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PURIFICATION AND PROPERTIES OF TWO ENZYMES FROM *Dichomitus squalens* WHICH EXHIBIT BOTH CELLOBIOHYDROLASE AND XYLANASE ACTIVITY

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ABSTRACT

Two fractions (Ex I and II), exhibiting activity towards *p*-nitrophenyl β-cellobioside (pNPC) have been isolated by chromatofocusing of the proteins obtained from the supernatant solution of a cellulose-containing culture of the white-rot fungus *Dichomitus squalens*. They were further purified up to 16.0- and 14.2-fold by chromatography on Phenyl-Sepharose CL-4B and ion-exchange chromatography on DEAE-Trisacryl. Each purified enzyme gave a single peak for protein and activity on chromatography on Ultrogel AcA-54 and a single protein band in disc gel electrophoresis, in the absence and presence of sodium dodecyl sulphate, and on isoelectrofocusing. The mol. wts. of Ex I and II were 39,000 and 36,000, respectively, and their isoelectric points were 4.6 and 4.5, respectively. Maximum activity towards pNPC was shown at pH 5.0 and 60°, and each enzyme was stable over the pH range 4.0–8.0, and up to 70° and 60° for Ex I and II, respectively. The enzymes cleaved pNPC, released mainly cellobiose from cellulose, were especially active towards xylan and *o*-nitrophenyl β-D-xylopyranoside, and exhibited strong transglycosylating activities.

INTRODUCTION

The extensive degradation of native (crystalline) cellulose is achieved mainly by the cooperative action of endo-glucanases [(1→4)-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4] and exo-glucanases [(1→4)-β-D-glucan-4-cellobiohydrolase, EC 3.2.1.91][1]. These enzymes are produced extracellularly by certain cellulolytic fungi when cultivated on cellulose or soluble inducers, and occur in many isoenzymic forms[2–4].

The detection and quantification of individual components from a cellulolytic

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complex involves the use of specific substrates. Soluble derivatives of cellulose, which allow viscometric measurement, are currently used[5] for endo-glucanases. Cellobiohydrolase (CBH) activity is more difficult to measure since cellobiose released by its action is also an end-product from the action of endo-glucanases. A method using *p*-nitrophenyl β-cellobioside (pNPC), recently developed[6], was suitable for the detection and measurement of the activity of CBH from *Sporotrichum pulverulentum* and *Trichoderma reesei*.

We have shown that activity towards pNPC occurred in a culture supernatant from a cellulose-containing liquid culture of the white-rot fungus *Dichomitus squalens*[7] and now report the fractionation of the extracellular proteins.

EXPERIMENTAL AND RESULTS

A "crude enzyme preparation" was obtained[7] from an avicel-containing culture of *D. squalens* (Karst) Reid CBS 432-34 and consisted essentially of ammonium sulphate-precipitated (80% saturation) and freeze-dried material from the cell-free supernatant. Xylan from larchwood (xylose:acidic residues 1:0.09)[8], carboxymethylcellulose (CM-cellulose, medium viscosity), dextran, cellobiose, arabinogalactan from larchwood, and lichenin from *Cetraria islandica* were purchased from Sigma, laminarin from *Laminaria sp.* from Fluka, and polygalacturonic acid from ICN. Arabinoglucuronoxylan (xylose:arabinose:acidic residues 1:0.13:0.04) was a gift from Dr. J. M. Brillouet (INRA-Nantes), barley glucan was a gift from Dr. B. Stone (La Trobe University, Australia), and waxy-maize beta-limit dextrin was a gift from Dr. P. Colonna (INRA-Nantes).

Carboxymethylcellulose–azure (CMC-azure) was prepared as described by Leisola *et al.*[9], except that the time of contact between CM-cellulose and Remazol Brilliant Blue was increased to 3 h. H_3PO_4-swollen cellulose was prepared according to the method of Wood[10] from Whatman cellulose CC41. Cello-oligosaccharides were prepared[11] by acetolysis of dewaxed cotton fibres and fractionated using preparative h.p.l.c. [R Sil C18 HL (Alltech Europe), elution with water].

Polybuffer 74, polybuffer exchanger (PBE) 94, and Phenyl-Sepharose CL-4B were purchased from Pharmacia (Sweden), DEAE-Trisacryl from IBF (France), Ultrogel AcA-54 from LKB (Sweden), and collagen dialysis bags from Soussana S.A. (France). Other chemicals used were of analytical grade.

