Mode of action of endoglucanase III from *Trichoderma reesei*

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Endoglucanase III (EG III) was purified to homogeneity from the culture medium of *Trichoderma reesei* QM 9414. It has a molecular mass of 48 kDa, and an isoelectric point of 5.1. Maximal activity was observed between pH 4 and 5. Cello-oligosaccharides and their chromophoric derivatives were used as substrates, and the reaction products were analysed by quantitative h.p.l.c. Nucleophilic competition experiments (between methanol and water) allowed unequivocal assessment of cleavage sites. EG III preferentially released cellobiose (or the corresponding glycoside) from the reducing end of the higher cellodextrins. A putative binding model containing five subsites is proposed. The pH-dependence of 4′-methylumbelliferyl β-cellotrioside hydrolysis indicates the presence of a protonated group with a pK 5.5 in the reaction mechanism, and the possible involvement of a carboxy group is corroborated by a temperature study ($\Delta H_{ion} = -15.9$ J/mol). This, together with independent evidence from affinity-labelling experiments [Tomme, Macarrón and Claeyssens (1991) Cellulose '91, New Orleans, Abstr. 32] and n.m.r. studies [Gebbler, Gilkes, Claeyssens, Wilson, Béguin, Wakarchuk, Kilburn, Miller, Warren and Withers (1992) J. Biol. Chem. **267**, 12559–12561], favours the assumption of a lysozyme-type (retention of configuration, two essential carboxy groups) mechanism for this family A cellulase.

INTRODUCTION

The cellulase system of *Trichoderma reesei* has been extensively studied (e.g. [1,2]). Its main components are two cellobiohydrolases (EC 3.2.1.91) (CBH I, CBH II), three endoglucanases (EC 3.2.1.4) (EG I, EG III and 'low-molecular-mass EG') and one β-D-glucosidase (EC 3.2.1.21). Usually, culture filtrates present more complicated compositions, mainly due to proteolysis [3,4], aggregation [5] and most probably other post-translational modifications of the gene products, such as deamidation or glycosylation.

The gene for EG III (named *egl3*) was cloned and sequenced some years ago [6]. At this time a purification method for the enzyme and a preliminary characterization were also reported. As for many other β-glycanases, two domains can be observed [7]: a catalytic core protein is linked by an *O*-glycosylated sequence to a cellulose-binding domain. The catalytic domain of EG III was isolated from the culture filtrate of *T. reesei* [3]. It was classified as a family A cellulase [8]. The cellulose-binding domain is typically conserved in all *T. reesei* cellulases and in EG III it is located in the N-terminal region as in CBH II [9].

Extensive information on the substrate specificity and action modes of the other principal components of the *T. reesei* cellulase system has been obtained in previous investigations [10,11]. The present study intends to enlarge our knowledge of EG III purified to apparent homogeneity by a new method. The data reported are interpreted in terms of a binding model, postulating five subsites and a lysozyme-like catalytic mechanism, involving two essential carboxy groups.

EXPERIMENTAL

Materials

DEAE-Sepharose CL-6B was from Pharmacia (Uppsala, Sweden). Ultrogel AcA-44 was purchased from LKB (Bromma, Sweden). CM-cellulose (medium viscosity, degree of substitution 0.8) was from Fluka (Buchs, Switzerland) and microcrystalline cellulose (Avicel PH 101) from FMC Corp. (Philadelphia, PA, U.S.A.). Chromophoric substrates were synthesized as previously described [12]. Cello-oligosaccharides were prepared conventionally [13]. Methyl β-glycosides were obtained by classical synthesis [14]. Purities of all compounds were checked by h.p.l.c. (see below) and t.l.c. (silica; ethyl acetate/acetic acid/water, 3:3:1 by vol.). Data obtained for their physical properties (melting points, specific rotations, proton n.m.r., chemical shifts and coupling constants) agree with published values [14,15].

All other reagents were analytical grade.

Enzyme purification

T. reesei QM 9414 was grown in wheat straw as previously described [16]. The culture supernatant was precipitated with $(NH_4)_2SO_4$ (20–60% saturation) at 4 °C. After centrifugation (12000 *g*, 15 min), the precipitate was desalted, lyophilized and loaded on to a DEAE-Sepharose CL-6B column (2.6 cm × 42 cm) equilibrated in 5 mM sodium phosphate, pH 7.0. Elution was carried out successively with 500 ml of this buffer, 250 ml of a linear NaCl gradient (0–500 mM) in the same buffer and 250 ml of buffer containing 500 mM NaCl. Flow rate was 60 ml/h and 6 ml fractions were collected and analysed for CM-cellulase and

Abbreviations used: CBH, cellobiohydrolase, 1,4-β-D-glucan cellobiohydrolase; EG, endoglucanase (1,4-β-D-glucan glucanohydrolase); IEF, isoelectric focusing; MeUmb, 4-methylumbelliferone; MeUmbGlc, 4′-methylumbelliferyl β-D-glucopyranoside; MeUmb(Glc)$_n$ (n = 2–5), 4′-methylumbelliferyl β-glycosides from cellobiose to cellopentaose; CNP, 2-chloro-4-nitrophenol; CNPGlc, 2′-chloro-4′-nitrophenyl β-D-glucopyranoside; CNP(Glc)$_3$, 2′-chloro-4′-nitrophenyl β-cellotrioside.

