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PURIFICATION AND PROPERTIES OF A CELLOBIOHYDROLASE FROM *Penicillium pinophilum*

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ABSTRACT

Cellobiohydrolase II, isolated from the extracellular cellulase system of *Penicillium pinophilum* by chromatography on DEAE-Sephadex and DEAE-Sepharose followed by chromatofocusing, gave a single homogeneous band in SDS-gel electrophoresis and gel electrofocusing. It had a molecular weight of 50,700 and a pI of 5.0, and was associated with 19% of carbohydrate. Cellobiose was the sole product of hydrolysis of the cellulosic materials, Avicel and H_3PO_4-swollen cellulose. No cross reaction was observed with antiserum prepared with another purified cellobiohydrolase (I) isolated from the same cellulase system. Cellobiohydrolase II showed no capacity for producing short fibres from filter paper. Avicel was hydrolysed extensively, but little or no hydrolysis of cotton fibre was apparent. However, cotton fibre was hydrolysed with a reconstituted mixture of the purified cellobiohydrolase II and the four major endo-(1→4)-β-D-glucanases isolated during fractionation. The action of cellobiohydrolase II on H_3PO_4-swollen cellulose was stimulated by high concentrations of cellobiose, but inhibited by high concentrations of D-glucose. Other notable inhibitors were Mn^{2+} and carbodi-imide. The properties of cellobiohydrolase II and the immunologically unrelated cellobiohydrolase I are compared.

INTRODUCTION

Studies of the extracellular cellulase from a few cellulolytic fungi have established that the hydrolysis of highly ordered, crystalline cellulose can be discussed in terms of the activities 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 (1→4)-β-D-glucan glucohydrolase, EC 3.2.1.74], and β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21). These enzymes, which are glycoproteins, appear in multiple forms in the main, but it is not known which are genetically determined and which are artefacts resulting from modification of the enzyme during culture by proteolysis or, perhaps, by the activities of carbohydrases. The nature of, the cause of, and the need for multiple forms of the

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same type of enzyme are the subject of much debate[1,2].

The heterogeneity of the cellobiohydrolase activity in cultures of the fungi *Fusarium solani*[3] and *Trichoderma* species[4,5] has been studied. A commercial cellulase preparation has been found to contain three cellobiohydrolases[5] which differed in the contents of neutral carbohydrates, but were similar in amino acid composition, molecular weight, and thermal activity. Immunological studies revealed cross reactivity between the three forms of cellobiohydrolase. Similar results have been obtained from immunological investigations made on the cellobiohydrolases of *Trichoderma reesei*. However, this species of *Trichoderma* synthesises several forms of another type of cellobiohydrolase (type II) which is immunologically distinct and gives no cross reaction[6] with antiserum prepared with cellobiohydrolase type I. No satisfactory explanation of why two immunologically distinct types of cellobiohydrolase are synthesised by *T. reesei* has been given[7], but it has been suggested that they may have definite roles to play in the process of cellulolysis[8].

The isolation and properties of a cellobiohydrolase from *Penicillium funiculosum* strain 87160 (now classified as *P. pinophilum*) has been described[9], and we now report on the isolation and characterisation of cellobiohydrolase II, which is immunologically unrelated to that (cellobiohydrolase I) previously discussed. Other studies suggest that co-operation between cellobiohydrolases I and II is an important feature of the cellulolysis of crystalline cellulose[10].

Part of this work has been presented in preliminary form[11].

EXPERIMENTAL

P. pinophilum IMI 87160 iii was obtained from the Commonwealth Mycological Institute (Kew, Surrey). Previously, this strain was called *P. funiculosum* IMI 87160 iii, but the two species are now considered to be sufficiently different to be assigned different taxa.

Molecular weight markers and the enzyme substrates were obtained from the sources previously indicated[3,12].

P. pinophilum *cellulase.* — Cultures and partially purified [20–80% saturated $(NH_4)_2SO_4$ fraction], concentrated (50-fold) cellulase preparations were produced from *P. pinophilum* 87160 iii as previously described[9].

H_3PO_4-swollen cellulose. — Avicel (grade PH101) was swollen[9,13] at 1° for 4 h.

Cello-oligosaccharides. — Cello-oligosaccharides up to cellohexaose were prepared by the acetolysis of Whatman cellulose powder (CC41) and isolated by gradient elution (0→35% ethanol) from a column of charcoal–acid-washed Celite (1:1).

Dyed H_3PO_4-swollen cellulose. — H_3PO_4-swollen cellulose was dyed[14] with Remazol Brilliant Blue R.

