Characteristics of Fungal Cellulases

Anil Goyal, Bijan Ghosh & Douglas Eveleigh

Abstract

Fungi colonize diverse ecologic niches, yet their cellulases are based on a central theme — exo- and endo-splitting glucanases acting co-operatively and in some instances, synergistically. The characterization of cellulases has proved to be complex. This is due in part to the ill-defined nature of the natural substrates, plus the multiple nature of cellulase components. The rationales for this multiplicity continues to be explored. Cloning studies have shown far less diversity at the genomic level. Additionally, the endo- and exo-splitting glucanases have been shown to be comprised of a tripartite structure with both enzyme forms containing a cellulose binding site, hinge region and catalytic domain. Oxidative enzymes (cellobiose quinone oxidoreductase, cellobiose oxidase and lactonase) though minor in quantitative terms, may have important roles in the regulation/initiation of cellulase synthesis per se.

Key words: Binding site, catalytic domain, cellobiose oxidase, cellobiose quinone oxidoreductase, cellulase, exoglucanase, endoglucanase, fungi, lactonase, oxidative enzymes, synergism.

INTRODUCTION

The fungi have been placed in a Super-Kingdom based in part on their absorptive metabolism (Whittaker & Margulis, 1978). Since cellulose is the world’s most abundant organic polymer (annual production of $4 \times 10^{10}$ tonnes per year), it is not surprising that cellulolysis occurs widely in diverse fungal classes. Cellulolysis is normally considered from an ecological perspective, an industrial application or with regard as the utilization of cellulose for growth. However, fungi such as the Oomycetes (Leptomitus, Pythium) and Ascomycetes (Ceratoxyris and its anamorph Verticiladiella) synthesize cellulases to allow extension of their cellulose containing mycelial walls, while diverse fungi produce cellulases in response to plant associations be it endomycorrhizae formation or as plant pathogens. Other fungi have evolved to fill such productive ecologic cellulolytic niches that they have become the ‘prey’ of secondary consumers as exemplified by Termomyces spp. in the termite fungal gardens (Cowie, 1988). Cellulolytic fungi in the rumen deserve a special mention given these anaerobes appear to be of special importance in dissociating plant cell wall fibers through the ramifying action of chytrid rhizoids (Heath, 1988; Wood, 1989). From the evolutionary principle of retention of energy efficiency one might predict that cellulases from the anaerobes will have a higher specific activity than aerobic cellulases since anaerobes have less energy available for protein synthesis (36 ATP compared to 2 ATP/mole sugar in aerobic versus anaerobic metabolism). Indeed, to date, anaerobic fungi have proved sources of excellent cellulase though they produce lower amounts of the enzymes (Wood et al., 1988). Surprisingly, cellulases from anaerobic fungi were only discovered in the 1970s. Perhaps this resulted from the illusion given in general biology texts that all fungi are aerobic, although fermentative fungi were definitively described in the 1960s (Aqualinderella ferments) (Emerson & Held, 1969) or well before for yeast — Pasteur, 1860! One can expect further interesting cellulases will be isolated from anaerobic fungi in niches other than the rumen. Already chytrids have been isolated from monogastrics (elephants and horses) and the hindgut of sheep. Pond muds and marshes are other untapped ecologic niches.

With such a diversity of cellulolytic niches, it seems likely that a wide spectrum of fungal cellulases will exist, differing according to their broad ranging roles. Such a biochemical cornucopia has yet to materialize and this is probably due to the...
enzyme's conserved mechanism of action in the hydrolysis of cellulose. It could also be artefactual and result from the restricted study of hypercellulolytic (hypersecretory?) species of industrial potential. Thus the available detailed descriptions of fungal cellulases are biased due to (a) the relatively few cellulase producers that are of industrial interest (Aspergillus, Penicillium, Schizophyllum, Trichoderma spp., Phanerochaete chrysosporium (syn. anamorph Sporotrichum pulverulentum) and Sclerotium rolfsii) and (b) characterization of 'soluble' commercial cellulases from acidic culture broths all of which have been 'purified' by removal of mycelium to yield preparations that lack cell-bound cellulase components such as membrane bound cellobiase (Umile & Kubicek, 1986) or even perhaps cellulosomes. Quizically, the concept of using cell-bound β-glucosidase as a source of immobilized enzyme, has rarely been addressed. Finally, it is rare to find a description of an alkaline cellulase, e.g. Humicola sp. with an optimum pH of 7–9 recently proposed for use in washing powders (Novo Co., 1987).

For a wider perspective of cellulolysis, the reader is directed to the variety of excellent reviews (Lutzen et al., 1983; Coughlan, 1985, 1989; Henrissat et al., 1985; Ljungdahl & Eriksson, 1985; Margaritis & Merchant, 1986; Marsden & Gray, 1986; Saddler, 1986; Béguin et al., 1987; Enari & Niku-Paavola, 1987; Eveleigh, 1987; Fan et al., 1987; Knowles et al., 1987; Aubert et al., 1988; Coughlan et al., 1988; Wood et al., 1988; Penttila et al., 1989; Pourquid & Desmarquest, 1989; Wood, 1989). Here we briefly review the nature of the components of fungal cellulases and recent molecular biological insights into their structure.