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Avicelase activities as described below. Protein concentration was monitored by absorbance readings at 280 nm.

Fractions containing EG III were pooled and concentrated (3 ml) by diafiltration (Diaflo PM-10 membrane; Amicon, Lexington, MA, U.S.A.) and applied to an Ultrogel AcA-44 column (1.6 cm × 95 cm) equilibrated with imidazole/HCl (8 mM, pH 6.5). Elution was at a rate of 10 ml/h and 2 ml fractions were collected. Molecular-mass standards used to calibrate the column were BSA (67 kDa), ovalbumin (45 kDa), soyabean trypsin inhibitor (20.1 kDa) and cytochrome *c* (12.3 kDa). Fractions containing CM-cellulose activity were pooled and applied to a DEAE-Sepharose CL-6B column (1.6 cm × 45 cm) equilibrated with imidazole/HCl (8 mM, pH 6.5). The column was washed with 50 ml of buffer before elution with a linear gradient of 0–300 mM NaCl (200 ml) in the equilibration buffer, at a flow rate of 30 ml/h. Fractions (3 ml) were collected and assayed for CM-cellulase activity.

All buffers contained 0.2 g/l sodium azide to avoid microbial contamination.

Analytical methods

Reducing sugars were determined with the 3,5-dinitrosalicylic acid reagent [17] using D-glucose as standard. Units of enzyme activity (i.u.) were expressed as μmol of reducing sugars released per min. During the enzyme purification, protein concentrations were determined by the method of Lowry et al. [18], with BSA (Sigma, St. Louis, MO, U.S.A.) as standard. The concentration of pure EG III was determined spectrophotometrically (molar absorption coefficient at 280 nm, 77000 $M^{-1} \cdot cm^{-1}$ [6]).

SDS/PAGE was performed as described by Laemmli [19] in an SE 600 vertical slab unit (Hoeffer, San Francisco, CA, U.S.A.) at 12.5% acrylamide concentration. Visualization was by Coomassie Blue staining.

Analytical isoelectric focusing (IEF) was performed using Ampholine PAG plates (pH range 3.5–9.5) from Pharmacia as described by the manufacturer. EG III active fractions were revealed by flooding the gel with MeUmb$(Glc)_3$ as described [6], before staining with Coomassie Blue.

The pH titration curve was obtained using Pharmacia Phast System precast IEF gels (pH range 3.5–9.5) as described by the manufacturer.

Activity on chromophoric substrates

Enzymic release of the chromophoric moiety [2-chloro-4-nitrophenol (CNP) or MeUmb] from CNP$(Glc)_3$ or MeUmb$(Glc)_3$ was measured spectrophotometrically or fluorimetrically as previously described [12]. MeUmb$(Glc)_3$ activity was routinely assayed by incubating EG III (50 nM) with the substrate (100 μM) in sodium acetate buffer (0.1 M, pH 5.0) at 25 °C for 10 min.

The hydrolysis of chromophoric substrates was also monitored by h.p.l.c. as described [20]. The assays were carried out at 25 °C and pH 5.0 (pyridine/acetic acid buffer, 10 mM) with 50 nM EG III. Substrate concentrations ranged between 10 and 500 μM. Values of kinetic constants were determined by fitting the experimental data to eqns. (A1) and (A2) (see the Appendix.) by the BMDP-AR program (BMDP Statistical Software, Los Angeles, CA, U.S.A.). The apparent kinetic parameters were determined by fitting theoretical values to eqns. (A3), (A4) and (A5).

In the temperature range 30–55 °C, pH-dependences of $k_{cat.}/K_m$ for the release of MeUmb from MeUmb$(Glc)_3$ were determined in buffers of different pH (4.5–6.3, 50 mM sodium acetate/50 mM sodium phosphate). The ionic strength was kept constant at 0.2 mol/l in all buffers, by addition of adequate amounts of NaCl. Values for specificity constants were determined by fitting initial-velocity data to eqn. (A7). pK_a and ΔH_{ion} values were obtained as described in [21].

Activity on cello-oligosaccharides

Enzymic hydrolysis of cello-oligosaccharides was monitored by h.p.l.c. (Waters, Eke, Belgium), using a Rsil Polyol (Bio-Rad, Richmond, CA, U.S.A.) column (0.46 cm × 25 cm) and acetonitrile/water (6:4, v/v) as mobile phase (flow rate 1 ml/min). Sugars were detected with a refractive-index detector (R 401, Waters). The assays were performed in 10 mM pyridine/acetic acid, pH 5.0, at 37 °C. Release of D-glucose from cellobiose and cellotriose was measured with the D-glucose oxidase/peroxidase reagent (Boehringer, Mannheim, Germany).

