Biodegradation **1**: 147–161, 1990.

Enzymology of cellulose degradation

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Key words: fungal cellulase, bacterial cellulase, mode of action, structure/activity relationships, synergism

Abstract

In the last few years there has been a considerable improvement in the understanding of the mechanisms involved in the microbial degradation of cellulose, but there are still many uncertainties. As presently understood, it would appear that different mechanisms may operate in the various types of microorganism. Thus degradation of crystalline cellulose is effected by anaerobic bacteria by large Ca-dependent and thiol-dependent multicomponent endoglucanase-containing complexes (cellulosomes) located on concerted action of endo- and exo-glucanases which act some distance from the cell which renders cellulose soluble. All of the endo- and exo-glucanases possess a bifunctional domain structure: one contains the catalytic site, the other is involved in binding the enzyme to crystalline cellulose.

Introduction

It would appear that a number of different mechanisms operate in the microbial solubilization of crystalline cellulose. Cellulolysis by soft rot and white rot aerobic fungi (Eriksson & Wood 1985) and some aerobic bacteria (Coughlan 1990) involves the synergistic action of enzymes, loosely defined as exoglucanases (normally cellobiohydrolases i.e. 1,4-β-D-glucan cellobiohydrolase), endoglucanases (endo-1,4-β-D-glucan-4-glucanohydrolase) and β-glucosidases (Eriksson & Wood 1985). Brown rot fungi, on the other hand, produce endoglucanases but no exoglucanases and may have a different mechanism, perhaps involving H_2O_2 (Koenings 1975). Some anaerobic bacteria (Lamed & Bayer 1988) and possibly anaerobic fungi (Wood et al. 1988) use a multicomponent enzyme complex which contains endoglucanases, but the exact composition of the complex remains to be described in each case.

Unfortunately, space does not permit a discussion of all these mechanisms. Of necessity, the review will focus mainly on those mechanisms and microorganisms which have been the subject of most research activity, namely, the aerobic fungi and the anaerobic bacteria. Moreover, discussion of fungal cellulases is restricted only to enzymes classified as endoglucanase and exoglucanase (cellobiohydrolase): enzymes such as glucohydrolase, cellobiose oxidase and cellobiose dehydrogenase, which are found in some culture filtrates as minor constituents (Eriksson & Wood 1985), are not discussed further. Thus this review is confined mainly to a discussion of those enzymes and enzymatic processes by which crystalline hydrogen bond-ordered cellulose is rendered soluble.

Much progress has been made in understanding the mechanism by which fungi and bacteria degrade hydrogen bond-ordered cellulose, but there are still many uncertainties. Some of the most recent interesting insights into the mechanism have been obtained from studies of the structure/function relationships provided by structural analysis of the enzymes, by studies on cloned genes and on enzymes resulting from expression of these cloned genes.

Fungal cellulases

Composition of the cellulase system

The distinguishing feature of the cellulase that can solubilize crystalline cellulose is that it contains a cellobiohydrolase. This is in addition to the randomly-acting endoglucanases and β-glucosidases/cellobiases found in all fungal culture filtrates. Only a few fungi synthesise and release into the culture medium appreciable amounts of the cellobiohydrolase enzyme. Notable in this regard are the fungi, *Trichoderma reesei, Trichoderma viride, Fusarium solani* and *Penicillium funiculosum/pinophilum* (Eriksson & Wood 1985).

All fungal cellulases studied so far have been shown to contain a multiplicity of enzyme components (Wood 1990; Coughlan 1985). The actual number of components depends on the source of the fungus and the manner in which it has been cultured. *Trichoderma viride* and *Trichoderma reesei* cellulases have been most extensively studied (Eriksson & Wood 1985; Wood 1990; Coughlan 1985). They have been shown to contain four to eight endoglucanases, two cellobiohydrolases and one to two β-glucosidases (Coughlan 1985; Wood 1990). *Penicillium funiculosum/pinophilum* cellulase contains two cellobiohydrolases (Wood et al. 1980; Wood & McCrae 1986a), five to eight endoglucanases (Bhat et al. 1989) and two β-glucosidases (Wood et al. 1980). Other cellulases are equally heterogeneous (Streamer et al. 1975; Wood 1990). It seems that only some of these components are genetically determined; others are artefacts resulting from differential glycosylation of a common polypeptide chain (Wood & McCrae 1972; Gum & Brown 1977), from partial proteolysis (Eriksson & Pettersson 1982), from aggregation of the enzymes with each other or with part of the fungal cell wall (Sprey & Lambert 1983), or from manipulation of the enzymes during purification (Enari & Niku Paavola 1987). These artefacts make elucidation of the mechanism of action extremely difficult: consequently there is considerable discussion on the substrate specificity of the enzymes, on the mode of action of the individual enzymes, particularly the cellobiohydrolases, and on the nature of the co-operation between the various enzymes. Currently, there is some agreement that the *extensive* conversion of crystalline cellulose to glucose can be discussed in terms of the co-operative action of two immunologically unrelated cellobiohydrolases (so-called CBH I and CBH II), one or more randomly-acting endoglucanases and at least one β-glucosidase (Wood 1990).