Enzyme assays. — Activity towards *p*-nitrophenyl β-cellobioside (pNPC), *p*-nitrophenyl β-D-glucopyranoside (pNPG), and *o*-nitrophenyl β-D-xylopyranoside (oNPX) was measured by incubating 0.1 mL of appropriately diluted enzyme with 1 mL of mM substrate in 0.05M sodium acetate buffer (pH 5.0) at 40° for 60 min. The reaction was stopped by the addition of 2 mL of M Na_2CO_3. Absorbance was measured at 400 nm, and activity was determined using a *p*-nitrophenol calibration curve and expressed as nkat (1 nkat of enzyme releases 1 nmol of product per s).

The activity on various polysaccharides and oligosaccharides was determined by measuring[12] the amount of sugar liberated: 1 mL of a 0.5% solution or suspen-

sion (1% for CM-cellulose, 0.02% for cello-oligosaccharides) of substrate in 0.05M sodium acetate buffer (pH 5.0) was incubated with 0.05 mL of diluted enzyme at 40° for 30 min. Reducing sugar was determined as liberated sugar equivalent, and activity was expressed as nkat.

Activity towards CMC-azure was measured as follows: 0.5 mL of 1% CMC-azure solution in 0.05M sodium acetate buffer (pH 5.0) was mixed with 0.05 mL of diluted enzyme and incubated at 40° for 30 min. The reaction was stopped by adding 1 mL of cold ethanol. The mixture was then centrifuged (9000*g*, 2 min) and the absorbance of the supernatant solution was measured at 595 nm. The activity was expressed as arbitrary units (AU) representing the amount of enzyme which increased the absorbance at 595 nm by 0.1 per min.

Protein determination. — Column chromatography was monitored at 280 nm. Protein concentration was measured by the method of Bradford[13] during the enzymic purification steps and by the method of Lowry[14] for the determination of specific activity towards various substrates. Bovine serum albumin was used as standard.

Electrophoresis and isoelectric focusing. — Disc gel electrophoresis was carried out in glycine–Tris buffer (pH 8.3), using 6% acrylamide gel, at 8 mA per gel (6.5 cm) at 25°.

Sodium dodecyl sulphate (SDS) disc gel electrophoresis was performed in sodium phosphate buffer (pH 7.2) containing 0.1% of SDS, using 5% acrylamide gel, at 2.5 mA per gel (6.5 cm) at 25°. Samples were dissolved in 0.08M sodium phosphate buffer (pH 7.2) containing 1.7% of SDS, 3% of 2-mercaptoethanol, and 80% of glycerol, and heated at 90° for 3 min. Cytochrome C (mol. wt. 12,500), trypsin inhibitor (21,500), pepsin (35,000), ovalbumin (45,000), and bovine serum albumin (68,000) were used as standards.

Analytical isoelectric focusing was performed on IEF agarose in the pH range 3–10. After focusing, pieces (0.5 cm) cut from the edge of the gel were incubated overnight at 4° in distilled water (1 mL). The pH of the supernatant solutions was measured and the pH gradient thus determined.

Proteins were stained with Coomassie Brilliant Blue, and gels destained with methanol–acetic acid–water.

Purification of enzymes. — All steps of purification were carried out at 4° (Table I).

(a) Chromatofocusing on PBE-94. A solution of an aliquot of the crude enzyme preparation (100 mg of protein) in 20 mL of 25mM imidazole-HCl buffer (pH 7.2) was dialysed overnight in collagen bags against the same buffer. The sample was then applied to a column (50 × 1.0 cm) of PBE-94 equilibrated with the same buffer. Bound proteins were eluted with 450 mL of 8-fold diluted polybuffer 74 adjusted to pH 3.7. Remaining bound proteins were removed by a linear gradient (100 mL) of 0→M sodium chloride in the polybuffer. Fractions (~5 mL) were assayed for pH, absorbance at 280 nm, and activity towards pNPC, pNPG, cellobiose, xylan, and polygalacturonic acid.

TABLE I

PURIFICATION OF TWO ENZYMES FROM *Dichomitus squalens*, WHICH ARE ACTIVE TOWARDS pNPC

Step		*Protein*[a] *(mg)*	*Total activity (nkat)*	*Specific activity (nkat.mg^{-1})*	*Yield (%)*	*Purification (fold)*
Crude enzyme		100	1420	14.2	100	1
Chromatofocusing on	Ex I	3.62	341	94.2	24.0	6.6
PBE 94	Ex II	6.08	295	48.5	20.8	3.4
Hydrophobic-interaction	Ex I	2.34	330	141.0	23.2	9.9
chromatography on	Ex II	2.52	126	50.0	8.9	3.5
Phenyl-Sepharose CL 4B						
Ion-exchange	Ex I	1.08	250	231.5	17.6	16.3
chromatography on	Ex II	0.38	73.8	194.2	5.2	13.7
DEAE-Trisacryl						
Gel filtration	Ex I	0.95	216	227.4	15.2	16.0
on Ultrogel AcA-54	Ex II	0.36	72.4	201.1	5.1	14.2

[a]Determined by the method of Bradford.