Chromatofocusing. — Polybuffer PBE94 exchanger packed into a column

and equilibrated with starting buffer was used according to the procedure recommended in the Pharmacia booklet. For the purification of cellobiohydrolase II, the starting buffer was 25mM histidine-HCl (pH 6.2) and the eluting buffer was Polybuffer 74 which had been diluted 9-fold and brought to pH 4.5 with conc. HCl.

For the purification of cellobiohydrolase I, the starting buffer was 25mM piperazine-HCl (pH 5.5) and the eluent was Polybuffer 74, diluted 10-fold and brought to pH 4.0 with HCl. The column (0.9 × 28.5 cm) of Polybuffer PBE54 carrying a load of 5.8 mg of cellobiohydrolase protein (desalted on Biogel P-2 and re-dissolved in the starting buffer), when eluted with 200 mL of the eluting buffer, provided a pH gradient covering the range 4.0–5.0. Cellobiohydrolase I, free from contaminating carbohydrase activities, was eluted at pH 4.4. Cellobiohydrolase I was precipitated with $(NH_4)_2SO_4$ (85% saturation) and isolated by centrifugation. The pellet was dissolved in 0.01M ammonium acetate buffer (pH 5.0), and the residual SO_4^{2-} was separated from the protein on a column of Biogel P-2. When purified in this way, cellobiohydrolase I gave only one protein band (Coomassie Blue stain) on isoelectric focusing in polyacrylamide gels and on electrophoresis in polyacrylamide gels in non-denaturing and denaturing conditions.

Analytical methods. — Reducing sugars were determined by the Somogyi–Nelson method[15] and expressed as glucose or cellobiose equivalent. D-Glucose was measured using D-glucose oxidase[16]. Protein was normally determined by the method of Lowry *et al.*[17], but the method of Bradford[18] was used in the chromatofocusing purification because of interference by the eluting buffer. Each reagent was calibrated using bovine serum albumin as standard.

Enzyme assays. — The substrate specificities of the principal enzymes of the cellulase system [cellobiohydrolase, endo-(1→4)-β-D-glucanase, and β-D-glucosidase] are known[19]. Thus, (*a*) endo-(1→4)-β-D-glucanase can be distinguished by the fact that it is the only enzyme that can hydrolyse soluble CM-cellulose significantly. (*b*) Cellobiohydrolase and endo-(1→4)-β-D-glucanase act synergistically to solubilise crystalline cellulose, but it has been claimed that cellobiohydrolase is the only enzyme, when acting alone, that can release a significant amount of reducing sugar from the microcrystalline Avicel[20]. Avicel is a better substrate than cotton fibre for measuring cellobiohydrolase activity, in that it has a low d.p. (~200) and therefore has many more non-reducing end-groups which can be attacked by the endwise-acting enzyme. (*c*) H_3PO_4-swollen cellulose is hydrolysed by endo-(1→4)-β-D-glucanase and cellobiohydrolase acting independently.

Activity towards cotton or H_3PO_4-swollen cellulose was determined by measuring either the residual cellulose after a 7-day incubation at 37° of a 2-mg sample of dewaxed cotton[21], or the soluble sugars released during 18 h at 37° from a 20-mg sample[12].

Activity towards Avicel was assayed by measuring the soluble sugars released during 2 h at 37° in a mixture containing 2 mL of 1% Avicel (PH101) in 0.1M

acetate buffer (pH 5.0), enzyme, and water to give a total volume of 2.1 mL.

Endo-(1→4)-β-D-glucanase was measured with CM-cellulose (d.s. 0.5; Cellofas B, I.C.I. Ltd.) by the reducing sugar method[3].

β-D-Glucosidase activities were measured with *o*-nitrophenyl β-D-glucopyranoside as substrate[22].

Xylanase activity was determined as follows. A solution of xylan (1 g, Sigma) in M NaOH (100 mL) was adjusted to pH 5.0 with M acetic acid and aqueous 2% NaN_3 (10 mL) was added followed by water to give a total volume of 1 L. The slightly cloudy solution was centrifuged (76,000*g*, 45 min), and the supernatant solution was stored at 4°.

Substrate solution (1.5 mL) was incubated at 37° for 1 h with enzyme in a total volume of 2 mL. The hydrolysis was stopped by the addition of Somogyi reagent[15] (2 mL), and the reducing sugar was determined[15]. The unit of activity is defined as the amount of enzyme required to liberate 1 nmol of xylose/min under the assay conditions.

