

## Isolation and characterization of the 1,4- $\beta$ -D-glucan glucohydrolases of *Talaromyces emersonii*

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1. Culture filtrates of *Talaromyces emersonii* were found to contain four endocellulases termed I, II, III and IV, the last having the greatest electrophoretic mobility towards the anode in homogeneous 5%-(w/v)-polyacrylamide gels at pH 4.5. 2. All four are glycoproteins, the carbohydrate contents being: I, 27.7%; II, 29.0%; III, 44.7%; IV, 50.8. 3. Each form is eluted as a single peak corresponding to an  $M_r$  value of 68 000 on gel filtration at pH 3.5 and as a single band corresponding to an  $M_r$  value of 35 000 on reductive sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis. However, we believe that the latter represents the native  $M_r$  value. 4. The pI values for each lie between pH 2.8 and 3.2. 5. Activity in each case is optimal at pH 5.5–5.8 and at 75–80°C. Half-life values at pH 5 and 75°C were from 2 to 4 h. 6. The specific activity with any individual substrate was much the same for each enzyme, as was the ratio of activity from one substrate to the next. 7. Possible reasons for the observation that plots of velocity versus substrate concentration are sigmoidal are discussed. 8. We believe that the finding of four endocellulases reflects differential glycosylation of a single enzyme form rather than genetically determined differences in primary structure.

Investigations on the properties of the enzyme systems that catalyse extensive hydrolysis of crystalline cellulose have been stimulated by the great potential of this most abundant of organic materials as a source of food and energy. The enzyme systems are comprised of endocellulases (1,4- $\beta$ -D-glucan glucohydrolase, EC 3.2.1.4), exocellulases (1,4- $\beta$ -D-glucan cellobiohydrolase, EC 3.2.1.91; and 1,4- $\beta$ -D-glucan glucohydrolase, EC 3.2.1.74),  $\beta$ -glucosidase ( $\beta$ -D-glucoside glucohydrolase, EC 3.2.1.21) and, at least in some instances, a low- $M_r$  non-protein component (see, e.g., Wilke, 1975; Bailey *et al.*, 1975; Gaden *et al.*, 1976; Coughlan & Folan, 1979; Goksøyr & Eriksen, 1980; Wood, 1980; Ljungdahl *et al.*, 1983; Enari, 1983; Griffin *et al.*, 1984). In fungal-culture filtrates each of the above enzymes is generally found to exist in multiple forms. Whether these multiple forms reflect the presence of true isoenzymes or merely differential glycosylation or

proteolytic modification after secretion has been the subject of much debate (see, e.g., the review by Gong & Tsao, 1979). More recently Labudova & Farkas (1983) have concluded that the cellulase from *Trichoderma reesei* is inherently a complex system containing multiple genetically determined forms of each enzyme type. Wood *et al.* (1984) have stated that multiplicity of forms is not surprising, in view of the steric problems confronting these enzymes and their need to work synergistically. *Talaromyces emersonii*, a thermophilic fungus, when grown on media containing cellulose produces a complete extracellular cellulase system containing four endocellulases, four or five exocellulases and three  $\beta$ -glucosidases (McHale & Coughlan, 1980, 1981*a,b,c*). Examination of the substrate specificities and other properties of the extracellular  $\beta$ -glucosidases and those of an intracellular form of this enzyme allowed of a tentative assignment of their functions in cellulose hydrolysis *in vivo* (McHale & Coughlan, 1981*c*; Coughlan *et al.*, 1984). In the present paper we examine the properties of each of the four endocellulases synthesized by *Talaromyces emersonii*.

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## Materials and methods

### Materials

Solka floc (BW 40; purified ball-milled spruce cellulose) was from Brown and Co., Berlin, NH, U.S.A.; CM-cellulose [viscosity of aq. 2% (w/v) solution at 25°C is 10–12cP], acrylamide, dinitrosalicylate, Coomassie Blue, glucono-D-lactone and methylcellulose were from Sigma Chemical Co., Poole, Dorset, U.K.; CM-cellulose 7H3XSF (degree of substitution 0.88) was from Hercules, Wilmington, DE, U.S.A.; sodium CM-cellulose (Cellofas B) with a degree of substitution of approx. 0.5 was from I.C.I. (Nobel Division), Stevenston, Ayrshire, Scotland; *NNN'N'*-tetramethylethylenediamine and Page Blue were from BDH Chemicals, Poole, Dorset, U.K.; corn-steep liquor was from Biocon, Carrigaline, Co. Cork, Ireland; Bio-Gel P-2, CM-cellulose (Cellex CM) and sodium dodecyl sulphate were from BioRad Laboratories, Watford, Herts., U.K.; t.l.c. silica gel 60 plates were supplied by Merck, Darmstadt, W. Germany; Sephadex G-150, DEAE-Sephadex G-50, Pharmalytes (pH range 2.5–5.0) and  $M_r$ -marker proteins were from Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K. Cello-oligosaccharides were prepared as described below.