The problem of classification of the enzymes

Table 1 summarizes views that have been widely held on the substrate specificities of the enzymes which are found in the cellulases that can degrade crystalline cellulose. However, as the properties of the isolated enzymes have been studied more closely, it is becoming increasingly clear that it is difficult to classify them strictly as endoglucanases and cellobiohydrolases. For example, some purified cellobiohydrolases are reported to attack barley β-glucans (Henrissat et al. 1985) and even CM-cellulose (Wood 1990), which are substrates long been held to be degradable only by enzymes classified as randomly-acting endoglucanases. On the other hand, some enzymes classified as endoglucanases are reported to be able to hydrolyse crystalline cellulose (Beldman et al. 1985; Enari & Niku-Paavola 1987), which of course is presumed to be a property of the

Table 1. Action of cellulase components on different substrates.

Enzyme	Crystalline cellulose	Amorphous 'swollen' cellulose	CM-Cellulose	Cello-oligosaccharides	Cellobiose
Cellobiohydrolase	Slow	Very active	Nil	Active	Nil
Endoglucanase	Nil	Very active	Very active	Active	Nil
β-Glucosidase	Nil	Nil	Nil	Active	Active

cellobiohydrolase. But the confusion does not end there: yet another endoglucanase is reported to have no action on amorphous cellulose prepared by milling cellulose powder in ethanol (Niku-Paavola et al. 1985). There are other apparent anomalies too frequent to report here.

Clearly, the substrate specificities of the various enzymes continues to be a contentious issue. However, the explanation may be quite simple, viz, either the enzymes have overlapping substrate specificities or some of the enzymes which have been reported to be pure obviously are not. The purity of the cellobiohydrolases in particular has been questioned and, as a consequence, the mode of action of these enzymes has been the subject of great debate.

Are the cellobiohydrolases endo- or exo-acting?

It has been held for many years that cellobiohydrolases (CBHs) are exoglucanases that remove cellobiose consecutively from the non-reducing end of the cellulose chain. Typically, crystalline and amorphous cellulose have been reported to be degraded to cellobiose, the rate depending on the degree of polymerisation, and the crystallinity of the cellulose (Wood & McCrae 1979; Wood 1990). Recently, however, there have been several reports which have indicated that the cellobiohydrolases may not attack exclusively the penultimate glycosidic link at the non-reducing end of the cellulose chain. Unfortunately, the case is weakened by the apparent lack of agreement as to whether it is CBH I or CBH II or both that possess this property. Thus, a CBH I from *T. reesei* attacked barley β-glucan in a random manner typical of an endoglucanase (Henrissat et al. 1985), but CBH I from *P. pinophilum* effected only a slow change in the degree of polymerisation of the β-glucan (Wood et al. 1989), as would be expected from an exo-acting enzyme. On the other hand, another CBH I from *T. reesei* hydrolysed chromophoric cello-oligosaccharides in a manner not typical of an exoglucanase (Claeyssens et al. 1989).

Support that the CBH I enzyme may not act from the end of the chain has been obtained by electron microscopy. Thus, Chanzy et al. (1984) noted that CBH I from *T. reesei*, labelled with colloidal gold, was found to be attached to microcrystals of the alga, *Valonia microphysa*, along the length of the microfibril. White & Brown (1981), in a similar study, but using cellulose from the bacterium *Acetobacter xylinum*, made the same observation.

Conclusions as to the mode of action of CBH II are equally diverse. Thus, electron microscopic evidence showing that CBH II from *T. reesei* attacked *Valonia* cellulose microcrystals only from the non-reducing end supports the claim that it is a true exoglucanase (Chanzy et al. 1985). Using biochemical studies, a similar mode of action was deduced for a CBH II from *P. pinophilum* purified by affinity chromatography (Wood et al. 1989). By contrast, Enari & Niku Paavola (1987) and Kyriacou et al. (1987), also using biochemical studies, conclude that CBH II from *T. reesei* are endo-acting, albeit 'less randomly-acting' than a typical endoglucanase.

How can these conflicting results be rationalised? Clearly, enzymes from different sources may indeed have different substrate specificities; and some of the variations at least may therefore be quite easily explained. However, when the same enzyme from the same source would appear to have completely different properties, another reason must be sought. One possibility is that the apparent differences may be a consequence of the existence of aggregates or enzyme-enzyme complexes between cellobiohydrolases and endoglucanases that are extremely difficult to break into their constituent parts. Such complexes have been shown to exist in a cellulase from *T. reesei*. In this case, electrophoretically homogeneous complexes between endoglucanase, xylanase and β-glucosidase were found to be heterogeneous after treatment with a urea/octyl glucoside dissociation reagent (Sprey & Lambert 1983). Similar complexes have been found to exist between cellobiohydrolases and endoglucanases in electrophoretically homogeneous enzyme preparations isolated from cultures of *P. pinophilum* and *T. reesei* (Wood et al. 1989). These complexes, which were homogeneous after extensive purification involving gel filtration, chromatofocusing and affinity chromatography on a column of cellulose, were found to be hetero-

geneous after affinity chromatography on a column that had been prepared by coupling *p*-aminobenzyl-1-thio-β-D-cellobioside to Affigel 10 (van Tilbeurgh et al. 1984). A CBH II from *P. pinophilum* purified in this way had the properties of a typical exoglucanase: the enzyme could effect only a slow decrease in the viscosity (a parameter related to chain length) of a solution of barley β-glucan in contrast to the rapid decrease shown by purified endoglucanases from the same fungus: CBH I and II from *T. reesei* prepared on the cellobioside column were similar in this respect (Tomme et al. 1988a).

