Studies of the cellulolytic system of the filamentous fungus *Trichoderma reesei* QM 9414

Substrate specificity and transfer activity of endoglucanase I

Marc CLAEYSSSENS,*§ Herman VAN TILBEURGH,* Johannes P. KAMERLING,† Jan BERG,† Maria VRSANSKA‡ and Peter BIELY‡

*Laboratorium voor Biochemie, Rijksuniversiteit Gent K.L. Ledeganckstraat 35, B-9000 Gent, Belgium,

†Afdeling Bio-Organische Chemie, Transitorium III, Rijksuniversiteit Utrecht Postbus 80.075, NL-3508 TB Utrecht,

The Netherlands, and ‡Chemický ústav Slovenskej akadémie vied, Dubravská cesta, 9, CS-8423 Bratislava, Czechoslovakia

Endoglucanase I from the filamentous fungus *Trichoderma reesei* catalyses hydrolysis and glycosyl-transfer reactions of cello-oligosaccharides. Initial bond-cleaving frequencies determined with 1-³H-labelled cello-oligosaccharides proved to be substrate-concentration-dependent. Using chromophoric glycosides and analysing the reaction products by h.p.l.c., kinetic data are obtained and, as typical for an endo-type depolymerase, apparent hydrolytic parameters (k_{cat} , k_{cat}/K_m) increase steadily as a function of the number of glucose residues. At high substrate concentrations, and for both free cellodextrins and their aromatic glycosides, complex patterns (transfer reactions) are, however, evident. In contrast with the corresponding lactosides and 1-thiocellobiosides, and in conflict with the expected specificity, aromatic 1-*O*- β -cellobiosides are apparently hydrolysed at both scissile bonds, yielding the glucoside as one of the main reaction products. Its formation rate is clearly non-hyperbolically related to the substrate concentration and, since the rate of D-glucose formation is substantially lower, strong indications for dismutation reactions (self-transfer) are again obtained. Evidence for transfer reactions catalysed by endoglucanase I further results from experiments using different acceptor and donor substrates. A main transfer product accumulating in a digest containing a chromophoric 1-thioxyloside was isolated and its structure elucidated by proton n.m.r. spectrometry (500 MHz). The β 1-4 configuration of the newly formed bond was proved.

INTRODUCTION

The filamentous fungus *Trichoderma reesei* secretes a very efficient cellulolytic system which contains several components. The endo-(1,4)- β -glucanases I and III have been purified to apparent homogeneity (Shoemaker & Brown, 1978; Hakansson *et al.*, 1979; Bhikhabhai & Pettersson, 1984; Saloheimo *et al.*, 1988), and primary structures of the encoding genes have been determined (Penttilä *et al.*, 1986; van Arsdell *et al.*, 1987; Saloheimo *et al.*, 1988). Endoglucanase I has been successfully cloned, e.g. in yeast (Penttilä *et al.*, 1988) and *Aspergillus* sp. (Barnett & Shoemaker, 1987).

We described the detection and differentiation of several enzymes of the cellulolytic complex from *Trichoderma reesei* using either chromogenic glycosides derived from glucose, cellobiose, cellotriose and lactose (van Tilbeurgh & Claeysens, 1985) or, alternatively, soluble, covalently dyed hydroxyethylcellulose and xylan (Biely & Markovic, 1988). Thus endoglucanase I was shown by analytical electrofocusing, in combination with specific detection, to be identical with a non-specific endoglucanase. In the culture filtrate of *Trichoderma reesei*, multiple forms with isoelectric points (pI) between 3.5 and 4.6 can be observed as distinct from those of the main exo-(1 $\rightarrow$ 4)- β -glucanase (cellobiohydrolase I; pI 3.9).

The amino acid sequence of the endoglucanase I shows considerable identity (> 45%) with that of the cellobiohydrolase I, and both enzymes are classified in the same family of cellulases

using criteria of secondary-structure analogies (Henrissat *et al.*, 1989). Their specific activities against common substrates, both native cellulose and its soluble derivatives (e.g. carboxymethyl-cellulose), are, however, completely different. The cellobiohydrolase I attacks very slowly and is adsorbed very strongly to (semi-)crystalline cellulose (Avicel), whereas endoglucanase I has been shown to act primarily on soluble cellulose derivatives and xylans (Niku-Paavola *et al.*, 1985). Both enzymes catalyse the hydrolysis of the lower members of the cellodextrin series (Claeysens *et al.*, 1989).