Activity on cellulose substrates

CM-cellulose activity was measured as previously described [16] except that 0.1 M sodium acetate, pH 5.0, was used as buffer. For the determination of kinetic constants, EG III (60 nM) was incubated at 30 °C with various concentrations of CM-cellulose (2–20 mg/ml) in the latter buffer. After 2, 4, 6, 8 and 10 min, samples were withdrawn and analysed for reducing sugar concentration. K_m and $k_{cat.}$ values were determined by fitting initial-velocity data to the Hanes equation [22].

Avicelase activity was assayed as described [23], except that the incubation temperature was 50 °C. Adsorption experiments were performed as described [24]. Reaction products from Avicel (10 mg/ml in 0.1 M sodium acetate, pH 5.0) obtained by EG III (8 μM)-catalysed hydrolysis at 37 °C were monitored by h.p.l.c. as described above. The samples withdrawn at different times were concentrated by lyophilization before injection.

RESULTS

Purification of endoglucanase III

The fractionation procedure and the resulting purification and yields are summarized in Table 1.

As reported earlier [25], β-D-glucosidase active fractions (not shown) are eluted in the flowthrough peak during the first chromatographic step on DEAE-Sepharose CL-6B. CM-cellulase fractions are eluted isocratically in two peaks (Figure 1a), one corresponding to a low-molecular-mass endoglucanase [26] and the other to the endoglucanase under study. The other cellulase components (CBH II, CBH I and EG I) are successively displaced by the NaCl gradient. The EG III fraction was clearly still heterogeneous (SDS/PAGE analysis not shown) and was further purified by gel filtration (Figure 1b, Table 1). The resulting preparation, homogeneous when analysed by SDS/PAGE, showed two bands when electrofocused (Figure 2a), both active against the chromophoric substrate MeUmb$(Glc)_3$ (Figure 2b). The higher-pI protein was identified as intact EG III, whereas the lower-pI component corresponds to the 'A1:2' fraction previously described [3] and probably represents a deamidated form of EG III. The two isoforms were separated by an additional DEAE-Sepharose CL-6B chromatography at pH 6.5 (Figure 1c). The resulting EG III preparation (purification factor approx. 13, yield 7% of total CM-cellulase activity in the culture supernatant) was homogeneous by SDS/PAGE and IEF/PAGE (Figure 2a).

Properties of the enzyme

Molecular mass as determined by gel filtration was 50 kDa,

Table 1 Purification of EG III

Fraction	Volume (ml)	Total protein (mg)	CM-cellulase activity: Total activity (units)	CM-cellulase activity: Specific activity (units/mg)	Activity yield (% of total)	Purification factor (fold)
Culture supernatant	750	645	17542	27.2	100	1.0
$(NH_4)_2SO_4$ precipitate	15	511	11093	21.7	63	0.8
DEAE-Sepharose, pH 7.0	126	8.5	2451	288	14	10.6
Ultrogel AcA-44	31	5.3	1800	340	10	12.5
DEAE-Sepharose, pH 6.5	29	3.7	1299	351	7	12.9