Fig. 1 shows a typical elution profile. Three peaks (Ex I–III) of activity towards pNPC were eluted at pH 5.6, 5.4, and 4.3, respectively. Ex III was overlapped by a peak of activity towards both pNPG and cellobiose (ratio of activity towards pNPG to activity towards cellobiose, 11.4). A peak of polygalacturonase activity was also associated with Ex III. Peaks of xylanase activity were also superimposed on those of Ex I–III. The ratio of activity towards pNPC to activity towards xylan was 0.12 for both Ex I and II, and 0.13 for Ex III. Ex I and II were combined as shown in Fig. 1, and purified further. The chromatofocusing step gave a 6.6- and 3.4-fold purification for Ex I and II, respectively.

(b) Chromatography on Phenyl-Sepharose CL-4B. Fractions Ex I and II from (*a*) were adjusted to pH 5.0 with acetic acid. Solid ammonium sulphate was added to M. Each sample was applied to a column (20 × 0.8 cm) of Phenyl-Sepharose CL-4B equilibrated with 25mM sodium acetate buffer (pH 5.0) containing M ammonium sulphate at 50 mL/h. Bound proteins were eluted by a linear gradient (200 mL) of 1→0M ammonium sulphate. Fractions (~5 mL) were assayed for absorbance at 280 nm and activity towards pNPC.

The elution profiles of Ex I and II are shown in Fig. 2. Enzymes active towards pNPC were eluted at 0.15 and 0.10M ammonium sulphate, respectively, for Ex I and II. Ex I was purified 1.5-fold by this step, with 97% recovery of activity, whereas the specific activity of Ex II did not increase since an early denaturation caused a loss of nearly 60% of activity and protein.

(c) Chromatography on DEAE-Trisacryl. The two fractions from (*b*) were dialysed overnight in collagen bags against 5 L of 10mM imidazole-HCl buffer (pH 7.5) and then each was applied to a column (10 × 0.9 cm) of DEAE-Trisacryl equilibrated with the same buffer at a flow rate of 25 mL/h. Bound proteins were eluted by a linear gradient (100 mL) of 0→0.4M sodium chloride in the starting

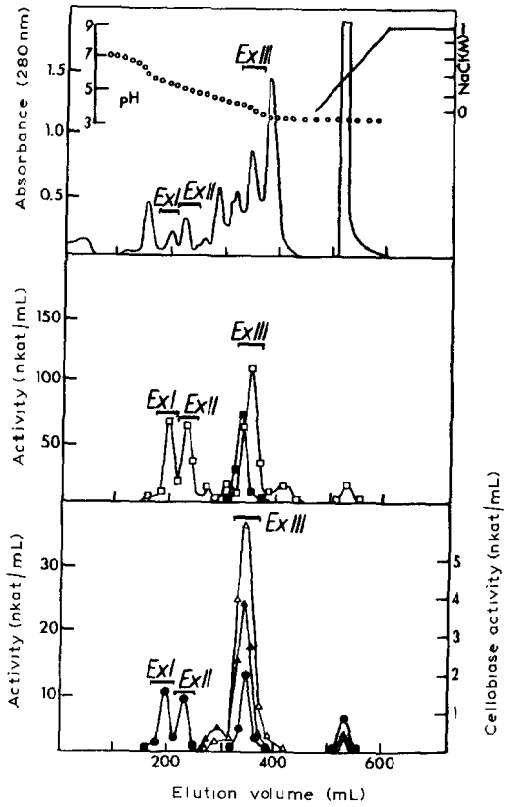

Fig. 1. Chromatofocusing on PBE-94 of the crude enzyme preparation from *D. squalens*: pH, ∞∞; absorbance at 280 nm, ———; activity towards pNPC (—●—), pNPG (—△—), cellobiose (—▲—), polygalacturonic acid (—■—), and xylan (—□—) were assayed.