The present paper deals with the characterization of the endoglucanase I acting on these low-molecular-mass substrates and ligands. Special attention is paid to the transglucosylation activity of this enzyme.

EXPERIMENTAL

Substrates and ligands

Cellobiose and lactose (Aldrich, Beerse, Belgium) were commercial. Cellotriose, cellotetraose and cellopentaose were obtained by acetolysis of cellulose, deacetylation and fractionation on Bio-Gel P-2 (Bio-Rad) columns (van Tilbeurgh *et al.*, 1982). 1-³H-Reducing-end-labelled cello-oligosaccharides were prepared as described by Evans *et al.* (1974). A mixture of unlabelled cellodextrins was taken into the reaction and individual 1-³H-labelled cello-oligosaccharides (cellobiose to cellohexaose) were purified by preparative t.l.c. (P. Biely & M. Vrsanska, un-

Abbreviations used: Glc, D-glucose (D-glucosyl); (Glc)_n ($n = 2-5$), cellobiose, cellotriose, cellotetraose, cellopentaose (cellobiosyl, cellotriosyl, cellotetraosyl, cellopentaosyl); MeUmb, 4-methylumbelliferone; MeUmbGlc, 4-methylumbelliferyl β -D-glucopyranoside; MeUmb(Glc)_n ($n = 2-5$), 4-methylumbelliferyl β -D-glycosides from cellobiose, cellotriose, cellotetraose and cellopentaose; MeUmbGal1-4Glc, 4-methylumbelliferyl β -D-lactoside; MeUmbXyl, 4-methylumbelliferyl β -D-xylopyranoside; E_t , total enzyme concentration.

§ To whom correspondence and reprint requests should be sent.

published work). Specific radioactivity was determined only for [^3H]cellobiose (14.6 MBq/mol, 394 Ci/mol). This value is considered to correspond to the specific radioactivity of all higher [^3H]cello-oligosaccharides tritiated in the same reaction mixture.

The 4-methylumbelliferyl β -D-glycopyranosides of glucose and cellobiose were commercial (Koch-Light), whereas the derivatives of the higher cello-oligosaccharides were prepared as described by van Tilbeurgh *et al.* (1982). Concentrations were determined from the molar absorption coefficient at 313 nm ($13\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$). The 4-methylumbelliferyl derivatives of lactose (De Boeck *et al.*, 1984), D-ribose (Claeysens *et al.*, 1978) and L-arabinose (Deleyn *et al.*, 1985) were synthesized as indicated. *p*-Nitrophenyl β -D-xylopyranosyl β 1-4-xylopyranoside was prepared enzymically (Claeysens *et al.*, 1966). The 2',4'-dinitrophenyl 1-thio- β -D-glycosides were prepared as described by Claeysens & De Bruyne (1972). Their molar absorption coefficient at 313 nm is $12\,300\text{ M}^{-1}\cdot\text{cm}^{-1}$.

Enzymes

Endoglucanase I (component with pI 4.6), cellobiohydrolase I and cellobiohydrolase II from *Trichoderma reesei* QM 9414 were isolated as described (Bhikhabhai & Pettersson, 1984; van Tilbeurgh *et al.*, 1984) and their purity was checked by SDS/PAGE (Laemmli, 1970) and gel isoelectric focusing (van Tilbeurgh & Claeysens, 1985).

Endocellulase I concentration was estimated by the absorption coefficient at 280 nm ($61\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$) as calculated (Beaven & Holliday, 1959) from the amino acid composition (Pentillä *et al.*, 1986).

β -Glucosidase from almonds was of commercial origin (Fluka). β -Xylosidase from *Penicillium wortmanni* was purified as described by Deleyn *et al.* (1985).