Effect of cellobiose and D-*glucose on cellobiohydrolase activity.* — A suspension (5 mL) of dyed H_3PO_4-swollen cellulose (4 mg/mL) was centrifuged. To part (3.85 mL) of the supernatant was added a solution (1.25 mL) of D-glucose or cellobiose in 0.2M acetate buffer (pH 4.0) followed by enzyme (0.1 mL) and water to give a total volume of 2.5 mL. The mixture was incubated at 50° for 24 h and centrifuged, and the absorbance of the supernatant was read at 595 nm. A calibration curve relating enzyme activity to absorbance was also prepared.

Effect of cellobiohydrolase on the d.p. of H_3PO_4-swollen cellulose. — The d.p. of a sample of H_3PO_4-swollen cellulose (40 mL) remaining after incubation at 37° with enzyme (50 μg of protein) was determined viscometrically[12] after dissolution in cadoxen.

Other methods. — Polyacrylamide-gel electrophoresis and electrofocusing, t.l.c., and the estimation of molecular weight by molecular sieve chromatography were all performed as described by Wood *et al.*[9].

For amino acid analysis, samples, each containing ~0.5 mg of purified enzyme, were hydrolysed in 6M HCl at 110° for 24 and 72 h, and then analysed in a Chromaspek amino acid analyser. Cysteine and methionine were determined as cysteic acid and methionine sulphone, respectively, after oxidation with performic acid. Serine and threonine were calculated by extrapolation to zero time of the values observed after hydrolysis for 24 and 72 h.

D-Glucose and cello-oligosaccharides produced by enzyme action were assayed by h.p.l.c. with a Perkin–Elmer Chromatograph, using a Shandon column (25 cm × 5 mm o.d.) packed with Partisil 10 PAC and fitted with a pre-column (2 cm × 2 mm i.d.). The mobile phase was water–acetonitrile (29:71) at 2 mL/min. A refractive index detector maintained at 25° was used.

Cellobiohydrolase antiserum. — A "water in oil" emulsion, prepared with Freund's complete Adjuvant (0.1 mL containing 100 μg of cellobiohydrolase protein), was injected intradermally into a rabbit at intervals of 3 weeks for the first

3 injections, and then at intervals of 6 weeks. Samples of blood (20 mL) were taken at intervals of ~4 weeks and the serum was removed after clotting. Immunoglobulins were precipitated with $(NH_4)_2SO_4$ (50% saturation) at 1°, isolated by centrifugation, redissolved in water, and dialysed at 4° against water and then 0.05M acetate buffer (pH 5.0). Insoluble lipoprotein was removed by centrifugation and the α-globulin fraction containing the antibodies was purified by ion-exchange chromatography according to the method described in the LKB Manual of Quantitative Immuno Electrophoresis, 1973.

The antibodies were precipitated with $(NH_4)_2SO_4$ (25 g/100 mL) at 1°, isolated by centrifugation, redissolved in a few mL of 0.1M NaCl which was 15mM with respect to NaN_3, and dialysed against the same buffer solution.

Ochterlony double immunodiffusion. — The technique of Ochterlony[23] was used with ionagar (1%) containing a 1:1 dilution of 0.28M NaCl/100mM Tris adjusted with HCl to pH 7.4 as the diffusion medium. Immunodiffusion was allowed to proceed at room temperature in a damp atmosphere until precipitin arcs appeared. When precipitation appeared to be complete, unprecipitated antibodies were removed from the gel by washing twice with aqueous 0.9% NaCl. The precipitin arcs were stained for protein with 0.1% Coomassie Brilliant Blue R-250 in ethanol–acetic acid–water (45:10:45), and destained after 15 min with ethanol–acetic acid–water (45:10:45).

Treatment of enzyme with carbodi-imide. — A solution (0.125 mL) of 0.5M pyridine–pyridinium chloride (pH 4.8), which was 0.2M with respect to 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate and 2M with respect to glycine methyl ester, was mixed with a solution (0.015 mL) of cellobiohydrolase II (50 μg of protein) and water to give a total volume of 0.25 mL. This mixture was incubated for 1, 2, or 3 h at 25° and the reaction was stopped by the addition of 0.25M acetate buffer (pH 4.8). The solution was stored for 15 min at 25° before a sample (0.4 mL) was added to dyed swollen cellulose. The residual activity was assayed, as indicated above, using a calibration curve relating enzyme activity to dyed soluble-fragments of chain released.

RESULTS

Fractionation of enzyme system by ion-exchange chromatography. — Cellobiohydrolase I and II were separated from the other enzyme components of the *P. pinophilum* cellulase system in two stages involving DEAE-Sephadex and DEAE-Sepharose. In the first stage, which was similar to that used previously[9], two protein components, accounting for 71% of the total protein isolated from the column (fractions 1 and 2) and endo-glucanase 2 were eluted with 0.06M acetate buffer (pH 4.8). By applying a gradient involving a simultaneous increase in ionic strength (0.06 → 0.1) and a decrease in pH (4.8 → 4.0), components 4 and 5 were separated. For the purposes of this investigation, only fractions 1 and 2 were purified further.

