Study of the mode of action and site-specificity of the endo-(1→4)- β -D-glucanases of the fungus *Penicillium pinophilum* with normal, 1-³H-labelled, reduced and chromogenic cello-oligosaccharides

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The modes of action of the five major endo-(1→4)- β -D-glucanases (I, II, III, IV and V) purified from *Penicillium pinophilum* cellulase were compared by h.p.l.c. analysis, with normal, 1-³H-labelled and reduced cello-oligosaccharides and 4-methylumbelliferyl glycosides as substrates. Significant differences were observed in the preferred site of cleavage even when substrates with the same number of glycosidic bonds were compared. Thus, although endoglucanase I was unable to attack normal cello-oligosaccharides shorter than degree of polymerization 6, it hydrolysed reduced cellopentaose to yield cellotriose and cellobi-itol, and it produced cellotriose and 4-methylumbelliferyl glucoside from 4-methylumbelliferyl cellotetraose. Endoglucanase IV hydrolysed [1-³H]cellotriose but did not attack either cellobi-itol or 4-methylumbelliferyl cellobioside. These and other anomalous results indicated clearly that modification of the reducing glycosyl residue on the cello-oligosaccharides induces in an apparent change in the mode of action of the endoglucanases. It is suggested that, although cello-oligosaccharide derivatives are useful for differentiating and classifying endoglucanases, conclusions on the mechanism of cellulase action resulting from these measurements should be treated cautiously. Unequivocal information on the mode of endoglucanase action on cello-oligosaccharides was obtained with radiolabelled cello-oligosaccharides of degree of polymerization 3 to 5. Indications that transglycosylation was a property of the endoglucanases were particularly evident with the 4-methylumbelliferyl cello-oligosaccharides. Turnover numbers for hydrolysis of the umbelliferyl cello-oligosaccharides were calculated, and these, along with the other analytical data collected on the products of hydrolysis of the normal, reduced and radiolabelled cello-oligosaccharides, suggested that the various endoglucanases had different roles to play in the overall hydrolysis of cellulose to sugars small enough to be transported through the cell membrane.

INTRODUCTION

The extracellular cellulase system of certain cellulolytic fungi consists of endo-(1→4)- β -D-glucanase [(1→4)- β -D-glucan glucanohydrolase, EC 3.2.1.4], exo-(1→4)- β -D-glucanase [(1→4)- β -D-glucan cellobiohydrolase, EC 3.2.1.91] and β -D-glucosidase (β -D-glucoside glucosylhydrolase, EC 3.2.1.21) activities, and each activity exists in multiple forms (Eriksson & Pettersson, 1975; Gong *et al.*, 1977). These enzymes and their isoforms are generally differentiated on the basis of their substrate-specificity and their mode of action with a variety of cellulosic substrates (Wood & McCrae, 1978; Shoemaker & Brown, 1978). However, cello-oligosaccharides and their chromophoric derivatives have also been used advantageously for identification and differentiation (van Tilbeurgh & Claeysens, 1985).

In recent years the cellulase systems of *Penicillium pinophilum* and other fungi have been studied in great

detail because of their commercial potential for generating glucose from cellulosic materials (Wood, 1985). In addition to two immunologically distinct cellobiohydrolases (Wood & McCrae, 1986a, b) and a glucosylhydrolase (Wood & McCrae, 1982), five major endoglucanases (Bhat *et al.*, 1989) have been purified from the culture filtrates of *P. pinophilum*. Studies involving the purified enzymes acting alone and in combination (Wood & McCrae, 1986a) have provided some understanding of the mechanism of synergistic action involved in solubilizing crystalline cellulose. However, in fact the mechanism by which the endoglucanases and cellobiohydrolases interact to solubilize crystalline cellulose is poorly defined and is still the subject of considerable debate. The nature of and the requirement for the many endoglucanases found in the culture filtrates has been discussed at some length (Wood, 1985). The present paper is an attempt (a) to demonstrate and understand the biochemical uniqueness of the five major

Abbreviations used: MeUmb(Glc)_n (*n* = 1–5), 4-methylumbelliferyl β -D-glycosides from glucose, cellobiose, cellotriose, cellotetraose and cellopentaose; MeUmbLac, 4-methylumbelliferyl β -D-glycoside from lactose; MeUmbXyl, 4-methylumbelliferyl β -D-glycoside from xylose; [1-³H](Glc)_n (*n* = 1–5), 1-³H-labelled glucose, cellobiose, cellotriose, cellotetraose and cellopentaose.

