Fungal cellulase systems

Comparison of the specificities of the cellobiohydrolases isolated from *Penicillium pinophilum* and *Trichoderma reesei*

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Reaction patterns for the hydrolysis of chromophoric glycosides from cello-oligosaccharides and lactose by the cellobiohydrolases (CBH I and CBH II) purified from *Trichoderma reesei* and *Penicillium pinophilum* were determined. They coincide with those found for the parent unsubstituted sugars. CBH I enzyme from both organisms attacks these substrates in a random manner. Turnover numbers are, however, low and do not increase appreciably as a function of the degree of polymerization of the substrates. The active-site topology of the CBH I from *T. reesei* was further probed by equilibrium binding experiments with cellobiose, cellotriose, lactose and some of their derivatives. These point to a single interaction site (ABC), spatially restricted as deduced from the apparent independency of the thermodynamic parameters. It appears that the putative subsite A can accommodate a galactopyranosyl or glucopyranosyl group, and subsite B a glucopyranosyl group, whereas in subsite C either a glucopyranosyl or a chromophoric group can be bound, scission occurring between subsites B and C. The apparent kinetic parameters (turnover numbers) for the hydrolysis of cello-oligosaccharides (and their derivatives) by the CBH II type enzyme increase as a function of chain length, indicative of an extended binding site (A–F). Its architecture allows for specific binding of β -(1→4)-glucopyranosyl groups in subsites A, B and C. Binding of a chromophore in subsite C produces a non-hydrolysable complex. The thermodynamic interaction parameters of some ligands common to both type of enzyme were compared: these substantiate the conclusions reached above.

INTRODUCTION

The presence of two immunologically unrelated cellobiohydrolases (CBH I and II, EC 3.2.1.91) in extracellular culture fluids of both *Trichoderma* sp. (Gritzali & Brown, 1979; Fagerstam & Pettersson, 1979; Montencourt, 1983) and *Penicillium pinophilum* (Wood & McCrae, 1980, 1982) has been well established. These enzymes are similar in their molecular masses (approx. 50 kDa), their pI values (acidic range) and their capacities for acting synergistically with reconstituted mixtures of endoglucanases in solubilizing microcrystalline cellulose (Henrissat *et al.*, 1985; Wood & McCrae, 1986). Furthermore, CBH I and CBH II from both species have been shown to act synergistically (Fagerstam & Pettersson, 1979; Wood & McCrae, 1986). The substrate specificities of CBH I and II have been studied mostly in relation to their capacities to catalyse the hydrolysis of soluble and insoluble cellulose derivatives (Enari & Niku-Paavola, 1987). However, the chromophoric (fluorophoric) substrates described in the present study proved to be useful in earlier studies of CBH I, CBH II and endocellulases from *Trichoderma reesei* (van Tilbeurgh & Claeysens, 1985), and to have some efficacy as probes of the active sites of the enzymes (van Tilbeurgh *et al.*,

1988). Thus these substrates have been used to provide information on structure–activity relationships of the component enzymes of the cellulase complex and in the differentiation of two domains (i.e. catalytic and binding) in CBH I and CBH II from *T. reesei* (van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988). Successful crystallization of the ‘core enzymes’ containing the active-site domains from *Trichoderma* CBH I and CBH II augurs well for the release of detailed structural information of these enzymes in the not too distant future (A. Jones, personal communication).

The present paper describes the parameters affecting enzymic activity on some substrates in a comparative study of the cellobiohydrolases purified from *T. reesei* and *P. pinophilum*. More detailed information on the active-site topology of CBH I (*T. reesei*) was obtained and is compared with the data collected in a previous study of CBH II (van Tilbeurgh *et al.*, 1985).

EXPERIMENTAL

Purification of the enzymes

The CBH I and CBH II from *Trichoderma reesei* were purified by affinity chromatography as reported by van Tilbeurgh *et al.* (1984). By this procedure mixtures of

Abbreviations used: CBH, cellobiohydrolase; EG, endoglucanase; MeUmb(Glc)_n ($n = 1-5$), 4-methylumbelliferyl β -D-glycosides from glucose, cellobiose, cellotriose, cellotetraose and cellopentaose; MeUmb, 4-methylumbelliferone; MeUmbLac, 4-methylumbelliferyl β -D-glycoside from lactose; N₂PhSC, 2',4'-dinitrophenyl 1-thio- β -D-cellobioside; CNPhLac, 2'-chloro-4'-nitrophenyl β -D-lactoside.