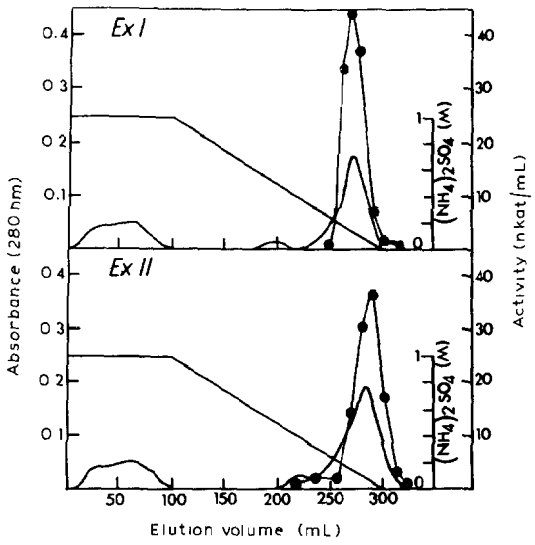

Fig. 2. Chromatography of Ex I and II on Phenyl-Sepharose CL-4B; absorbance at 280 nm, ———; activity towards pNPC, —●—.

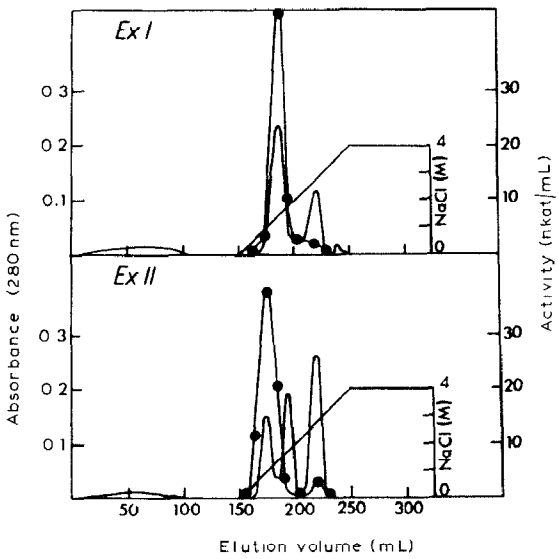

Fig. 3. Chromatography of Ex I and II on DEAE-Trisacryl; absorbance at 280 nm, ———, activity towards pNPC, —●—.

buffer. Fractions (~2.5 mL) were assayed for absorbance at 280 nm and activity towards pNPC.

Nearly all of the injected protein was bound to the gel at pH 7.5. Activity was mainly eluted as a single peak at 0.15 and 0.1M sodium chloride for Ex I and II, respectively. The fractions containing activity towards pNPC, when combined, accounted for 76% and 59% of the injected activity and 46% and 15% of the injected proteins, respectively, for Ex I and Ex II (Fig. 3). The purification factor obtained by this step was 1.6 for Ex I and 3.9 for Ex II.

(d) Chromatography on Ultrogel AcA-54. The fractions from (*c*) were dialysed overnight in collagen bags against 5 L of 5mM sodium acetate buffer (pH 5.0) and freeze-dried. Solutions of samples in 1.5 mL of 0.05M sodium acetate buffer (pH 5.0), containing 0.1M sodium chloride, were each applied to a column (37 × 1.6 cm) of Ultrogel AcA-54 equilibrated with the same buffer at 7.5 mL/h. Fractions (~2 mL) were assayed for absorbance at 280 nm and activity on pNPC.

Both Ex I and II were eluted as a single peak, either for activity or proteins, as shown in Fig. 4. The protein yield was 88% and 95% of injected fractions for Ex I and II, respectively, and 86% and 98%, respectively, of the activity was recovered.

The purified fractions Ex I and II still exhibited activity towards xylan. The final ratio between the activities against pNPC and xylan were 0.13 for Ex I and 0.14 for Ex II.

The procedure (*a*)–(*d*) purified (Table I) Ex I 16.0-fold and Ex II 14.2-fold, each of which gave a single band in disc gel electrophoresis in the presence and absence of SDS, and in agarose isoelectric focusing (Fig. 5).

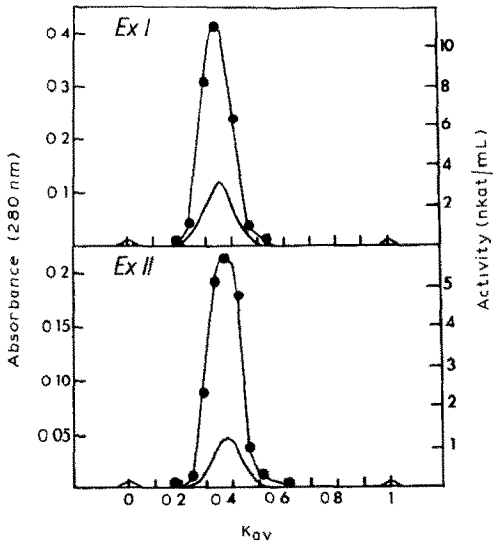

Fig. 4. Gel filtration of Ex I and II on Ultrogel AcA-54 gel; absorbance at 280 nm, ———; activity towards pNPC, —●—.

