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A new appraisal of the endoglucanases of the fungus *Trichoderma reesei*

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The properties and enzymic activity of endoglucanases (EC 3.2.1.4) of the fungus *Trichoderma reesei* were studied by means of immunological methods and by using polyglycosidic substrates. Endoglucanases exist in the culture liquid as a series of immunologically related components. The most active endoglucanase component has an M_r of 43000 and pI value of 4.0. The most abundant components have a value of pI about 5.0, an M_r of 56000–67000 and specific activity only one-fifth of that of the pI-4.0 component. During purification and storage the endoglucanases are spontaneously modified; the relative proportion of components having greater M_r values, more alkaline pI values and lower specific activities is increased. The hexose content of the endoglucanase components is 2–7%. Endoglucanases hydrolyse soluble β-1,4 glycans. The enzymes described here differ from endoglucanase preparations described previously in not showing activity towards insoluble substrates. The role of endoglucanases in wood hydrolysis is consequently limited to the stage where wood constituents are already in soluble form.

INTRODUCTION

The cellulolytic enzymes of the fungus *Trichoderma reesei* have been studied for many years because of their potential value in biotechnology. Their biochemical properties appear to be variable, and this has resulted in the publication of confusing and contradictary information. It is, however, generally accepted that there are three different groups of cellulolytic enzymes, cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21), which all participate in the hydrolysis of cellulose to yield glucose. A synergistic effect in the hydrolysis has been observed between and within different groups of these enzymes. The role of the different enzymes is further confused by the existence of several isoenzyme forms of each of the groups of the cellulolytic enzymes. The continued use of an outdated nomenclature causes further misunderstanding (cf. Enari, 1983).

Our previous studies concerning the cellulolytic system from *Trichoderma reesei* showed that a cellobiohydrolase was able alone to hydrolyse native cotton (Nummi *et al.*, 1983). Moreover, the endoglucanases were active only towards substituted cellulose derivatives and soluble barley β-glucan. They possessed only negligible activity towards pure insoluble amorphous cellulose and did not act synergistically with cellobiohydrolase in this hydrolysis. Thus endoglucanases cannot play an important role in the hydrolysis of pure cellulose.

Accurate knowledge of the biochemical properties of cellulolytic enzymes is essential for the application of genetic-engineering technology to problems related to biomass. The studies presented here concentrate on the purification and properties of the different endoglucanase forms produced by *Trichoderma reesei*. The characteristics of these enzymes were investigated by using immunological methods. The role of endoglucanases in the hydrolysis of cellulose was studied by analysing the activity of endoglucanases towards natural cellulosic materials: native cotton, amorphous cellulose, amorphous spruce and birch pulps, insoluble xylan, soluble glucomannan, polygalactomannan, cellohexaose and cellobiose.

MATERIALS AND METHODS

Preparation of endoglucanase antigen

The mutant strain of VTT-D-80133 *Trichoderma reesei* was cultivated on sulphite cellulose and distiller's spent grain for 3–4 days at 29 °C. The proteins secreted into the cultivation medium were separated into two fractions: the cellobiohydrolase fraction adsorbed on amorphous cellulose (Whatman CF 11, ball-milled; Whatman, Maidstone, Kent, U.K.), and the fraction not adsorbed (Nummi *et al.*, 1981). The latter was concentrated by ultrafiltration (Amicon YM-2 filter and fractionated by chromatofocusing.

PGE 94 ion-exchanger (Pharmacia, Uppsala, Sweden) (20 ml) stabilized in 25 mM-histidine/HCl buffer, pH 6.5, was used to start the focusing. The sample (20–200 mg) was eluted with diluted (1:10) PB 76 buffer (Pharmacia) with a gradient from pH 5.0 to 3.0. Fractions were collected and the activities of cellulolytic enzymes were assayed. The fraction corresponding to the highest specific endoglucanase activity, pI 4.0, was collected and used as an antigen.

Preparation of endoglucanase antiserum

During 3 months' immunization, 1.0 mg of antigen was injected intracutaneously once a month into two rabbits and the sera were collected and pooled. The immunoglobulin fraction containing the antibodies was purified from the sera with precipitation at 0–30% saturation of $(NH_4)_2SO_4$. The precipitate was dissolved in 0.01 M-phosphate buffer, pH 6.8, and subjected to ion-exchange chromatography with 200 ml of Whatman

Abbreviations used: CBH, cellobiohydrolase; HEC, hydroxyethylcellulose; CMC, carboxymethylcellulose; SDS, sodium dodecyl sulphate.