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endoglucanases of *P. pinophilum*, as revealed by a study of their mode of action and site specificity with normal, 1-³H-reducing-end-labelled, reduced and chromophoric cello-oligosaccharides as substrates, and (b) to examine the possibility that these substrates can be used interchangeably for studies on the mechanism of cellulase action.

EXPERIMENTAL

Materials

Endoglucanases I, II, III, IV and V were purified preparations from the culture filtrates of *P. pinophilum* as described previously (Bhat *et al.*, 1989). Cello-oligosaccharides (Glc₃–Glc₅) (Miller *et al.*, 1960), reduced cello-oligosaccharides (Abdel-Akher *et al.*, 1951), MeUmb(Glc)_n (*n* = 1–5) (van Tilbeurgh *et al.*, 1982), MeUmbLac, MeUmbXyl and *p*-nitrophenyl xylobioside (Claeysens *et al.*, 1980) were prepared as described below. A mixture of reducing-end-labelled [1-³H]cello-oligosaccharides (specific radioactivity approx. 10.5 GBq/mmol), kindly donated by Dr. P. Biely (Bratislava, Czechoslovakia), were fractionated by preparative h.p.l.c., as described below. The octadecylsilyl-bonded silica column, the RSil Polyol 5 µm column and the 10 PAC stainless-steel column were purchased from Hichrom (Reading, Berks., U.K.), Alltech Research Science Laboratory (Eko, Belgium) and Jones Chromatography (Hengoed, Glamorgan, U.K.) respectively. Nylon-66 filter (0.2 µm pore size) were bought from Rainin Instrument Co. (Woburn, MA, U.S.A.). Scintillation fluid and acetonitrile (h.p.l.c. grade) were from Alltech Applied Science Laboratory. All chemicals used were AnalaR grade or equivalent and purchased either from BDH Chemicals (Poole, Dorset, U.K.) or Sigma Chemical Co. (Poole, Dorset, U.K.).

Preparation of cello-oligosaccharides

These were prepared (Miller *et al.*, 1960) from dewaxed cotton by acetolysis and separated by gradient elution [0–35 % (v/v) ethanol] of a column (40 cm × 3.5 cm) of charcoal (acid-washed; BDH)/Celite (1:1, w/w).

Preparation of reduced cello-oligosaccharides

Approx. 50 mg of cello-oligosaccharide [(Glc)_n (*n* = 3–5)] was dissolved in 10 ml of distilled water, 10 mg of NaBH₄ was added and the solution was left to stand at room temperature overnight. The solution was acidified with 1 M-acetic acid and evaporated to dryness under vacuum. The solid was suspended in acidified methanol and dried under vacuum. The process was repeated five or six times. The sample was redissolved in distilled water and deionized by passage through anion-exchange [IR-120 (H⁺ form)] and cation-exchange resins [Dowex 1-X8 (HCO₃⁻ form)] columns in series, and the eluent and washings (water) were combined and freeze-dried.

Preparation of 4-methylumbelliferyl β-glycosides and *p*-nitrophenyl β-D-xylobioside

The 4-methylumbelliferyl β-glycosides of the cello-oligosaccharides [MeUmb(Glc)_n (*n* = 1–5)] (van Tilbeurgh *et al.*, 1982), lactose (MeUmbLac) and xylose (MeUmbXyl) (van Tilbeurgh & Claeysens, 1985) and *p*-nitrophenyl β-D-xylobioside [*p*-nitrophenyl β-D-xylo-

pyranosyl-β(1→4)-xylopyranoside] were prepared as described previously (Claeysens *et al.*, 1980).

Purification of reducing-end-labelled [1-³H]cello-oligosaccharides

A 100 µl [1-³H]cello-oligosaccharide mixture (10⁶–10⁷ c.p.m.) was diluted with 900 µl of 5 mM pure unlabelled cello-oligosaccharide and then diluted with 2 ml of 65 % (v/v) acetonitrile in water. A 100 µl sample was chromatographed on an RSil Polyol 5 µm column (0.3 cm × 25 cm) with 65 % (v/v) acetonitrile in water as mobile phase at a flow rate of 1.0 ml/min. Apparently pure oligosaccharide fractions were collected and the process was repeated several times. The combined chromatographically pure samples were flushed with N₂ and then freeze-dried.

Action of endoglucanases on normal cello-oligosaccharides

(a) **Enzymic hydrolysis.** A 0.5 ml reaction mixture containing 0.125 ml of cello-oligosaccharide [(Glc)_n (*n* = 2–5)] (final concn. 2.5 mM), 0.25 ml of 0.1 M-sodium acetate buffer, pH 5.0, and 0.125 ml of diluted enzyme (2.5 µg of protein) was incubated at 50 °C for 5, 10, 15, 20 and 30 min. The reaction was terminated by immersing the tubes in a boiling-water bath for 10 min.