### Micro-organism

*Talaromyces emersonii* C.B.S. 814.70 was obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. The organism was routinely grown at 45°C in the mineral salts/2% (w/v) Solka floc/1% (w/v) ammonium nitrate/0.5% (w/v) corn-steep liquor/0.1% (w/v) yeast extract/0.5% (w/v)  $\text{KH}_2\text{PO}_4$  medium containing neomycin (100 µg/ml) and adjusted to pH 4.5 (Folan & Coughlan, 1978, 1981; Moloney *et al.*, 1983). Cultures maintained on Sabouraud dextrose/agar plates for 5 days were subcultured in 50 ml of the above liquid medium in 250 ml flasks and incubated at 45°C with shaking for 48 h. Growth in these flasks was used to inoculate 10-litre fermentations, during which the temperature was maintained at 45°C, aeration at 10 litres/min and agitation at 400 rev./min. Culture fluids were harvested at 75 h and filtered through Celite. When necessary the filtrate was freeze-dried and resolubilized in 0.1 M-sodium acetate buffer, pH 5, for use.

### Assays

CM-cellulase (endocellulase) activity was determined by measuring the release of reducing equivalents by the dinitrosalicylate method of Miller (1959) after incubation at 60°C of 5 µl samples of enzyme with 6% (w/v) low-viscosity CM-cellulose in 0.1 M-sodium acetate buffer, pH 5 (final vol.

2 ml), for 10 min, or less, depending on linearity. Alternatively, the reducing equivalents released were measured by the Somogyi-Nelson method (Nelson, 1952). Endocellulase activity was also measured by determining the decrease in viscosity (i.e. the increase in fluidity) of a solution of Cellofas B (1%, w/v) in 0.1 M-sodium acetate buffer, pH 5.4, containing 0.01% (w/v) merthiolate after incubation with a sample of enzyme at 30°C for 30 min (Wood & McCrae, 1972). Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Protein-bound carbohydrate was measured by the phenol/ $\text{H}_2\text{SO}_4$  method of Dubois *et al.* (1965), and sialic acid was measured as described by Jourdan *et al.* (1971). For carbohydrate determination samples (0.2 ml) of each enzyme solution were incubated at 28°C for 15 min with 10 µl of phenol/water (4:1, w/v) and 1 ml of  $\text{H}_2\text{SO}_4$  (sp. gr. 1.84). The  $A_{490}$  of the mixture was measured, and the carbohydrate content as mannose equivalents was calculated by reference to a mannose standard curve. Under the conditions used 10 µg of mannose gave an  $A_{490}$  of 0.62.

### Electrophoresis (preparative and analytical)

Endocellulase fractions were examined for homogeneity at all stages of purification by electrophoresis, adapted to the cassette technique, in homogeneous 5%-(w/v)-polyacrylamide gels and the buffer system described by Reisfeld *et al.* (1962). Samples were applied in 10% (w/v) sucrose in the electrode buffer. Bromocresol Purple (0.4%, w/v) was used as the marker dye, and representative portions (see below) of the gels were stained for protein with 0.1% (w/v) Coomassie Blue in 50% (w/v) trichloroacetic acid, and for glycoprotein with the periodic acid/Schiff reagent of Zacharius *et al.* (1969). The above electrophoretic procedure was also used in a preparative manner.

### $M_r$ and subunit composition

Subunit  $M_r$  values of the endocellulases were determined by subjecting samples of the purified preparations to sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis (Margolis & Kenrick, 1968) after pretreatment of unknowns and of calibration standards as recommended by Pharmacia (Publication no. 11-B-037-01, February 1980). Thus unknowns and standards were dissolved in 10 mM-Tris/HCl, buffer, pH 8, containing 1 mM-EDTA, 2.5% (w/v) sodium dodecyl sulphate and 5% (w/v) 2-mercaptoethanol and heated at 100°C for 5 min. The electrophoresis buffer was 40 mM-Tris/HCl buffer, pH 7.4, containing 20 mM-sodium acetate, 2 mM-EDTA and 2% (w/v) sodium dodecyl sulphate, and the

gradient gels were Pharmacia PAA 4130 type. Samples were allowed to run into the gels at 300 V for 10 min, and electrophoresis was continued for 300 V·h until the Bromophenol Blue tracking dye had just run off the gel. Gels were fixed for 60 min in 10% (w/v) trichloroacetic acid containing 5% (w/v) sulphosalicylic acid, and stained with 0.2% (w/v) Page Blue 83 in water/methanol/acetic acid (6:3:1, by vol.). Gels were destained in water/methanol/acetic acid (6:3:1, by vol.) until the background was clear. The subunit  $M_r$  values of the endocellulases were calculated by comparing their mobilities with those of the standard proteins under the above conditions. The standards used were phosphorylase *b* ( $M_r$  94000), albumin ( $M_r$  67000), ovalbumin ( $M_r$  43000), carbonic anhydrase ( $M_r$  30000), trypsin inhibitor ( $M_r$  20000) and  $\alpha$ -lactalbumin ( $M_r$  14400).  $M_r$  values were also measured by gel filtration on a Sephadex G-150 column (4.5 cm  $\times$  72 cm) equilibrated with 20 mM-Tris/borate buffer, pH 8.2, or with 0.1 M-potassium acetate buffer, pH 3.5, containing 0.1 M-NaCl. Standards and unknowns eluted with the same buffer were detected by their absorbance at 280 nm. The void volume was determined with Blue Dextran.