DE 52 ion-exchanger equilibrated with phosphate buffer, pH 6.8 (Livingston, 1974). Elution was performed stepwise by using first 300 ml of the equilibrium buffer and then the same buffer (300 ml) but at 0.05 M. The fractions were analysed by immunodiffusion against endoglucanase pI-4.0 component. Fractions giving the highest titre were used for preparation of immunoadsorbent for immunoaffinity chromatography (Livingston, 1974).

Immunoaffinity chromatography

The immunoadsorbent consisted of 20 ml of CNBr-activated Sepharose 4B gel (Pharmacia) to which 200 mg of purified specific endoglucanase antibodies were bound (Livingston, 1974). The culture liquid of *Trichoderma* was adsorbed on the gel at pH 7.0 in 50 mM-histidine/HCl buffer containing 0.5 M-NaCl.

Non-specifically bound proteins were eluted with 0.1 M-sodium acetate buffer, pH 4.5, containing 0.5 M-NaCl. Endoglucanase was released at pH 5.0 with 4 M-$MgCl_2$, dialysed against 50 mM-citrate buffer, pH 5.0, and concentrated by ultrafiltration (Amicon YM 2 membrane). Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as standard. The capacity of the immunoadsorbent column was 2.7 mg of endoglucanase protein.

Activity determinations

Determinations were performed in 50 mM-sodium citrate buffer, pH 5.0, as described previously (Nummi *et al.*, 1983). CBH activity was measured nephelometrically with amorphous cellulose as substrate. The same method was used for measuring activity towards insoluble birch xylan (Nummi *et al.*, 1985) and ball-milled spruce and birch pulp (The Finnish Pulp and Paper Research Institute, Espoo, Finland).

Endoglucanase activity was measured with HEC (D.S.O.9; Fluka, Buchs, Switzerland) as substrate, using the dinitrosalicylic acid method and calculating activities on the basis of liberation of reducing sugars measured as glucose (Nummi *et al.*, 1983).

Activity towards barley β-glucan (Biocon, Eardiston, Tenbury Wells, Worcs., U.K.), glucomannan (Serva, Heidelberg, Germany), polygalactomannan (Baker, Deventer, The Netherlands) and laminarin (Roth, Karlsruhe, Germany), was measured by the same method.

Endoglucanase activity was also measured with CMC (D.S.0.88 type 7H3SXF; Hercules, Wilmington, DE, U.S.A.) as substrate in a viscosimetric method (Almin *et al.*, 1967).

β-Glucosidase activity was measured with cellobiose (Fluka) as substrate and determination of the glucose liberated with GOD-Perid reagent (Boehringer, Mannheim, Germany) by glucose oxidase.

Activity towards cellohexaose (Nummi *et al.*, 1983) was measured from the hydrolysis products by liquid chromatography (Optilab 931 HSRI apparatus equipped with Miltiref 902C; Tecator, Vällingby, Sweden) on an Aminex HPX-87C column in water at 65 °C.

Endoglucanase and β-glucosidase activities are expressed as katals (1 kat is the amount of enzyme that can catalyse the transformation of 1 mol of substrate/s under the conditions specified).

Analysis of hydrolysis products

The hydrolysis products from amorphous cellulose, insoluble xylan, spruce and birch pulp and glucomannan after 20 h hydrolysis by endoglucanase were analysed by liquid chromatography. Hydrolysis products from CMC, HEC and barley β-glucan were analysed by gel chromatography on a column (150 ml) of Sephacryl S-200 (Pharmacia) at pH 7.00 in 0.05 M-sodium phosphate buffer containing 0.5 M-NaCl. The carbohydrate content of fractions was analysed by the anthrone/H_2SO_4 method (Whistler & Wolfrom, 1962). The column was calibrated with Blue Dextran (Pharmacia), cellobiose (Fluka) and glucose (Marck, Darmstadt, Germany). The presence of glucose was confirmed by use of the GOD-Perid reagent. Hydrolysis of native cotton (Suomen Vanutehdas, Jokela, Finland) was studied by analysing the formation of short fibres by measurement of A_{620} during 20 days' incubation. The soluble sugars released into the medium were determined by the anthrone/H_2SO_4 method from the supernatant. The insoluble remainder was dried at 104 °C and weighed.