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iso-components were obtained that are enzymically indistinguishable from the purified main fraction (pI 3.9 for CBH I and pI 5.9 for CBH II). The molar absorption coefficients (280 nm) are $73\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for CBH I and $75\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for CBH II (Tomme *et al.*, 1988).

The cellobiohydrolases from *Penicillium pinophilum* were purified as described by Wood & McCrae (1980, 1982) or alternatively by affinity chromatography (van Tilbeurgh *et al.*, 1984). Properties of these enzymes are as reported previously (Wood, 1988).

Substrates and ligands

Cellobiose and lactose were commercial products. Cello-oligosaccharides were prepared from cellulose (Whatman CC41) acetolysates and fractionation of the deacetylated mixtures by gel filtration (Bio-Gel P-2) with water as eluent (65 °C). Reducing-end-labelled ($1\text{-}^3\text{H}$) sugars were fractionated from a cello-oligosaccharide mixture, prepared at the U.V.V.R. (Prague, Czechoslovakia, courtesy of Dr. Peter Biely of Bratislava) by preparative h.p.l.c. (see below). The specific radioactivities were 10.5 GBq/mmol and samples were diluted with non-labelled sugars as appropriate. 4-Methylumbelliferyl, 2'-chloro-4'-nitrophenyl and 2',4'-dinitrophenyl 1-thioglycosides of cello-oligosaccharides and lactose were prepared as described previously (van Tilbeurgh *et al.*, 1988).

Enzyme assays

The enzymic hydrolysis of the chromophoric substrates was followed by h.p.l.c. (van Tilbeurgh *et al.*, 1982). A $25\text{ cm}\times 0.46\text{ cm}$ RSil-Polyol $5\text{ }\mu\text{m}$ -particle-size column (Alltech) and a Waters chromatographic system were used [eluent acetonitrile/water (39:11, v/v) at 1.5 ml/min]. Samples ($20\text{ }\mu\text{l}$) were injected (Valco) from a reaction mixture (in 50 mM -sodium acetate/acetic acid buffer, pH 5.0, at $25\text{ }^{\circ}\text{C}$) diluted 1:3 in acetonitrile. The concentration of chromophoric reaction products was determined at 313 nm with a Waters 440 detector (peak heights). The unmodified cello-oligosaccharides were also analysed by h.p.l.c. [same column, eluent acetonitrile/water 3:2 (v/v)] and monitored with a Waters 400 refractive-index detector. Radioactive fractions ($50\text{--}100\text{ }\mu\text{l}$) were collected and their radioactivities counted conventionally (Tri-Carb spectrometer) after addition of a scintillation fluid (4 ml of Aqualuma; Lumac).

With $\text{MeUmb}(\text{Glc})_2$ and MeUmbLac as substrate enzymic activities were monitored continuously by recording the liberated MeUmb at 347 nm (Perkin-Elmer B55 spectrophotometer) or discontinuously by fluorimetry at pH 10 (Vitatron photometer, excitation at 366 nm and emission at $>400\text{ nm}$).

The 2'-chloro-4'-nitrophenyl derivatives were used in a continuous assay (405 nm) at pH 5.7 (50 mM -sodium acetate buffer), and the increase in concentration of reaction product (2-chloro-4-nitrophenol) as a function of time was calculated from the molar absorption coefficient under these conditions ($9500\text{ M}^{-1}\cdot\text{cm}^{-1}$).

When cellobiose or one of the higher cello-oligosaccharides was used as substrate, glucose was determined by the glucose oxidase/peroxidase method (Bergmeyer, 1974).

Binding experiments

Equilibrium dialysis was performed by forced-flow diafiltration, as described previously (Claeysens *et al.*, 1985).