(b) **Sample preparation for h.p.l.c. analysis.** A 0.3 ml sample from the 0.5 ml enzyme digest was applied to a column (0.4 ml) of cation-exchange resin [IR-120 (H⁺ form; 14–52 mesh)] and the column was eluted five times with 0.4 ml of distilled water. The eluent and washings were then passed through a column (0.4 ml) of anion-exchange column [Dowex 1-X8 (HCO₃⁻ form; 18–52 mesh)] and the residue was isolated by freeze-drying. The freeze-dried sample was dissolved in 0.15 ml of water, filter-centrifuged through nylon-66 filters (pore size 0.2 µm) and analysed by h.p.l.c.

(c) **Quantitative separation of hydrolysate by h.p.l.c.** This was carried out on a Partisil 10 PAC stainless-steel column (25 cm × 0.5 cm) with 71 % (v/v) acetonitrile in water as mobile phase at a flow rate of 1.5 ml/min. The sugars were detected and quantified by means of a refractive-index detector (LKB-2142) and a recording integrator (LKB-2220).

Action of endoglucanases on [1-³H]cello-oligosaccharides

A 150 µl reaction mixture containing 100 µl of unlabelled (2.0 mM) and labelled (25000–30000 c.p.m.) cello-oligosaccharides (final concn. 2.0 mM), 40 µl of distilled water and 10 µl of enzyme (1–10 µg) was incubated at 50 °C. Samples (20 µl) were withdrawn at different time intervals and the reaction was stopped by adding 40 µl of unlabelled cello-oligosaccharides mixture (1 mM each) prepared in 65 % (v/v) acetonitrile in water. A 20 µl sample of each was separated on an RSil Polyol 5 µm column (25 cm × 0.5 cm) with 65 % (v/v) acetonitrile in water as mobile phase at a flow rate of 1.0 ml/min. Peaks corresponding to each oligosaccharide were detected (Waters 401 refractive-index detector), collected in scintillation vials containing 5.0 ml of scintillation fluid and mixed, and the radioactivities were quantified by using an LKB liquid-scintillation counter. Analysis of the frequency of bond cleavage was carried out as described elsewhere (Walker & Dewar, 1978).

Action of endoglucanases on reduced cello-oligosaccharides

(a) **Enzymic digestion.** A 0.5 ml mixture containing 0.125 ml of reduced cello-oligosaccharide [(Glc)_n (*n* = 3–5)] (final concn. 2.5 mM), 0.25 ml of 0.1 M-sodium acetate buffer, pH 5.0, and 0.125 ml of diluted enzyme (2.5–10.0 µg) was incubated at 50 °C for 30 min. The reaction was terminated by placing the tubes in a boiling-water bath for 10 min.

(b) **Sample preparation for h.p.l.c. analysis.** Samples (0.25 ml) were deionized by passage through cation-exchange [IR-120 (H⁺ form; 14–52 mesh)] and anion-exchange resins [Dowex 1-X8 (HCO₃⁻ form; 18–52 mesh)] as described above and then freeze-dried. After dissolution in 2.5 ml of pyridine and 1 ml of phenyl isocyanate and mixing, the samples were placed in a water bath at 50–60 °C for 1 h, then cooled in crushed ice, and 0.5 ml methanol was added dropwise to each with rapid mixing (Björkqvist, 1981). The samples were then filtered through 0.2 µm-pore-size nylon filters and analysed by h.p.l.c.

(c) **Quantitative separation of reaction products by h.p.l.c.** Samples (2–5 µl) were separated on an octadecylsilyl-bonded silica (5 ODS 2) column (100 mm × 4.6 mm) fitted to a similar pre-column (20 mm × 2 mm). The elution was effected by using a convex gradient [50–90% (v/v) acetonitrile in 10 mM-potassium phosphate buffer, pH 7.0] at a flow rate of 1.5 ml/min. The modified sugars detected at 240 nm were quantified by using an HP 3396A integrator, previously calibrated with standard cello-oligosaccharide derivatives.