**CELLULASE – THE GENERAL FUNGAL MODEL**

The term cellulase encompasses a melange of hydrolytic plus some oxidative enzymes, that interactively promote the degradation of cellulose. The classic fungal system based primarily on *Trichoderma* cellulase includes endo-glucanases (1,4-β-d-glucan 4 glucanohydrolase, EC 3.2.1.4 — abbreviated to EG), cellobiohydrolase (1,4-β-d-glucan cellobiohydrolase, — EC 3.2.1.91 — abbreviated to CBH), exoglucohydrolase (1,4-β-d-glucan glucohydrolase, EC 3.2.1.74 — abbreviated to EXG), and cellobiase (β-d-glucoside glucohydrolase, EC 3.2.1.21 — abbreviated to CB). This classification rests mainly on assumed substrate specificity, a theoretical criterion fraught with exceptions. The classical action of cellulase is envisioned as an initial attack by endoglucanases, followed by the combined action of cellobiohydrolyses and endoglucanases, with final hydrolysis of the small oligosaccharides to glucose by cellobiase (Fig. 1).

Exoglucohydrolyses also act on soluble oligosaccharides (Wood & McCrae, 1982; Rao & Mishra, 1989) while cellobiases with relaxed specificity can release glucose from oligomers (cellobiose to cello-octaose) (Schmid & Wandrey, 1987). This cellulase model of sequential and cooperative attack gives a central foundation. Alternate routes of cellulose degradation are probable. Marsden and Gray (1986) suggest by-

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**Fig. 1.** Generalized scheme for cellulolysis. Cellulose is initially attacked in amorphous zones by endoglucanases, (A:A'), thus generating multiple sites for attack by cellobiohydrolyses (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 cellobiohydrolyase and endoglucanase is synergistic. Most individual enzymes do not promote effective hydrolysis, although certain cellobiohydrolyses 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 stereoisomeric forms of the cellobiosyl unit in the chain. In contrast, in the attack of soluble oligomers such as by exoglucohydrolase (D) only one enzyme is necessary as the enzyme can initiate attack from either 'side' of the substrate. Reproduced with permission of the Royal Society (Eveleigh, 1987).
passing cellobiose as an intermediate, through the oligosaccharide products of endoglucanase(s) action being directly converted to glucose via the action of exo-glucanases with broad specificity. Niku-Päävola et al. (1985) opine that the combined action of CBHs can yield complete hydrolysis of cellulose and thus endoglucanases show little action on insoluble substrates and have a role limited to acting only on solubilized substrates. Each model has merit and perhaps each is at least transiently dominant during hydrolysis. EGs initiate attack (especially in amorphous regions) creating additional sites for hydrolysis by CBH (Fig. 1). Furthermore, removal of cellobiose by cellobiase or other cellobiose-transforming enzymes relieves its end-product inhibition towards both EG and CBH. Cellobiase, is analogously inhibited by its product glucose, and here relief of end-product inhibition is gained by subsequent oxidation via glucose oxidase or by glucose uptake by the fungus. Synergism occurs between EGs and CBHs, between cellobiohydrolase components and occasionally between endoglucanase and β-glucosidase components (Uzioie & Sasaki, 1987). Synergism between cellulases of different species is a further exciting concept (Macris et al., 1985; Szakyác Dobóz et al., 1985). For a full discussion see J. Woodward, this issue.

**Substrates**

Selective assays for the different classes of enzymes are available (Sharrock, 1988; Wood & Kellog, 1988) (Table 1, and see J. Puls & T. M. Wood, this issue).

EGs randomly hydrolyze internal glycosidic bonds and can be assayed using soluble substituted celluloses by measuring the production of reducing sugars. We prefer non-ionic hydroxyethyl cellulose as this substrate is independent of ionic strength, pH and polyvalent cations (Child et al., 1973). The decrease in the degree of polymerization of these substrates can also be monitored through loss of staining with complexing agents such as Congo red, or directly through solubilization of dyed substrates.

The assay for CBH is interesting in that it is theoretically defined as an enzyme splitting cellobiose moieties from the non-reducing end of the substrate and shows greatest activity towards longer oligosaccharides. However, there is no specific assay for CBH although commercial crystalline celluloses (e.g. Avicel) have proved adequate as a substrate even though they also

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**Table 1. Assay substrates for cellulases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay substrate</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cellulase</td>
<td>Crystalline cellulose</td>
<td>RS⁺, d</td>
</tr>
<tr>
<td></td>
<td>Practical substrates</td>
<td>RS⁺, d</td>
</tr>
<tr>
<td>Cellobiohydrolase</td>
<td>Avicel</td>
<td>RS⁺</td>
</tr>
<tr>
<td></td>
<td>Methyl-umbelliferyl-β-D-cellobioside</td>
<td>Fluorescence</td>
</tr>
<tr>
<td></td>
<td>Methyl-umbelliferyl-β-D-lactoside</td>
<td></td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>Celloxotins</td>
<td>RS⁺⁻, b</td>
</tr>
<tr>
<td></td>
<td>Substituted soluble celluloses (HEC, CMC)</td>
<td>V⁻, RS</td>
</tr>
<tr>
<td></td>
<td>Barley-β-glucan</td>
<td>Color</td>
</tr>
<tr>
<td></td>
<td>Trinitrophenyl-cellulose</td>
<td>Color</td>
</tr>
<tr>
<td></td>
<td>Ostazin Brilliant Red H3B-HEC</td>
<td></td>
</tr>
<tr>
<td>Glucohydrolase</td>
<td>Oligodextrins</td>
<td>RS⁺, d, l</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>Cellobiose</td>
<td>RS⁻, d</td>
</tr>
<tr>
<td></td>
<td>p-Nitrophenyl-β-D-glucoside</td>
<td>Color</td>
</tr>
<tr>
<td></td>
<td>X-Glu (5-bromo-4-chloro-3-indolyl-β-D-glucoside)</td>
<td>Color</td>
</tr>
<tr>
<td></td>
<td>Methyl-umbelliferyl-β-D-glucoside</td>
<td>Fluorescence</td>
</tr>
</tbody>
</table>