Carbohydrate determination

The amount of carbohydrate in the purified endoglucanase was determined by the anthrone/H_2SO_4 method.

Chromatographic methods

The purified endoglucanase was fractionated on a column of Sephacryl S-200 (Pharmacia) under the same conditions at pH 7.0 as were used for the hydrolysis products. The column was calibrated with a mixture containing Blue Dextran (Pharmacia), human serum albumin (Sigma), ovalbumin (Schwarz/Mann, Orangeburg, NY, U.S.A.) and cytochrome *c* (Sigma). Affinity chromatography for glucoproteins were performed with a 1 ml column of concanavalin A (Pharmacia) equilibrated with 0.05 M-phosphate buffer, pH 7.2, containing 0.5 M-NaCl, 5 mM-$CaCl_2$ and 5 mM-$MnCl_2$. The elution was performed with 10 mM-α-methyl D-mannoside (Sigma).

Electrophoretic methods

Electrophoresis was performed on 10%-(w/v)-polyacrylamide slabs gel at pH 8.5 with Tris/HCl buffer according to Laemmli (1970), but without SDS.

The activity was demonstrated with overlay plates containing 1% HEC and 1% agarose. After incubation for 2 h at 50 °C the substrate plate was washed for 30 min in haemoglobin solution (haemolysed red cells; Finnish Red Cross Blood service) and for 10 min with water. The activity was seen as red–brown spots where haemoglobin was bound to hydrolysis products formed by glucanase activity.

The separation on acrylamide gel was analysed by immunodiffusion against the antiserum prepared against the pI-4.0 endoglucanase. The acrylamide-gel strips containing the proteins separated by electrophoresis were embedded in agarose in barbital buffer, pH 8.2. The antisera were applied in wells cut alongside the acrylamide gel. The diffusion and washing steps were identical with those used after conventional immunoelectrophoresis. In those experiments where enzymes in the culture medium were compared with the purified enzyme components, electrophoresis was performed for

the sample of culture medium as described above. However, after they had been embedded in agarose the purified enzyme samples were added to holes bored in appropriate places on the agarose and antiserum was added as described above.

Proteins were stained with Coomassie Brilliant Blue R-250 (Serva) (Diezel *et al.*, 1972) and silver stain (Bio-Rad, Utrecht, The Netherlands) (Merril *et al.*, 1981).

Electrophoresis under denaturating conditions

Electrophoresis was carried out in a 10%-polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). The low-M_r calibration mixture (Pharmacia) was used as reference.

Attempts to eliminate the heterogeneity of endoglucanase preparation

Elimination of possible sialic acid residues was performed with neuraminidase (from *Clostridium perfringens*; Boehringer) in 0.1 M-sodium acetate buffer, pH 4.5, at room temperature for 20 h (Cummings *et al.*, 1983). Phosphoric acid residues were eliminated with alkaline phosphatase (from calf intestine; Boehringer) in 0.1 M-Tris/HCl buffer, pH 8.5, at room temperature for 20 h (Hashimoto *et al.*, 1981). Reduction of disulphide bridges was performed by adding 0.05 M-iodoacetamide to the sample buffer before SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

$NaBH_4$ treatment to eliminate the carbohydrate was performed by using 0.3 M-$NaBH_4$ in 0.1 M-NaOH for 20 h at 45 °C. The reaction was terminated by addition of acetic acid (Bertolini & Pigman, 1967).

Oxidation with periodate was performed with 0.12 M-sodium periodate in 40 mM-sodium acetate buffer, pH 5.0, in the dark at 4 °C for 20 h. The reaction was terminated by adding ethylene glycol in 11-fold excess over periodate (Spiro, 1966). After treatment, the endoglucanase was analysed by SDS/polyacrylamide-gel electrophoresis.