Assays

The reactions with ^3H -labelled cello-oligosaccharides were carried out in 0.05 M-pyridine/acetate buffer, pH 5.0, at 30 °C and 0.25 mM substrate concentration. Hydrolysis was monitored by t.l.c., followed by measuring the radioactivity in products and remaining substrate by liquid-scintillation counting in Aquasol (P. Biély & M. Vrsanska, unpublished work). The product ratios of ^3H -labelled cello-oligosaccharides were used for determination of initial-bond-cleavage frequencies as described by Robyt & French (1970).

The reactions with the chromophoric oligosaccharides (0.01 M-sodium acetate/acetic acid buffer, pH 5.0) were monitored (25 °C) by quantitative h.p.l.c. as reported by van Tilbeurgh *et al.* (1982). Concentrations of the 4-methylumbelliferyl and 2',4'-dinitrophenyl 1-thioglycosides were measured at 313 nm (peak heights).

Glucose was measured with the glucose oxidase/peroxidase reagent (Bergmeyer & Bernt, 1974) and fluorimetric determination of 4-methylumbelliferone (MeUmb) was performed at pH 7.0 (0.1 M-glycine/NaOH) with a Vitatron MP photometer (excitation 336 nm, emission > 455 nm).

Reaction velocities as a function of substrate concentration were fitted hyperbolically according to the Michaelis-Menten equation (Sakoda & Hiromi, 1976).

Isolation of transfer products

Small amounts of chromophoric transfer products were obtained by preparative h.p.l.c. (van Tilbeurgh *et al.*, 1982) from a digest (0.1 M-acetate buffer, pH 5.0) containing cellotriose (2 mM), 4-methylumbelliferyl β -D-xylopyranoside (0.9 mM) and endoglucanase I (0.11 μM). The main transfer product was obtained in larger amounts when a mixture of cello-

oligosaccharides (5 g), 2,4-dinitrophenyl 1-thio- β -D-xylopyranoside (1 g) and endoglucanase I (1 mg) in 300 ml of 0.1 M-acetate buffer, pH 5.0, was incubated. After 20 h at 40 °C the digest was concentrated *in vacuo* and the residue dissolved in *NN*-dimethylformamide (20 ml), followed by filtration through a Celite bed. After washing the Celite with ethyl acetate (200 ml), the combined filtrates were evaporated to dryness and the residue was fractionated on a column (4 cm $\times$ 100 cm) of Bio-Gel P-2 using distilled water (16 ml/h). The eluates were monitored continuously at 280 nm and the fractions containing the main transfer product were collected. After freeze-drying, the resulting powder (10 mg) was chromatographically pure (h.p.l.c.).

N.m.r. spectroscopy

Carbohydrates were repeatedly exchanged in $^2\text{H}_2\text{O}$ (99–96 atom % ^2H ; Aldrich) with intermediate freeze-drying. ^1H n.m.r. spectra were recorded on a Bruker WM-500 spectrometer (SON hf-n.m.r.-facility, Department of Biophysical Chemistry, University of Nijmegen, Nijmegen, The Netherlands) operating at 500 MHz in the Fourier-transform mode at a probe temperature of 27 °C. Chemical shifts (δ) are given relative to disuccinyl suberate, but were actually measured indirectly to acetone in $^2\text{H}_2\text{O}$ ($\delta = 2.225\text{ p.p.m.}$). Two reference compounds and their chemical-shift and relevant coupling-constant values are: methyl β -D-glucopyranoside (H-1, 4.375; H-2, 3.256; H-3, 3.486; H-4, 3.374; H-5, 3.460; H-6, 3.924; H-6', 3.720; OCH_3 , 3.569) and 2',4'-dinitrophenyl 1-thio- β -D-xylopyranoside (H-1, 5.137; $J_{1,2}$, 9.2 Hz; H-2, 3.625; H-3, 3.575; H-4, 3.749; H-5 ax., 3.573; H-5, eq., 4.103; aromatic protons, 7.964, 8.483, 9.116).

RESULTS

Specificity of endoglucanase I against cello-oligosaccharides and their 4-methylumbelliferyl glycosides

Initial-bond-cleavage frequencies of [^3H]cello-oligosaccharides were determined at 0.25 mM substrate concentration and enzyme concentration in the range 0.05–10 μM (Fig. 1).