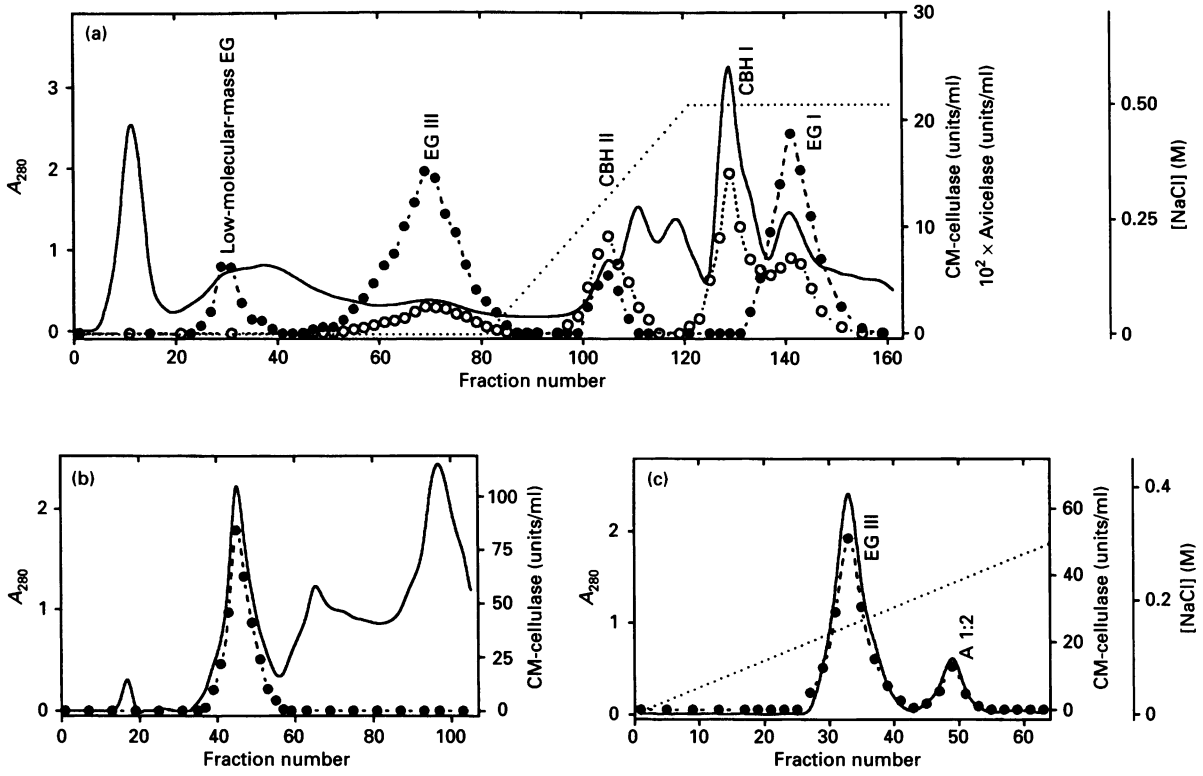


Figure 1 Purification of EG III

(**a**) DEAE-Sepharose CL-6B anion-exchange chromatography (pH 7.0) of $(NH_4)_2SO_4$-precipitated culture supernatant. (**b**) Gel filtration on Ultrogel AcA-44 of EG III fraction from the previous step. (**c**) DEAE-Sepharose anion-exchange chromatography (pH 6.5) of CM-cellulase-active fraction from gel filtration. Details of chromatographic runs and enzymic assays are described in the Materials and methods section. ——, A_{280}; (· · · ·), NaCl concentrations; ●, CM-cellulase activity; ○, Avicelase activity.

whereas SDS/PAGE yielded a value of 48 kDa. The latter value is identical with that previously reported [6]. The identity of this endoglucanase with the *egl3* gene product [6] was confirmed by peptide mapping with CNBr and sequencing of tryptic fragments (not shown). The isoelectric point of EG III was estimated as 5.1 and differs slightly from the value reported earlier [6]. EG III exhibits a low net charge at pH values above its pI, as can be observed in the pH titration curve (Figure 3).

EG III was stable in the pH range 3.0–8.0 at 30 °C, but only between pH 4.0 and 6.3 at 55 °C. At pH 5.0 the enzyme was inactivated at temperatures higher than 60 °C, although it retained 90 % of its activity when incubated for 30 min at 65 °C.

For MeUmb(Glc)$_3$, CNP(Glc)$_3$ and cellotriose, maximal activity was in the broad pH range 4.0–5.0. Only CM-cellulase activity showed a distinct behaviour, with a punctual maximum at pH 4.8, and this is probably due to the ionic nature of CM-cellulose.

Activity on chromophoric substrates

As pointed out in previous work [6,20], EG III is the only cellulase from *T. reesei* that hydrolyses MeUmb(Glc)$_3$ at the heterosidic bond, and the fluorescent phenol (MeUmb) liberated is particularly suited for sensitive assay. Alternatively, CNP(Glc)$_3$ can be used in a continuous colorimetric method since the chromophore released (CNP) exhibits a convenient pK_a value

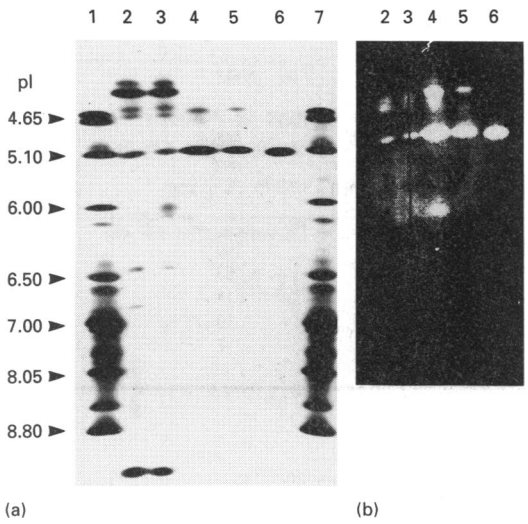


Figure 2 IEF of the preparations obtained at various stages of EG III purification

(**a**) IEF on polyacrylamide gel (pH 3.5–9.5, Coomassie Blue stained): lane 2, culture supernatant (50 μg); lane 3, $(NH_4)_2SO_4$ precipitate (50 μg); lane 4, EG III pool from DEAE-Sepharose at pH 7.0 (25 μg); lane 5, CM-cellulase pool from gel filtration (20 μg); lane 6, first CM-cellulase peak from DEAE-Sepharose at pH 6.5 (15 μg); lanes 1 and 7, isoelectric point standards. (**b**) Activity detected fluorimetrically on the IEF gel. Before staining, the gel was flooded with a MeUmb(Glc)$_3$ solution (5 mM, pH 5.0) and viewed under u.v. illumination [6]. Lane numbers as in (**a**).