RESULTS AND DISCUSSION

Purification procedures

The culture liquid of *Trichoderma reesei* contains four electrophoretically distinct proteins that hydrolyse HEC (Fig. 1*a*, arrows). To separate these from each other, chromatofocusing chromatography was performed by

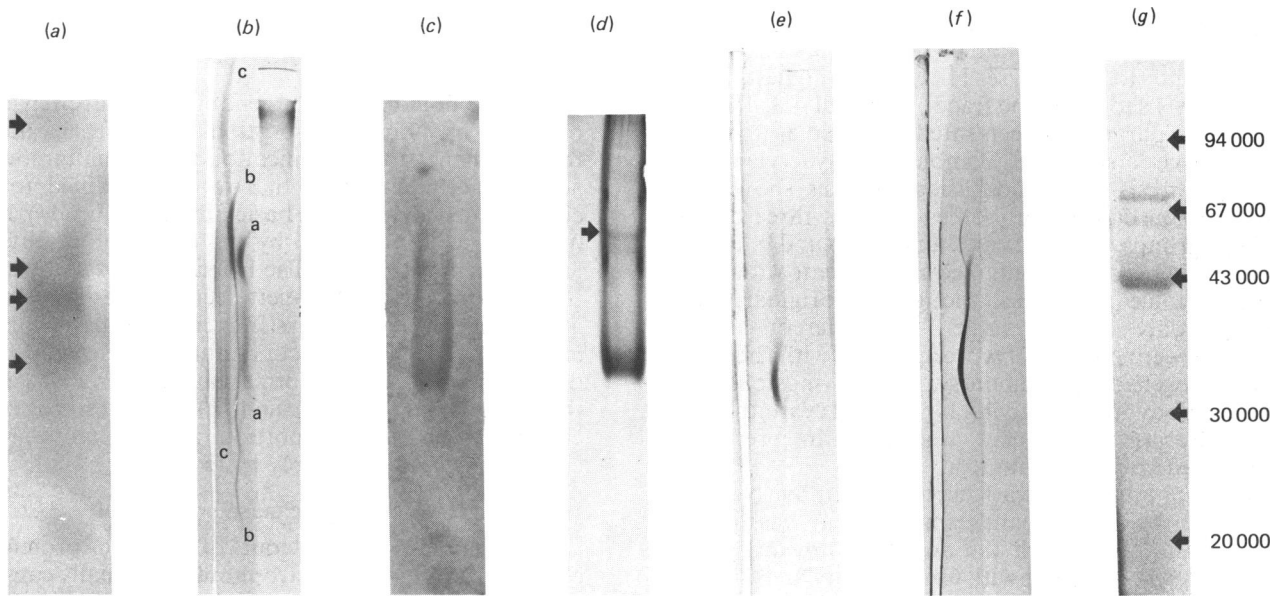

Fig. 1. Electrophoresis of endoglucanase components

(*a*) Polyacrylamide-gel electrophoresis on 10%-polyacrylamide gel, pH 8.5. The sample was culture liquid (100 μg of protein); activity determination was with a 1%-HEC overlay plate for 2 h at 50 °C. Precipitation of reaction products was with haemoglobin solution. Arrows show the active zones: (*b*) Combined polyacrylamide-gel electrophoresis–immunodiffusion: polyacrylamide-gel electrophoresis was on 10%-polyacrylamide gel at pH 8.5; gel strips were embedded in agarose at pH 8.2 (0.025 M-barbital buffer) and analysed by immunodiffusion against antiserum evoked by endoglucanase pI-4.0 component. The sample was culture liquid (100 μg of protein). Proteins were stained with Coomassie Brilliant Blue. Distinct protein arcs are indicated by a, b and c. (*c*) Polyacrylamide-gel electrophoresis on 10%-polyacrylamide gel at pH 8.5. The sample was endoglucanase preparation after immunoadsorption (15 μg of protein); activity determination was with 1% HEC (as in *a*). (*d*) Polyacrylamide-gel electrophoresis on 10%-polyacrylamide gel at pH 8.5. The sample was endoglucanase preparations after immunoadsorption (15 μg of protein); the gel was silver-stained. The arrow points to the cathodic impurity in the preparation. (*e*) Polyacrylamide-gel electrophoresis-immunodiffusion: endoglucanase preparation after immunoadsorption (15 μg of protein); staining was with Coomassie Brilliant Blue. (*f*) Polyacrylamide-gel electrophoresis–immunodiffusion: endoglucanase after immunoadsorption and chromatofocusing; fraction with pI 4.0 (25 μg of protein); staining was with Coomassie Brilliant Blue. (*g*) SDS/polyacrylamide-gel electrophoresis: endoglucanase after immunoadsorption and chromatofocusing (fraction with pI 4.0; 40 μg); staining was with Coomassie Brilliant Blue. The M_r value was calculated from Pharmacia low-M_r standards (indicated by arrows).