Cellobiose was shown to be hydrolysed at a rate approx. 1000 times lower than that at which cellotriose was hydrolysed and, with the latter, substrate formation of both labelled glucose and cellobiose could be demonstrated (bond-cleavage frequencies respectively 0.96 and 0.06). At higher substrate concentration (5 mM), a shift in the initial bond-cleavage frequencies was observed (0.75 and 0.25 respectively). This is indicative of secondary reactions occurring, as will be discussed further.

More detailed quantitative results were obtained with the chromophoric substrates (Fig. 2). MeUmb(Glc)₂ was apparently hydrolysed at both scissile bonds. Kinetic data could be obtained for the hydrolysis of the phenolic bond, and the

Fig. 1. Hydrolysis of cello-oligosaccharides catalysed by endoglucanase I

^3H -labelled cello-oligosaccharides were used, and bond-breaking frequencies were determined (pH 5.0, 30 °C) as described by Robyt & French (1970). □, β 1-4 glucopyranosyl residue; ▨, reducing-end residue.

Substrate	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (M ⁻¹ ·min ⁻¹)
1.4	454	3.2×10^5
1.8	547	3.0×10^5
0.11	2240	2.0×10^7
(1) 0.11 (2) 0.18	658 1265	6.0×10^6 7.0×10^6
0.034	1570	4.6×10^6

Fig. 2. Hydrolysis of the 4-methylumbelliferyl β -glycosides of cello-oligosaccharides catalysed by endoglucanase I

Apparent kinetic parameters (pH 5.0, 25 °C) were determined as described in the Experimental section. The glucosyl moieties and the 4-methylumbelliferyl groups are represented respectively by open rectangles ($\square$) and closed circles ($\bullet$). The open triangle (Δ) represents a galactopyranosyl moiety in lactose. The arrows indicate the bonds cleaved; for those marked with an asterisk (*), relevant kinetic data are given in the right-hand columns; (a), apparent cleavage site due to transfer activity (see the Results section).

Fig. 3. MeUmb(Glc)₂ as a substrate of endoglucanase I (pH 5.0, 25 °C)

Velocities of product formation (v/E_t) as a function of substrate concentration [MeUmb ($\times$) and MeUmbGlc ($\circ$)] were determined as described in the Experimental section. In the inset D-glucose formation as a function of time [at 1.5 mM-MeUmb(Glc)₂] was monitored by an enzymic method (Bergmeyer & Bernt, 1974).

relationship between the rate of MeUmb formation and substrate concentration is apparently hyperbolic (Fig. 3). By contrast, the relationship for MeUmbGlc formation (h.p.l.c. analysis) is clearly non-hyperbolic. When a thioglycoside such as 2',4'-dinitrophenyl 1-thio- β -cellobioside was used, hydrolysis could not be observed either at the 1-thio position or at the holosidic bond, suggesting that the chromophoric glucoside in the previous case is not a primary reaction product. When D-glucose formation (glucose oxidase/peroxidase reagent) was monitored at a particular MeUmb(Glc)₂ concentration, a lag phase was observed (Fig. 3,

inset), and the final rate (4 min⁻¹) did not compare with that observed for MeUmbGlc formation (300 min⁻¹) under these conditions. These findings undoubtedly point to the fact that D-glucose and MeUmbGlc are formed as secondary reaction products by dismutation (self-transfer) of the substrate MeUmb(Glc)₂, as will be discussed below.

MeUmbGal1-4Glc was solely hydrolysed at the phenolic bond. No deviations of the Michaelis-Menten function were observed for this substrate. The K_m and the k_{cat} were very similar to the values obtained for MeUmb(Glc)₂ and cellotriose ($K_m = 2.4$ mM, $k_{cat} = 567$ min⁻¹). Lactose itself was not a substrate.

With MeUmb(Glc)₃ the relationship between velocities of MeUmbGlc liberation and substrate concentration was hyperbolic, although substrate activation was observed at higher concentrations (> 1.8 mM) (results not shown).

Three chromophoric reaction products were detected with MeUmb(Glc)₄. MeUmb could be a secondary reaction product [hydrolysis of MeUmb(Glc)₂]. In the experimentally attainable substrate concentration range, velocities of both MeUmb(Glc)₂ and MeUmbGlc formation could be fitted hyperbolically, and the resulting apparent parameters are listed (Fig. 2).