Whether or not enzyme-enzyme complexes can explain the differing opinions on the substrate specificity and mode of action, it is clear that at least some of the confusion regarding the properties of the cellobiohydrolase enzymes is caused by the difficulty in obtaining single enzyme species. Expression of the cellulase genes in a heterologous host makes it possible to produce each enzyme free of contaminating glycosidases. It may therefore be significant that a CBH I gene from *T. reesei*, expressed in yeast, showed no capacity to hydrolyse barley β-glucan (Knowles et al. 1988b), which is readily degraded by endoglucanases (Wood et al. 1989), while a recombinant CBH II did. The implication is that CBH I from *T. reesei* may indeed be an exocellobiohydrolase and CBH II may have some endo-type action. The fact that the recombinant CBH II had no apparent activity to CM-cellulose, which is typical of an endoglucanase (Table 1), however confuses the issue. Perhaps this observation will enforce a more critical evaluation of the use of non-cellulosic substrates (barley β-glucan) and unnatural substrates (CM-cellulose) in studies of the mode of action.

Chromophoric cello-oligosaccharides as substrates

To some extent, the difficulty in reaching a consensus on the mode of action of the cellulase components is a direct consequence of having to use substrates which are poorly characterized and assay methods which lack sensitivity. In an attempt to overcome these problems, the use of chromophoric glycosides from cello-oligosaccharides and lactose substrates in conjuction with fluorescence and HPLC methods has been pioneered by van Tilbeurgh et al. (1982). This elegant procedure, in demonstrating the preferred site of hydrolysis, has provided interesting insights into the mode of action of CBH I and II of *T. reesei* and *P. pinophilum* (Claeyssens et al. 1989). Thus the results have been interpreted to indicate that the site of attack of all four cellobiohydrolase enzymes is not restricted to the penultimate glycosidic linkage at the non-reducing end, suggesting that there is a degree of randomness in the attack. However, as the CBH I and II of *P. pinophilum* produced cellobiose almost exclusively when cellulose swollen in H_3PO_4 was the substrate, and effected only a slow fall in the degree of polymerisation, one clearly must be cautious in drawing conclusions on the mechanism of action from only one substrate (Wood et al. 1980; Wood & McCrae 1986a, b).

Despite these reservations, a range of chromophoric glycosides has been extremely useful in classifying the cellulase components of *T. reesei*. Thus, 4-methylumbelliferyl cellobioside and the corresponding lactoside are hydrolysed by CBH I or an endoglucanase designated EG I (Claeyssens & Tomme 1989), but not by CBH II or EG III (Saloheimo et al. 1988). The structure/activity relationships discussed below show the significance of this classification.

Thus the controversy as to the mode of action and the substrate specificity of the components of the cellulase system of fungi continues.

Synergism

When the observed action of two or more enzymes acting together in solution is greater than the sum of the individual actions, it is concluded that the enzymes act synergistically. There are two types of synergistic action involved in the process by which crystalline cellulose is rendered soluble, i.e. co-operation between endoglucanase and cellobiohydrolase (so-called, endo-exo synergism) (Wood & McCrae 1972; 1979) and co-operation between two cellobiohydrolases (so-called, exo-exo synergism)

(Fagerstam & Pettersson 1980). However, despite intense research activity the molecular basis for synergistic action is not well understood. It is possible that the lack of agreement is a direct consequence of the wide diversity of opinion regarding the individual roles of the 'purified' enzymes. However, the choice of substrate as an example of crystalline cellulose has also been responsible for some confusion. This was elegantly demonstrated by Henrissat et al. (1985) who showed that the degree of synergistic activity observed between cellobiohydrolase and endoglucanase varies with substrate used as well as with the ratio of cellobiohydrolase to endoglucanase. It appears, however, that synergism is most marked when crystalline cellulose is the substrate, that it is low or non-existent with amorphous-highly hydrate cellulose, and that it is absent with soluble cellulose derivatives (Wood & McCrae 1979).

The original model for synergistic activity between enzyme components envisaged an enzyme (so-called C_1) whose sole function was to cause some relaxation in the intramolecular hydrogen bonding as a preliminary to action by the hydrolytic enzymes (Reese et al. 1950). No one now believes that such an enzyme exists, but there is no doubt that the disaggregation and subsequent hydration of the closely packed cellulose chains in the cellulose crystallite is an essential prerequisite of cleavage of the glycosidic bond by cellulase enzymes. The discovery that cellobiohydrolase and endoglucanase consist of two domains, one binding and one hydrolytic (Tomme et al. 1988b) suggests that the swelling and hydrolytic function may reside in one enzyme. Knowles et al. (1988) envisage the binding domain to 'unzip' the individual chains as a preliminary to hydrolysis of the cellulose by the hydrolytic domain. This suggestion though relates only to action by one enzyme.