#### *Isoelectric focusing*

The pI values of the endoglucanases were determined by isoelectric focusing for 3400 V·h in 5%-(w/v)-polyacrylamide gels with Pharmalytes (at a final dilution of 1:16) covering the pH range 2.5–5.0 and the procedure recommended by Pharmacia (Publication no. 11-B-045-01). In conjunction with the unknowns, the following standard pH markers were also run: soya-bean trypsin inhibitor (pI 4.55), glucose oxidase (pI 4.15), amyloglucosidase (pI 3.5) and pepsinogen (pI 2.8). Gels were fixed, stained for protein and destained as above.

#### *Preparation of cello-oligosaccharides*

Cello-oligosaccharides were prepared by acetylation/deacetylation of Solka floc (Miller *et al.*, 1960) followed by fractionation of the products by gel filtration on Bio-Gel P-2. Oligosaccharides eluted from the column with distilled water were detected by the dinitrosalicylate method indicated above, and their identities were established by t.l.c. on silica-gel plates as described by Wood & McCrae (1978) except that plates were developed by exposure to I<sub>2</sub> vapour (Dawson *et al.*, 1972). The fractions corresponding to the individual oligosaccharides were judiciously pooled and freeze-dried. The products of action of endocellulases with cello-oligosaccharides or acid-swollen cellulose as substrate were identified by t.l.c. as above.

## Results and discussion

### *Enzyme purification*

(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added with stirring to culture filtrate at 4°C to give 80% saturation. After stirring for 1 h the precipitated protein was collected by centrifugation at 2500g, resuspended in distilled water, dialysed overnight against distilled water and freeze-dried. The dialysis tubing used was not degraded by the filtrate at 4°C. Approx. 1g of freeze-dried material was redissolved in 0.1 M-potassium acetate buffer, pH 3.5, containing 0.1 M-NaCl and subjected to gel filtration on a column (4.5 cm  $\times$  72 cm) of Sephadex G-150. The column was irrigated at 4°C with the above buffer at a flow rate of 50 ml/h. Endocellulase activity was eluted as a single symmetrical peak (Fig. 1). The appropriate fractions were pooled, concentrated approx. 10-fold by using an Amicon ultrafiltration device with a PM-10 membrane and desalted by passage of several volumes of distilled water through the apparatus. A 50 mg portion of the freeze-dried concentrate was redissolved in 50 mM-sodium acetate buffer, pH 5, containing 0.15 M-NaCl and applied to a DEAE-Sephadex A-50 ion-exchange column (1.7 cm  $\times$  30 cm). The column was irrigated at 4°C with 3–4 column volumes of the above buffer and then with 500 ml of an exponential NaCl gradient (0.15 to 0.35 M) in 50 mM-sodium acetate buffer, pH 5. Fractions exhibiting endoglucanase activity were eluted as a symmetrical peak (Fig. 2). These were pooled, dialysed against distilled water and freeze-dried. This material was redissolved in 50 mM-sodium acetate buffer, pH 4.3, containing 0.1 M-NaCl and applied to a DEAE-Sephadex A-50 ion-exchange column (1.7 cm  $\times$  45 cm) equilibrated with the same buffer. Approx. 2 column volumes of starting buffer were run through at 4°C, and then 600 ml of a linear gradient of NaCl (0.11 to 0.17 M) in 50 mM-sodium acetate buffer, pH 4.3, was applied. Four overlapping peaks of endoglucanase activity were eluted (Fig. 3). Fractions corresponding to the best cut from each peak were pooled separately, dialysed, freeze-dried and resuspended in the electrode buffer described by Reisfeld *et al.* (1962), and subjected to preparative electrophoresis for 1200 V·h. Thin strips cut from each edge of the developed gel were stained for protein and realigned with the unstained gel. Transverse strips corresponding to each of the major protein bands were now cut from the gel. These were homogenized, in a Potter-Elvehjem homogenizer, in 5 ml of 0.1 M-sodium acetate buffer, pH 5. The homogenates were left at 4°C for 4 h so as to allow protein to leach into solution, and then centrifuged at 13000g for 1 h at 4°C. The pellet was resuspended in buffer, homogenized as before and