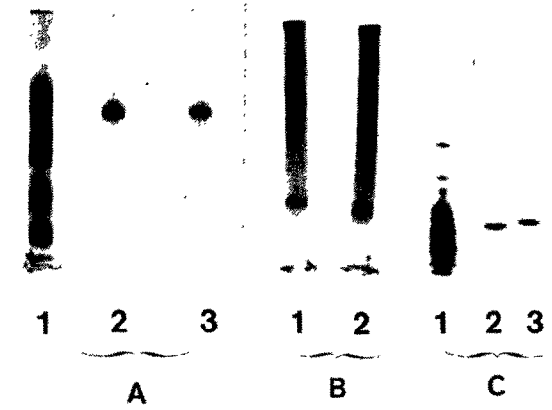

Fig. 5. A. Disc gel electrophoresis of 1, crude enzyme; 2, Ex I; 3, Ex II. B. SDS-disc gel electrophoresis of 1, Ex I; 2, Ex II. C. Isoelectric focusing on agarose of 1, crude enzyme; 2, Ex II; 3, Ex I.

The purified enzymes were dialysed overnight against 2 L of 5mM sodium acetate buffer (pH 5.0), freeze-dried, and stored at −20°.

Properties of the purified enzymes. — (a) Molecular weights. The mol. wts. of Ex I and II, as determined by SDS/polyacrylamide gel electrophoresis (according to the relative mobilities of various standard proteins), were estimated to be 39,000 and 36,000, respectively (Fig. 6). The occurrence of a single protein band for each enzyme indicated the absence of sub-units.

(b) Isoelectric points. These, determined by agarose isoelectrofocusing in the pH range 3–10, were 4.6 and 4.5 for Ex I and II, respectively.

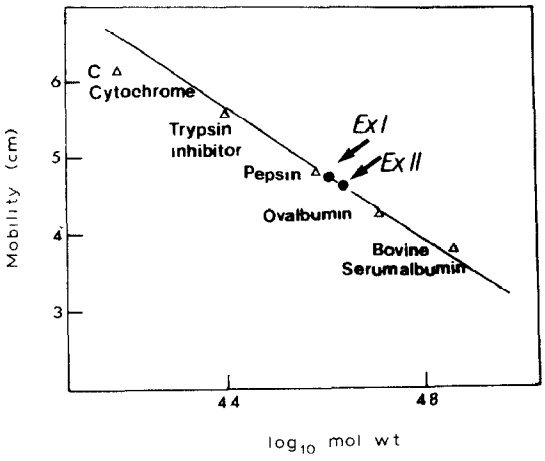

Fig. 6 Determination of the mol wt of Ex I and II.

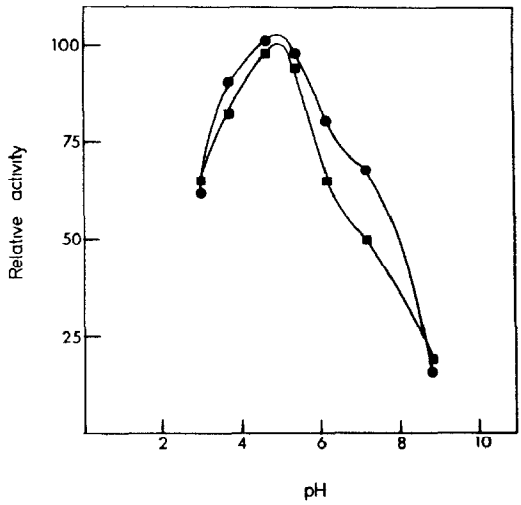

Fig. 7. Effect of pH on the activity of Ex I (—●—) and Ex II (—■—).

(c) Optimum pH. The activity towards pNPC in citrate–phosphate buffers was a maximum at pH 5.0 for both Ex I and II (Fig. 7). A ~40% decrease in the maximum activity was observed at pH 3.0, and a similar loss occurred at pH 7.5 for Ex I and pH 6.2 for Ex II.

(d) pH stability. Ex I and II were stable in Universal Buffer over the pH range 4.0–8.0, for 17 h at 30° (Fig. 8). The solutions were dialysed against 0.05M sodium acetate buffer (pH 5.0) and the residual activity was measured under standard conditions. Ex I and II were readily inactivated at pH 2.5, but only 25% of the activity was lost at pH 9.5.