Difference absorption spectra were obtained with a UVICON 810 double-beam spectrophotometer (Kontron) and tandem mixing cuvettes (Yankeelov, 1963). Titrations were performed with two $1\text{ cm}\times 1\text{ cm}\times 4.5\text{ cm}$ cuvettes, equipped with motor-driven stirrers and connected to two Hamilton syringes that deliver a solution of a non-binding sugar (sucrose) to the reference cuvette and a solution of the appropriate ligand to the sample cuvette (De Boeck *et al.*, 1984). The cuvettes were first filled with buffer and then the ligands were added. The resulting difference spectrum was stored. The cuvettes were then filled with enzyme solution and the net difference spectra recorded after the addition of the ligands. Difference absorption titrations were recorded at the maxima of the difference spectra. The results were treated as described previously (De Boeck *et al.*, 1984).

RESULTS

Specificity of cellobiohydrolases type I

H.p.l.c. analysis (van Tilbeurgh *et al.*, 1982) of the enzymic hydrolysis of the MeUmbLac and $\text{MeUmb}(\text{Glc})_n$ by CBH I (*T. reesei* and *P. pinophilum*) produces activity patterns as shown in Fig. 1(a). Relevant kinetic data (turnover numbers, i.e. catalytic-centre activities) are also indicated. These are only apparent values for those substrates that yield more than one product. The similarities observed for both CBH I enzymes, isolated from two different organisms, are striking.

Only in the case of the first two homologues, $\text{MeUmb}(\text{Glc})_2$ and $\text{MeUmb}(\text{Glc})_3$, are specificities as expected for a cellobiohydrolase. However, with some CBH I samples appreciable hydrolysis at the bond between the glucosidic moieties of $\text{MeUmb}(\text{Glc})_2$ was also observed. This activity was almost completely absent for enzyme samples further purified by affinity chromatography (van Tilbeurgh *et al.*, 1984), suggesting that a contaminating endocellulose (van Tilbeurgh *et al.*, 1982) or exoglucanase (Wood & McCrae, 1982) was present.

More complex degradation patterns were obvious for the higher homologues, $\text{MeUmb}(\text{Glc})_n$. Thus from the tetraoside MeUmb , MeUmbGlc and $\text{MeUmb}(\text{Glc})_2$ were released in early attack with a linear formation rate of the first two reaction products. Initial reaction velocities were independent and linear in the substrate concentration range tested ($10\text{--}2000\text{ }\mu\text{M}$). The inhibitory effects of cellobiose and lactose on each reaction product formation were similar, which is preliminary evidence for a single active site.

Hydrolysis of $\text{MeUmb}(\text{Glc})_5$ is as complex as the hydrolysis of the tetraoside. The release of each reaction product was linear from the start, and no substrate concentration ($20\text{--}300\text{ }\mu\text{M}$)-dependency of the reaction velocities could be demonstrated. The MeUmb released is probably a secondary reaction product of the $\text{MeUmb}(\text{Glc})_2$ hydrolysis.

Deviant specificity could also be demonstrated with cello-oligosaccharides such as cellotetraose, which yielded significant amounts of glucose (glucose oxidase/peroxidase reagent). As the position of the cleaved bond cannot be unambiguously assigned by using normal cello-oligosaccharides, reducing-end-labelled derivatives were used. This is illustrated here for the cellopentaose (Fig. 2). The positional and kinetic similarities in the

Fig. 1. Degradation patterns of cello-oligosaccharides and their chromophoric glycosides by (a) CBH I and (b) CBH II from *T. reesei* and *P. pinophilum*

Mixtures of substrates (30–500 μM) and CBH I (0.1–1 μM) or CBH II (0.05–0.5 μM) were incubated (at pH 5.0 at 25 °C) and analysed (by h.p.l.c.). Arrows indicate bonds hydrolysed as deduced from the chromophoric or radioactive reaction products observed. The numbers corresponding to the turnover values (min^{-1}) were calculated from the linear rates of the reactions monitored (5–30 min) as described in the Experimental section. The values in parentheses are those found for the *P. pinophilum* enzymes (*, turnover numbers not determined). Symbols: Δ , β -(1 $\rightarrow$ 4)-galactopyranosyl; $\square$, β -(1 $\rightarrow$ 4)-glucopyranosyl; ∇ , reducing-end glucopyranosyl; $\circ$, 4-methylumbelliferyl group. Letters A–F refer to the putative binding sites on the CBH I.