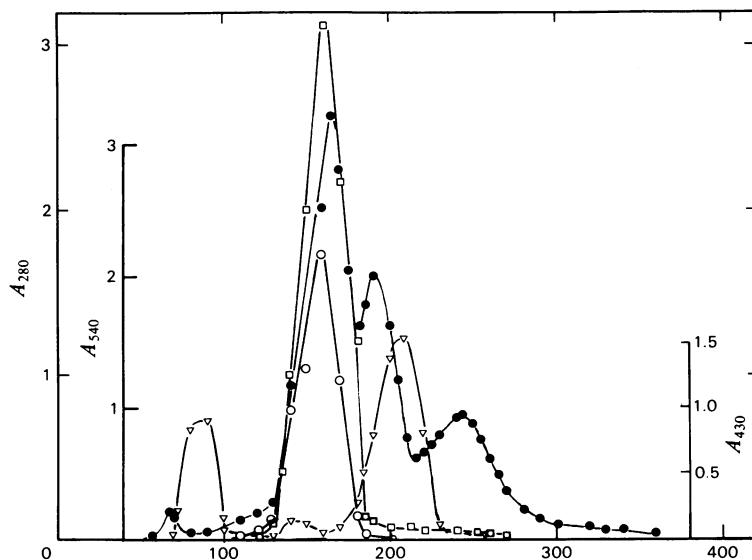


Fig. 1. Gel filtration of culture filtrate of *Talaromyces emersonii* on Sephadex G-150 at pH 3.5. For experimental details see the text. Fractions (5ml) were assayed for protein concentration ( $A_{280}$ , ●),  $\beta$ -glucosidase activity ( $A_{430}$ , ▽), exocellulase activity, as measured by the ability to hydrolyse acid-swollen cellulose but not CM-cellulose ( $A_{540}$ , □), and endocellulase (CM-cellulase) activity ( $A_{540}$ , ○). Fractions 130–180 were pooled for further fractionation.

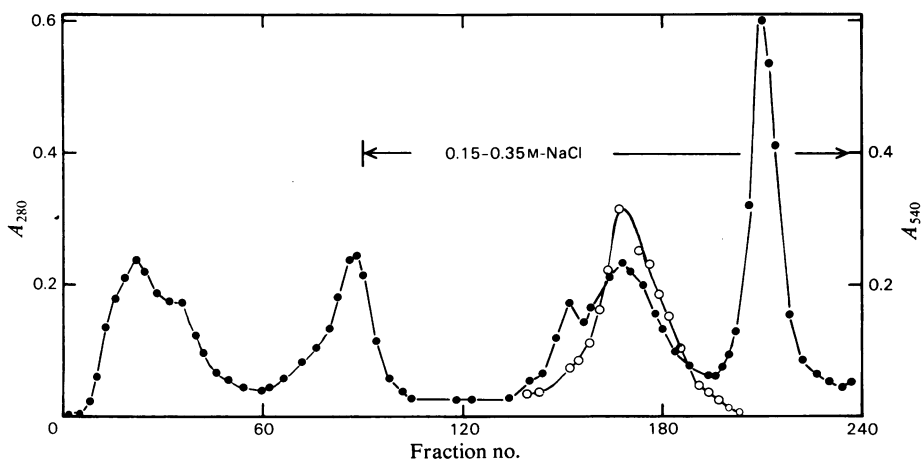


Fig. 2. First ion-exchange chromatography on DEAE-Sephadex A-50 in the purification of endocellulases from *Talaromyces emersonii*.

For experimental details see the text. Fractions (3ml) were assayed for protein ( $A_{280}$ , ●) and for endocellulase activity ( $A_{540}$ , ○). Fractions 140–190 were pooled for further purification.

kept overnight at 4°C, and then again centrifuged. After a total of four washes enzyme activity could no longer be extracted from the homogenized gels. Typically, at least 60% of the protein applied in the preparative electrophoresis step could be recovered. A sample of each of these pooled supernatants was dialysed against distilled water,

freeze-dried and again subjected to polyacrylamide-gel electrophoresis. Staining for protein indicated the presence of but a single band in each solution. The four endocellulases were designated I to IV, the distance of migration of each towards the anode under the conditions used being as follows: I, 4.8 cm; II, 5.35 cm; III, 5.9 cm; IV, 6.2 cm.

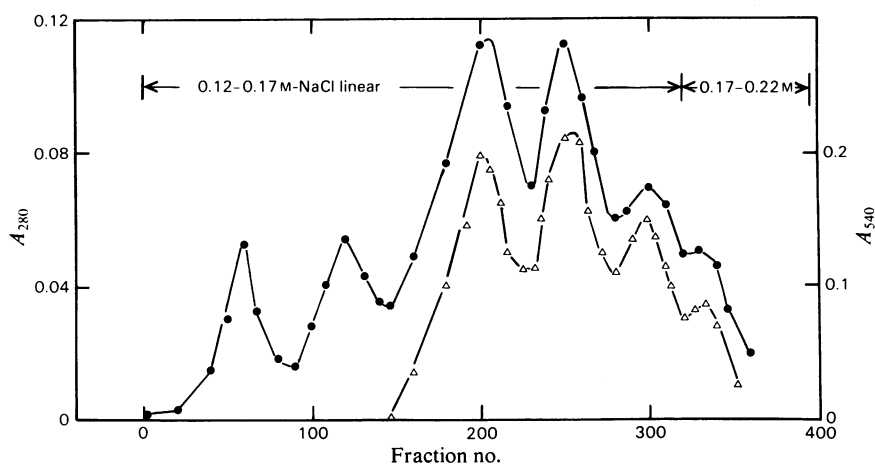


Fig. 3. Second ion-exchange chromatography on DEAE-Sephadex A-50 in the purification of endocellulases from *Talaromyces emersonii*