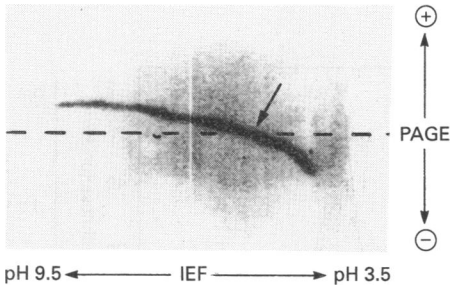


Figure 3 pH titration curve of pure EG III

The protein (5 μg) was loaded (broken line) on to a prefocused IEF gel (first dimension, pH 3.5–9.5) and subjected to electrophoresis (second dimension). The arrow indicates the pI of the enzyme. Activity [MeUmb(Glc)$_3$] (not shown) coincides with Coomassie Blue staining (shown).

(5.5) for activity measurements at slightly acidic or neutral pH [12].

In fact, h.p.l.c. analysis proves that EG III catalyses the hydrolysis of these chromophoric substrates at both the holosidic and heterosidic bonds (Figure 4). A possible analysis of the complex kinetics is proposed in the Appendix and the results of activity measurements by h.p.l.c. analysis are presented in Figure 4. The apparent kinetic constants indicate a preference for cleavage at the heterosidic bond in MeUmb(Glc)$_3$, in contrast with the CNP derivative where the holosidic bond is preferentially attacked. This could indicate different binding affinities (K_m) of these substrates for the enzyme's active (sub)site(s) (see below).

Mixtures of methyl β-glycosides and reducing sugars were obtained as reaction products for both chromophoric substrates in partition experiments using methanol (2.5 M) as nucleophile in competition with water. The ratios of methyl β-cellotrioside/cellotriose and methyl β-cellobioside/cellobiose were independent of the substrate used and constant: 0.42 ± 0.03. This is sufficient evidence for the presence of a common glycosyl intermediate [27]. Neither reaction rates nor K_m values for MeUmb(Glc)$_3$ hydrolysis were affected by the presence of the alcohol, whereas a twofold increase in CNP release and an unchanged rate of CNPGlc formation from CNP(Glc)$_3$ were observed. However, K_m values for both catalytic pathways remained constant. These findings are consistent with a difference in rate-limiting step [27], namely deglycosylation in the case of cleavage at the CNP bond and formation of the glycosyl intermediates in all other cases.

Some data concerning the EG III-catalysed hydrolysis of methylumbelliferyl derivatives of higher cello-oligosaccharides are included in Figure 4. Product inhibition undoubtedly influences the kinetic parameters of these substrates since the cello-oligosaccharides released are good substrates for the enzymes.

The hydrolysis patterns were not affected by changing the pH. The dependence of $k_{cat.}/K_m$ values for the formation of MeUmb from MeUmb(Glc)$_3$ in the range pH 4.5–6.3 was studied at different temperatures from 30 to 55 °C. In all cases the plot of log ($k_{cat.}/K_m$) against pH showed a similar shape (Figure 5a), with a decrease at the alkaline side. The delimiting slope was -1, indicating that a single group in the free enzyme must be protonated for catalysis. The pK_a of this group was determined as 5.46 ± 0.03 at 55 °C. The corresponding enthalpy of ionization was found to be -15.9 ± 2.6 J/mol (Figure 5b), pointing to the possible involvement of a carboxy residue [28].

Activity on cello-oligosaccharides

EG III-catalysed hydrolysis patterns of unsubstituted cello-oligosaccharides are illustrated in Figure 6. The higher sensitivity limit set by the detection method (refractive index) allowed only apparent rates of substrate depletion to be obtained at high initial substrate concentrations ($\geqslant 10$ mM). Approximate $k_{cat.}$ values are given (Figure 6). In the case of cellobiose and cellotriose, D-glucose could be determined enzymically (D-glucose oxidase/peroxidase) and more accurate parameters could be calculated (Figure 6).

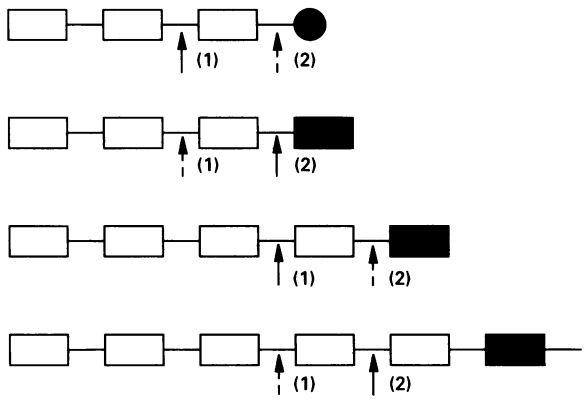
No evidence (h.p.l.c.) was obtained for transglycosidation (self-transfer with formation of higher oligomers) even after prolonged incubations at high substrate concentrations.