¹Cotton. Valonia cell walls.
²RS = Reducing sugars.
³Decrease in turbidity, loss of dry weight.
⁴HPLC analysis for cellobiose or glucose.
⁵Filter paper, Avicel, bacterial cellulose, acid swollen cellulose.
⁶Methyl-umbelliferyl glycosides are variably susceptible to different enzymes; CBHI attacks both holo- and aglycone-bonds; CBHII binds to MUC but will not cleave it.
⁷Carboxymethyl-cellulose and hydroxyethyl-cellulose.
⁸V = Viscometric assay.
⁹Analysis of glucose production by glucose oxidase.
appear susceptible to some endoglucanases. Immunologic assay of celllobiohydrolase is an alternative approach that is specific and highly sensitive, (Riske et al., 1986; Mischak et al., 1989) although it can yield positive results with inactivated enzymes. For cellobiose, celllobiase is the absolute substrate. Modified/colorimetric substrates facilitate purification and have given many insights into the mechanism of action (Claeyssens, 1988; see also J. Puls & T. M. Wood, this issue), but final evaluation of cellulolysis has to be with practical cellulosic substrates. It is emphasized that assessment of cellulase efficiency in biomass studies is based on 90–95% hydrolysis, rather than extrapolating the biochemist’s traditional enzyme characterization based on the initial rates of reaction.

**Multiplicity of components**

Cellulase components occur in multiple forms and though this was initially surprising, it is now recognized as a general phenomenon (Labudová & Farkas, 1983). A typical *T. reesei* commercial cellulase (Maxazyme, Gist Brocades) contains six endoglucanases, three celllobiohydrodrolases and one cellobiase (Beldman et al., 1985). The basis for such multiplicity is ill-defined and can either be artefactual or based on genetic factors, related to cellulase synthesis under various cultural regimes at the transcriptional/translational level, or can arise through post-translational modification.

The artefactual origin of the multiple forms has included multiplicity arising from protein complexes with Ampholyte carrier proteins (Farkas et al., 1987). This resulted lessened their prior emphasis of the biologic origin of these multiple forms (Labudová & Farkas, 1983). The binding of carbohydrates to cellulases can also result in multiple forms (Alurralde & Ellenrieder, 1984). The different fungal hydrolases also bind to form complexes which are only separated by strong disruptive treatments. Thus Sprey and Lambert (1983) could resolve an apparently pure cellulase, obtained by isoelectric focusing, into six components using electrophoresis in the presence of urea and octylglucoside. The study also indicated a lectin-type binding of these complexes to cellulose. Higher molecular weight aggregates are also known (I. Lubudová, pers. comm.).

The types of biological modifications resulting in the multiplicity of cellulases are diverse. Multiplicity can arise from the degree of glycosylation, for example with *Trichoderma* CBH (Gum & Brown, 1977) or expression of recombinant forms of EGI in yeast (Van Arsdell et al., 1987). In *Schizophyllum commune* (Willick & Seligy, 1985) variable glycosylation yields multiple cellulase forms. However these latter workers also demonstrated that heterogeneity can arise at the level of translatable mRNA via transcript heterogeneity, though it was not resolved if this was due to differential RNA splicing or multiple initiation sites.

Proteolysis has been directly suggested as a source of multiple enzyme forms (Nakayama et al., 1976; Gong & Tsao, 1979; Kelleher, 1981; Mo & Hayashida, 1988). In the study by Nakayama et al. (1976), the derived enzymes gained new substrate specificities. Eriksson and Pettersson (1982) showed proteolytic activation of endoglucanases but whether this was due to modification of the hydrolase or destruction of a proteinaceous inhibitor was not clarified. The role of proteolysis has taken on enhanced significance with the recent demonstration of cleavage of *T. reesei* CBH1 into two components, a catalytic core (55 kDa) lacking its binding domain (and inability to bind to crystalline cellulose) and yet still active towards soluble substrates, plus a small glycopeptide (10 kDa) containing the binding site (Van Tilbeurgh et al., 1986). This emphasizes the concept of post-translational modification to yield novel enzymes with new specificities. On this basis it has been suggested that optimal cellulolysis can be gained by the native enzyme initially acting towards the insoluble substrate, and subsequently discrete hydrolysis of those soluble oligosaccharides through the action of the proteolytically derived core enzymes (Knowles et al., 1988; Mo & Hayashida, 1988; Yablonsky et al., 1989). Even so, extracellular proteolysis may not be a general phenomenon, for Kammel and Kubicek (1985) in a careful study of *T. reesei* cellulases, showed that these glycoproteins, once released to the culture fluid, were not further proteolytically modified. The Vienna group did point out that multiple forms can still arise through variable glycosylation during secretion at the endoplasmic reticulum (Messner & Kubicek, 1985).