Attempts to explain the concerted action of endoglucanase and cellobiohydrolase have discussed the mechanism in terms of sequential action, where a randomly acting endoglucanase initiates the attack in the amorphous areas of the cellulose to create non-reducing ends for the endwise-acting cellobiohydrolase (Wood & McCrae 1972). However, this model is granted only qualified acceptance and it is generally regarded as an oversimplification. It does not account, for example, either for the fact that there is little or no synergism observed between some endoglucanases and cellobiohydrolases (Wood 1975) or that synergism exists between two cellobiohydrolases (Fagerstam & Pettersson 1980).

There is no doubt that adsorption of the enzyme on the cellulose is essential for solubilisation (Coughlan 1985; Klyosov 1988). Klyosov and his colleagues (Klyosov 1988) conclude that only those endoglucanases that have a strong affinity for crystalline cellulose can act synergistically with the cellobiohydrolase. Ryu et al. (1984) are of the opinion that exo-endo synergism can be described in terms of competitive adsorption of the two types of enzyme, optimum co-operation being manifested when the enzymes were present in the ratio in which they were present in the culture filtrate. Woodward et al. (1988a, 1988b), on the other hand, showed that the concentration of the mixture of cellobiohydrolase and endoglucanase from *T. reesei* was more important than the ratio of the enzymes. As the concentration of the enzyme mixture decreased, the degree of synergism increased to a maximum and then decreased with further increases in enzyme concentration.

Fagerstam & Pettersson (1980) were the first to demonstrate that purified CBH I and CBH II from *T. reesei* co-operated to effect hydrolysis of crystalline cellulose. This unexpected finding was confirmed by Henrissat et al. (1985) and Kyriacou et al. (1987) working on the same cellulase and by Wood & McCrae (1986b) using CBH I and II from *P. pinophilum*. Henrissat et al. (1985) envisaged that competitive adsorption or the formation of a binary complex between CBH I and CBH II might result in the enhancement of the turnover of the enzymes. There is now some evidence that such complexes do in fact exist (Tomme et al. 1990).

Synergism between two cellobiohydrolases acting at the ends of the substrate is difficult to explain. Wood & McCrae (1986b) have postulated that the mechanism can be discussed in terms of the stereochemistry of the cellulose chains, based on the fact that there are likely to be two different naturally-occurring configurations of non-reducing

end group in the cellulose crystallite. In essence, they envisage that CBH I and CBH II may differ in their substrate stereospecificities, and that the apparent cooperation may be discussed in terms of CBH I attacking only one of the two stereospecifically-different non-reducing end groups, while CBH II attacks the other. Thus, synergistic action would be observed if the sequential removal of cellobiose from one type of non-reducing end by CBH I exposed, on a neighbouring chain, a non-reducing chain of different configuration which would be a substrate for CBH II, and vice versa.

Clearly, there is a great deal of debate and uncertainty regarding the mechanism of synergistic action between the various enzymes and rationalisation is difficult. However, it is abundantly clear that there can be no agreement on the matter until there is a consensus of opinion regarding the substrate specificity and mode of action of the cellobiohydrolases. As purification techniques improve, so views on the properties of the individual enzymes may be modified. Of interest in this regard has been the use of affinity chromatography on a column of *p*-aminobenzyl-1-thio-β-cellobioside, which is a technique developed by Van Tilbeurgh et al. 1984). Thus, as mentioned above, Wood et al. (1989) have shown that preparations of *P. pinophilum* CBH II, isolated by conventional separation techniques including affinity chromatography on a column of cellulose, and shown to be electrophoretically homogeneous, were in fact contaminated by trace amounts of endoglucanase. When the contaminating endoglucanase was removed on the affinity column, no synergism was observed between CBH I and II nor between a mixture of CBH I and II and either of the endoglucanases, when crystalline cellulose in the form of the cotton fibre was used as substrate. Synergistic action was only apparent when CBH I and II and a specific endoglucanase were present in admixture (Fig. 1). The optimum ratio of the cellobiohydrolase components was 1 : 1 and, significantly, the addition of a trace of endoglucanase was needed for extensive degradation of the substrate. Thus, it appears that when the enzymes are highly purified, three enzymes, and not two as previously believed, are required for a reasonable rate of hydrolysis of crystalline cellulose in the form of cotton fibre.

The implication of these new results is that many of the contradictory statements in the literature may be the result of incomplete resolution of enzyme complexes. The authors report (Wood et al. 1989; Wood 1989) that an electrophoretically homogeneous preparation of *T. reesei* CBH II, prepared in their laboratory, could be further purified in the same way. Cellobiohydrolase preparations from *T. reesei* purified in different ways have been shown to be able to degrade cotton fibre without the need for the addition of endoglucanase (Enari & Niku Paavola 1987): perhaps, in this case, one or other of the enzymes were complexed with small amounts of contaminating endoglucanase which acted synergistically with the cellobiohydrolase.

Bacterial cellulases

In comparison with the cellulases of the fungi, very little is known about the mechanisms by which bacteria degrade cellulose. To a large extent this is a consequence of the fact that many bacteria, unlike fungi, degrade the cellulosic fibre by erosion of the surface and use cell-bound enzymes. It appears possible, however, that 'fungal-like' prokaryotes such as the *Actinomycetes* and the *Corynebacteria* might degrade cellulose using a mechanism similar to that operating in the fungi (Beguin 1990). It has been suggested that this can be rationalized to indicate that cell-bound enzymes will be more efficient in some situations (Yablonsky et al. 1988). These include situations where the microorganism will be exposed to predatory microorganisms, or where it is operating in an aquatic environment or in ecosystems such as that in the rumen. Cell-free enzymes, on the other hand, would be more effective in aiding the spread of mycelia through the plant cell wall by predigestion by the extracellular enzymes.