However, as with the chromophoric substrates, transfer reactions were observed when methanol was added to the reaction mixture of cellodextrins and EG III. The formation of methyl β-glycosides could unequivocably be demonstrated (h.p.l.c.), allowing unambiguous identification of cleavage sites. The hydrolysis rates were not accelerated in the presence of methanol, showing that glycosylation is the rate-limiting step in these reactions.

Cellobiose was slowly hydrolysed by EG III and a possible involvement of a contaminating β-D-glucosidase was discounted since specific inhibitors (e.g. δ-gluconolactone) had no effect. Whereas D-glucose concentrations up to 200 mM had no effect, 50 mM cellobiose reduced the hydrolysis rates of the chromophoric substrates MeUmb(Glc)$_3$ (100 μM) or CNP(Glc)$_3$ (500 μM) by 10 or 50 % respectively. No alterations in hydrolysis patterns were observed.

Activity on cellulose substrates

EG III showed a very high CM-cellulase activity (K_m = 5 mg/ml, $k_{cat.} = 2.2 \times 10^3$ min^{-1}, at pH 5.0, 30 °C). Nevertheless, this



	$k_{cat.}$ (min^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($min^{-1} \cdot M^{-1}$)	$(k_{cat.})_{app.}$ (min^{-1})	$(K_m)_{app.}$ (μM)	v_1/v_2
(1)	85	119	7.1×10^5	69	97	1.3
(2)*	286	521	5.5×10^5	53		
(1)	6	51	1.2×10^5	4.5	38	0.5
(2)	36	152	2.4×10^5	9		
(1)	n.d.	n.d.	8.8×10^5	n.d.	n.d.	3.5
						0.6

Figure 4 Hydrolysis of chromophoric substrates catalysed by EG III

Kinetic parameters (pH 5.0, 25 °C) were determined as described in the Materials and methods section. Errors were in all cases less than 10%. The filled circle symbolizes a CNP group. The D-glucosyl moieties and the 4-methylumbelliferyl groups are represented respectively by open and filled rectangles. The arrows indicate the bonds cleaved; dashed arrows indicate secondary hydrolysis sites. Relevant kinetic data are given in the right-hand columns. *The values reported for this pathway are not true $k_{cat.}$ and K_m values, since the rate-limiting step of the reaction is deglycosylation (see the Results section). n.d., not determined.

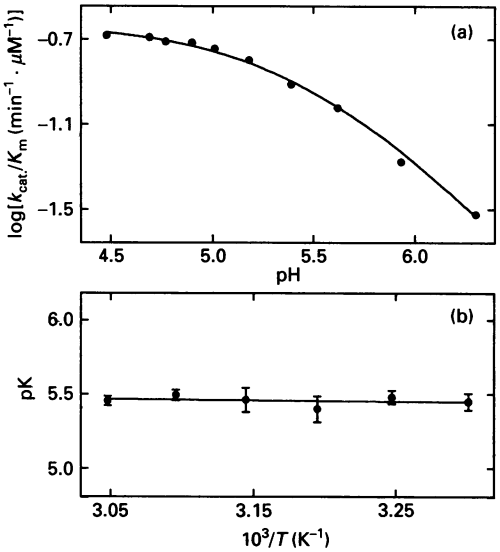


Figure 5 Effect of pH and temperature on $k_{cat.}/K_m$ for MeUmb(Glc)$_3$ hydrolysis

(**a**) pH variation of log ($k_{cat.}/K_m$) at 55 °C, (**b**) temperature-dependence of pK_a values from $k_{cat.}/K_m$ profiles. MeUmb produced by enzymic hydrolysis was measured fluorimetrically as described in the Materials and methods section.

	$k_{cat.}$ (min^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($min^{-1} \cdot M^{-1}$)	v_1/v_2
	2	20	1×10^2	-
(1) (2)	15	1	1.5×10^4	0.4
(1) (2)	800*	n.d.	-	1.8
	200*	n.d.	-	-
Me (1) (2)	500*	n.d.	-	0.9
Me (1) (2)	500*	n.d.	-	0.3

Figure 6 Hydrolysis of cello-oligosaccharides and their methyl β-glycosides catalysed by EG III

Kinetic parameters (pH 5.0, 37 °C) and hydrolysis patterns were determined as described in the Materials and methods section. Open rectangles (▭) represent D-glucosyl moieties, and crossed rectangles (⧄) reducing end residues. The arrows indicate the bonds cleaved; dashed arrows indicate secondary hydrolysis sites. Relevant kinetic data are given in the right-hand columns. *Substrate depletion rates at 10 mM substrate concentration. n.d., not determined.

substrate is not suitable for kinetic studies since the dilution curve (activity versus enzyme concentration) is non-linear.