Gene cloning has shown there to be relatively few fungal cellulase genes (at least in comparison to *Clostridium thermocellum*). Thus the multiple cellulase components arise primarily via artefactual or biosynthetic routes, or through enzyme/substrate or enzyme/enzyme interactions. Rationales for such diversity/variety of components are rampant, the most attractive being to gain a series of cellulases of differing specificity...
That, in combination, optimize cellulolysis. Wood et al. (1988) have also emphasized that as the repeating unit cellobiose occurs as two distinct stereochemical forms in cellulose, two analogous stereospecific forms of CBH and of EG can be envisioned to yield optimal cellulolysis.

Transglycosylation
Transglycosylation occurs commonly with hydro-lases and cellulases are no exception. T. reesei β-glucosidase promotes formation of sophorose and laminaribiose from gentiobiose (Vaheri et al., 1978). A different concept has been proposed for the activity of endoglucanase II-b from T. viride (Meiselase) which produces high amounts of glucose from Avicel or cotton (Okada & Tanaka, 1988). From its preferential attack on β-D-cellobiose over that of α-D-cellobiose, and with the production of glucose in a sigmoidal manner over time, it is proposed that endo-II-b first converts cellobiose to cellotetraose, and this product is subsequently hydrolyzed to cellotriose and glucose, with subsequent production of glucose from the triose. The authors suggest that this type of enzyme has an important role in glucose production from native cellulose. The difference in susceptibility of the anomeric forms of cellobiose to endo II-b support the ‘Wood concept’ of two stereospecific forms of the classes of cellulases (Wood et al., 1988). A specific transglycosylase has also been reported from T. longibrachiatum which lacks both cellulase and β-glucosidase activity, but reacts with cellopentaose or higher oligomers to yield a range of insoluble and new soluble oligomers (Tanaka & Oi, 1984).

The term ‘complete’ cellulase is used for extracellular preparations from culture broths that are effective in attacking crystalline cellulose in contrast to those that only attack amorphous or soluble forms. To date the ‘complete’ fungal preparations all contain major cellulobioligohydrolase components. However, there is still the possibility that efficient cellulases exist that lack CBH, and some endoglucanases with limited activity on crystalline cellulose have been discovered (Sahasrabude et al., 1987). Finally, we reiterate that it is certain that the handful of well-described fungal cellulases are not responsible for recycling the vast amounts of the world’s crystalline cellulose. Further potential candidates will minimally include the 1600 unstudied Basidiomycete species.

**CELLULASE COMPONENTS**

Trichoderma cellulase
Current commercial cellulase production using submerged culture rather than solid substrate cultivation yields preparations of greater uniformity than were previously marketed. Several excellent biochemical characterizations of *Trichoderma* cellulases have been based on commercial ‘T. viride’ preparations. However, caution should be exercised in making absolute comparisons between such studies, because, although *T. viride*

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**Table 2. Range of nomenclature of Trichoderma reesei endoglucanases**

<table>
<thead>
<tr>
<th>Enzyme commission notation</th>
<th>pI</th>
<th>Author’s notation</th>
<th>Substrates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGI</td>
<td>4.0-4.7</td>
<td>EGI</td>
<td>CMC (+ +), xylan (- -)</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>EndoII</td>
<td>MUL (-), MUC (-)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGI</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>EGII</td>
<td>5.5</td>
<td>EGI</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGIV</td>
<td>CMC (+), xylan (+)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGIII</td>
<td>MUC (+)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>EndoIII</td>
<td>CMC (+), xylan (+)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGII</td>
<td>MUC (+)</td>
<td>3</td>
</tr>
<tr>
<td>EGIII</td>
<td>7.7</td>
<td>Endo IV</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>EndoI</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Others</td>
<td>7.4</td>
<td>EGIII</td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

*Modified from Teeri (1987) and Kyriacou et al. (1987).*


+ = attacked; - = not attacked.

*MUL and MUC — methylumbelliferyl-lactoside and cellobioside.*
is a valid species aggregate (Rifai, 1969), it has also been used as a 'catch-all' for unknown *Trichoderma* species. It is thus possible that some variations in cellulase preparations can be attributed to the use of different 'species' (Shoemaker & Brown, 1978; Gong & Tsao, 1979; Beldman et al., 1988). Furthermore, from a genetic standpoint, it should be remembered that all commercial cellulases from *T. reesei* are derived from a single isolate (QM6a) from the Solomon Islands (for discussion see Eveleigh (1985)). There is also renewed discussion as to whether *T. reesei* is a valid sub-species of *T. longibrachiatum* (Bissett, 1984). Beyond such taxonomic hassles, there is a need for gaining effective genetic crossing, e.g. between *T. reesei* and *T. harzianum* as has been initiated by Toyama et al. (1984). These cautionary notes apply to other cellulolytic fungi, with the exception of the well-developed genetic system of the cellulolytic *Neurospora crassa* (Deshpande et al., 1986).