Table 1. Fate of endoglucanase activity during purification

Results are calculated from 15 purification batches. Values correspond to the purification of 16 mg of crude culture protein collected from six successive immunoaffinity chromatography runs. Endoglucanase activity was determined with HEC as substrate; the protein content was determined by the method of Lowry *et al.* (1951).

Purification step	Total protein (mg)	Protein yield (%)	Total activity (nkat)	Activity yield (%)	Specific activity (nkat/mg)	Purification (fold)
Culture liquid	1200		192000		160	
Immunoaffinity chromatography	16	1.3	10432	5.4	652	4.0
Gel chromatography*	6.7	0.6	4288	2.2	640	4.0
Chromatofocusing chromatography*						
pI 6.5	0.33	0.03	0	0	0	
pI 5.0	5.92	0.5	403	0.2	68	0.4
pI 4.0	5.45	0.4	2305	1.2	423	2.6
Affinity chromatography for glucoproteins (pI-4.0 component)						
Non-carbohydrate fraction	0.53	0.04	53	0.03	101	0.6
Carbohydrate fraction	0.46	0.04	90	0.05	195	1.2

* Chromatography was performed on the preparation obtained after immunoadsorption.

using culture liquid from which CBH had been eliminated by adsorption on amorphous cellulose (Nummi *et al.*, 1983). Eight different fractions, separated by chromatofocusing and having pI values between pH 6.5 and 4.0, were found to hydrolyse HEC. The highest specific activity was shown by the fraction with pI 4.0. This fraction was separated, concentrated and used as an antigen to produce antiserum. Combined polyacrylamide-gel electrophoresis–immunodiffusion analysis showed that this fraction evoked antibodies towards three different protein groups of the culture liquid (a, b and c, Fig. 1*b*). Each of these groups contained components with heterogeneous ionic properties, though the original antigen showed only one pI value. The different antibody fractions were prepared by precipitation with $(NH_4)_2SO_4$ and ion-exchange chromatography (Livingston, 1974). The antibody fraction giving highest titre with the pI-4.0 endoglucanase component was used to prepare an immunoadsorbent (Livingston, 1974).

By using immunoaffinity chromatography (Nummi *et al.*, 1983), an endoglucanase preparation was obtained that did not react with the antiserum towards CBH I (Nummi *et al.*, 1983), or with CBH II or xylanase antisera prepared previously in our laboratory. This shows that endoglucanase is free of the above-mentioned enzyme contaminants and that it is a clearly different enzyme from them.

The endoglucanase gave one active band on electrophoresis (Fig. 1*c*). Sensitive protein staining revealed, however, the presence of a more-cathodic component (Fig. 1*d*, arrow) in the preparation. On immunoelectrophoresis the heterogeneity of the purified endoglucanase preparation is seen as an elongation of the immunoarc towards the cathode (Fig. 1*e*).

To eliminate the cathodic component from the endoglucanase preparation, different fractionation methods were used after immunoadsorption, namely gel chromatography, chromatofocusing and affinity chromatography for glucoproteins. Even if all these steps were performed successively in the same experiment, final homogeneity of the endoglucanase protein was never obtained, but the endoglucanase was gradually inactivated. Endoglucanase was fractionated in all the chromatographies tested into a number of components with low specific activity and one with higher specific activity (Table 1).

It was noted that the endoglucanase preparation obtained by immunoadsorption (Figs. 1*c*, 1*d* and 1*e*) retained in the further purification all the immunologically active determinants but that it was modified to yield components having more basic pI values (Fig. 1*f*), higher M_r values (as measured by gel chromatography) and lower specific activities. The heterogeneity was also seen on SDS/polyacrylamide-gel electrophoresis performed after each purification step (Fig. 1*g*). The modification of endoglucanase also occurs during cultivation: the different endoglucanase forms can already be detected in the culture liquid (Figs. 1*a* and 1*b*). During storage of the culture liquid the proportion of the more-alkaline components was noticeably increased.