Cell-bound enzymes are more difficult to study. Some of the most efficient cellulolytic bacteria, such as *Sporocytophaga myxococcoides*, release practically no extracellular enzyme. Most bacteria

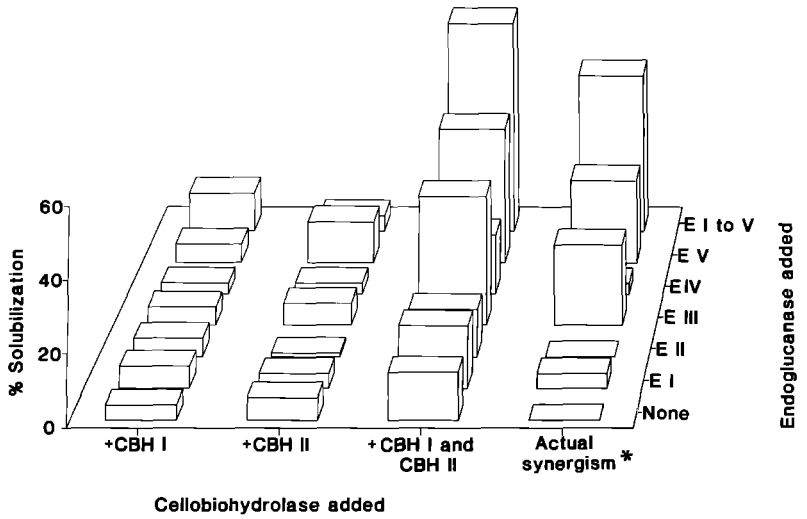

Fig. 1. Synergism between cellobiohydrolases and endoglucanases in solubilizing cellulose (cotton). Actual synergism means the solubilization effected by a mixture of CBH I and CBH II minus the solubilization recorded by a mixture of the two cellobiohydrolases. From: Wood 1989

that do secrete cellulases, in fact release only a variety of endoglucanases which show little activity to crystalline cellulose. Exceptionally, cell-free enzymes with activity towards crystalline cellulose are found in cultures of the anaerobes: *Clostridium thermocellum* (Lamed et al. 1985; Johnson et al. 1982), *Acetivibrio cellulolyticus* (McKenzie et al. 1987) and *Clostridium stercorarium* (Creuzet et al. 1983), and the aerobe *Microbispora bispora* (Yablonski et al. 1988).

The activity of cellulases from *C. thermocellum* (Johnson et al. 1982) and *A. cellulolyticus* (McKenzie et al. 1987) towards crystalline cellulose appears to depend on the presence of Ca^{2+} and thiol reducing agents (Johnson et al. 1982). In the case of *C. thermocellum*, activity towards crystalline cellulose is found in a tightly associated multimolecular complex containing endoglucanases but, as far as is known, no cellobiohydrolase. Cellobiohydrolases (or at least exoglucanases) have been identified in culture filtrates of *Cellulomonas fimi* (Miller et al. 1988), *Clostridium stercorarium* (Creuzet et al. 1983), *Ruminococcus flavefaciens* (Gardner et al. 1987), *Microbispora bispora* (Yablonsky et al. 1988), *Streptomyces flavogriseus* (McKenzie et al. 1984), on the basis of their capacity to hydrolyse nitrophenyl- or 4-methylumbelliferyl-cellobioside, the corresponding lactosides, or amorphous cellulose, but only two (*C. stercorarium; M. bispora*) have been characterised as 'fungal-like' cellobiohydrolases, which release cellobiose virtually exclusively from crystalline cellulose and which act synergistically with the endoglucanases in solubilizing crystalline cellulose.

Only a few bacterial extracellular cellulases have been studied in any detail: of those, *C. thermocellum* cellulase has received most attention (Lamed & Bayer 1988). The multicomponent cellulase complex of *C. thermocellum* is a very stable structure, comprising 14 to 18 polypeptides (Lamed et al. 1983a, b). The attachment of the complex, which has been termed the cellulosome (Lamed et al. 1983b), may be mediated by a non-cellulolytic binding factor (S_L) of molecular mass 250 kDa (Wu et al. 1988). Polycellulosomal protuberances cover the surface of the bacterial cell at periodic intervals (Bayer & Lamed 1986; Lamed & Bayer 1988). The cellulosomes dissociate from the cell after a time and are to be found in clusters covering the whole of the residual cellulose (Mayer 1988; Mayer et al. 1987). A number of other aerobic, anaerobic, mesophilic and thermophilic cellulolytic bacteria

have been shown to have these cellulosomes (Coughlan & Ljungdahl 1988; Beguin 1990): however, no polycellulosomal protuberances are found on non-cellulolytic bacteria. Each cellulosome is characterized by the presence of a polypeptide of molecular mass 200 to 220 kDa (Coughlan & Ljungdahl 1988; Ljungdahl 1989), which may be involved in binding the cellulosome to the cellulose. Thus, bacteria which utilise a multicomponent cellulase for hydrolysis of crystalline cellulose have similar structural features and enzymic properties.