**Hydrolases**

The characteristics of the *Trichoderma* system are discussed as this is the best characterized cellulase. The consensus of the cellulase components of *T. reesei* based on biochemical and genetic criteria include endoglucanases, cellobiohydrolases, glucohydrolases and cellobiases (Tables 1, 2). The nomenclature of the endoglucanases is confused as individual laboratories, in general, sequentially numbered these enzymes according to when they were first discovered. In contrast the International Enzyme Commission has recommended that such enzymes should be numbered according to their relative mobilities in isoelectric focusing gels, i.e. according to their isoelectric points. The naming of the two *Trichoderma* CBHs followed this recommendation, but there is considerable confusion regarding the designations for EGs (Table 2). To avoid misunderstandings citations in this review are according to the authors' designations (Table 2 — third column).

**Cellbiohydrolases**

*T. reesei* has two distinct cellbiohydrolases, CBHI and CBHII (Gritzali & Brown, 1979; Fagerstam & Pettersson, 1980; Nummi et al., 1983). CBHI is the dominant enzyme forming up to 60% of the secreted proteins. Purified CBHI from several sources, range in molecular weight from 42-72 kDa with carbohydrate content from 1-4-10-4%, and iso-electric points reported from 3-5-4.2. These ranges in part reflect the analytical methodologies employed. Isoelectric focusing has also been used to illustrate several iso-components from a single source (Bhikhabhai et al., 1984; Merivuori et al., 1985a). The CBHI amino acid sequence was first determined by Fagerstam et al. (1984) and is essentially in agreement with that deduced through cloning and sequencing of the gene which yielded a 496 amino acid protein (52,214 Da) (Shoemaker et al., 1983a; Teeri et al., 1983). Bhikhabhai and Pettersson (1984) reported that CBHI has 12 disulfide bridges and thus there were no free cysteine residues. From the nucleotide sequence, there are four putative N-glycosylation sites (Asn-X-Ser/Thr), probably three of which are glycosylated. Salovuori et al. (1987) analyzed the glycoprotein nature of CBHI via biosynthesis combined with the use of tunicamycin (an inhibitor of N-glycosylation), and also degradatively using endo H glycosidase (which cleaves N-acetylgalactosaminate of 'high mannose type glycans') and via β-elimination of derived glycopeptides plus further degradation using α-mannosidase. Analyses were facilitated by monitoring the release of 3H mannose. The N-glycosylated sites were shown to contain (man)₉(GNAc)₂ and (man)₅(GNAc)₂ chains (ratio 0-7:2-3), while the O-glycosylation was in the ratio of 2-2 mono-, 3-9 di- and 1-4 tri/tetrasaccharide chains comprised mainly of mannose. The 'high mannose type N-glycosylation' is analogous to that in animal cells, a positive feature in consideration of *Trichoderma* as the host for expression of eukaryotic heterologous proteins. About eight O-linked chains were estimated. *In toto* this yields 4000 Da for N-glycans and 2700 Da for the O-glycans, which plus the core protein sums to 59000 Da in close agreement to the values determined by Bhikhabhai et al. (1985) and Nummi et al. (1983). The molecular biological approaches have resolved much of the ambiguity in the characterization of CBHI. For further discussion see General Structure below.

CBHII forms roughly 20% of the secreted protein with molecular weights ranging from 50-58 kDa, its pIs from 5-0-6-3 and it shows 8-18% glycosylation. CBHII does not act as a truly theoretical cellbiohydrolase for besides releasing cellbiose from amorphous cellulose, it shows low activity towards CMC and causes short fiber formation (Kyriacou et al., 1987). Its classification as a cellbiohydrolase is thus debatable. More importantly, these combined exo- and endo-splitting characteristics lead to a re-inter-
pretation and perhaps an endo-splitting role of CBHIII in the exo/endo-synergism found between CBHI and CBHII (Fagerstam & Pettersson, 1980). An alternative explanation of synergism due to competition via adsorption and resultant high enzyme turnover, is not excluded (Henrissat et al., 1985). Differences in the action of CBHI and CBHII are illustrated in the ability of CBHII to promote small particle formation from Solka floc, a property not shared with CBHI.

Cloning of the genes coding for CBHI and CBHII in Saccharomyces cerevisiae has resulted in greater understanding of the cellulase system (Penttila et al., 1987). CBHI and II expressed in S. cerevisiae were found to be highly glycosylated and consequently of greater molecular weight than the native CBHs. Substrate specificity of the cloned enzymes indicates that CBHII is active towards a barley β-glucan in plate assays (endoactivity, cf clearing of this glucan by EG), whereas CBHI is inactive in this assay. This confirms the earlier indications of the unique characteristics of CBHII. Cloned CBHII was found to bind to crystalline cellulose but with less affinity than the native enzyme. Though this cloned CBHII is slightly less active than the native enzyme it will probably be quite adequate for direct application in yeast for the preparation of beverages (glucan haze removal) and the preparation of food products with modified texture.