Fraction 2 has already been demonstrated[3] to consist of a cellobiohydrolase (now termed I) and a small proportion of a glucohydrolase. Purified cellobiohydrolase I was obtained by chromatofocusing (see Experimental).

Fraction 1 was associated with 81% of the xylanase, 36% of the β-D-glucosidase, 34% of the endo-(1→4)-β-D-glucanase (CM-cellulase), and 36% of the protein eluted from the column, and was further fractionated on DEAE-Sepharose (Fig. 1). The starting conditions (0.01M phosphate buffer, pH 6.9) were such that many of the enzyme activities previously eluted, without adsorption, from the DEAE-Sephadex (0.06M acetate buffer, pH 4.8) column were now bound to the column. Elution with the starting buffer resulted in the complete separation of a xylanase (Fig. 1, *A*) and an endoglucanase (*B*). Stepwise increases of buffer molarity yielded another major endoglucanase (*C*), two peaks of xylanase activity (*E* and *F*), and a large protein component (*G*) which was associated with traces of xylanase and endoglucanase activity but which was rich in activity towards Avicel.

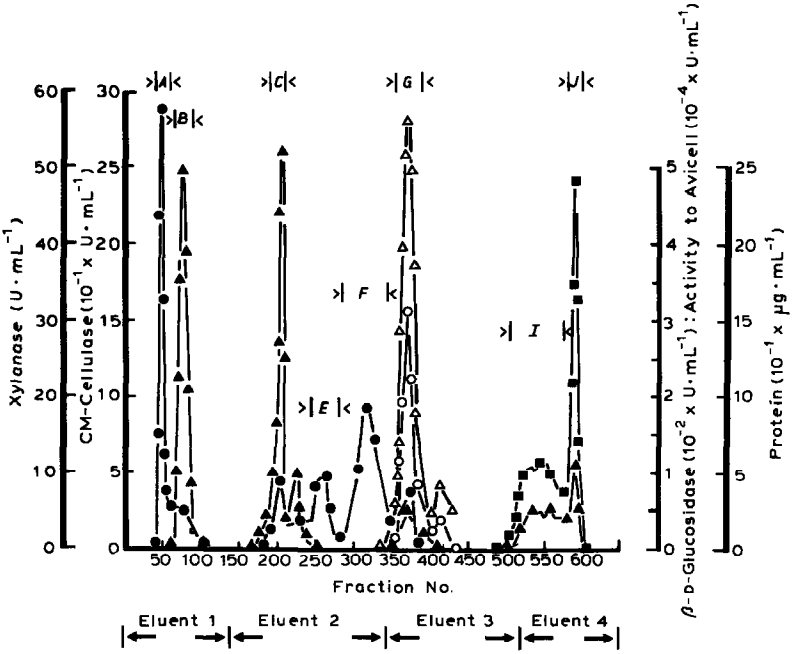

Fig. 1. Purification of enzyme components on DEAE-Sepharose CL-6B. The first major protein component eluted from the column of DEAE-Sephadex (see Results) was precipitated with $(NH_4)_2SO_4$ (85% saturation), centrifuged, redissolved in 0.01M ammonium acetate buffer (pH 5.0), desalted on Biogel P-2, freeze-dried, redissolved in 0.01M phosphate buffer (pH 6.9), and applied to a column (2.5 × 30.0 cm) of DEAE-Sepharose CL-6B equilibrated with 0.01M phosphate buffer (pH 6.9). The column was eluted at 15 mL/h with stepwise changes in buffer concentration as shown. Fractions (2 mL) were collected, and endoglucanase (CM-cellulase) (▲), xylanase (●), β-D-glucosidase (■), protein (△), and activity to Avicel (○) were determined. The peaks (*A–J*) were combined as shown. Eluents: 1, 0.01M phosphate buffer (pH 6.9); 2, 0.05M phosphate buffer (pH 6.9); 3, 0.1M phosphate buffer (pH 6.9); 4, 0.2M phosphate buffer (pH 5.6).

An increase in molarity and a decrease in pH of the eluent was necessary to effect the removal of the β-D-glucosidase (*I* and *J*).

As the major protein component (*G*) possessed cellulose-hydrolysing activities previously found to be associated with cellobiohydrolase activity[20] in fungal cellulase systems, it was purified further.