Action of endoglucanases on MeUmbcello-oligosaccharides

A reaction mixture containing 200 µl of 50 mM-sodium acetate buffer, pH 5.0, 50 µl of substrate [MeUmb-β-(Glc)_n (*n* = 1–5)] (between 50 µM and 250 µM) and a 5 µl enzyme sample (50–1000 ng of protein) was incubated at 25 °C. Samples (50 µl) were withdrawn after 1, 5, 10, 15 and 20 min, and the reaction was stopped by adding 100 µl of acetonitrile. A 20 µl sample of each dilution was analysed on an RSil Polyol 5 µm column (25 cm × 0.5 cm) with 65% (v/v) acetonitrile in water as mobile phase. The 4-methylumbelliferyl derivatives were detected at 313 nm (Waters 440 detector) and quantified on the basis of peak height with the use of standard methylumbelliferyl glycosides. *K_m* and *V_{max}* were determined from a plot of [S]/*v* versus [S].

Action of endoglucanases on MeUmbLac, MeUmbXyl and *p*-nitrophenyl xylobioside

The mode of action of endoglucanases on these three compounds was studied with 500 ng of enzyme protein and 50 µM-*p*-nitrophenyl xylobioside, 50 µM-MeUmbLac or 500 µM-MeUmbXyl, as described above for MeUmbcello-oligosaccharides.

Measurement of transferase activity of endoglucanases with cellotetraose as donor and MeUmb(Glc)₂ or MeUmbXyl as acceptor

A reaction mixture containing 125 µl of 1 mM-MeUmb(Glc)₂ or 1 mM-MeUmbXyl, 5 µl of 50 mM-

cellotetraose, 125 µl of 0.1 M-sodium acetate buffer, pH 5.0, and 5 µl of enzyme (1 µg) was incubated at 25 °C. Samples (50 µl) were withdrawn after 1, 30, 60, 90 and 120 min, mixed with 100 µl of acetonitrile and analysed as above.

RESULTS

Mode of action of endoglucanases on normal cello-oligosaccharides

The pattern of hydrolysis of cello-oligosaccharides at the concentration given and by the same amount of each endoglucanase was studied by h.p.l.c. analysis of the hydrolysis products at different time intervals. None of the endoglucanases was active on cellobiose, but endoglucanases III and IV effected significant hydrolysis of cellotriose. The rate of attack of endoglucanases III and IV on cellotriose was very rapid in the first 10 min of incubation (Figs. 1*a* and 1*b*); endoglucanase III effected almost complete hydrolysis in 30 min under the conditions tested.

Only endoglucanases II, III and IV were active on cellotetraose. After 30 min incubation, endoglucanase III effected approx. 95% degradation of cellotetraose (Fig. 1*d*), but only 30–50% hydrolysis was noted with endoglucanases II and IV (Figs. 1*c* and 1*e*). Cellobiose was the predominant product in all three cases. The release of cellotriose without equal amounts of glucose by endoglucanases II and IV by their action on cellotetraose is difficult to explain unless transglycosylation is involved.

Endoglucanases II, III and IV hydrolysed cello-pentaose very rapidly within the first 5 min of incubation, but endoglucanase V had effected only 50% hydrolysis in 30 min (Figs. 1*f*–1*i*). The reaction products of endoglucanases II and V (Figs. 1*f* and 1*i*) consisted of equimolar amounts of cellobiose and cellotriose, but the hydrolysates obtained with endoglucanases III and IV (Figs. 1*g* and 1*h*) also contained cellobiose, cellotriose and glucose. In the hydrolysates obtained with endoglucanases III and IV the concentration of cellotriose increased initially (Figs. 1*g* and 1*h*) and then decreased as it was degraded to glucose and cellobiose. Clearly, all four endoglucanases attacked cello-pentaose initially at either the second or third glycosidic bond, releasing cellobiose and cellotriose; only endoglucanases III and IV degraded the cellotriose to glucose and cellobiose.

Cellohexaose was the shortest cello-oligosaccharide attacked by endoglucanase I (Bhat *et al.*, 1989).

Mode of action of endoglucanases on [1-³H]cello-oligosaccharides

[1-³H]Cello-oligosaccharides were used in order to obtain unequivocal information on the actual glycosidic bond cleaved. Analysis of the frequency of bond cleavage (Walker & Dewar, 1978) showed that endoglucanase III hydrolysed the first and second glycosidic bonds from the non-reducing end of [1-³H]cellotriose to the extent of 33% and 67% respectively, but the frequency of the attack on the same bonds by endoglucanase IV was 56% and 44% respectively (Table 1). Thus the results show that endoglucanase III has a high degree of preference for the second glycosidic bond of [1-³H]cellotriose from the non-reducing end, whereas endoglucanase IV attacks the first glycosidic bond more effectively.

