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The Isolation, Purification and Properties of the Cellobiohydrolase Component of *Penicillium funiculosum* Cellulase

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1. A cellobiohydrolase component was isolated from a *Penicillium funiculosum* cellulase preparation by chromatography on DEAE-Sephadex, and purifed by isoelectric focusing. 2. Purified in this way, the enzyme was homogeneous as judged by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels and isoelectric focusing in polyacrylamide gels. 3. Acting in isolation, the enzyme had little hydrolytic activity to highly ordered celluloses such as cotton fibre, but, when recombined in the original proportions with the other components [endo-(1→4)-β-D-glucanase and β-D-glucosidase] of the complex, 98% of the original activity was recovered. 4. Synergistic effects were also observed when the enzyme was acting in concert with endo-(1→4)-β-D-glucanase from other fungal sources. 5. Less-well-ordered celluloses, such as that swollen in H_3PO_4, were extensively hydrolysed, the principal product being cellobiose. 6. Attack on carboxymethyl-cellulose (CM-cellulose), which is the substrate normally used to assay for endo-(1→4)-β-D-glucanase activity, was minimal. 7. The enzyme was associated with 9% of neutral sugar, 88% of which was mannose. It was isoelectric at pH 4.36 (4°C) and had a mol.wt. of 46300 (determined by gel chromatography on a calibrated column of Ultrogel). 8. The enzyme was specific for the β-(1→4)-linkage.

The cellulolytic fungi *Trichoderma viride* (Mandels & Reese, 1964; Ogawa & Toyama, 1965; Selby & Maitland, 1967; Okada *et al.*, 1968), *Trichoderma koningii* (Toyama, 1958; Halliwell, 1965; Wood, 1968), *Fusarium solani* (Wood & Phillips, 1969; Wood, 1969), *Sporotrichum pulverulentum* (formerly called *Sporotrichum pruinosum*) (Eriksson & Rzedowski, 1969) and *Penicillium funiculosum* (Selby, 1968; Wood & McCrae, 1977*a*), when grown on cellulose as the sole carbon source, synthesize and release into solution an enzyme system that can solubilize all forms of cellulose. In contrast, cell-free culture filtrates from most other cellulolytic fungi can hydrolyse soluble (CM-cellulose) or 'swollen' cellulose (H_3PO_4-swollen cellulose), but attack on highly ordered cellulose, as typified by the cotton fibre, is minimal. The distinguishing feature of the cellulase capable of solubilizing highly ordered cellulose is that it contains an exo-(1→4)-β-D-glucanase (also called C_1; Wood & McCrae, 1972; Halliwell & Griffin, 1973) that acts in synergism with the endo-(1→4)-β-D-glucanases (also called C_x; Reese *et al.*, 1950) and β-D-glucosidases in solubilizing crystalline cellulose.

When highly purified, neither the exo-(1→4)-β-D-glucanase nor the endo-(1→4)-β-D-glucanase nor the β-D-glucosidase components can solubilize 'crystalline' cellulose to any significant extent, but reconstituted mixtures of these enzymes are capable of solubilizing 'crystalline' cellulose extensively (Mandels & Reese, 1964; Selby & Maitland, 1967; Wood, 1968, 1969; Streamer *et al.*, 1975). Quantitative recoveries of the activity to cotton manifested by the unfractionated enzyme system liberated by *T. koningii* (Wood, 1968; Wood & McCrae, 1972), *T. viride* (Selby & Maitland, 1967) and *F. solani* (Wood & McCrae, 1977*b*), have been obtained by recombining exo-(1→4)-β-D-glucanase, endo-(1→4)-β-D-glucanase and β-D-glucosidase enzymes in their original proportions.

Much of the discussion on the mechanism of cellulase action has centred on the exo-(1→4)-β-D-glucanase component. In the cellulases of *T. koningii* (Wood & McCrae, 1972; Halliwell & Griffin, 1973), *T. viride* (Emert *et al.*, 1974) and *F. solani* (Wood & McCrae, 1977*b*) it is a cellobiohydrolase. A minority view (Reese, 1975) is that cellobiohydrolase and C_1 [defined as the enzyme that must be present along with the endo-(1→4)-β-D-glucanases to solubilize highly ordered cellulose] may be separate enzymes that co-chromato-

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graph under all conditions tested, and that C_1 acts by splitting a few covalent bonds of the surface of the crystallite as a preliminary to action by the endo-(1→4)-β-D-glucanases and cellobiohydrolase. There is, however, as yet, no evidence to support this hypothesis.