The cellulosome of *C. thermocellum* can be fragmented into a number of subunits using SDS under different conditions of temperature and concentration (Lamed & Bayer 1988; Bayer et al. 1985; Lamed et al. 1985). One subunit (S1) is of special interest (Lamed et al. 1983a). It is highly antigenic, it contains about 40% carbohydrate, it is non-cellulolytic and it is relatively easily removed from the rest of the cellulosome by low concentrations of sodium dodecylsulphate (SDS) (Lamed & Bayer 1988). A cellulose-binding role has been considered for S1. With higher concentrations of SDS, the intact cellulosome dissociates readily into a number of subunits, some of which show endoglucanase activity after renaturation, but activity to crystalline cellulose is lost completely even when the dissociating reagents are removed and a multicomponent cellulase complex is reformed (Lamed & Bayer 1988). Using fairly drastic conditions, Wu et al. (1988) were able to effect the dissociation of the cellulosome into a number of components, two of which had M_r value 82 kDa (designated S_s) and 250 kDa (S_L), respectively. Only S_s showed CM-cellulase activity, but neither could degrade crystalline cellulose in the form of Avicel when acting alone. However, when S_s and S_L were recombined some of the activity was recovered. Neither Ljungdahl (1989) or Lamed & Bayer (1988) have isolated a subunit of this molecular weight; consequently it is not clear if this S_L relates to subunit S1.

A yellow affinity substance (YAS), which has a high affinity both for the cellulose and the cellulosome, also appears to have a role to play in binding (Ljungdahl et al. 1983). Ljungdahl et al. (1988) speculate that YAS may be a factor used by the bacterial cell to recognize cellulose. The structure of the YAS is not known precisely, but there is some evidence that it may be a carotinoid (Ljungdahl 1989).

It is obvious that the mechanism by which the cellulosome hydrolyses cellulose is not well understood, but it would appear to be quite different to that operating in the aerobic fungi. On the basis of evidence obtained in the electron microscope, Mayer et al. (1987) have proposed a model (Fig. 2) in which the subunits of the cellulosome attack the cellulose chains simultaneously at every eight glycosidic linkage to release cello-oligosaccharides which are four cellobiose (C_4) units in length. This model was deduced when it was observed that the average distance between the polypeptides was 4 nm, which corresponds in length to a cello-oligosaccharide of four cellobiose units. Subsequently, the C_4 units may be degraded to cellotetraose or cellobiose by rows of subunits which are smaller in size. The proposers do not make it clear how the large variety of endoglucanases resulting from expression of the multitude of genes found in C. thermocellum fit into this model.

Structure/activity relationships in fungal and bacterial cellulases

All cellulolytic microorganisms of fungal or bacterial origin which have been studied so far contain multiple genes. Remarkably, fifteen different endoglucanase genes, two xylanase genes and two β-glucosidase genes of *C. thermocellum* have been cloned in *E. coli* (Hazlewood et al. 1988; Grabnitz & Staudenbauer 1988) and several have been sequenced [celA, celB, celC, celD, celF, xynZ and bglB – (Beguin 1990)]. The products of these have been purified and one (endoglucanase D) has been crystallised (Joliff et al. 1986). Other bacteria contain a similar multiplicity in the genes. Thus, 10 have been cloned from *Ruminococcus albus* 8 (Howard & White 1988). Other examples of multiple genes in bacteria have been found in *M. bispora* (Yablonsky et al. 1988, 1989), *Erwinia chrysanthemi* (Chippaux 1988) and *C. fimi* (Miller et al. 1988). Of the cellulolytic fungi, the molecular genetics of

T. reesei have been studied in most detail and two cellobiohydrolases [CBH I (Shoemaker et al. 1983; Teeri et al. 1983), CBH II (Teeri et al. 1987b; Chen et al. 1987)] two endoglucanases [EG I (Penttila et al. 1986; van Arsdell et al. 1987) and EG III (Saloheimo et al. 1988)] have now been cloned, sequenced and the primary structure deduced.

A comparison of primary protein sequences derived from the nucleotide sequences has shown that cellulase components from different sources have a common design in both fungi and bacteria. Thus, all cellulase components from these widely different sources have a common design in both fungi and bacteria. Thus, all cellulase components appear to be composed of two separate functional domains, which are conserved to different degrees and integrated into the proteins in different orders. Each enzyme appears to consist of a non-conserved catalytic core protein which is linked by a flexible hinge region, usually rich in proline, threonine and serine, to a highly conserved tail region which is situated at either the C- or N-terminal end of the molecule.

The domain structure has now been characterized to different degrees in the fungi *T. reesei* (Knowles et al. 1988b; Teeri et al. 1987b) and *Sporotrichum pulverulentum* (Johanson et al. 1989), and there is some evidence for it in *P. pinophilum* (Claeyssens & Tomme 1989). Among the bacteria, it has been demonstrated in *C. fimi* (Warren et al. 1986; Gilkes et al. 1988), *M. bispora* (Yablonsky et al. 1988), *Bacteroides succinogenes* (McGavin & Forsberg 1989), *C. thermocellum* (Hall et al. 1988; Faure et al. 1989) and *Thermononospora fusca* (Changas et al. 1988).