Fig. 1. Hydrolysis of cello-oligosaccharides by endoglucanases as a function of time

For experimental details see the Experimental section. With cellotriose as substrate: (a) endoglucanase III; (b) endoglucanase IV. With cellotetraose as substrate: (c) endoglucanase II; (d) endoglucanase III; (e) endoglucanase IV. With cellopentaose as substrate: (f) endoglucanase II; (g) endoglucanase III; (h) endoglucanase IV; (i) endoglucanase V. Symbols: Δ , glucose (Glc); $\circ$, cellobiose (Glc₂); $\bullet$, cellotriose (Glc₃); $\square$, cellotetraose (Glc₄); $\blacksquare$, cellopentaose (Glc₅).

Table 1. Bond cleavage frequencies for the hydrolysis of [1-³H]cello-oligosaccharides by endoglucanases

Bond cleavage frequencies were determined (Walker & Dewar, 1978) by plotting product ratios (radioactivity of each product/total radioactivity of the sample) versus extent of reaction (total radioactivity of the products/total radioactivity of the sample). The slope of each line represented the cleavage frequency for the bond giving rise to the product of particular chain length.

Endoglucanase	Substrate	Bond no. from non-reducing end ...	Bond cleavage frequency (%)			
			1	2	3	4
II	[1- ³ H]Cellotetraose	—	—	90	10	—
	[1- ³ H]Cellopentaose	—	—	16	84	—
III	[1- ³ H]Cellotriose	33	67	—	—	—
	[1- ³ H]Cellotetraose	7	90	3	—	—
	[1- ³ H]Cellopentaose	10	25	65	5	—
IV	[1- ³ H]Cellotriose	56	44	—	—	—
	[1- ³ H]Cellotetraose	10	85	5	—	—
	[1- ³ H]Cellopentaose	6	24	58	11	—
V	[1- ³ H]Cellopentaose	—	—	45	45	10

Another interesting observation made with the radiolabelled cellotriose in the same experiment was the appearance of oligomers with the same retention times as [1-³H]cellopentaose and [1-³H]cellohexaose in the early stages of hydrolysis. However, the concentrations of these sugars decreased rapidly on prolonged incubation. Clearly, in certain conditions endoglucanase III catalyses transglycosylation reactions.

When [1-³H]cellotetraose was the substrate, endoglucanases II, III and IV released predominantly [1-³H]cellobiose (Table 1), but there were differences in the mode of attack. Thus endoglucanase III and endoglucanase IV reaction mixtures contained a small amount of [1-³H]glucose and [1-³H]cellotriose, but the hydrolysate produced by endoglucanase II, surprisingly, contained only [1-³H]glucose. Determination of the frequency of bond cleavage revealed that with all three enzymes the major proportion (85–90 %) of hydrolysis was effected at the central glycosidic bond of [1-³H]cellotetraose and only a small amount of degradation (10–15 %) was observed at the other two linkages (Table 1).

[1-³H]Cellopentaose was hydrolysed by all four endoglucanases: endoglucanases III and IV degraded the substrate by approx. 60 % in 2 min and had effected 95 % hydrolysis in 10 min, but endoglucanases II and V effected only about 60 % hydrolysis in 10 min. At the end of the incubation period tested, the reaction mixtures containing endoglucanase III or endoglucanase IV comprised 7–10 % [1-³H]glucose, 50–58 % [1-³H]cellobiose, 23–30 % [1-³H]cellotriose and 5–8 % [1-³H]cellotetraose. In contrast, endoglucanase II released about 45 % [1-³H]cellobiose, but only 8 % of [1-³H]cellotriose and no labelled glucose. Interestingly, endoglucanase V was quite different from the other three endoglucanases in its action on [1-³H]cellopentaose and it released almost equal amounts of [1-³H]cellobiose and [1-³H]cellotriose and only a small amount of [1-³H]glucose. Analysis of bond cleavage frequency revealed that endoglucanases II, III and IV all acted mainly on the third linkage of [1-³H]cellopentaose from the non-reducing end (Table 1). Endoglucanase V, however, attacked both the second and the third linkages from the non-reducing end equally (Table 1). Thus in most cases the endoglucanases preferentially cleaved these linkages that result in the release of [1-³H]cellobiose.

Mode of action of endoglucanases on reduced cello-oligosaccharides

Gum & Brown (1977) reported the separation of reduced and normal cello-oligosaccharides on a Whatman PXS-1025 PAC column. Using identical conditions, we have found that although glucose could be readily separated from glucitol the other cello-oligosaccharides could not be separated from the reduced forms. Carbanilated (Björkqvist, 1981) normal and reduced sugars, however, were readily separated, and this method was used to analyse the products of hydrolysis and determine the site of attack of the endoglucanases on the reduced cello-oligosaccharides. However, because of the complexity of the carbanilation procedure only reaction mixtures obtained after one incubation period (30 min) were analysed.