Heterogeneity of endoglucanase preparation

The anomalous behaviour of purified proteins on chromatography and electrophoresis is usually caused by sialic acid, phosphoric acid, thiol groups or carbohydrate constituents (Cummings *et al.*, 1983; Hashimoto *et al.*, 1981).

The carbohydrate content of the endoglucanase preparation after immunoadsorption was 2–7%, as determined by the anthrone reaction. To eliminate this and the other possible agents causing heterogeneity, more conventional methods were used. The endoglucanase preparation was treated with neuraminidase, alkaline phosphatase, reduced with mercaptoethanol in the presence of iodoacetamide, reduced with borohydride under alkaline conditions and, on the other hand, oxidized with periodate. However, even with this conventional procedure, considerable heterogeneity was still present.

The endoglucanases in the culture liquid were shown to migrate together with two other proteins (Fig. 1*b*, arcs a, b and c). Immunological comparison between these

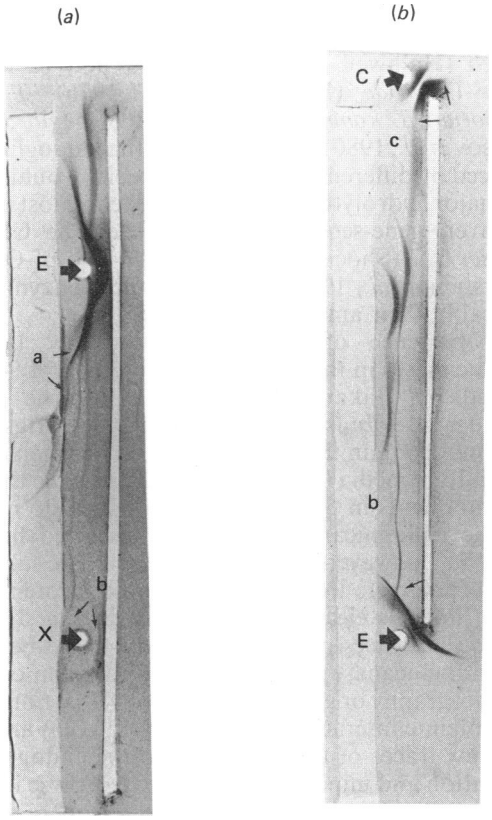

Fig. 2. Identification of the endoglucanase among the contaminating proteins of unfractionated culture liquid

Combined polyacrylamide-gel electrophoresis–immunodiffusion was used. Polyacrylamide-gel electrophoresis was on 10%-polyacrylamide gel at pH 8.5; the sample was culture liquid containing 100 μg of protein. (*a*) After the gel strips had been embedded, purified enzymes were added (thick arrows): E, endoglucanase pI-4.0 component (24 μg); X, xylanase (7 μg). (*b*) After the gel strips had been embedded, purified enzymes were added (thick arrows): E, endoglucanase 24 μg; C, CBH II (12 μg). Distinct protein arcs from the culture liquid are indicated by a, b, c. Thin arrows point to the areas showing immunological identity (between a and E, between c and C) and non-identity (between b and X, between b and E). Proteins were stained with Coomassie Brilliant Blue.

proteins from the culture medium and endoglucanase component with pI 4.0 and purified CBH II and xylanase fractions (preparations from our laboratory) showed (Figs. 2*a* and 2*b*) that arc a represents the endoglucanase; arcs b and c are contaminating components present also in the xylanase and CBH II preparations. The similar physicochemical properties of the contaminating proteins make the purification of endoglucanase extremely difficult by methods based on ionic properties and molecular size, as has been discussed elsewhere (Sprey & Lambert, 1983). Endoglucanase preparations will always contain these contaminants if more-rigorous methods are not used.