Purification of cellobiohydrolase by chromatofocusing. — Fractions (354–388, *G* in Fig. 1) were combined, the protein was precipitated with $(NH_4)_2SO_4$ (85% saturation) and isolated by centrifugation. A solution in 0.01M ammonium acetate buffer (pH 5.0, 5 mL) was desalted on a column of Biogel P-2 equilibrated with 0.01M ammonium acetate buffer (pH 5.0), and the enzyme was isolated by freeze-drying. A solution in 25mM histidine–HCl buffer (pH 6.4, 6 mL) was applied to a column of PBE 94 chromatofocusing-medium and eluted with Polybuffer 74 (see Experimental). The pH gradient produced covered the range 4.8–6.4 (Fig. 2), and this resulted in the elution of the cellobiohydrolase as a single protein peak at pH 5.45 and another component at pH 4.58 with u.v. absorption at 280 nm but insignificant cellulase activity. The small amount of xylanase activity associated with the cellobiohydrolase component after chromatography on DEAE-Sepharose (Fig. 1) was eluted at pH 5.3 (fractions 56–61, Fig. 2), and was well separated from the cellobiohydrolase component (not shown in Fig. 2).

Isoelectric focusing in a polyacrylamide gel was carried out on an aliquot (2.5 μL) of each of the fractions (46–56) containing the cellobiohydrolase, using

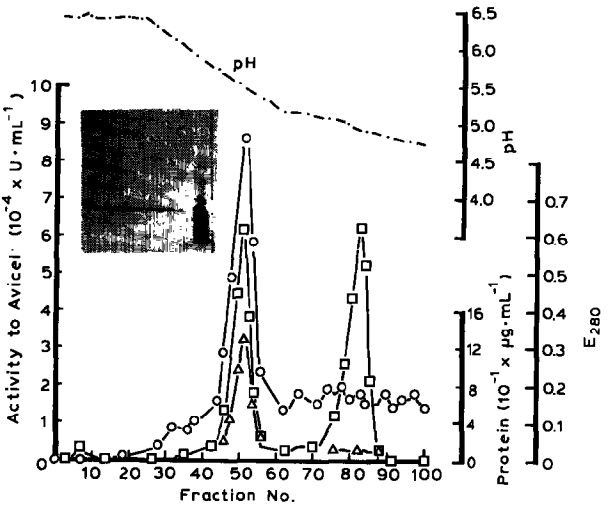

Fig. 2. Purification of cellobiohydrolase II by chromatofocusing. Peak *G* (Fig. 1) was prepared for chromatofocusing as described in the text, applied to a column (0.9 × 28.5 cm) of PBE94 equilibrated with 25mM histidine–HCl (pH 6.2) (see Experimental), and eluted (15 mL/h) with Polybuffer 24 (200 mL) which had been diluted 1:9 and brought to pH 4.5 with HCl. Fractions (2 mL) were collected and assayed for activity towards Avicel (○), protein (△), $E_{280\,nm}$ (□), and pH (· — ·). Inset shows isoelectric focusing (ampholyte pH 3.5–10.0) of the original unfractionated enzyme and fractions 46–56 (left to right).

carrier ampholyte covering the pH range 3.5–10, and showed only one protein band when stained with Coomassie Blue (inset Fig. 2). These fractions were therefore combined and the enzyme was precipitated with $(NH_4)_2SO_4$ (85% saturation) and isolated by centrifugation. A solution of the pellet in ammonium acetate buffer (pH 5.0) was desalted on a column of Biogel P2 equilibrated with 0.01M ammonium acetate buffer (pH 5.0), freeze-dried, and dissolved in the minimum of 0.01M ammonium acetate buffer (pH 5.0). Typically, 80–85% of the protein applied to the column was recovered.

Electrophoresis of the purified and concentrated enzyme under non-denaturing and denaturing conditions in polyacrylamide gels showed only one band on staining for protein (Coomassie Blue).

The nature of the component eluted at pH 4.9 (fractions 75–86, Fig. 2) is still under investigation. Although it absorbs u.v. light at 280 nm, it did not stain with Coomassie Blue, or react with the same dye using the conditions recommended by Bradford[18], and is unlikely to be a protein. The amount of this component obtained relative to the cellobiohydrolase protein was very variable. In some chromatofocusing runs, only a trace was found. A similar component was separable from one of the major endoglucanase components (Fig. 1) by the chromatofocusing technique[34].