In the present paper we examine the cellulase of *P. funiculosum* and present evidence showing that the enzyme that solubilizes 'crystalline' cellulose when acting in synergism with the endo-(1→4)-β-D-glucanases and β-D-glucosidases (Selby, 1968) is also a cellobiohydrolase.

Part of this work has been presented in preliminary form (Wood & McCrae, 1977*a*, 1979).

Experimental

Materials

All strains of *P. funiculosum* were obtained from the Commonwealth Mycological Institute, Kew, Surrey, U.K. The sources of the reagents, the enzyme substrates and the separation media and apparatus were as previously indicated (Wood & McCrae, 1972, 1978). Dextran (clinical grade) and Celite were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Preparative methods

Preparation of P. funiculosum cellulase. Cell-free culture filtrates were prepared from 34-day stationary cultures of the fungus grown in a medium containing cotton as the sole carbon source (Saunders *et al.*, 1948); the method used was similar to that previously described for the preparation of *T. koningii* cellulase (Wood, 1968).

Of those culture filtrates prepared, that from strain I.M.I. 81760 was the most active in terms of the capacity for solubilizing cotton cellulose (Table 1) and was selected for further study.

A large batch (6500ml) of cell-free culture filtrate from strain I.M.I. 81760 was prepared (50 Roux bottles) and the cellulase was partially purified by precipitation with $(NH_4)_2SO_4$ between the limits of 20 and 80% saturation. The precipitate was dissolved in 0.1M-sodium acetate buffer, pH 5.0, 0.02% with respect to NaN_3, to give a 50-fold enzyme concentrate. The solution was stored at −18°C.

A 50-fold dilution showed the same capacity for solubilizing cotton fibre (71%), under the conditions of the standard assay (Wood, 1969), as the original cell-free culture filtrate.

Preparation of H_3PO_4-swollen cellulose. A suspension of Avicel (5g) in phosphoric acid (88%, w/v) was kept, with occasional stirring, at 1°C for 4h. The gelatinous product was worked up as already described (Wood, 1971).

Preparation of cello-oligosaccharides. These were prepared from Whatman cellulose powder (CC 41) by acetolysis, and separated by gradient elution [0–35% (v/v) ethanol] from a column of charcoal/Celite (535) (1:1, w/w).

Isoelectric focusing. Electrofocusing was carried out in an LKB electrofocusing column (110ml) at 5°C with a sucrose density gradient with 1% (w/v) Ampholine carrier ampholytes covering the pH range 3–5. The column was filled and the sample added by the method described by Wood & McCrae (1972). The pH values of the fractions were measured at 5°C with a Corning–EEL pH-meter fitted with a combination electrode.

Analytical methods

Activity to H_3PO_4-swollen cellulose. Activity to a 22mg sample of H_3PO_4-swollen cellulose was measured, unless stated otherwise, after an 18h incubation with enzyme (Wood & McCrae, 1977*b*). The soluble sugars were assayed reductimetrically by the Somogyi–Nelson method (Nelson, 1952) or by the phenol/H_2SO_4 method (Dubois *et al.*, 1956) and expressed as glucose equivalent.

Cellulase (cotton-solubilizing) activity. Cellulase activity was determined by measuring either the residual cellulose left after a 7-day incubation of 2mg of dewaxed (Corbett, 1963) cotton (Wood, 1969) or the soluble sugars released in 18h from 20mg of cotton (Wood & McCrae, 1978).

Dextranase. The assay mixture contained 0.4ml of a solution (0.8%) of dextran in 1M-sodium acetate buffer, pH 5.4, enzyme and water to give a total volume of 2ml. After incubation for 30min at 37°C the enzyme action was stopped by the addition of Somogyi copper reagent (Nelson, 1952) and the reducing sugar was measured (Nelson, 1952).

Other assays. CM-cellulase (reducing sugar) (Wood & McCrae, 1977*b*), β-D-glucosidase (Wood, 1968) and cellobiase (Wood, 1969) activities were determined as previously described.