With MeUmb(Glc)₅, four chromophoric reaction products were found: MeUmb, MeUmb(Glc)₂, MeUmb(Glc)₃ and MeUmbGlc. For the last-named product and for substrate concentrations up to 500 μ M, apparent Michaelis-Menten constants could be determined (Fig. 2).

Thus the specificities determined with either the radiolabelled sugars or the chromophoric derivatives seem to coincide (Figs. 1 and 2), validating the use of the latter in these studies.

Transfer reactions catalysed by endoglucanase I

1-Thioglycosides such as 2',4'-dinitrophenyl 1-thio- β -cellobioside were not hydrolysed by endoglucanase I (see above). However, when the reaction mixture was supplemented with a substrate (e.g. cellotriose), the corresponding 1-thio- β -D-glucoside was rapidly formed (as indicated by h.p.l.c.).

Fig. 4. H.p.l.c. analysis of the transfer activity of endoglucanase I (pH 5.0, 25 °C)

Cellotriose (2 mM) was incubated in the presence of MeUmbXyl (0.9 mM) and enzyme (0.11 μ M). Analysis before (0 min) and after 2 min and 16 min incubation. Peak identification: 1, MeUmb; 2, MeUmbXyl; 3, transfer product A; 4, transfer product B. Spectrophotometric detection was at 313 nm. R_t , retention time; INJ., point of injection.

Evidence for secondary reactions occurring during the enzymic breakdown of some substrates [such as cellotriose and MeUmb(Glc)₂] had already been obtained (see above). Thus the possibility of (self)-transfer reactions catalysed by the endoglucanase I has to be envisaged. Indeed, chromatographic analysis of mixtures containing a non-chromophoric substrate (e.g. cellotriose) and MeUmbXyl revealed the formation of chromophoric transfer products (Fig. 4). With MeUmb(Glc)₂ or

cellotetraose as substrate and using the same acceptor, MeUmbXyl, similar patterns were obtained. Thus the nature of the transfer products formed is apparently independent of the substrate, and lactosides serve as glycosyl donors in these transfer reactions, but lactose or 1-thiolactosides are not acceptors. Preliminary chromatographic evidence (retention times) pointed to the formation of transfer products with disaccharide, trisaccharide and tetrasaccharide structures.

The inhibitions by lactose and cellobiose were determined (25 °C) with MeUmb(Glc)₂ as substrate (fluorimetric MeUmb determination). Lactose inhibited competitively ($K_i = 54$ mM), whereas the inhibition pattern for cellobiose was complex (results not shown). It seems logical to suggest that the particular behaviour of the latter is a consequence of the transfer activity of the endoglucanase I. Cellobiose is, but lactose is not, an acceptor in these reactions. As shown above, MeUmbGlc is formed by initial transfer of a cellobiosyl unit to the substrate, MeUmb(Glc)₂ (see also the Discussion section). When potential acceptors (e.g. cellobiose) other than a second substrate molecule are added, several transfer products (e.g. cellotetraose) will be formed competitively. Therefore the ratio of production of MeUmb to MeUmbGlc should increase as acceptor is added. The effect of lactose (0–100 mM) was almost nil (ratio 1.3–1.5), whereas the presence of cellobiose (0–10 mM) had an appreciable influence, with the ratio increasing steadily (1.3–4.0).

The acceptor specificity of this enzyme was further investigated (by h.p.l.c.) using cellotriose as substrate (3 mM) and a series of chromophoric (MeUmb or *p*-nitrophenyl) glycopyranosides as acceptors (1–2 mM): α -D-mannoside, α -L-arabinoside, β -D-galactoside, β -D-ribose, β -D-lactoside, β -D-glucoside, β -D-xyloside, β 1-4 xylobioside and β -1-thiocellobioside. Only with the glucosyl and xylosyl derivatives was there evidence for transfer-product formation.