Fig. 2. Time course of hydrolysis of [1- ^3H]cellopentaose by CBH I from *T. reesei*

[1- ^3H]Cellopentaose (5 mM; 0.1 GBq/mol) (at pH 5.0 at 25 °C) was incubated with CBH I (0.1 μM), and samples (10 μl) were removed at times given and diluted (1:4) in acetonitrile/water (4:1, v/v) containing a mixture of unlabelled cello-oligosaccharides (degrees of polymerization 1–5, 1 mM each) as reference. After injection (20 μl) on to the h.p.l.c. column (see the Experimental section), fractions (approx. 0.05–0.5 ml) corresponding to each oligosaccharide (refractive-index detection) were collected and their radioactivities were determined (by liquid-scintillation counting).

degradation of the cello-dextrins compared with their chromophoric derivatives provide evidence for equivalencies in binding of glucosyl moieties, as shown (Fig. 1a). The phenolic residue in MeUmb(Glc)₂ then seems to occupy subsite C, which is, in the case of cellotriose, filled by the reducing-end glucosyl residue.

With MeUmbLac and MeUmb(Glc)₂ as substrates, normal Michaelis–Menten kinetics for the release of MeUmb were found (Table 1). The K_m value of the lactoside was about 10 times higher than that for the cellobioside. The catalytic efficiencies of the enzyme for both types of substrates were nearly identical; hydrolysis of cellotriose was, however, much less efficient.

The 2'-chloro-4'-nitrophenyl derivatives are useful substrates for continuous assays, and the kinetic parameters for the lactoside are given in Table 1. These compounds are also hydrolysed by some endoglucanases isolated from *T. reesei* (Claeysens, 1988) and *P. pinophilum* (K. Bhat, T. M. Wood & M. Claeysens, unpublished work), and for comparative purposes the kinetic parameters for endoglucanase I from *T. reesei* are included.

Specificity of cellobiohydrolases type II

The specificity of CBH II from *T. reesei* for the hydrolysis of MeUmb glycosides derived from the cello-oligosaccharides was described in a previous study (van

Tilbeurgh *et al.*, 1985). The data presented here (Fig. 1b) update these results and compare the specificities of the two fungal type II cellobiohydrolases. The formation of MeUmb was never observed, and the binding scheme proposed seems to be valid for both enzymes, namely that three contiguous sites occupied by glucosyl moieties are a prerequisite for hydrolysis at the bond located penultimate to the non-reducing end.

For the unmodified cellobiose and cellopentaose the bonds cleaved were determined by using the sugars radioactively labelled at the reducing end, as reported above for CBH I. Turnover numbers for MeUmb(Glc)₃ and cellobiose were identical, proving that the presence of the MeUmb group does not appear to influence the overall reaction rate. In contrast with CBH I, the influence of the aglycon on the association constants is, however, important (Table 2), indicating that the aromatic group is strongly implicated in the interaction of the chromophoric ligands and substrates with the CBH II. The concomitant quenching of the fluorescence was used to determine the association constants and thermodynamic binding parameters of some of these ligands (van Tilbeurgh *et al.*, 1985).

Table 1. Comparison of kinetic constants for some substrates of CBH I and EG I from *T. reesei*

Activity measurements were performed as described in the Experimental section (at pH 5.0 at 25 °C). Abbreviation: N.D., not determined.

Substrate	CBH I		EG I	
	K_m (μM)	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)
MeUmbLac	260	6.1×10^4	1.4	3.2×10^5
MeUmb(Glc) ₂	20	4.5×10^4	1.8	3.0×10^5
CNPhLac*	504	2.2×10^4	N.D.	N.D.
Cellobiose†	184	4.3×10^3	2.4	2.3×10^5

* At pH 5.7 (at 25 °C).

† Glucose determined.