(e) Optimum temperature. The two enzymes exhibited optimum activity at 60°, but there was a shoulder between 70° and 80° (Fig. 9). Thus, 75% and 60% of

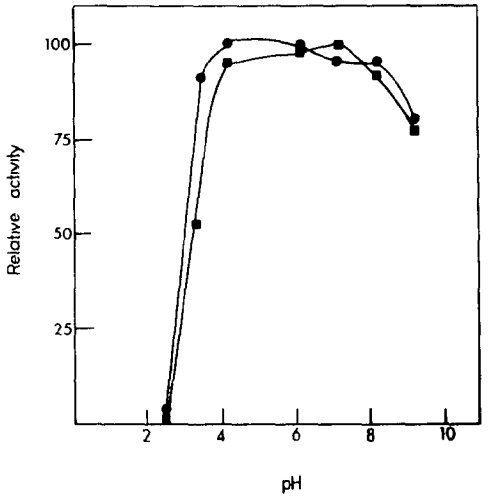

Fig. 8. Effect of pH on the stability of Ex I (—●—) and Ex II (—■—).

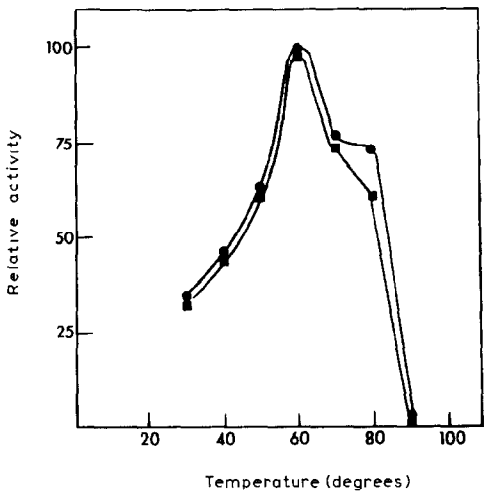

Fig. 9. Effect of temperature on the activity of Ex I (—●—) and Ex II (—■—).

the activities for Ex I and II, respectively, were retained even at 80°. The activation energy, as calculated from the Arrhenius plot, was ~3.3 kcal/mol for each enzyme. The Q10 values between 30° and 40° were 1.5 for Ex I and 1.4 for Ex II.

(f) Temperature stability. Ex I was stable in 0.05M sodium acetate buffer (pH 5.0) up to 70° for 30 min, whereas denaturation of Ex II began beyond 60° (Fig. 10). A 50% loss of activity occurred at 77° for Ex I and at 68° for Ex II. Each enzyme was inactivated when stored for 30 min at 90°. Enzyme solutions were cooled at 4° for 10 min before the residual activity was determined.

(g) Effect of D-*glucose and cellobiose.* Ex I or II (5 nkat) was incubated with

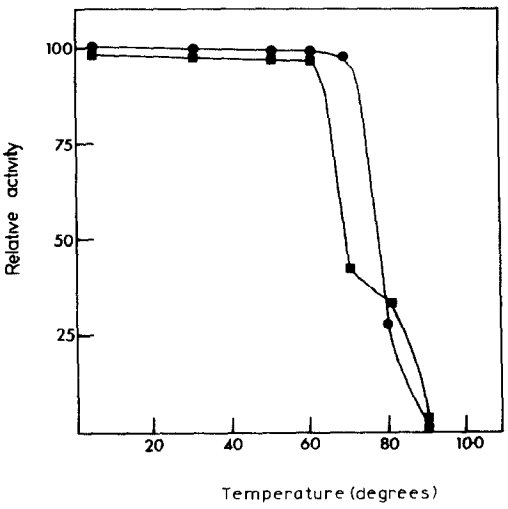

Fig. 10. Effect of temperature on the stability of Ex I (—●—) and Ex II (—■—).

0.25 mL of 2mM pNPC in 0.05M sodium acetate buffer (pH 5.0) and 0.25 mL of the same buffer containing D-glucose or cellobiose in the concentration range 0–50 g/L. Activity was measured under standard conditions. The activity of Ex I and II was not modified by D-glucose up to 0.28M, but cellobiose had a noticeable effect (Table II). Thus, 0.04M cellobiose caused 10% inhibition and 0.15M cellobiose caused 35% loss of activity.

(h) Activity towards various substrates. Ex I and II had no action against inulin, dextran, starch, arabinogalactan, laminarin, cellotriose, cellobiose, or pNPG, but were active towards substrates containing β-(1→4) linkages (Table III). Their activities on CM-cellulose, CMC-azure, H_3PO_4-swollen cellulose, and cellopentaose were, respectively, ~10-, ~20-, ~5-, and ~5-fold less than that observed for an endo-glucanase (En I) purified from the same fungus[15]. Barley glucan and lichenin were degraded to approximately the same extent by each of the three enzymes. Substrates containing xylosyl residues were hydrolysed more rapidly by Ex I and II than those containing glucosyl residue. The highest specific activities were obtained against oNPX and larchwood xylan.