For experimental details see the text. Fractions (2ml) were assayed for protein ( $A_{280}$ , ●) and for endocellulase activity ( $A_{540}$ , ○).

#### Carbohydrate content

In previous publications we showed that all components of the *Talaromyces emersonii* cellulase system gave a positive reaction when stained for glycoprotein (McHale & Coughlan, 1981a,b,c, 1982). We can now confirm, as perhaps expected of extracellular enzymes, that each of the purified endocellulases is a glycoprotein, but we note that the differences in carbohydrate contents are considerable. Expressed as a percentage of total weight, the values for the various forms are: I, 27.7; II, 29.0; III, 44.7; IV, 50.8. Assuming that the  $M_r$  of each endocellulase is 35000 (see below), one may calculate the carbohydrate contents as molar equivalents of mannose per mole of enzyme to be: I, 54, II, 56; III, 87; IV, 99. Sialic acid was not detected in any of the four enzyme preparations.

#### $M_r$ and subunit composition

Because of the fact that the endocellulases are glycoproteins, one must treat with caution the  $M_r$  values obtained by gel filtration (see Andrews, 1965). Bearing this in mind, one notes that a mixture of all four enzyme forms were eluted from Sephadex G-150 as a single symmetrical peak (see, e.g., Fig. 1) at a volume consistent with an  $M_r$  value of 68000 at pH 3.5 or of 58000 at pH 8.2. On sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis of fully reduced enzyme each form exhibited only one band, with an  $M_r$  value of 35000. Moreover, when subjected to polyacrylamide-gradient-gel electrophoresis under non-reductive and supposedly non-denaturing conditions, i.e. in the absence of mercaptoethanol

and sodium dodecyl sulphate, each endoglucanase was seen to migrate off the gel. This, according to the Pharmacia Laboratory Manual (Publication no. 11-B-037-01, February 1980), indicated an  $M_r$  value of less than 40000. These results would suggest that each enzyme *in vivo* exists as a dimer ( $M_r$  68000) of similar, if not identical, subunits ( $M_r$  35000). However, there are indications (see below) that the native enzymes have  $M_r$  values of 35000 but that they associate under certain conditions to form dimers and possibly higher- $M_r$  aggregates.

#### Isoelectric points

Each of the endocellulase preparations was found to be homogeneous as judged by polyacrylamide-gel electrophoresis and by reductive sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis (see above). This was also found to be the case with forms I, II and IV when analysed by isoelectric focusing in polyacrylamide gel. The pI values for these forms were determined to be as follows: I, 3.19; II, 3.08; IV, 2.86. Endocellulase type III migrated not as a discrete band but as a smear corresponding to pI values from 2.93 to 3.0. This 'microheterogeneity' and indeed the differences between the isoelectric points of the four endocellulases may reflect minor variations in the carbohydrate content or composition of these enzymes rather than differences in primary structure. One notes, however, that the carbohydrate moieties of the cellulase enzymes from *Trichoderma reesei* are comprised for the most part of neutral sugars (Shoemaker & Brown, 1978). The same may be true of the *Talaromyces* enzymes.

If so, the findings obtained by Hayashi & Nakamura (1981) may be relevant. They reported that the protein moieties of glucose oxidase enzymes of different pI values were identical and that the enzymes with the more-acidic pI values had a greater content of neutral sugar. They tentatively concluded that differences in the structures of the carbohydrate moieties affected dissociable groups in neighbouring amino acid residues differently and so gave rise to isoelectric multiplicity.

#### pH and temperature optima

Each of the endocellulases investigated, namely types I, II and III, was optimally active at pH values between 5.5 and 5.8 with low-viscosity CM-cellulose as substrate and an incubation temperature of 60°C.

Activity declined sharply on either side of this pH range, but especially on the acid side with little or no conversion of substrate at pH values below 3.5. At pH 5 the optimum temperature for hydrolysis of CM-cellulose by each enzyme form was 75–80°C. At pH 5 at 75°C the activity of endocellulases I, II and III decayed in a first-order manner, as expected. However, loss of activity in the case of type IV was second-order, suggesting

perhaps a degree of association/dissociation. Half-life values under these conditions (namely pH 5, 75°C) were determined to be: I, 104 min; II, 93 min; III, 75 min; IV, 66 min. At pH 5, 70°C, the half-life values were in each case some 4–5-fold greater. Hayashida & Yoshioka (1980) have shown that the carbohydrate moieties of the cellulases from *Humicola insolens* contribute significantly to their thermal stabilities. This may also be true for the endocellulases from *T. emersonii*, although, as we can see above, the half-life values of the four forms are inversely related to their carbohydrate contents.