Properties of cellobiohydrolase II. — The enzyme showed an optimum pH of 4.2, using 0.05M citrate–phosphate buffer and H_3PO_4-swollen cellulose as substrate. However, with the highly crystalline Avicel as substrate, the pH optimum covered the range 4.0–5.2. At pH 4.2, the optimum assay temperature was 55° using H_3PO_4-swollen cellulose, but only 50° using Avicel. Heating for 50 min at 60° resulted in the loss of 95% of the activity (75% lost in 20 min).

On SDS/polyacrylamide-gel electrophoresis, the purified cellobiohydrolase gave a single polypeptide band corresponding to a mol. wt. of 50,700. Antibodies to purified cellobiohydrolase I did not react with purified cellobiohydrolase II (see Experimental).

The pI value of the cellobiohydrolase, when focused in an LKB column, was 5.0 at 4°. The enzyme was associated with 19% of carbohydrate, as determined by the phenol–H_2SO_4 method[24], and effected only a slow change in the d.p. of H_3PO_4-swollen cellulose (zero, 3600; 30 min, 3460; 1 h, 3370; 2 h, 3214; 3 h, 2500; 24 h, 2500) (see Experimental). The endoglucanase of *P. pinophilum*, in contrast, causes a rapid fall in the d.p. of H_3PO_4-swollen cellulose[9].

Suitable concentrations of *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide completely inhibited cellobiohydrolase II (Table I). Mn^{2+} and EDTA were inhibitory at 50mM and D-glucose at 200mM, but cellobiose stimulated activity (Fig. 3); the opposite effect was observed with cellobiohydrolase I (Fig. 3).

The amino acid compositions of cellobiohydrolases I and II are presented in Table II. The cellobiohydrolases were high in acidic and low in basic amino acid contents.

Specificity. — The specificity of purified cellobiohydrolase II was tested using

TABLE I

INHIBITION[a] OF CELLOBIOHYDROLASE II

Compound	*Concentration used in assay (mM)*	*Inhibition (%)*
Co^{2+}	50	2
Mn^{2+}	50	35
Mg^{2+}	50	2
Cu^{2+}	50	7
EDTA	50	17
N-Bromosuccinimide[b]	5	100
2-Hydroxy-5-nitrobenzyl bromide	10	9
	50	100
1-Cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate[c]	100	51[d]

[a]The enzyme + additive (0.25 mL, total volume) were preincubated at 25° for 30 min before the residual activity of a sample (0.2 mL) was determined by the standard assay for measuring activity to H_3PO_4-swollen cellulose (see Experimental). [b]Dissolved in aqueous acetone (20:1) before incubation. No inhibition was observed with 10mM D-glucono-1,5-lactone, 50mM Zn^{2+}, 50mM Ba^{2+}, 50mM Ca^{2+}, 50mM Hg^{2+}, and 50mM *N*-acetylimidazole. [c]See Experimental. [d]Treatment for 2 h.

a number of (1→4)-linked β-D-glucose substrates (Table III). Cotton fibre was almost completely resistant under all the conditions used. On the other hand, cotton fibre swollen in H_3PO_4 was readily degraded. Avicel was the most easily hydrolysed of the crystalline celluloses tested; on prolonged incubation, 24% was solubilised. Cellobiose was the sole product in these hydrolyses (h.p.l.c.). The specific activities of the enzyme on Avicel and H_3PO_4-swollen cellulose were 0.080 and 0.574 μmol of cellobiose $min^{-1}.mg^{-1}$, respectively, at 37°.

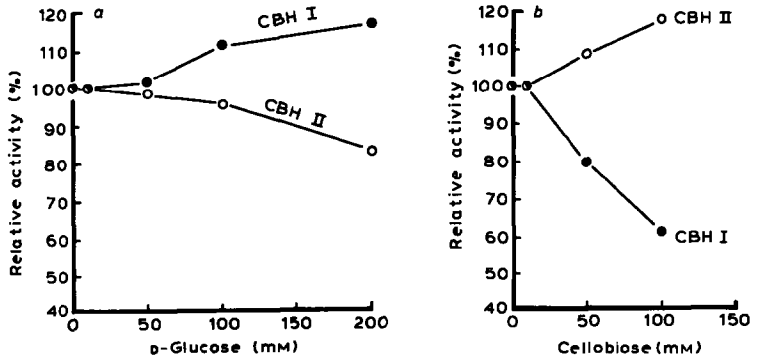

Fig. 3. Effect of D-glucose and cellobiose on the activity of cellobiohydrolase I and II of *P. pinophilum* on H_3PO_4-swollen cellulose (see Experimental): effects of (*a*) D-glucose and (*b*) cellobiose; CBH I, cellobiohydrolase I; CBH II, cellobiohydrolase II.