Endoglucanases

Endoglucanases preferentially attack internal glycosidic linkages of substrates such as acid-swollen cellulose and soluble barley glucan, besides the modified substrates carboxymethyl cellulose and hydroxyethyl cellulose (Table 1). Endoglucanases have received wide, yet often superficial study perhaps reflecting the initial ease of assay via viscometric means combined with educational institute's research programs being based on the use of broken tipped pipettes plus a wristwatch. This approach belies the complexity of cellulolysis and has yielded a mass of conflicting data. Even with sophisticated analytical methods there is some confusion regarding the nomenclature of the T. reesei endoglucanases (Table 2).

Three endoglucanase groupings based on the isoelectric focusing classification scheme, can be recognized (Table 2). The endoglucanase grouping I (EGI) is fairly well substantiated. EGI is a major cellulase component comprising 6–10% of the secreted protein (54 kDa, pl 4-7 and carbo-hydrate content of 4% (Shoemaker et al., 1983b; Bhikhabhai et al., 1984; Henrissat et al., 1985; Niku-Päävola et al., 1985). Analysis of the glycoprotein nature of EGI showed a glycan distribution with 73% of mannose in the O-linked glycan, and these chains were longer than those occurring in CBHI. The egI gene has been cloned and sequenced (Chanzy et al., 1983; Van Arsdell et al., 1987; see General Structure below).

A second major endoglucanase grouping ‘EGII’, appears common to Trichoderma spp. EGIII is illustrative (Saloheimo et al., 1988) and has been characterized through cloning and sequencing. It has 397 amino acids (42.5 kDa) plus 15% glycosylation (47 hexose residues) to yield a total molecular weight of 49.8 kDa (Saloheimo et al., 1988). Its Km values for 4-methyl-glycosides are in the 10–60 μM range, compared to those of EGI in the millimolar range. EGIII lacks transferase activity. Saloheimo et al. (1988) noted that the amino acid composition resembles that of Endo IV (Shoemaker & Brown, 1978). Though the nucleotide sequence analysis of egI3 is quite distinct from the other Trichoderma egI1, cbh1 and cbh2 genes, it does show remarkable homology to that of an endoglucanase gene from Schizophyllum commune (Saloheimo et al., 1988). The gene is also remarkable for having relatively long introns (123 and 174 base pairs) in comparison to other fungi. EGIII alone hydrolyses the aglycone linkage of 4-MU-cellotrioside (Saloheimo et al., 1988). Other endoglucanases probably in this group are listed in Table 2, e.g. EGII (48 kDa with 6% glycosylation) (Bhikhabhai et al., 1984; Henrissat et al., 1985).

The endoglucanase III grouping is comprised of smaller molecular weight enzymes (20–23.5 kDa), which being relatively labile, have been harder to characterize, e.g. endo IV (Beldman et al., 1985). There are additional cellulase components which require further characterization in order to place them in the appropriate category. It is emphasized that the classification presented is for convenience, and changes are anticipated including gaining international nomenclatorial agreement.

Specificity of endoglucanases

Though apparently showing no attack on crystal-line cellulose, EGI acts synergistically with CBHI and CBHII towards this substrate. The other EGs have similar general endo-cleavage properties as EGI towards CMC but each has a unique specificity. EGII also shows synergism with CBHI and
CBHII in attack of crystalline cellulose. In an extension of this aspect, an elegant electron microscopic analysis using the highly crystalline Valonia cellulose clearly showed endo-attack specifically at bent fibril locations (Chanzy et al., 1983; Chanzy & Henrisat, 1985). EGI, II and III all attack CMC and can disaggregate Solka Floc to yield small particles (Kyriacou et al., 1987). EGII and EGIII also show relaxed substrate specificity, as they also attack the aglycone bond of MUC and MUL.

The proposed theoretical specificity of cellulase components does not always agree with the experimental results. Certain endoglucanases show little activity towards carboxymethyl cellulose, e.g. Avicelase II of Y-94 (Yamanobe et al., 1988), EGIb (syn, CBHII) of T. reesei (Kyriacou et al., 1987). Other EGs show relaxed specificity and are also active on xylans, e.g. T. reesei endo IV, V and VI and Exo I (as defined by Beldman et al. (1985) all attack xylan), and T. reesei EGII and EGIII (Kyriacou et al., 1987). Whether these dual cellulolytic and xylanolytic activities are due to different active sites has yet to be determined.

Glycosylation

During the analysis of the endoglucanases, a variety of effects have been ascribed to the type (O- or N-) and degree of glycosylation of the proteins, including suggestions of modified stability, changed susceptibility to proteolysis and resultant or indirect effects on enzyme activity. Decreased N-glycosylation obtained through culture in the presence of tunicamycin (an N-glycosylation inhibitor), had little effect on the specific activity of cellulase (Merivuori et al., 1985b, 1987; Murphy-Holland & Eveleigh, 1985) in studies using the Rutgers mutant P37 (subsequently described as RL-P37). Merivuori et al. (1985b, 1987) recorded a slight decrease in the thermal stability of the cellulase, though Murphy-Holland and Eveleigh (1985) were unable to detect such a change in any of the cellulase components. As tunicamycin probably does not result in complete N-deglycosylation, the effects of N-glycosylation on cellulase remains equivocal. Hyperglycosylation of recombinant derived EGI occurs in yeast and in this instance Van Arsdell et al. (1987) commented on greater thermal stability, though the parameters of enhanced stability were not cited. As most cellulases are generally glycoproteins, the carbohydrate moieties have been suggested to have specific functions. However, when cellulases are expressed in E. coli they are not glycosylated and yet are still active towards soluble substrates. Conversely, overglycosylation of Trichoderma cellulases produced in yeast still yielded active enzymes though with lower substrate affinity. Using selective inhibition of N- and O-glycosylation using tunicamycin and 2-deoxyglucose, Kubicek et al. (1987) showed that in T. reesei, O-linked glycosylation is necessary for secretion of endoglucanases I and II.