#### Substrate specificity

As judged by the release of product, none of the purified endocellulases acted on cotton, Avicel, filter paper, phosphocellulose, DEAE-cellulose, dextran, DEAE-Sephadex, inulin, laminarin, xylan, *p*-nitrophenyl  $\beta$ -D-xyloside, cellobiose or *p*-nitrophenyl  $\beta$ -D-glucoside, even with long incubation times. By contrast, phosphoric acid-swollen filter paper (McHale & Coughlan, 1980), CM-cellulose from various suppliers, methylcellulose and cello-oligosaccharides from cellohexaose to cellotriose were active substrates (Tables 1 and 2). The specific activity with any single substrate was

Table 1. *Relative rates of hydrolysis of CM-celluloses by the endocellulases from Talaromyces emersonii*  
For experimental details see the text. The rate of hydrolysis of each substrate, at the indicated concentration, was measured by the release of reducing sugars and expressed as a percentage of that obtained with Sigma CM-cellulose as substrate. Specific activities (mequiv. of reducing sugar released/min per mg of enzyme) with this substrate were: I, 414; II, 425; III, 385; IV, 325. At 25°C the viscosities of 2% (w/v) solutions of Sigma CM-cellulose and methylcellulose were 10–20 cP and 25 cP respectively, and that of the Hercules substrate would have been one to two orders of magnitude greater. The BioRad CM-cellulose used was an insoluble cross-linked ion-exchange resin.

Substrate	Concn. (mg/ml of assay medium)	Relative rate of hydrolysis (%)			
		I	II	III	IV
CM-cellulose (Sigma)	60	100	100	100	100
CM-cellulose (Hercules)	10	21.8	26.1	24.7	25.5
CM-cellulose (BioRad)	60	32.9	15.6	18.9	20.0
Methylcellulose	20	11.8	15.2	10.1	12.8

Table 2. *Relative rates of hydrolysis of cello-oligosaccharides by the endocellulases from Talaromyces emersonii*  
For experimental details see the text. The rate of hydrolysis of each substrate (1 mM) at 60°C at pH 5 relative to that of cellopentaose under the same conditions was determined by measuring the release of reducing sugars or of glucose. Relative rates based on the former measurements are given without parentheses and those based on glucose determination are given in parentheses. The relative rates of hydrolysis of cellopentaose by endocellulase forms I, II, III and IV were 0.77, 1.0, 1.0 and 0.72 respectively.

Oligosaccharide	Relative rate of hydrolysis (%)			
	I	II	III	IV
Cellohexaose	90.3 (53.8)	71.4 (61.5)	89.5 (75.0)	71.8 (50.0)
Cellopentaose	100	100	100	100
Cellotetraose	73.7 (69.2)	66.2 (91.7)	65.4 (91.7)	66.7 (81.3)
Cellotriose	39.8 (61.5)	58.6 (70.8)	52.6 (70.8)	30.2 (62.5)
Cellobiose	0	0	0	0

essentially the same for each enzyme, as was the ratio of activity from one substrate to the next. The real differences in the ability of any one enzyme form to hydrolyse the various CM-celluloses may be ascribed to the differences between the degrees of polymerization or between the degrees or types of substitution of the substrates (Table 1). On the other hand, it would appear that release of product from the cello-oligosaccharides is to some extent governed by chain length (Table 2). In the context of substrate specificity we note that glucose (2mM), cellobiose (1.25mM), methylcellulose (0.5%, w/v) and gluconolactone (100mM) did not inhibit the hydrolysis of CM-cellulose by any of the endocellulases. That cellobiose was not an inhibitor is of particular interest in view of the well-established fact that accumulation of cellobiose, when  $\beta$ -glucosidase activity is insufficient, is one of the factors that impede the practical saccharification of cellulose (see, e.g., Sternberg *et al.*, 1977). Thus the observed inhibition by cellobiose of cellulose hydrolysis by *Talaromyces emersonii* culture filtrates (Folan & Coughlan, 1978) must be directed mainly against the exocellulase components. This has indeed been shown to be the case with respect to the exocellulases from *Trichoderma* sp. (Gong & Tsao, 1979).