Table 2. Thermodynamic parameters for binding of some chromophoric and non-chromophoric ligands on to CBH I and CBH II from *T. reesei* (at pH 5.0)

Association constants were determined either by ^adiafiltration (Claeysens *et al.*, 1985), ^bligand difference spectrophotometry, ^cprotein difference spectrophotometry, ^dligand fluorescence quenching titrations (van Tilbeurgh *et al.*, 1985) or ^edisplacement titrations of ligand-fluorescence quenching (van Tilbeurgh *et al.*, 1985).

Ligand	K (at 25 °C) (M^{-1})	ΔG^0 (at 25 °C) ($\text{kJ} \cdot \text{mol}^{-1}$)	ΔH^0 ($\text{kJ} \cdot \text{mol}^{-1}$)	ΔS^0 ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)
(a) CBH I				
N ₂ PhS(Glc) ₂	$(6.1 \pm 0.4) \times 10^{4\text{ab}}$	-27.0 ± 0.1	-58 ± 1	-103 ± 4
Cellobiose	$(5.4 \pm 0.7) \times 10^{4\text{c}}$	-26.7 ± 0.5	-47 ± 8	-68 ± 28
Cellobiose	$(8.0 \pm 0.2) \times 10^{4\text{c}}$	-27.9 ± 0.4	-33 ± 9	-17 ± 12
Lactose	$(3.2 \pm 0.1) \times 10^{3\text{c}}$	-20.0 ± 0.6	-53 ± 6	-110 ± 55
(b) CBH II				
MeUmb(Glc) ₂	$(2.00 \pm 0.03) \times 10^{5\text{d}}$	-30.40 ± 0.05	-11.6 ± 1.7	63 ± 6
Cellobiose	$(5.40 \pm 0.05) \times 10^{2\text{e}}$	-15.60 ± 0.04	-28.3 ± 3.3	-42 ± 13
Cellobiose	$(6.2 \pm 0.1) \times 10^4$	-27.40 ± 0.04	-34 ± 2	-24 ± 7

Fig. 3. Ligand difference absorption spectra for N₂PhSC and N₂PhSLac binding to CBH I from *T. reesei*

Spectra were measured (at 15.8 °C) in tandem mixing cuvettes (Yankeelov, 1963). (a) 39 μM -N₂PhSC and 50 μM -CBH I: spectrum a, baseline (before mixing); spectrum b, spectrum after mixing of sample cuvette; spectrum c, after mixing of both sample and reference cuvettes; spectrum d, after adding saturating amounts of cellobiose (0.1 M) to the reference cuvette. (b) 32 μM -N₂PhSLac and 50 μM -CBH I: spectrum a, baseline; spectrum b, after mixing of sample cuvette; spectrum c after mixing sample and reference cuvettes; spectrum d, after the addition of cellobiose (0.1 M) to the reference cuvette.

Fig. 4. Protein difference spectrum for the binding of cellobiose (a) and lactose (b) to CBH I from *T. reesei*

Conditions were as indicated in Fig. 3 legend. (a) Spectrum a, baseline recorded with 0.72 mM-cellobiose in the sample cuvette and 0.72 mM-sucrose in the reference cuvette; spectrum b, difference spectrum recorded after the addition of CBH I ($9.3 \mu\text{M}$) to both cuvettes. (b) Spectrum a, baseline recorded with 7.2 mM-lactose in the sample cuvette and 7.2 mM-sucrose in the reference cuvette; spectrum b, difference spectrum recorded after the addition of CBH I ($9.3 \mu\text{M}$) to both cuvettes.

Thermodynamics of the binding of small ligands

As in the case of CBH II (van Tilbeurgh *et al.*, 1985), either chromophoric ligands were tested as indicator ligands in binding studies of CBH I, or unmodified cello-oligosaccharides were used as perturbants of the protein spectrum.

The determination of the number of sites of interaction for chromophoric ligands such as 2',4'-dinitrophenyl 1-thio-D-cellobioside was carried out by forced-flow diafiltration, as described for the parent lactoside

Fig. 5. Titration of the difference absorption spectrum of CBH I and cellobiose

Conditions were as described in the Experimental section. Both cuvettes contained 2.141 ml of CBH I solution ($12.4 \mu\text{M}$), and 0–125 μl of cellobiose (5 mM) or 0–125 μl of sucrose (5 mM) was added to the sample and reference cuvette respectively (at pH 5.0 at 29.4°C). The sum of the changes in absorption at 290 nm and 294 nm (A) and the free ligand concentration ($[L]$) were used in the calculations (De Boeck *et al.*, 1984). The linearized titration curve is shown. From the slope $K = (2.7 \pm 0.1) \times 10^4 \text{ M}^{-1}$ is computed. The intercept on the ΔA axis corresponds to $\Delta A_{\text{max.}} = (4.67 \pm 0.2) \times 10^{-3}$.