Structure of transfer products

Using cellotriose as donor and MeUmbXyl as acceptor a main transfer product, A, (retention time: 2.2 min) and a minor

Fig. 5. ¹H n.m.r. spectrum (500 MHz)

Conditions were as given in the Experimental section. Chemical shifts (p.p.m.) of the transfer product 2',4'-dinitrophenyl β -D-glucopyranosyl-(1 $\rightarrow$ 4)-1-thio- β -D-xylopyranoside: Xyl, H-1, 5.157 ($J_{1,2}$ 9.4 Hz); H-2, 3.665; H-3, 3.722; H-4, 3.967; H-5ax., 3.653; H-Seq., 4.251; Glc, H-1, 4.571 ($J_{1,2}$ 8.0 Hz); H-2, 3.305; H-3, 3.499; H-4, 3.406; H-5, 3.481; H-6, 3.933; H-6', 3.737. These are compared with those of the reference compounds (see the Experimental section).

compound, B (retention time: 3.9 min), were isolated by preparative h.p.l.c. (Fig. 4). Preliminary evidence for their structure resulted from enzymic analysis: examination of a digest of A with β -glucosidase from almond (*Amygdalus communis*) indicated rapid formation of a chromophoric glycoside, which upon subsequent treatment with β -xylosidase from *Penicillium wortmanni* generated the free phenol (MeUmb); in the case of transfer product B, a chromophoric compound with the same retention time as A was intermediately formed. Compound A was a substrate neither of cellobiohydrolase I nor of cellobiohydrolase II (*Trichoderma reesei*), and with B, formation of MeUmbXyl was observed for both enzymes. Taking into account the established specificities (Claeysens *et al.*, 1989) the structures of the transfer products were tentatively defined as:

A: 1-*O*-4'-methylumbelliferyl-(β -D-glucopyranosyl)- β -D-xylopyranoside

and

B: 1-*O*-4'-methylumbelliferyl-(β -cellobiosyl)- β -D-xylopyranoside

Definite proof of the structure of the main transfer product (corresponding to A) accumulating in a digest containing cello-oligosaccharides and 2',4'-dinitrophenyl 1-thio- β -D-xyloside (see the Experimental section) was obtained by 500 MHz ^1H -n.m.r. spectroscopy.

The assignments of the various signals in the spectrum of the transfer product (Fig. 5) were made on the basis of the comparison with the ^1H n.m.r. data from the two reference compounds (see the Experimental section) and selective ^1H -decoupling experiments. In the aromatic region (not shown), three signals at δ 7.963, 8.490 and 9.110 p.p.m. are observable, corresponding to the three aromatic H atoms of the 2',4'-dinitrophenyl group, which indicate that the isolated product contains the acceptor molecule. The anomeric region of the spectrum shows two doublets of equal intensity at δ 5.157 p.p.m. ($J_{1,2}$ 9.4 Hz) and δ 4.571 p.p.m. ($J_{1,2}$ 8.0 Hz), in favour of a disaccharide structure. The large coupling constants correlate with diaxial vicinal H-1 and H-2 atoms for both monosaccharide residues in their pyranose form, demonstrating β -D-glycosidic linkages only. When comparing the chemical-shift values of H-2, H-3 and H-4 of 2',4'-dinitrophenyl 1-thio- β -D-xylopyranoside and the Xyl constituent of the transfer product, the chemical-shift value of H-4 gave rise to the largest downfield shift ($\Delta\delta$, 0.218 p.p.m.), establishing the glycosidic linkage of this disaccharide derivative to be 1-4. A similar $\Delta\delta$ value (0.232 p.p.m.) for Xyl H-4 was observed going from β -Xyl1 $\rightarrow$ 0 Ser to β -Gal1-4- β -Xyl1-0 Ser (van Halbeek *et al.*, 1982).

DISCUSSION

The chromophoric glycosides derived from the cello-oligosaccharides and lactose proved to be very useful as substrates and active-site probes in specificity studies of cellobiohydrolases I and II from *Trichoderma reesei* (van Tilbeurgh *et al.*, 1984, 1989; Claeysens *et al.*, 1989). Some preliminary results for the endoglucanases I and III from the same organism have also been reported (Saloheimo *et al.*, 1988). The specificities of endoglucanase I and cellobiohydrolase I for small substrates are qualitatively very similar, although relevant kinetic data differ significantly (Claeysens *et al.*, 1989).