#### Mode of action

After 52h incubation with any one of the endocellulases, the products of hydrolysis of phosphoric acid-swollen filter paper were shown by t.l.c. to be glucose and cellobiose with small

amounts of higher oligomers. The ratio of reducing sugars to glucose in the hydrolysates was much the same (namely 3.5 to 4.2) for each enzyme form. Similarly, the products of hydrolysis of cello-oligomers, from cellohexaose to cellobiose, after 4h at pH5 at 60°C (Table 2), were found to be mainly glucose and cellobiose. Since cellobiose is not a substrate for any of the enzyme forms (see above), such findings are consistent with these enzymes being true endocellulases, i.e. they participate in cellulose hydrolysis by cleavage of internal glycosidic linkages at random (see, e.g., Gong & Tsao, 1979). This being so, one should expect the rate of release of reducing sugars on hydrolysis of CM-cellulose to be linearly related to the rate of decrease in viscosity of the solution. Such was indeed found to be the case (Fig. 4). Moreover, the ratio of reducing groups released to increase in fluidity of solution, i.e. the slope of the line, was the same for each endocellulase. By contrast, different slopes are given by each of the endocellulases of *Trichoderma koningii* (Wood & McCrae, 1978).

#### Kinetics

The effects of substrate (i.e. low-viscosity CM-cellulose) concentration on the velocity of the reaction (i.e. the rate of release of reducing equivalents) was essentially the same for each enzyme. Results typical of those obtained with endocellulase type III are shown in Fig. 5. Surprisingly, the plot of velocity versus substrate concentration was sigmoidal (Fig. 5a). A reciprocal plot of the data curves upwards as substrate concentration decreases (Fig. 5b). By taking the  $V_{max}$  value to be that indicated in Fig. 9(a) and plotting the experimental results in accordance with the method of Hill (Dawes, 1972) one obtains a straight line (Fig. 5c). From this one may calculate the  $K'$  value of endocellulase type III for CM-cellulose under the conditions used to be 1.47% (w/v). In the same way one may calculate the  $K'$  values for endocellulases I, II and IV to be 1.85% (w/v), 1.68% (w/v) and 1.54% (w/v) respectively.

The Hill coefficient values obtained were as follows: I, 1.53; II, 1.85; III, 1.76; IV, 1.83. Such values would generally be interpreted as indicating the presence of two binding sites on each enzyme and that the binding of substrate at one site positively influences binding of substrate at the second site. However, there is, *a priori*, no reason to expect extracellular hydrolytic enzymes to be allosteric. Thus one suspects that the sigmoidal kinetics above do not reflect the situation that obtains *in vivo*. We are reasonably certain that the observations cannot be ascribed to the presence of more than one enzyme, since the preparations used were homogeneous according to various electrophoretic criteria (see above). Moreover, the

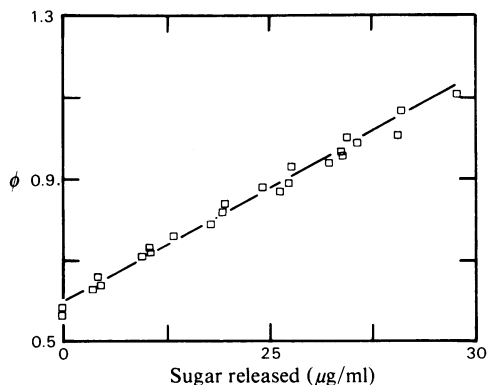


Fig. 4. Plot of the increase in fluidity ( $\phi$ ) versus the release of reducing sugars after hydrolysis of CM-cellulose by the endocellulases from *Talaromyces emersonii*

For experimental details see the text. The values shown ( $\square$ ) represent experimental observations with each of the purified endocellulases.