Oxidative enzymes

Eriksson and coworkers proposed an oxidative mechanism for the initiation of cellulose degradation (Eriksson et al., 1974; Eriksson, 1981). This concept was based on enhanced degradation by crude and/or reconstituted mixtures of cellulases under aerobic compared to anaerobic conditions. Oxidative enzymes (cellobiose quinone oxidoreductase, lactonase and cellobiose oxidase) have since been recognized and, though occurring in relatively minor amounts, may be of crucial importance.

Cellobiose quinone oxidoreductase (CBQOR) (cellobiose dehydrogenase — EC 1.1.5.1) was discovered in Phanerochaete chrysosporium by Westermark and Eriksson (1974) and later found in Monilia sp. (Dekker, 1980). CBQOR oxidizes cellobiose (or higher oligosaccharides) to cellobiono-δ-lactone. Westermark and Eriksson (1974) suggested that CBQOR facilitated lignin degradation by preventing the repolymerization of quinones and phenoxyradicals, by their reduction with the concomitant oxidation of cellobiose. This suggestion has recently been refuted by Odier et al. (1988), who were unable to substantiate such effects of CBQOR on the phenoxy-radical coupling produced by lignin peroxidase. However, K. Eriksson has alternate proof of the co-operative role of CBQOR in lignin transformations (pers. comm., 1989). Furthermore, Morpeth (1985) notes that the P. chrysosporium CBQOR oxidizes ‘cellulose’ (besides xylan, chitin and agarose) to yield the superoxide anion which could participate in ligninolysis. Iyayi et al. (1989) have shown the important role of cellbionolactone (CBL) in inducing de novo in T. reesei a distinct set of cellulase components required for the degradation of crystalline cellulose. In this vein, Graf et al. (1989) suggest that CBQOR and lactonase act co-ordinately in inducing cellulase synthesis in fungi including non-lignolytic forms such as Trichoderma. Cellulase induction was promoted by low concentrations (2.9 × 10⁻⁴ M) of CBL, while higher concentrations (8.8 × 10⁻³ M)
inhibited the activity of all components of *T. reesei* cellulase. With CBL arising through the action of CBQOR, they postulate that lactonase acts as a 'cellulase inducer' through attacking and thereby optimizing the inductive concentration of CBL. Thus they consider lactonase and CBQOR, though present in relatively low concentrations, to be essential components of 'cellulase'. CBQOR is currently commercially available from Industrial Microbiological Services, Seattle, WA.

Lactonase (D-glucono-1,5-lactono hydrolase — EC 3.1.1.17) catalyzes the hydrolysis of glucono-lactone and cellobiolactone. It is present in Novozym 188 a commercial preparation based on enzymes from *T. reesei* and *Aspergillus niger* (Bruchmann et al., 1987). Lactonase, besides having an inductive role in cellulase synthesis as suggested above, can also promote cellulolysis by removing lactones which are inhibitors of β-glucosidase.

Cellobiose oxidase (EC 1.1.99.18) from *P. chrysosporium* oxidizes cellobiose and oligosaccharides, with resultant disruption of the crystalline substrate through formation of uronic residues in addition to the production of H₂O₂ (Morpeth, 1985). Hydrogen peroxide has previously been suggested to be a key factor in cellulose degradation by brown rot fungi (Koenigs, 1974; Highley, 1980; Bratt et al., 1988). The latter author suggested that in brown rot fungi, Fe²⁺/H₂O₂ (Fenton’s reagent) catalyzes polysaccharide degradation. H₂O₂ can be produced from several enzyme reactions, and while Fe³⁺ is generally in sufficient supply, it would require reduction to Fe²⁺. It is surprising that this area has attracted relatively little study. Cellobiose oxidase and glucose oxidase (EC 1.1.3.4) can also be considered as integral components of 'cellulase' as they relieve end-product inhibition respectively of the endoglucanase and cellobiase.

**STRUCTURE-ACTIVITY STUDIES**

**General structure**

Fungal cellulases appear to be generally comprised of distinct binding, hinge and catalytic domains. This concept arose from analysis of peptide fragments (56 kDa and 45 kDa respectively) produced by partial proteolysis (by papain) of *T. reesei* CBHI (65 kDa) and CBHII (58 kDa) (Van Tilbeurgh et al., 1986; Tomme et al., 1988). These fragments showed loss of activity and of adsorption towards microcrystalline cellulose, completely for CBHI and partially for CBHII. Activity towards soluble substrates was maintained, while the activity towards amorphous cellulose was lost only in the case of the CBHII core fragment. It was suggested that these novel characteristics of the peptide cores, namely general retention of activity through with decrease in activity and inability to adsorb onto crystalline cellulose, were a result of loss of fragments containing the cellulase's binding domains. In support of this concept, the smaller glycosylated peptides from the C-terminus of CBHI and from the N-terminus of CBHII showed binding but no hydrolytic activity towards crystalline cellulose.