Although the enzyme adsorbs strongly to Avicel (binding capacity = 0.2 μmol of protein/g of Avicel), this substrate is only slightly hydrolysed, probably at amorphous regions.

Only D-glucose and cellobiose were detected as products from Avicel hydrolysis. After 1 h of incubation at 37 °C, pH 5.0 (EG III 8 μM; Avicel 10 mg/ml), 0.1 mM D-glucose and 0.2 mM cellobiose were formed; the concentrations found after 22 h of incubation were 0.5 mM and 0.3 mM respectively. These sugars could be either primary reaction products or formed from longer cellodextrins released immediately.

DISCUSSION

Several purification procedures for *Trichoderma* cellulases have been described (e.g. [23,29,30]). The modified DEAE-Sepharose chromatographic steps presented in this study improve considerably the fractionation of the complex mixture and the isolation of pure EG III isoenzyme components. Incidentally, it is also shown that the composition of the cellulase system is independent of the carbon source used for fungal growth, as already suggested for the endoglucanase components [5]. Growth conditions and culture aging, however, could influence the formation of isocomponents or proteolysis products. Thus, under

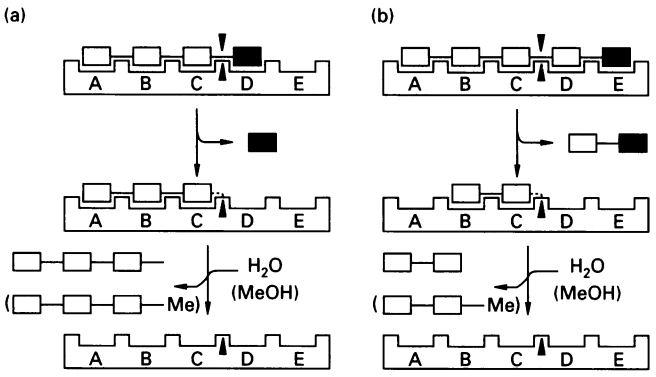


Figure 7 Schematic representation of the postulated model of EG III active centre and its binding subsites

The two hydrolysis pathways (**a** and **b**) for MeUmb(Glc)$_3$ are shown as examples. The possibility of methanol transfer is also indicated. The indentations in the block and the letters (A–E) represent the sugar-binding subsites; the arrowheads indicate catalytic groups of the enzyme. Open rectangles (□) represent D-glucosyl moieties and filled rectangles (■) represent 4-methylumbelliferyl groups.

the conditions described in this work, no evidence for EG III core protein formation was found, in contrast with the situation described earlier [3].

The final EG III preparation corresponding to the intact enzyme [6] is purified to apparent homogeneity and represents 0.5% of the initial protein content of the culture supernatant. Its particular behaviour on DEAE-Sepharose chromatography suggests that the protein is weakly charged at neutral pH values, as is demonstrated by pH titration (Figure 3). Consequently re-evaluation by isoelectric focusing of the published pI (5.6 in [6]) using longer equilibration times led to a corrected value of 5.1 (Figure 2a).

Study of the pH and temperature effects on MeUmb(Glc)$_3$ hydrolysis, catalysed by EG III, reveals the involvement of an essential carboxy residue (pK_a 5.5) in the active centre. As in hens'-egg white lysozyme Glu-35, this group must be protonated and assists in general acid-catalysed departure of the aglycone moiety of the substrate [31]. Although no definite proof has been obtained, this catalytic residue should be identified with Glu-218 present in the conserved Gln-Glu-Pro triad sequence found in almost every cellulase from family A. Site-directed mutagenesis experiments with three members of this family have confirmed the essential role played by this conserved glutamate and in all cases it was recognized as a potential proton donor [32,33]. Information about a putative basic group could not be found in this study since the enzyme was rapidly and irreversibly inactivated below pH 4.0. However, independent evidence resulting from affinity-labelling experiments [34] points to the involvement of a dissociated carboxy group corresponding to Asp-52 in hens'-egg white lysozyme [31]. Therefore a lysozyme-type mechanism can be proposed for EG III.

In contrast with lysozyme [35] and also with another endoglucanase (EG I) from *T. reesei* [11], EG III does not catalyse transfer reactions to other sugars or its own substrates (self-transfer). On the other hand, simple aliphatic alcohols do act as acceptors of glycosyl residues. As this is characteristic of many glycosidases operating with retention of configuration in the reaction products [31], one may wonder if this is also typical of all other cellulases from family A [8]. It has indeed recently been shown that EG III and several other members of this family operate by retaining configuration in the reaction products [36], adding new evidence to the classification proposed earlier [8].

Recent results from X-ray-diffraction analysis of crystallized cellulases from two other families (B and E) point to alternative reaction mechanisms: CBH II from *T. reesei* [37] and endoglucanase D [38] are inverting enzymes. In these enzymes also two active-site carboxy groups are operative and their roles must certainly be different from those present in the EG III active site. Thus no general cellulase mechanism can be advanced.