(van Tilbeurgh *et al.*, 1982): an association constant $K = 6.1 \times 10^4 \pm 0.4 \times 10^4 \text{ M}^{-1}$ (at 25°C) for 1.2 ± 0.05 binding sites was obtained. These results correspond to the findings by Aluralde & Ellenrieder (1985), who report a single binding site for cellobiose on CBH I ($K = 2.3 \times 10^4 \text{ M}^{-1}$, at 30°C).

U.v.-absorption spectra of the same chromophoric compounds show distinct shifts on binding (Fig. 3), which could suggest a different micro-environment for the two ligands (cellobioside and lactoside). Both difference spectra, however, disappear after the addition of either cellobiose or lactose.

Binding of non-chromophoric ligands to CBH I produces very similar protein difference spectra (Fig. 4). In all cases maxima were observed at 285 nm and 290 nm and a minimum at 294 nm.

Association constants for cellobiose were obtained by titration of the CBH I difference spectra at 290 and 294 nm (Fig. 5); cellotriose binding was measured at 291 nm (results not shown). Hydrolysis of this ligand could be neglected under the conditions used. The temperature-dependence of these constants for both ligands is shown in Fig. 6. Values include association constants found by kinetic measurements [cellobiose as competitive inhibitor of MeUmb(Glc)₂] or by equilibrium binding experiments as described here or as reported in the literature (Aluralde & Ellenrieder, 1985). Thermodynamic parameters for these cello-oligosaccharides and lactose are listed in Table 2. Binding parameters were determined for the latter ligand by titration of difference spectra at 285 nm and 291 nm (results not shown). Titrations of cellotetraose and cellopentaose did not allow linearization according to a simple binding model, probably owing to complicating hydrolysis reactions. However, titrations at lower temperature indicate tentatively that the association constants do not appreciably increase as a function of the chain length in this oligosaccharide series.

Fig. 6. van't Hoff plots for the binding of (a) cellobiose and (b) cellobiose to CBH I from *T. reesei*

For (a) the values of K were obtained by (1) protein difference absorption titrations as in Fig. 5 (●) and (2) steady-state measurements with cellobiose as competitive inhibitor of the hydrolysis of MeUmb(Glc)₂ or CNPhLac (▲). For (b) the K values were obtained by protein difference absorption titrations (not shown).

DISCUSSION

The chromophoric derivatives of the cello-oligosaccharides and lactose are sensitive tools in the comparative studies of the cellobiohydrolases and endocellulases of fungal and bacterial origin (Claeysens, 1988). In the present study a clear differentiation between the CBH I and CBH II enzymes, isolated from either *T. reesei* or *P. pinophilum*, was obtained with these chromophoric substrates (Figs. 1a and 1b).

The specificities of the CBH I enzymes are identical for both organisms and the kinetic constants are very similar. The same conclusion is valid for CBH II enzymes. Thus both types of CBH seem to be functionally conserved, and this is supported by strong evidence resulting from structural studies on the enzymes from both sources (M. Claeysens, unpublished work).

As demonstrated in previous work (Tomme *et al.*, 1988), CBH I and CBH II (*T. reesei*) are both strongly adsorbed on insoluble cellulose, but from the present study important differences in substrate specificities become evident. As also reported before (van Tilbeurgh *et al.*, 1985), the specificity of the CBH II is more strict,