In the present study, reducing-end (radioactively)-labelled as well as chromophoric derivatives are used in order to prove unequivocally the scission sites of endoglucanase I attacking the lower homologues of the cello-oligosaccharide series. Bond-breaking frequencies (Fig. 1) are dependent on substrate con-

centration, as shown in the case of cellotriose, and further detailed elucidation of the reaction mechanism would require double-labelling experiments, as exemplified with several other transferring glycanases (see, e.g., Allen & Thoma, 1978).

As was demonstrated for several endoglucanases from *Penicillium pinophilum* (Bhat *et al.*, 1990), differing properties of the underivatized sugars and their aromatic glycosides could have inherent consequences for the mode of attack by specific enzymes. This seems not a major problem here.

However, endoglucanase I seems to catalyse multiple reaction pathways of substrate degradation or dismutation. Regardless of these complications, some quantitative results were obtained for the MeUmb glycosides (Fig. 2). An apparent increase in k_{cat} and catalytic efficiency with increasing number of glucose residues in the substrates is observed as typical for an endo-type depolymerase. The kinetic parameters for the chromophoric cellobioside and lactoside, as well as for cellotriose, are very similar, which could indicate that the aromatic leaving group does not influence the catalysis. For MeUmb(Glc)₃, however, the catalytic efficiency increases 100-fold, which could be due to an extra contribution of an additional D-glucose residue in the binding energy. With the higher homologues, multiple reactions have to be considered, and the resulting parameters are only apparent.

Closer examination shows important deviations from the Michaelis-Menten relationship, even for the smaller substrates (Fig. 3). Thus for MeUmb(Glc)₂ the sigmoidal dependency of the rates of MeUmbGlc formation on substrate concentration, as well as the lag phase observed for the concomitant D-glucose formation, can be explained by the reaction sequence shown in Scheme 1.

Scheme 1.

An intermediary cellobiosyl-enzyme complex $[\text{E}(\text{Glc})_2]$ is formed (reaction 1) and the glucose residue is then transferred to another substrate molecule (reaction 2). The cellotetraoside formed is rapidly hydrolysed (reaction 3) to cellotriose and the glucoside. Cellotriose is ultimately degraded to cellobiose and D-glucose, and this accounts for the lag phase observed for the formation of the latter reaction product (Fig. 3).

Alternative sequences could be considered, e.g. allowing for glucose transfer in the first steps. There is indeed some evidence that this could be possible (see below).

From these results it becomes evident that, within the global reaction mechanism of endoglucanase I, transfer reactions play a significant role. Analogously to other depolymerizing enzymes the active site of endoglucanase I probably possesses several subsites, each capable of binding a glucose residue. Several binding modes exist for most substrates [e.g. $\text{MeUmb}(\text{Glc})_4$]. During the hydrolysis reaction glycosyl intermediates are formed and these can be transferred to acceptor molecules (substrates, glucosides, xylosides).

The structures of two transfer products, a disaccharide (A) and a trisaccharide (B), accumulating in enzymic digests containing a chromophoric xyloside, are described here. By enzymic and n.m.r. spectroscopic investigation it was shown that only β 1-4 bonds were newly synthesized, and this points to the strict stereoselectivity of these transfer reactions. It characterizes the endoglucanase I as a retaining glycosylase (double inversion mechanism).

The formation of transfer product A could be rationalized as originating from direct glucosyl transfer. Alternatively a primary dismutation reaction of the donor substrate (e.g. cellotriose) could be postulated (Scheme 2), the intermediately formed

Scheme 2.

transfer product, cellopentaose, giving rise to the transient generation – by cellotriosyl transfer to the xyloside – of a tetraoside. This would be rapidly hydrolysed to either A or B.

Other possible pathways could be envisaged as the enzyme obviously exhibits very special properties of cleavage and synthesis of glycosidic linkages.

Further work is, however, needed to elucidate the reaction mechanisms of endoglucanase I in detail and the use of end-labelled (both reducing and non-reducing) sugars could be rewarding, as already demonstrated in this work. Also the possibility of xylo-oligosaccharides functioning as substrates and acceptors should be further investigated. This will open new aspects in the study of cellulose degradation by fungal enzymes in the natural habitat, where the polymer is intimately associated with hemicellulose components.

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