TABLE II

EFFECT OF CELLOBIOSE ON THE ACTIVITY OF PURIFIED ENZYMES TOWARDS pNPC

Final cellobiose concentration (mM)	*Inhibition (%)*	
	Ex I	*Ex II*
7.3	0	0
36.5	10.3	15.0
73.1	16 7	17.0
146.0	36.2	33 0

TABLE III

ACTIVITY OF THE PURIFIED ENZYMES TOWARDS VARIOUS SUBSTRATES, AS COMPARED WITH THE PURE ENDO-GLUCANASE En I FROM *Dichomitus squalens*[15]

Substrate	*Specific activity*[a] *(nkat/mg)*		
	Ex I	*Ex II*	*En I*
CM-cellulose	97	87	959
CMC-azure[b]	6	4	117
H_3PO_4-swollen cellulose	14	20	86
Avicel	0.2	0.3	0.3
Barley glucan	20	35	34
Lichenin	19	35	36
Xylan	292	311	0
pNPC	42	42	1
oNPX	199	174	ND
Cellotetraose	ε	ε	8
Cellopentaose	27	35	155

[a]Protein was measured by the method of Lowry. [b]Specific activity on CMC-azure was expressed as $AU.mg^{-1}$

(i) Analysis of hydrolysis products. Suspensions of H_3PO_4-swollen cellulose and xylan, or 0.5% solutions of lichenin, barley glucan, pNPC, oNPX, or cello-oligosaccharides, in 0.01M sodium acetate buffer (pH 5.0) were incubated with 5 nkat of Ex I and II for appropriate periods of time. After centrifugation if necessary, the mixtures were deionized with Dowex 1-X8 ($HCOO^-$) and Amberlite IR-120 (H^+) resins and freeze-dried. The residues were subjected to t.l.c. on Silica Gel F 1500 (Schleicher and Schüll), using 1-propanol–ethyl acetate–ethanol–pyridine–acetic acid–water (7:3:3:2:2:1). Cello-oligosaccharides were detected by

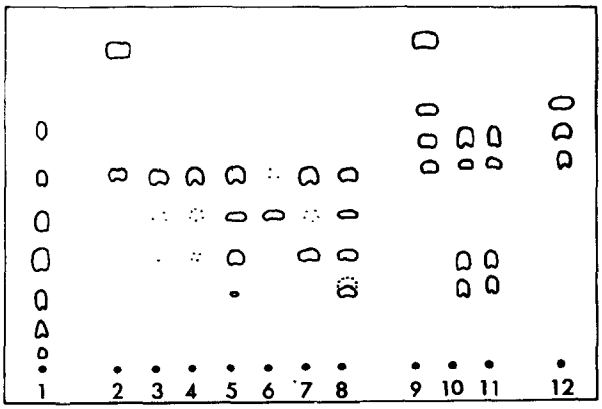

Fig. 11. Thin-layer chromatogram of the products formed by the action of Ex I or II on (2) pNPC, (3) H_3PO_4-swollen cellulose, (4) barley glucan, (5) lichenin, (6) cellotriose, (7) cellotetraose, (8) cello-pentaose, (9) oNPX, (10) larchwood glucuronoxylan, (11) wheat-straw arabinoglucuronoxylan (1, standard cello-oligosaccharides; 12, standard xylo-oligosaccharides).

charring with sulphuric acid. The same products were formed on hydrolysis of various substrates by Ex I and II (Fig. 11), and cellobiose but no glucose was formed from pNPC. Cellobiose was also the main product of hydrolysis of cellulose and barley glucan together with traces of cellotriose and cellotetraose. Oligomers of higher d.p. were found in the lichenin hydrolysates.

Ex I and II did not attack cellotriose, and cellotetraose was degraded only to a small extent to give cellobiose. Cellopentaose was more extensively hydrolysed and oligosaccharides of d.p. 2–5 were detected as well as some transglycosylation products. Glucose was not detected in any of the hydrolysates.

Hydrolysis of glucuronoxylan from larchwood and arabinoglucuronoxylan from wheat straw by Ex I or II gave xylobiose, xylotriose, and two main oligosaccharides of lower mobility which were not identified, but were suspected to contain a uronic acid residue in spite of the treatment with resin. Incubation of oNPX with Ex I and II even for a short time produced xylose, xylobiose, and xylotriose, indicating strong transglycosylating activities.