Studies on the domains of *T. reesei* cellulase components have been particularly thorough (Shoemaker et al. 1983; Teeri et al. 1983; Pentilla et al. 1986; Chen et al. 1987; van Arsdell et al. 1987; Teeri et al. 1987a; Teeri et al. 1987b; Saloheimo et al. 1988). Thus, it has been shown that CBH I, CBH II, EG I and EG III contain highly conserved regions (designated A, B in Fig. 3) which are either at the C-terminal (CBH I, EG I) or the N-terminal (CBH II, EG III) end of the enzymes (Fig. 3). Block A, which comprises approx. 30 amino acid residues is the better conserved in all four components (70% homology). This block is rich in glycine and cysteine and is stabilized by 2 or 3 disulphide bridges (Bhikhabai & Pettersson 1984): it exists as a small domain which is separate from the core or catalytic domain. The domain structure of CBH I (Schmuck et al. 1986; Abuja et al. 1988a) and CBH II (Abuja et al. 1988b) was shown to consist of a large ellipsoid head and a long tail reminiscent of a tadpole (Fig. 4). The small domain (block A) is joined to the rest of the protein by region B (Fig. 4). This region, which is rich in proline, serine and threonine (approx 40%) is heavily *O*-glycosylated (Fagerstam et al. 1984; Bhikhabhai & Pettersson 1984; van Tilbeurgh et al. 1986; Tomme et al. 1988b), which may protect against proteolytic attack (Claeyssens & Tomme 1989). Homology in region B is 50–60%. This region is assumed to function as a flexible hinge linking the small terminal domain to the larger domain (Knowles et al. 1988b). A recombinant endoglucanase (ngCenA) from *C. fimi* expressed in *E. coli* has a gross structural and functional organization similar to that of CBH I and CBH II of *T. reesei*, but the model incorporates a constrained angle of 135° between the long axes of the core and tail regions (Pilz et al. 1990). The binding domain of the recombinant enzyme could be excised precisely by an extracellular *C. fimi* protease (Gilkes et al. 1988).

Studies involving partial proteolysis have provided evidence of the possible functions of each do-

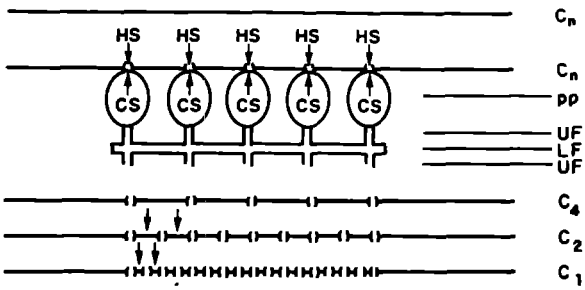

Fig. 2. Diagrammatic representation of the mode of action used by the cellulosome of *Clostridium thermocellum* to degrade cellulose.
Abbreviations: CS – catalytic site; HS – site of hydrolysis of cellulose; LF – central string of unknown material; C_n, C_4, C_2, C_1 – cellodextrins of various lengths. C_n is cellulose; C_1 is cellobiose. From: Mayer et al. 1987

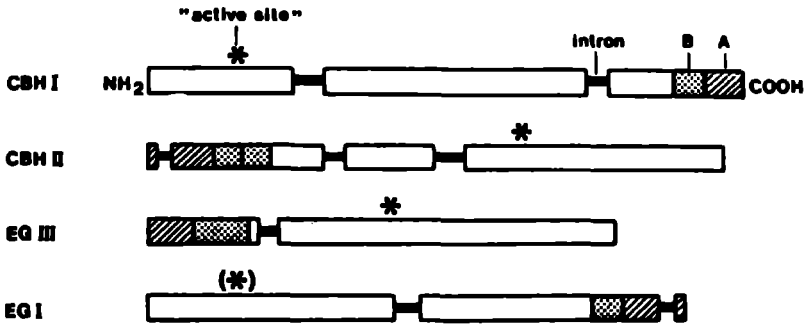

Fig. 3. Diagrammatic representation of four *Trichoderma* cellulase genes. Each enzyme comprises a tail domain (block *A*) linked to a flexible 'hinge' region (block *B*) to a catalytic domain (open boxes). The terminal domains are represented by striped boxes and the putative hinge regions by dotted boxes. A star indicates a putative active site. Intron positions are shown by solid bars. From: Knowles et al. 1988b.

main. Thus, the conserved region appears to be involved in substrate binding and the core in catalysis. This was readily demonstrated in *T. reesei* CBH I and CBH II when it was found that removal of blocks A and B from both by limited proteolysis had a dramatic effect on the capacity of the core proteins of both CBH I and II to degrade microcrystalline cellulose (Tomme et al. 1988b). Indeed, the specific activity of the CBH I core was only 10% of that shown by the intact enzyme: CBH II core retained 40% of the original activity. However, the cores retained much of the capacity (93% in the case of CBH I core) of the intact enzyme to bind to amorphous cellulose: all the activity to small soluble cello-oligosaccharides was retained. From these data it was concluded that the tails are involved in binding the intact enzymes to the crystalline substrate.

The core protein released from *T. reesei* CBH I by proteolysis was 56 kDa, the smaller domain 10 kDa (van Tilbeurgh et al. 1986): the large and small domains in CBH II were 58 kDa and 13 kDa, respectively. Monoclonal antibodies raised against the respective cores of CBH I and II were highly specific (Mischak et al. 1989); no cross reactivities were observed.