Hydrolytic activity of endoglucanase

Table 2 shows the activities of the endoglucanase preparation prepared by immunoadsorption towards some soluble carbohydrates containing β-linkages. The endoglucanase fractions from different purification steps gave the same activity pattern with the substrates used. Chromatography of the end products after hydrolysis of HEC and CMC and barley β-glucan on Sephacryl gel show that endoglucanase attacks its substrate by splitting the inner β-1,4 linkages. This is seen in the decrease of the molecular size of the substrate as a function of hydrolysis time (Fig. 3). At the final stage of reaction, the amount of glucose produced by different endoglucanase fractions suggested that there were no significant differences in the relative amounts of end products formed. It is seen (Table 2) that barley β-glucan is a more accessable substrate for endoglucanase than are the commonly used substituted celluloses. Only β-1,4 linkages were hydrolysed; laminarin, which contains only β-1,3 linkages, was not attacked. In addition, the heteropolysaccharide containing glucose, glucomannan, was hydrolysed, whereas polygalactomannan was not. Liquid chromatography of the end products after hydrolysis of glucomannan revealed the presence of cellobiose and glucose. Cellohexaose was hydrolysed to yield these sugars at the same rate as were polysaccharides, but cellobiose was not hydrolysed.

In 10 min the endoglucanase component with pI 4.0 did not detectably hydrolyse insoluble polysaccharides (Table 3). When the hydrolysis time was extended to 20 h,

Table 2. Activity of endoglucanase towards soluble β-glycans

The enzyme preparation was endoglucanase after immunoadsorption. Activities were determined with viscosimetry (1), as reducing groups with glucose as standard (2), with liquid chromatography, calculated from cellobiose (3), as 3, but calculated from glucose (4), and with glucose oxidase (5).

Substrate	Concn. (mg/ml)	Method of activity determination	Activity (nkat/mg)
CMC	2.6	1	16
HEC	10	2	652
Barley β-glucan	1	2	1253
Glucomannan	1	2	313
Polygalactomannan	1	2	0
Laminarin	1	2	0.1
Cellohexaose	0.2	3	292
		4	83
Cellobiose	8.8*	5	0.05

* mM, not mg/ml.

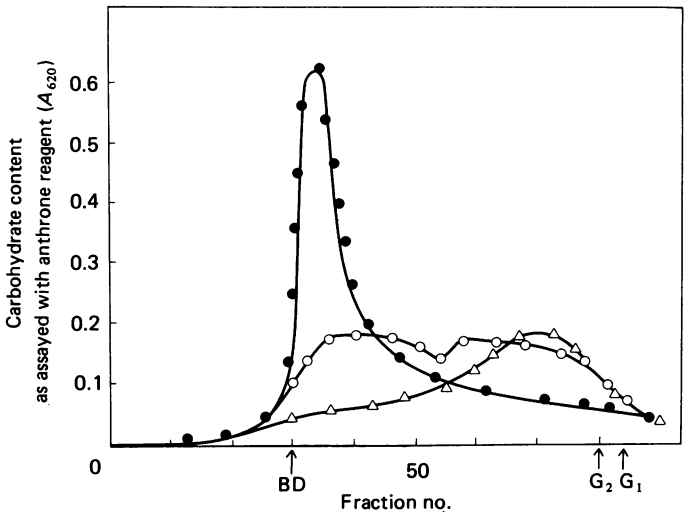

Fig. 3. Gel chromatography of end products after hydrolysis of CMC

The column (150 ml) contained Sephacryl S-200 in 0.05 M-sodium phosphate/0.5-M NaCl, pH 7.0. The sample was 60 mg of CMC hydrolysed by 2.5 μg of endoglucanase preparation after immunoadsorption. ●, Non-hydrolysed substrate; ○, hydrolysate after 10 min; △, after 20 h. Carbohydrate determination was with the anthrone/H_2SO_4 reagent. The elution volumes of Blue Dextran (BD), cellobiose (G_2) and glucose (G) are indicated by arrows.

activities 10^5-fold less than those obtained with soluble substrates were observed. Ball-milled spruce and birch pulps gave traces of cellobiose after 7 days' hydrolysis. Even during 20 days' hydrolysis of native cotton, neither short fibres nor soluble sugars could be detected. In the hydrolysis of amorphous cellulose or native cotton, no synergy was observed between any or all of the endoglucanase components pI 4.0–6.5 prepared in this work and cellobiohydrolase I or II.