TABLE II

AMINO ACID COMPOSITION (MOL %)[a] OF CELLOBIOHYDROLASES I AND II

Amino acid	*Cellobiohydrolase II*	*Cellobiohydrolase I*[b]
Aspartic acid	11.5	12.2
Threonine	11.5	13.6
Serine	10.2	10.6
Glutamic acid	6.6	5.5
Proline	6.7	4.1
Glycine	8.0	12.6
Alanine	15.5	7.9
Half-cystine	0.6	4.9
Valine	5.5	6.1
Methionine	0.4	1.5
Isoleucine	3.8	3.1
Leucine	7.0	4.6
Tyrosine	4.5	4.6
Phenylalanine	2.6	3.0
Histidine	1.2	1.6
Lysine	3.1	2.1
Arginine	1.5	2.2

[a]Tryptophan was not determined. [b]Data from ref. 9.

Early enzymic attack on the cotton fibre can be detected by measuring[25] the increase in the capacity of cotton fibres for swelling in 18% NaOH or by the production of short fibres[26]. Cellobiohydrolase II produced no such changes in cotton fibre or Whatman filter paper even after 7 days at 37°.

The purified cellobiohydrolase did not hydrolyse soluble CM-cellulose to a significant extent. However, the soluble β-D-glucan from oats, which contains

TABLE III

HYDROLYSIS[a] OF(1→4)-LINKED AND A MIXED β-D-GLUCAN BY CELLOBIOHYDROLASE II

Substrate	*Amount of substrate used (mg)*	*Hydrolysis time (h)*	*Reducing sugar (μg glucose equivalent)*	*Products of hydrolysis (t.l.c., h.p.l.c.)*
Cotton	20	18	12	N.t.[b]
	2	500	24	N.t.
Whatman cellulose powder (CC41)	20	18	42	G_2 only[c]
Avicel (PH 101)	20	18	146	G_2 only
	2	168	596	G_2 only
	2	500	598	G_2 only
H_3PO_4-swollen cellulose	20	18	1230	G_2 only
CM-cellulose	20	18	20	N.t.
Oat β-D-glucan	20	18	842	N.t.

[a]The substrates were incubated, for the times given, in a mixture consisting of 2.5 mL of acetate buffer (pH 5.0, 2.5 mL), enzyme protein (50 μg), aqueous 0.02% NaN_3 (0.1 mL), and water to give a total volume of 5 mL. [b]Not tested. [c]Cellobiose.

mixed (1→3)- and (1→4)-linkages was readily hydrolysed. Soluble laminarin, a (1→3)-linked β-D-glucan, was not attacked. Other non-substrates were the β-D-glucans, lutean and CM-pachyman, and the α-D-glucans, dextran, starch, and nigeran. Xylan from oat straw was not attacked.

The action of cellobiohydrolase II on 0.01M cellobiose–cellohexaose was tested. As shown in Table IV, cellobiose was not hydrolysed, the action on cellotriose was minimal, but cellotetraose, cellopentaose, and cellohexaose were readily hydrolysed. The tetraose gave cellobiose, but the pentaose and hexaose each yielded cellobiose and cellotriose; no glucose was detected (t.l.c., h.p.l.c.) in any hydrolysate.

Synergism with endoglucanase activity in solubilising cotton. — When cellobiohydrolase II (50 μg) was mixed with a reconstituted mixture (1000 units) made up of equal amounts of the endoglucanases (Fig. 1), a 2-mg sample of cotton was 31% solubilised after 7 days at 37°.

DISCUSSION

The purified cellulase component, which was isolated by ion-exchange chromatography and by chromatofocusing, has many properties consistent with a cellobiohydrolase. Thus, it hydrolysed, to different degrees and exclusively to cellobiose, amorphous H_3PO_4-swollen cellulose and crystalline cellulose such as Avicel, effected only a slow change in the d.p. of H_3PO_4-swollen cellulose, and had no effect on the viscosity (a parameter related to chain length) of a solution of CM-cellulose. Other properties, such as the lack of inhibition by low concentrations of D-glucono-1,5-lactone, the absence of transferase activity, and the high degree of specificity for the β-D-(1→4) linkage, satisfied some of the criteria[27] for exo-glucanase activity.