None of the endoglucanases was active on cellotri-itol, but endoglucanases II, III and IV attacked cellotetraitol to release roughly equimolar amounts of cellobiose and cellobi-itol. With the same substrate, hydrolysates obtained with endoglucanase III additionally yielded glucose, glucitol, cellotriose and cellotri-itol, and endoglucanase IV also produced glucose and cellotri-itol. Surprisingly, endoglucanase II appeared to release substantial amounts of cellotri-itol and cellotriose from cellotetraitol but no glucose and glucitol, and this may again suggest the involvement of some transglycosylation. The quantitative analyses of the products of hydrolysis of these substrates and of cellopentaitol are given in Table 2, and the significance of these results in terms of bonds cleaved are given in Table 1. Notable were the observations (a) of the remarkable diversity in the modes of action, (b) that cellopentaitol was a substrate for endoglucanase I but cellopentaose was not, and (c) that cellopentaitol was not hydrolysed by endoglucanase V but cellopentaose was readily degraded.

Mode of action of endoglucanases on MeUmbcello-oligosaccharides

None of the endoglucanases was active on MeUmbGlc and MeUmb(Glc)₂, but endoglucanases II, III, IV and V were active on MeUmb(Glc)₃, and all five endoglucanases attacked MeUmb(Glc)₄ and MeUmb(Glc)₅ (Fig. 2). In each case the products of hydrolysis detected at 313 nm were either MeUmbGlc or MeUmb(Glc)₂ or both. This

Table 2. Sugars released from cello-oligosaccharides by endoglucanases

Time of incubation was 30 min. For details see the Experimental section.

Endoglucanase	Substrate	Hydrolysis products (μM)					
		Glucose	Glucitol	Cellobiose	Cellobi-itol	Cellotriose	Cellotri-itol
I	Cellopentaitol	Nil	Nil	Nil	0.25	0.29	Nil
II	Cellotetraitol	Nil	Nil	0.26	0.30	0.24	0.22
	Cellopentaitol	Nil	Nil	0.65	0.65	0.83	0.70
III	Cellotetraitol	0.15	0.04	0.64	0.67	0.17	0.16
	Cellopentaitol	Nil	Nil	0.99	0.49	0.40	0.88
IV	Cellotetraitol	0.37	Nil	0.88	1.00	Nil	0.12
	Cellopentaitol	0.6	0.29	1.6	0.53	Nil	0.40

Fig. 2. Site-specificity of endoglucanases for the hydrolysis of $1\text{-}^3\text{H}$ -labelled, reduced and chromophoric cello-oligosaccharides

□, Glucose residue; ■, $1\text{-}^3\text{H}$ -labelled glucose residue; ○, reduced glucose residue; ●, 4-methylumbelliferyl glucoside. The bonds cleaved preferentially (↓) and those cleaved non-preferentially (↓) are indicated. Abbreviation: N.A., cello-oligosaccharide not attacked.

sensitive method of detection allowed initial rates of product formation to be calculated.

Some kinetic constants for endoglucanase action on $\text{MeUmb}(\text{Glc})_3$, $\text{MeUmb}(\text{Glc})_4$ and $\text{MeUmb}(\text{Glc})_5$ are presented in Tables 3(a), 3(b) and 3(c) respectively. These data were obtained from the quantitative separation by h.p.l.c. of the hydrolysis products during the initial rate of the reaction. It should be noted that these constants are apparent in those cases where more than one reaction product is observed.

It can be seen (Table 3a) that with the $\text{MeUmb}(\text{Glc})_3$ as substrate endoglucanase II released both MeUmbGlc and $\text{MeUmb}(\text{Glc})_2$, with apparent specific activities of 0.71 and 0.18 $\mu\text{mol}/\text{min}$ per mg of protein respectively, whereas endoglucanases III, IV and V released MeUmbGlc as the sole product of hydrolysis, with specific activities of 3.4, 1.9 and 3.8 $\mu\text{mol}/\text{min}$ per mg of protein respectively. However, the turnover number for the release of MeUmbGlc by endoglucanase II from the trioside was 4 times higher than for the release of $\text{MeUmb}(\text{Glc})_2$, suggesting that this enzyme preferentially attacked the second glycosidic bond from the aglycone of $\text{MeUmb}(\text{Glc})_3$ (Fig. 2). The highest turnover number was found with endoglucanase III acting on

$\text{MeUmb}(\text{Glc})_3$ (Table 3a), but endoglucanase V showed maximum specific activity.