DISCUSSION

Chromatofocusing is a powerful technique for the fractionation of extracellular proteins from fungi[16,17]. Three endo-glucanases from *D. squalens* (En I–III) have thus been separated[15]. It is shown here that at least one β-D-glucosidase, one polygalacturonase, and three xylanases occur among the extracellular enzymes produced by this fungus.

Three peaks of activity towards *p*-nitrophenyl β-cellobioside (pNPC) were also detected. This substrate allows the detection of enzymes which release cellobiose units, so that cellobiohydrolases (CBH) should be active towards this molecule, as demonstrated[6] with enzymes from *Trichoderma reesei*.

Two enzymes from *D. squalens*, responsible for the activity towards pNPC, were purified to homogeneity. Hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B, which allowed the elimination of contaminating ampholines, purified Ex I 1.5-fold, but there was marked loss of protein and enzymic activity with Ex II, perhaps caused by denaturation in the presence of ammonium sulphate as already reported for xylanases from other fungi[18,19]. Ion-exchange chromatography on DEAE-Trisacryl purified Ex I and II since no more fractionation was obtained by the last gel filtration. The ratios between activities towards pNPC and xylan showed only small differences before and after the purification procedure, suggesting that the same proteins were responsible for these two activities since the homogeneity of Ex I and II was demonstraed by several criteria. This raised the question as to whether Ex I and II were exo-glucanases or xylanases. Although their behaviour as a function of pH and temperature and their isoelectric points were similar to those of many other fungal depolymerases, their molecular weights were lower than those of the endo-glucanases from the same organism[15]. CBH exhibited molecular weights usually higher[20,21] and xylanase lower[22] than those of endo-glucanases.

The end-product of CBH action, *i.e.*, cellobiose, had little effect on the hydrolysis of pNPC by Ex I and II. A concentration of cellobiose incompatible with *in vivo* conditions must be reached to cause appreciable inhibition. This is in favour of the xylanolytic nature of Ex I and II.

The activity of Ex I and II was relatively higher on H_3PO_4-swollen cellulose than on CM-cellulose or on CMC-azure, as compared to that of the main endo-glucanase (En I) from *D. squalens*. This is the behaviour of an exo-enzyme for which substituent groups impede recurrent action along the β-D-glucan chain. Ex I and II were weakly active against microcrystalline cellulose. They had activities similar to that of En I towards barley glucan and lichenin, substrates containing β-(1→3) and β-(1→4) linkages, respectively, although none of these enzymes was able to split (1→3) linkages, since no activity was observed towards laminarin. The main product released from cellulose was cellobiose, but other oligosaccharides were detectable when barley glucan or lichenin was used as substrate.

Ex I and II should be considered as xylanases, taking into account their high activity towards xylan. The end-products of the hydrolysis of larchwood glucurono-xylan or wheat-straw arabinoglucuronoxylan were xylobiose and xylotriose plus some, presumably acidic, oligosaccharides. Nevertheless, the endo or exo mode of action of the enzymes cannot be demonstrated with certainty, mainly because of their potential for transglycosylation already observed during the hydrolysis of cellopentaose.

Transglycosylation occurred when enzymes acted on *o*-nitrophenyl β-D-xyloside (oNPX), since oligomers of d.p. 2 and 3 were produced together with xylose. Biely *et al.*[23] reported that the release of nitrophenol from oNPX by an endo-xylanase from *Cryptococcus albus* occurred through a complex pathway including several steps of transglycosylation. Similar reactions were observed with an exo-cellulase from *Irpex lacteus*[24]. It is surprising that Ex I and II, which acted mainly on xylan and to a lesser extent on cellulose, had no effect at all on pNPG but released nitrophenol from pNPC without the occurrence of transglycosylation. Cellotriose was not hydrolysed and cellotetraose only to a limited extent. Thus, the specificity and mode of action of the enzymes remain unclear.

The physicochemical properties and catalytic activities of Ex I and II were almost identical, indicating a close relationship. Ex I and II could be different glycosylated forms of the same protein moiety, as proposed to explain the multiplicity of CBH from *T. viride*[25,26]. A partial proteolysis of Ex I could also explain the occurrence of Ex II which has a lower molecular weight. Such a mechanism has been demonstrated for the multiple glucanase isoenzymes in culture supernatants from *T. viride*[27].

Preliminary experiments (not shown) did not provide evidence for a synergistic effect of Ex I or II with any of the purified endo-glucanases from *D. squalens* and they are concluded to be xylanases.

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