TABLE IV

ACTION OF CELLOBIOHYDROLASE II ON SOLUBLE CELLO-OLIGOSACCHARIDES[a]

Substrate	*Reducing sugar (μg glucose equivalent)*	*D-Glucose (μg) by D-glucose oxidase*	*Products of hydrolysis (t.l.c., h.p.l.c.)*
Cellobiose	Nil	Nil	G_2
Cellotriose	14	3	G_1, G_2 (both traces only)
Cellotetraose	181	Nil	G_2
Cellopentaose	66	1	G_2, G_3
Cellohexaose	361	4	G_2, G_3

[a]Equimolar (0.01M) amounts of the cello-oligosaccharides were incubated for 2 h at 37° with enzyme (5 μg of protein) at pH 5.0 (acetate buffer) in a total volume of 1 mL. After 2 h, the mixture was boiled for 5 min and assayed for D-glucose (by D-glucose oxidase) and reducing sugar. The remainder was freeze-dried, redissolved in the minimum of water, and examined by t.l.c. and h.p.l.c. (see Experimental). [b]G_1, glucose; G_2, cellobiose; G_3 cellotriose.

Cellobiohydrolase II and I[9] were similar in respect of their mol. wts. (II, 50,700; I, 46,300), pI (II, 5.0; I, 4.36 at 4°), and in their abilities to act synergistically with a reconstituted mixture of the endoglucanase in solubilising cotton fibre. The cellobiohydrolases differed in their carbohydrate contents (II, 19%; I, 9%), their heat stabilities (destroyed during 40 min at 60°: II, 97%; I, 65%), and their pH optima (II, 4.5; I, 2.5). Further, cellobiohydrolase II did not cross react with anticellobiohydrolase I antiserum, and their amino acid compositions were different particularly for cystine, alanine, and methionine.

Cellobiohydrolase I and II differed in their activities towards cello-oligosaccharides. Cellobiohydrolase I, but not II, had appreciable activity on cellotriose[9]. The higher cello-oligosaccharides were hydrolysed more rapidly by cellobiohydrolase II than I. The activity of cellobiohydrolase II towards cellohexaose was much higher than on any other cello-oligosaccharide. A cellobiohydrolase isolated from *T. reesei* had no capacity for hydrolysing cellohexaose[28].

The results of inhibitor tests showed that 50mM Cu^{2+} causes 7% inhibition of cellobiohydrolase II (*cf.* 27% for cellobiohydrolase I[9]). At 50mM, Mn^{2+} inhibited cellobiohydrolase II but slightly stimulated cellobiohydrolase I[9]. 200mM D-Glucose stimulated the action of cellobiohydrolase I on H_3PO_4-swollen cellulose, but inhibited cellobiohydrolase II. The converse was true for cellobiose.

5mM 2-Hydroxy-5-nitrobenzyl bromide did not inhibit[9] cellobiohydrolase I, but we have now found that inhibition occurs at 50mM. Cellobiohydrolase II was similar in this respect, and both enzymes were completely inhibited by *N*-bromosuccinimide.

Tryptophan is important for cellulase activity[29], and although the inhibition of cellobiohydrolases I and II by *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide would support the involvement of tryptophan, these reagents also modify tyrosine. However, the lack of inhibition by *N*-acetylimidazole[30] indicates that tyrosine may not be required for activity. *N*-Bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide also inhibit the cellobiohydrolase of *F. solani*[3].

In lysozyme, the tryptophan residues participate in binding, and aspartic and glutamic acid residues in catalysis[31]. Recently, a sequence analysis of an endo-(1→4)-β-D-glucanase of the fungus *Schizophyllum commune*[32] has revealed some homology with the active-site sequences of various hen egg-white lysozymes. It has also been inferred that similarities in sequences may extend to the cellobiohydrolase of *T. reesei*[33]. The observations made here that both cellobiohydrolase I and II are inhibited by carbodi-imide, which is known to react with carboxyl groups, may support the involvement of aspartic and glutamic acid residues in the activity.

Purified cellobiohydrolase II of *P. pinophilum* is similar to the cellobiohydrolases isolated from *T. reesei*[7], *T. viride*[5], *T. koningii*[4], and *F. solani*[3] in mol. wt. and glycoprotein nature. However, cellobiohydrolase II differs from the cellobiohydrolase of *T. reesei*[28] in the extent to which highly ordered cotton fibre is hydrolysed. Whereas the ability to hydrolyse cotton extensively and to produce short fibres characterises a cellobiohydrolase[28] of *T. reesei* and *T. viride*[20], cellobio-

hydrolase II of *P. pinophilum* produced no short fibres, and was unable to hydrolyse cotton fibre significantly even after prolonged incubation. Thus, there are significant differences in the substrate specificities of the various cellobiohydrolases isolated from different fungi.

Avicel, unlike the highly ordered cellulose in cotton fibre, was solubilised easily by cellobiohydrolase II. Indeed, extensive hydrolysis was effected on prolonged incubation. In this respect, cellobiohydrolase II was similar to a cellobiohydrolase of *T. viride*[20,28].

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