From $\text{MeUmb}(\text{Glc})_4$, endoglucanases II, III, IV and V released MeUmbGlc and $\text{MeUmb}(\text{Glc})_2$, whereas endoglucanase I released only MeUmbGlc . Calculation of the turnover numbers (Table 3b) revealed that endoglucanase II hydrolysed, with equal efficiency, the second and third glycosidic bonds from the aglycone end of $\text{MeUmb}(\text{Glc})_4$ (Fig. 2) whereas endoglucanases II, IV and V preferentially attacked the third glycosidic bond from the aglycone (Fig. 2).

When $\text{MeUmb}(\text{Glc})_5$ was the substrate MeUmbGlc was the only glycoside released by endoglucanase III, whereas endoglucanases I, II, IV and V released both MeUmbGlc and $\text{MeUmb}(\text{Glc})_2$. Kinetic data presented in Table 3(c) show that endoglucanases I, II and IV preferentially attacked the third glycosidic bond from the aglycone, whereas endoglucanase V preferred the second glycosidic bond.

The kinetic data presented in Tables 3(a)–3(c) demonstrate that endoglucanases I and II show an increased specific activity and turnover number as chain length of the MeUmb cello-oligosaccharides increases from three to five. In contrast, endoglucanase III showed decreased specific activity and turnover number. The remaining two endoglucanases (IV and V) showed a higher degree of preference for the second glycosidic bond from the aglycone as chain length increased.

Action of endoglucanases on other chromophoric glycosides

None of the endoglucanases was active on MeUmbXyl and *p*-nitrophenyl xylobioside. However, endoglucanases III, IV and V were slightly active against MeUmbLac under the conditions used. Clearly, the endoglucanases of *P. pinophilum* are very specific for the MeUmb cello-oligosaccharides.

Transglycosylation activity of endoglucanases

All the endoglucanases catalysed transglycosylation reactions in mixtures containing cellotetraose as a donor and $\text{MeUmb}(\text{Glc})_2$ as an acceptor. The products were equimolar amounts of oligomers with the same retention time as $\text{MeUmb}(\text{Glc})_3$ and $\text{MeUmb}(\text{Glc})_4$ in all cases except with endoglucanase I, which produced mainly $\text{MeUmb}(\text{Glc})_3$. However, when MeUmbXyl replaced $\text{MeUmb}(\text{Glc})_2$ as an acceptor only endoglucanases III, IV and V showed transglycosylation activity: the main product was supposedly $\text{MeUmbXyl}(\text{Glc})_2$ in all three cases [retention time as for $\text{MeUmb}(\text{Glc})_3$].

DISCUSSION

Like the endoglucanases of other fungi (Wood & McCrae, 1978; Moloney *et al.*, 1985), none of the five major (I, II, III, IV and V) endoglucanases of *P. pinophilum* was active on cellobiose. However, higher cello-oligosaccharides ($\text{Glc}_3\text{--Glc}_5$) were hydrolysed by all the endoglucanases except endoglucanase I. There were, however, significant differences in the mode of action. Thus endoglucanases III and IV were active on cellotriose and higher cello-oligosaccharides, but endoglucanases II, V and I required respectively at least four, five and six glucose residues in the chain before hydrolysis was effected (Bhat *et al.*, 1989).

Another interesting observation was made with the

Table 3. Some kinetic constants of endoglucanases for MeUmbcello-oligosaccharides

In (i) and (ii) the kinetic constants were obtained by measuring the release of MeUmbGlc and MeUmb(Glc)₂ respectively.

Endoglucanase		K_m (μM)	$10^4 \times V_{max}$ ($\mu mol/min$)	Specific activity ($\mu mol/min$ per mg)	Turnover number (min^{-1})
(a) MeUmb(Glc) ₃					
II	(i)	450	7.1	0.71	28
	(ii)	400	1.8	0.18	7
III	(i)	125	16.8	3.4	206
IV	(i)	95	9.4	1.9	100
V	(i)	190	9.4	3.8	165
(b) MeUmb(Glc) ₄					
I	(i)	85	0.08	0.008	2
II	(i)	162.5	23.9	2.4	92
	(ii)	125	23.7	2.4	91
III	(i)	150	14.8	3.0	181
	(ii)	140	29.8	6.0	366
IV	(i)	75	12.7	5.1	270
	(ii)	225	43.9	17.5	927
V	(i)	62.5	6.4	2.6	112
	(ii)	100	19.6	7.6	344
(c) MeUmb(Glc) ₅					
I	(i)	245	3.1	0.6	15
	(ii)	215	13.0	2.6	64
II	(i)	1250	17.1	17.1	654
	(ii)	500	25.5	25.5	976
III	(i)	15	1.0	1.0	63
IV	(i)	32.5	3.3	6.6	351
	(ii)	60	4.3	8.7	460
V	(i)	125	2.8	5.6	250
	(ii)	75	2.0	4.1	182