This 'hinge-model' with the binding domain (A) linked through a hinge region (B) to the catalytic domain (C) is further supported by structural studies employing small angle X-ray scattering (SAXS) of native CBHI and CBHII and also molecular biological analysis of the protein structure. The X-ray scattering data of *T. reesei* cellobiohydrolase I (Schmuk et al., 1986; Abuja et al., 1988b), indicate that CBHI is tadpole shaped, 18 nm long, and shows potential regions that can be correlated with the proposed catalytic, hinge and binding domains (Fig. 2). In contrast, CBHII was resolved to contain a double 'B' zone which correlates with the structure proposed from the amino acid sequence (Abuja et al., 1988a). From molecular biological studies, the primary sequence of CBHI (Shoemaker et al., 1983a; Teeri et al., 1983) and EGI (Penttila et al., 1986) reveals a similar peptide sequence (A/B block) at their C-termini whereas an equivalent sequence (A/BB' and A/B respectively) is present at the N-termini of CBHII and EGI (Chen et al., 1987; Teeri et al., 1987; Saloheimo et al., 1988; Fig. 3). The hinge B (B') region is rich in threonine–glycine residues and is heavily O-glycosylated.

Analogously, a core protein (core 38 kDa) arising via natural proteolysis of endoglucanase III (50 kDa) shows complete activity on soluble sub-

![Fig. 2. Model of Trichoderma reesei Cellobiohydrolase 1 based on small angle X-ray scattering. The proposed domains are: A = binding, B = hinge and C = catalytic core domains. Reproduced from Schmuck et al. (1986) with permission.](image-url)
strates but markedly reduced activity and adsorption on microcrystalline cellulose (Stahlberg et al., 1988). Analysis of the activities of the peptide products arising from partial proteolysis of CBHI and CBHII of *Penicillium pinophilum*, provides evidence for similar catalytic and binding domains in other fungi (Claeyssens & Tomme, 1989).

These studies also clarify the mode of synergism between *T. reesei* CBHI and CBHII. The synergism found between the CBHI core and CBHII or CBHII core, support the hypothesis that this is based on the adsorptive capacity of CBHI in yielding new sites for attack by CBHII (and not hydrolytic activity *per se*) (Claeyssens & Tomme, 1989).

**Mechanisms**

Lysozyme-like mechanisms have long been postulated for cellulases (Vernon & Banks, 1963), but to date supportive evidence is intriguing but quite meagre (Yaguchi et al., 1983; Paice et al., 1984). Modification of cellulases and their study in the presence or absence of substrate and/or inhibitor have provided some evidence regarding the involvement of carboxyl groups in the active site. Furthermore, using N-bromosuccinimide as the modifying carboiimide, it has been deduced (Clarke & Yaguchi, 1985; Clarke, 1987) that two tryptophan residues in the proposed active cleft of *Schizophyllum commune* endoglucanase are involved in catalysis, one residue binding to the
substrate while the other forms part of the active site. Tomme and Claeyssens (1989) using Woodward's K reagent (N-ethyl-5-fenyl-isoxazolium-3'-sulfonate) specifically modified T. reesei CBHI. With evidence from isolation of a catalytic peptide, they postulated the involvement of glutamic acid 126 (as a proton donor) in the catalytic site of CBHI, this being equivalent to glutamic acid 35 of hen egg white lysozyme (HEWL). Glu-127 was proposed as the equivalent active residue of EGI.

Site directed mutagenic modification of the proposed active site is also being used to resolve which amino acids are directly involved in hydrolysis. Knowles' group postulated that glutamyl 244 of T. reesei CBHIII is potentially in the active site by analogy to the lysozyme model. However, Shoemaker (1988) replaced this residue with glutamine and as the enzyme was still active, surmised that the 244 glutamyl residue is not in the active site. Shoemaker's group proposed (Shoemaker, 1988) that one of the two aspartyl residues (173 or 175) of CBHII is active in catalysis, and proved that one of them is involved, as replacing both of them with asparagine residues resulted in a protein enzyme inactive towards swollen cellulose.

**SUMMARY**

The understanding of fungal cellulases has advanced considerably over the last decade through a combination of biochemical and molecular biological approaches. The hydrolytic mechanism of certain exo- and endoglucanases appears similar to that of lysozyme, with formation of a carbonyl ion intermediate stabilized through an ionized aspartate carboxylate ion, followed by general acid catalyzed removal of the alcohol by the unionized glutamic carboxyl. Cloning has defined that there are relatively few fungal cellulase genes per host and thus emphasizes alternate origins for the multiple, heterogeneous cellulase components. It also allows study of individual gene products without the background interference of other cellulase components. The definition of discrete binding, hinge and active site domains is a major conceptual advance and allows more rational insights into cellulolysis including clarifying the mechanism and importance of enzyme adsorption to the substrate and perhaps to deciphering the mechanisms of de-crystallization. Finally, cellulolytic yeast, obtained through recombinant DNA means, appear as well defined, useful microbes which will find application in the beverage and food industry.

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