swollen cellulose} \]

to varying degrees. Such enzyme preparations contain endoglucanases but little exoglucanase activity. The cellulase of the anaerobe *C. thermocellum* has been the most widely studied but it is not well characterized. One cell-free enzyme preparation of *C. thermocellum* is reported to be able to hydrolyze highly hydrogen-bond-ordered cellulose as efficiently as the cellulase of *T. reesei* (Johnson et al., 1982), but most preparations do not exhibit this property. The composition of this cellulase system has not been reported as yet.

*C. thermocellum* produces a yellow substance when grown on cellulose (Ljungdahl et al., 1983). This yellow substance, which is of low *M* and having *M* but most preparations such as cellulase preparations from *T. reesei* which is of low *M* and having *M* may, however, support this hypothesis (Griffin et al., 1984).