since the presence of three or four contiguous β -(1→4)-linked glucosyl residues is required and no cleavage at the heterosidic bonds is observed. The use of reducing end-labelled (radioactive) cello-oligosaccharide made it possible to establish that a similarity existed in the degradation pattern of chromophoric substrates (Fig. 1b). If the binding model presented in our earlier study (van Tilbeurgh *et al.*, 1985) is still valid, subsites A, B and C are specific for glucosyl residues, whereas the aglycone group binds in subsite D [e.g. for MeUmb(Glc)₃]. The hydrolytic cleavage site is situated between subsites B and C. Subsite C can also accommodate a chromophoric group, but in this case no hydrolysis occurs [e.g. for MeUmb(Glc)₂]. The apparent kinetic parameters show low and almost constant values for K_m and increasing turnover numbers for substrates of higher degree of polymerization. The absolute values exceed those found in the case of CBH I, and in this respect CBH II is similar to some endocellulases. Thus the mode of action on insoluble substrates differs from action on soluble substrates.

The CBH I enzymes hydrolyse both cellobiosides and lactosides and attack at sites other than the non-reducing-end cellobiosyl residues in the higher homologues [MeUmb(Glc)_n, $n > 3$]. That this is not an artifact (due to the presence of chromophoric groups) is proven by the fact that the unsubstituted cello-oligosaccharides show similar degradation patterns and kinetic parameters (Fig. 1a).

The influence of the chain length on the kinetic parameters of the substrates shows no definite trend. As the K_m values are experimentally not measurable ($< 10 \mu\text{M}$) the apparent turnover numbers are indicative of the catalytic efficiency. In all cases where they were determined it appears that these numbers are very low when compared with other enzymes such as endoglucanase, e.g. EG I from *T. reesei* (Table 1). EG I and CBH I from *T. reesei* show a strong amino acid sequence homology (Bhikhabhai & Pettersson, 1984) and share, together with other components from the cellulase complex, a conserved C-terminal peptide (Knowles *et al.*, 1988). The data presented here suggest that the enzymes also resemble each other in specificity, and thus probably the homology extends to the active site of the enzymes. In its action on native cellulose, however, CBH I operates in a different manner from EG I, and this is almost certainly due to the presence of the C-terminal peptide, which functions as a substrate-recognition domain in CBH I (Tomme *et al.*, 1988), but whose function in EG I has still to be established.

The active-site topology of CBH I was further studied by equilibrium binding experiments. The data suggest that a single binding site exists for lactosides and cellobiosides, and this was supported by the identical protein difference spectra obtained with lactose and cellobiose (Fig. 4) respectively. This specific binding site is spatially restricted, as indicated by the apparent independency of the thermodynamic constants from the degree of polymerization of the cello-oligosaccharides. It is probable, however, that lactosides bind in a slightly different conformation, since specific parameters (Table 1) and distinct ligand difference spectra (Fig. 3) were found for 1-thio analogues of these substrates.

The interaction constants and thermodynamic parameters for the binding of some ligands common to CBH I and CBH II (*T. reesei*) are compared in Table 2.

Different active-site architecture and/or interaction modes can be deduced from these data, corroborating the conclusions reached above.

For the binding of lactose to CBH I the entropy contribution dramatically decreases compared with cellobiose and cellotriose. This could indicate different conformations of the enzyme-ligand complexes, leading to a less stable intermediate for lactosides when bound in subsites ABC.

The high interaction energy of chromophoric cellobiosides to CBH II is particularly dominated by its entropy term. The binding mode in the non-productive complex must be totally different from that of a true substrate (e.g. cellotriose).

The binding constants of cellobiose differ widely for the two cellobiohydrolases, whereas those from cellotriose are comparable. The effects of this reaction product on the hydrolysis of native cellulose are typical and have indeed been shown to differ from both enzymes (van Tilbeurgh *et al.*, 1988).

In conclusion, it is clear that the specificities of CBH I and CBH II from both *Trichoderma* and *Penicillium*, classified as exocellobiohydrolases, can be better defined by the use of low-molecular-mass substrates and ligands. With the use of these substrates some information concerning the reaction mechanisms of the enzymes and the binding modes for small ligands emerged, but no answer to the question of the nature of the synergistic action of the enzymes on native cellulose was found. Therefore some hypotheses put forward (Wood & McCrae, 1986) may still be valid. Further work is required to establish more detailed data on the adsorption characteristics of these enzymes and the reaction mechanisms involved in solubilizing microcrystalline cellulose.

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