Further studies carried out on the binding domain of *T. reesei* CBH I have established that the three dimensional structure of the binding peptide must be retained intact for biological function (Johansson et al. 1989). Using NMR spectroscopy, it has been shown that the binding domain has the shape of a wedge, with overall dimensions 30 × 18 × 10 Å (Teeri et al. 1990). One surface is flat and hydrophobic (Kraulis et al. 1989), the other is flat and hydrophilic. It has been postulated that this structure could interact with crystalline cellulose to effectively solubilize as a preliminary to hydrolysis by the core enzyme (Teeri et al. 1990).

Attention is now being directed at the core protein. Crystals of both CBH I and II cores have been obtained (Bergfors et al. 1989), but only the crystal structure of CBH II core protein has been elucidated (Rouvinen et al. 1989). The core is approximately 50 Å in diameter and consists of a seven-strand-

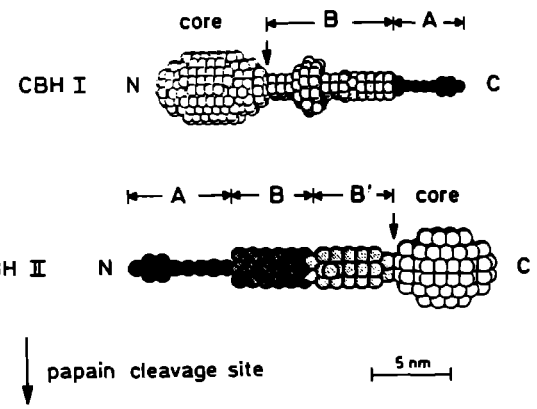

Fig. 4. Tertiary structure of CBH I and CBH II from *Trichoderma reesei* as deduced by small angle X-ray scattering. *A* and *B* relate to *A* and *B* in Fig. 3. From: Abuja et al. 1988b.

ed singly wound α-β-barrel. It seems that substrate binding occurs in a large channel which is formed by extended loops from the barrel (Teeri et al. 1990). The active site of the enzyme has been located at the COOH end of the β-sheets using an inhibitor diffused in the crystal (Teeri et al. 1990). It is not clear yet which amino acids constitute the active site but, as discussed below, progress has been made in this area.

Active site studies

Using chemical modification studies, Tomme & Claeyssens (1989) have identified Glu-126 to be a catalytically important carboxyl residue in CBH I of *T. reesei*: Glu-127 was proposed as a potential active site in EG I. A suggestion, based on limited homologies of the primary structures of the cellulases to the active sites of different lysozymes (Knowles et al. 1987), that Glu-65 and Asp-74 were involved in the active site was not supported by chemical modification studies. Glu-126 in *T. reesei* CBH I is reported to be located in a hydrophobic region between two large domains and to be equivalent to Glu-35 in egg white lysozyme.

Henrissat & Mornon (1990) have used hydrophobic cluster analysis to identify essential amino acids in a variety of cellulases. A histidyl residue appears to be involved in the active site of *C. thermocellum* (Claeyssens & Tomme 1989).

Stereochemical course of catalysis by cellulases

According to Reese et al. (1967), exoglucanases act by inversion of configuration. The question as to whether cellulases act by retention or inversion of anomeric configuration has been approached from time to time, as this has an important bearing on our conception of the mode of action of the cellobiohydrolases in particular. It now seems that this problem has been resolved. NMR spectroscopy of the hydrolysis of β-D-cellobiosylfluoride (Knowles et al. 1988a) suggests that the two cellobiohydrolases have different mechanisms: CBH I acts by retention of configuration while CBH II inverts. Similar conclusions have been reported by Claeyssens et al. (1990): CBH I of *T. reesei* liberated β-cellobiose from β-methyl cellobiose while CBH II, in contrast, liberated α-cellobiose from cello-oligosaccharides. EG I from *T. reesei*, which is structurally very similar to CBH I (see above), also acts by retention of configuration. Thus, while CBH I and EG I have a hydrolytic double inversion mechanism similar to that operating in lysozyme, CBH II may utilise a β-amylase type mechanism involving single displacement (Claeyssens & Tomme 1989). An exoglucanase from *C. fimi* acts by retention of anomeric configuration, while an endoglucanase from the same bacterium inverts (Withers et al. 1986).

Conclusions

Clearly, remarkable advances have been made in the last few years in the understanding of the cellulases and the study is in the middle of a very exciting phase. Further rapid advances in our understanding of the individual enzymes are in prospect now that,

- crystallisation of cellobiohydrolase cases has been possible and,
- attention is being directed at the development of an efficient host-vector system which will permit the reintroduction and expression of *Trichoderma* genes, modified by site specific mutagenesis, into cellulase negative mutants of *Trichoderma* (Teeri et al. 1990).

This will ensure effective enzyme processing and glycosylation and circumvent the problems associated with expression in a heterologous host. A study of the recombinant cellulase components which will be free from other contaminating cellulase components promises to provide unequivocal information on the hydrolytic properties of the individual enzymes.

Acknowledgements

The authors acknowledge the receipt of funding

from the Commission of the European Communities (contract MA1D 0014 UK [BA] and contract SC 1000205). They also wish to express their thanks to many colleagues who sent reprints and preprints of their work.

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