normal cello-oligosaccharide substrates, namely, the release of cellotriose from cellotetraose by endoglucanases II and IV without the apparent production of measurable amounts of glucose. A similar observation was made earlier by Shoemaker & Brown (1978), working with *Trichoderma reesei* endoglucanase: they suggested that the cellotriose released could in fact be a hydrolysis product of celohexaose that had been synthesized by transglycosylation. In the present study some evidence for transglycosylation was obtained when endoglucanase III was incubated with [1-³H]cellotriose, but transglycosylation was observed more clearly when methylumbelliferyl cello-oligosaccharides were used. Thus oligomers with the same retention time as MeUmb(Glc)₃ and MeUmb(Glc)₄ were detected in reaction mixtures containing endoglucanase II and mixtures of cellotetraose and MeUmb(Glc)₂. Evidence for transglycosylation was also obtained with all the other endoglucanases.

Normal cello-oligosaccharides are clearly useful for distinguishing and characterizing enzymes, but the results obtained in some cases are equivocal in terms of the actual linkage cleaved. By using radiolabelled [1-³H]cello-oligosaccharides it was possible to demonstrate the mode

of action of endoglucanases more precisely. Thus the study revealed that endoglucanases III and IV attacked all the glucosidic bonds of the [1-³H]cello-oligosaccharides tested but that endoglucanases II and V were more specific (Fig. 1). Analysis of bond cleavage frequency showed that the principal product of hydrolysis was [1-³H]cellobiose with all endoglucanases except endoglucanase III, which released [1-³H]glucose from [1-³H]cellotriose, and endoglucanase V, which liberated equal amounts of [1-³H]cellobiose and [1-³H]cellotriose from [1-³H]cellopentaose. Thus it would appear that many of the endoglucanases of *P. pinophilum* preferentially attack the linkage releasing [1-³H]cellobiose from [1-³H]cello-oligosaccharides; i.e. they are cellobiohydrolases with a preference for the reducing end of the molecules.

Attempts were made to characterize the endoglucanases further and to deduce their modes of action with reduced and MeUmbcello-oligosaccharides as substrates. However, the use of these substrates introduces the problem that the modified cello-oligosaccharides have quite different properties from the normal cello-oligosaccharides. The mode of attack on

normal cello-oligosaccharides may therefore be quite different from attack on the cello-oligosaccharide derivatives. The effect of reduction of the cello-oligosaccharide of the specificity of the enzyme was shown clearly with endoglucanases III and IV: both were active on cellotriose but neither had any apparent action on cellotri-itol. Remarkably, endoglucanase V, which had no effect on cellotetraose or cellotetraitol, was active on MeUmb(Glc)₃ (Fig. 2).

Methylumbelliferyl glycosides have been used to differentiate the cellulase components of *T. reesei* (van Tilbeurgh *et al.*, 1982; Saloheimo *et al.*, 1988). Some clearly defined results were obtained with *P. pinophilum* endoglucanases with these substrates, as shown in Fig. 2. There was, however, at least one significant difference between the modes of action of the endoglucanases of the two fungi: whereas none of the endoglucanases of *P. pinophilum* released the free phenol from MeUmb(Glc)₃ or higher oligomers, one of the endoglucanases of *T. reesei* (van Tilbeurgh *et al.*, 1982; Saloheimo *et al.*, 1988) did. The analysis of kinetic data showed that the internal glycosidic bonds of MeUmbcello-oligosaccharides were certainly the preferred sites of attack by each of the endoglucanases.

In conclusion, it would appear that the cello-oligosaccharide derivatives are suitable model substrates for classifying and characterizing endoglucanases and for providing some limited information on the mode of action. However, it is apparent that as there is not always a good correlation between attack on these compounds and the normal cello-oligosaccharides. It is obvious that it is not possible to extrapolate from measurements made on one substrate and make assumptions on the mode of action on another.

The diversity of attack on the normal and modified cello-oligosaccharides suggests that the various endoglucanases have specific roles to play in cellulose hydrolysis.

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