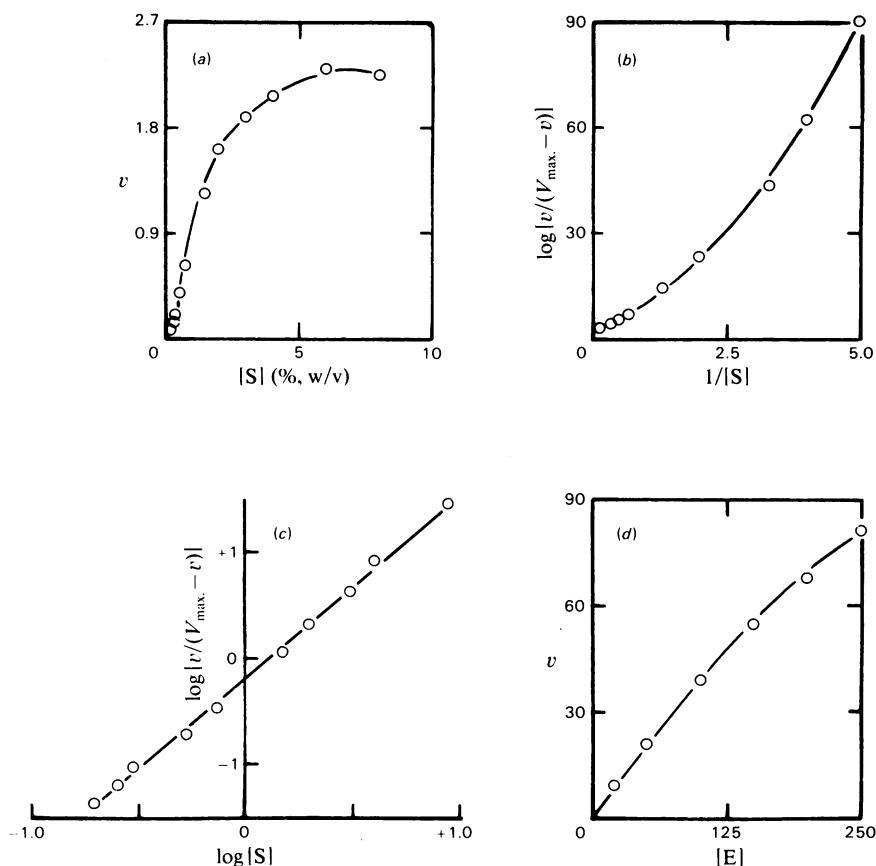


Fig. 5. Kinetics of CM-cellulose hydrolysis by endocellulase III from *Talaromyces emersonii*. For experimental details see the text. (a) Plot of mequiv. of reducing sugar released/min per ml of enzyme as a function of CM-cellulose (low-viscosity material) concentration. From this plot the  $V_{\max}$  value was determined to be 2.34 mequiv./min per ml. (b) Reciprocal plot of the data in (a). (c) Hill plot of the data in (a). (d) Plot of  $\mu\text{g}$  of reducing sugar in a 2ml assay mixture after 30min incubation with the indicated volumes ( $\mu\text{l}$ ) of enzyme.

velocities measured represent true initial rates, since they were obtained while reaction rates remained linear. However, examination of the effects of enzyme concentration versus velocity of the reaction (Fig. 5d) suggests a possible explanation. Thus it can be seen that the increase in activity does not keep pace with the increase in enzyme concentration. Rather, it decreases gradually with the increase in enzyme concentration. This would suggest that the enzyme molecules undergo some form of aggregation at high concentrations and that the aggregated forms are not as active as the monomers. Such a conclusion is not at variance with other findings reported above. Thus we suggest that substrate-induced aggregation, especially at high substrate concentrations, may be a possible explanation for the observed kinetic pattern.

### Synergism

The hydrolysis of crystalline cellulose results from the combined and possibly concomitant actions of endo- and exo-cellulases, the former providing a chain end from which the latter removes cellobiose or, less often, glucose (Wood, 1975, 1980; Wood & McCrae, 1978). The resultant cellobiose, an inhibitor of cellulase action, is then converted into glucose by the  $\beta$ -glucosidase present. In subsequent studies (see, e.g., Wood, 1980; Wood *et al.*, 1984) the ability of endocellulases from one source to show 'cross-synergism' with exocellulases from another source, and vice versa, has been examined in more detail. That some endo-/exo-cellulase pairs, even when from the same source, did not act in this way has also been noted (Wood *et al.*, 1984). In an earlier



Table 3. Synergistic hydrolysis of crystalline cellulose by combined exo- and endo-cellulases of *Talaromyces emersonii*. Samples of the appropriate enzyme combinations were incubated at 60°C with 5% (w/v) Avicel in 0.1M-sodium acetate buffer, pH 5, in sealed tubes. After 75 h the amounts of reducing sugars released were measured.

Enzymes present	Relative amount of reducing sugar released
Exocellulase plus total endocellulase fraction	100.0
Exocellulase plus endocellulase I	87.5
Exocellulase plus endocellulase II	82.7
Exocellulase plus endocellulase III	87.5
Exocellulase plus endocellulase IV	93.5
Exocellulase alone	40.0
Endocellulases alone	0

investigation we demonstrated the synergism between the bulk endo- and exo-cellulase fractions of *Talaromyces emersonii* in the hydrolysis of crystalline cellulose (McHale & Coughlan, 1980). We can now go a step further by saying that, with respect to the degree of synergism shown with the unfractionated exocellulase component, any one of the individual endocellulases produced by this organism is as good as the others, and indeed almost as good as the total endocellulase fraction at the same activity (Table 3). Wood (1980) has considered the possibility that endo- and exocellulases may have to form ternary complexes with the crystalline substrate in order to effect hydrolysis. In view of this, one wonders whether the apparent tendency of each endocellulase to dimerize (see above) may reflect the need to associate with an exocellulase *in vivo*.

### Conclusion

In previous publications we showed that culture filtrates of *Talaromyces emersonii* grown under the conditions outlined above exhibit very little proteinase activity (McHale & Coughlan, 1980) and that multiple forms of endocellulase are present in such filtrates even as early as 24 h (McHale & Coughlan, 1981b). Thus the multiplicity of forms of this enzyme does not appear to be due to proteolysis after secretion. However, since each enzyme is so similar to each of the others in all properties examined, it is difficult to believe that the observed multiplicity is genetically determined. Rather, we are of the opinion that the only difference between these forms is the content or composition of the carbohydrate moiety. This opinion must remain speculative until further investigation has been carried out.

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