The inability of endoglucanase to hydrolyse insoluble substrates alone or to enhance the hydrolysis rate in cooperation with the other cellulolytic enzymes is contradictory to previous observations on endoglucanases from various *Trichoderma* species, e.g. *Trichoderma koningii* (Halliwell & Riaz, 1970; Wood & McCrae, 1978), *T. viride* (Berghem *et al.*, 1976), *T. reesei*, *T. lignorum*, *T. koningii*, *T. viride* and *T. longibrachiatum* (Klyosov *et al.*, 1980). The possibility that endoglucanases produced by different *Trichoderma* species would differ in their major hydrolytic characteristics seems most unlikely. Moreover, gene-sequencing analysis of the CBH I of *T. reesei* L27 (Shoemaker *et al.*, 1983) and of QM 9414 (Fägerstam *et al.*, 1984) has shown these enzymes to be identical in their amino acid sequence.

The presence of two immunologically unrelated endoglucanases in the enzyme of *T. reesei* QM 9414 was reported by Håkansson *et al.* (1979). The purified endoglucanase obtained in the present work (Figs. 1*c* and 1*e*) did not contain the most cathodic component present in the culture media (Fig. 1*a*). It is thus possible that there are components in the culture liquid which do not react with the endoglucanase antiserum prepared here, but which do, however, hydrolyse HEC. The ability of endoglucanases to interact with the other proteins of the culture liquid is clearly demonstrated in Fig. 1(*b*). It is obvious that traces of proteins b and c could be present in the endoglucanase preparation purified by ion-exchange chromatography or gel chromatography. It is noteworthy that protein c interacts with CBH II (Fig. 2*b*) and could thus draw traces of this enzyme into the endoglucanase preparation and impart the ability to hydrolyse insoluble cellulose. The endoglucanase purified here has no significant role in the hydrolysis of insoluble wood constituents. It is, however, noteworthy that soluble polysaccharides with similar configuration to the hemicellulose fractions are readily hydrolysed by this endoglucanase.

Concluding remarks

The endoglucanases of *Trichoderma reesei* exist as a series of immunologically related components. These were shown to be one and the same endoglucanase modified and in interaction with the other components of the culture liquid. These endoglucanase components seem to represent the previously described 'isoenzyme' forms of endoglucanase. The molecular properties [molecular size, relative specific activity, ratios of carbohydrate contents and pI values (Okada *et al.*, 1968; Shoemaker

Table 3. Hydrolysis of insoluble β-glycans by endoglucanase pI-4.0 component

End products were analysed by liquid chromatography (1) and by the anthrone/H_2SO_4 method (2).

Substrate	Method of analysis	Hydrolysis time	Hydrolysis products	Hydrolysis products (μg/20 μg of enzyme)	Activity (nkat/mg of enzyme)
Amorphous cellulose	1	10 min	None		
		20 h	Cellobiose	32	0.06
			Glucose	16	0.06
Insoluble xylan	1	10 min	Xylobiose	26	7
		20 h	Xylobiose	45	0.1
			Xylose	67	0.3
Ball-milled birch pulp	1	7 days	Cellobiose	< 10	
Ball-milled spruce pulp	1	7 days	Cellobiose	< 15	
Natural cotton	2	20 days	Soluble sugar	7	

& Brown, 1978; Wood & McCrae, 1978; Håkansson *et al.*, 1979)] reported for the 'isoenzymes' are in agreement with the properties described here for the modification products. It is noteworthy that the endoglucanase component showing here the highest specific activity (pI-4.0 component, M_r 43000) has only seldom been described (Shoemaker & Brown, 1978), whereas the components with M_r 56000–67000 are more often reported in the literature (Okada *et al.*, 1968; Gong *et al.*, 1979; Håkansson *et al.*, 1979). Possible explanations are the differences in the purification procedures and in the age of the culture liquids, which will affect the relative composition of modification products. For example, during chromatofocusing, the endoglucanase preparation was 65% modified into the components with pI 5.0–6.5 (representing M_r 56000–67000), and in the aged culture liquids only the more-alkaline components were present.

The amino acid compositions published for different purified endoglucanase preparations of *Trichoderma reesei* (Okada *et al.*, 1968; Håkansson *et al.*, 1979; Shoemaker & Brown, 1978; Bhikhabhai & Petterson, 1984) give no clue to the anomalous behaviour of these proteins in purification procedures. The content of acidic and basic amino acids are similar in the endoglucanase components. Nevertheless, the high content of acidic and hydroxylated amino acids suggest that other proteins may interact with the enzymes studied to complicate interpretation of the results.

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