On the other hand, cellulases, as typical depolymerases, must have extended substrate-binding sites and this is consistently found in structures of both endo- [38] and exo- [37] cellulases. Although the non-carbohydrate aglycone of the chromogenic substrates may alter the subsite preferences of the saccharide moiety [39], the present results could indicate a subsite arrangement in the EG III active centre and a tentative model is proposed (Figure 7). In the five-subsite structure (ABCDE), catalytic residues could be situated between C and D. In the case of MeUmb(Glc)$_3$ the chromophore would complex in D, yielding the free phenol and cellotriose, or in E, resulting in the D-glucoside and cellobiose. Similar binding modes could explain the nature of the other reaction products formed. Partition experiments (water or methanol as acceptors) point to the fact that glycosylation is the rate-limiting step in these reactions, although with CNP(Glc)$_3$ deglycosylation becomes rate-limiting probably due to the presence of the activated phenolic group. This situation is similar to that encountered with, for example, *Escherichia coli* β-galactosidase [27].

In conclusion, the use of cello-oligosaccharides and their chromophoric and non-chromophoric β-glycosides as substrates of EG III from *T. reesei* allowed a detailed description of its specificity, and partition experiments and a pH study led to the proposal of a reaction mechanism. The enzyme probably contains an extended substrate-binding site and exhibits a double-displacement mechanism, distinguishing it from some other endocellulases and cellobiohydrolases.

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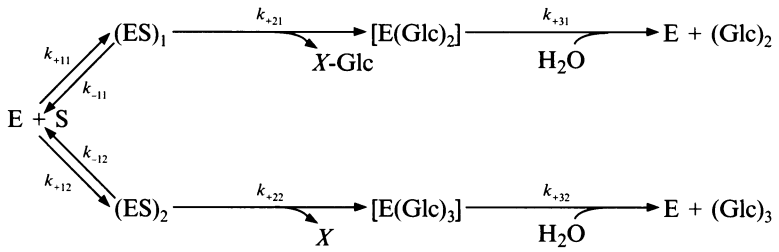
APPENDIX

Kinetic parameters for the hydrolysis of two substrates of EG III, MeUmb(Glc)$_3$ and CNP(Glc)$_3$, were determined assuming the Scheme A1, where X is either CNP or MeUmb when S is either CNP(Glc)$_3$ or MeUmb(Glc)$_3$, E is EG III, and k_{ij} (i = 1, 2 or 3, and j = 1 or 2) are the first-order rate constants for each reaction i and pathway j.



Scheme A1

This minimal mechanism is sufficient to explain the experimental data reported in this paper, but the nature of the proposed intermediates is unknown, and the possible existence of more steps is not discarded. If glycosylation is the rate-limiting step ($k_{+3j} > k_{+2j}$), it may be shown, by applying the steady-state approximation to the concentration of $(ES)_1$ and $(ES)_2$, that the rate of X-Glc formation, v_1, and X formation, v_2, are

$$v_1 = (k_{cat.1}[E]_0[S])/\{K_{m1} + [S](1 + K_{m1}/K_{m2})\} \quad (A1)$$

and

$$v_2 = (k_{cat.2}[E]_0[S])/\{K_{m2} + [S](1 + K_{m2}/K_{m1})\} \quad (A2)$$

where $k_{cat.j}$ is k_{+2j}, $[E]_0$ is the initial enzyme concentration, $K_{m1} = (k_{+21} + k_{-11})/k_{+11}$, and $K_{m2} = (k_{+22} + k_{-12})/k_{+12}$. Partitioning experiments reported in this paper showed that only in the case of CNP formation is deglycosylation slower than glycosylation, and thus the approximation is not valid.

The apparent reaction parameters, i.e. the parameters that can be determined from the experimental data considering each pathway individually and following Michaelis–Menten kinetics, are:

$$(k_{cat.1})_{app.} = k_{cat.1}/(1 + K_{m1}/K_{m2}) \quad (A3)$$

$$(k_{cat.2})_{app.} = k_{cat.2}/(1 + K_{m2}/K_{m1}) \quad (A4)$$

$$(K_{m1})_{app.} = (K_{m2})_{app.} = K_{m1}K_{m2}/(K_{m1} + K_{m2}) \quad (A5)$$

It can be deduced that the ratio v_1/v_2 is constant with respect to the substrate concentration, being related to the other kinetic parameters by the following equations:

$$v_1/v_2 = (k_{cat.1})_{app.}/(k_{cat.2})_{app.} = k_{cat.1}K_{m2}/(k_{cat.2}K_{m1}) \quad (A6)$$

Linearization of eqns. (A1) or (A2) permits calculations of $k_{cat.j}/K_{mj}$ (j = 1 or 2 respectively)

$$[S]/v_j = K_{mj}/(k_{cat.j}[E]_0) + [S]/\{(k_{cat.j})_{app.}[E]_0\} \quad (A7)$$

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