Reducing sugars were measured by the Somogyi–Nelson method (Nelson, 1952) or by the modified

Table 1. *Cellulase activity in cell-free culture filtrates from various strains of P. funiculosum*

Assays were performed with 2mg of dewaxed cotton and 1ml of crude culture filtrate (see the Experimental section). For experimental details see the text.

Strain	Solubilization of cotton (%)	
	In 7 days	In 14 days
I.M.I. 28044	1	1
I.M.I. 40235	37	45
I.M.I. 61383	46	56
I.M.I. 48843	49	58
I.M.I. 63903	61	70
I.M.I. 81760	71	85

Park–Johnson method (Wood & McCrae, 1978). Total carbohydrate was determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956) and glucose by the modified glucose oxidase method (Lloyd & Whelan, 1969). In each case the reagents were calibrated with D-glucose as standard.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Effect of enzymes on degree of polymerization (d.p.) of H_3PO_4-swollen cellulose. The d.p. of cellulose remaining after incubation with enzyme was determined viscometrically after dissolution in Cadoxen (Wood & McCrae, 1978).

Polyacrylamide-gel electrophoresis and electrofocusing. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Weber & Osborn (1969) with 7.5% gels. Proteins were stained with Coomassie Blue (0.01%) in methanol/water/acetic acid (1:1:0.1, by vol); destaining was effected with methanol/acetic acid/water (2:3:35, by vol.).

Analytical electrofocusing was performed in 7.5% gels containing 1% (w/v) ampholyte, with 2mA/tube. The gels were washed with 5% (w/v) trichloroacetic acid, stained with 0.2% Coomassie Blue in ethanol/water/acetic acid (5:5:1, by vol.) and then destained with ethanol/water/acetic acid (3:8:1, by vol.).

Gels were scanned at 585nm on a Gilford 250 spectrophotometer.

Carbohydrate composition. A sample of enzyme (2–3mg) was mixed with 1.25ml of 2M-HCl in a screw-capped tube and the tube was flushed out with N_2. Hydrolysis was allowed to proceed for 3h at 100°C; neutralization (pH7.0) was effected with 10% (w/v) NaOH solution. After the addition of inositol as internal standard (0.5mg to 0.5ml of water) the neutral sugars were determined by g.l.c. as their alditol acetates (Sloneker, 1971) by using a 1.5m × 6.0mm glass column of 5% SP 2340 coated on Gas-Chrom Q in a Pye Unicam model 104 gas chromatograph. An Infotronics CR5 309 microprocessor was used to integrate the peak responses.

Amino acid analysis. The Chemical and Physical Analysis Department of this Institute carried out amino acid analyses on hydrolysates prepared with 6M-HCl at 110°C for 24h by using a modified procedure based on the method of Spackman *et al.* (1958) and a Locarte amino acid analyser.

Thin-layer chromatography. Plates coated with a 0.25mm-thick layer of Kieselgel G were developed (two ascents) in ethyl acetate/propan-2-ol/water (18:13:9, by vol.), dried at room temperature and sprayed with anisaldehyde/H_2SO_4 (Stahl & Kaltenbach, 1961).

Estimation of the molecular weight by molecular-sieve chromatography. Chromatography was performed on an Ultrogel AcA-44 column (83.5cm × 1.5cm) calibrated by using the proteins cytochrome *c* (mol.wt. 12400), chymotrypsinogen (mol.wt. 24800), ovalbumin (mol.wt. 45000), and bovine serum albumin (mol.wt. 67000). The molecular weight was calculated from a plot of V_e/V_0 against the log(molecular weight).

Results

General properties of P. funiculosum cellulase

In addition to cotton-solubilizing activity, the partially purified [20–80%.-satn.-$(NH_4)_2SO_4$ fraction] showed an ability to hydrolyse Avicel, H_3PO_4-swollen cellulose, filter paper, CM-cellulose, xylan from larch wood, dextran, Sephadex G-25, cellobiose and *o*-nitrophenyl β-D-glucoside. It had a broad pH optimum extending from pH4 to pH5 in both phosphate/citrate and acetate buffers with cotton cellulose as substrate (7-day assay; see the Experimental section), but a sharp optimum at pH4.5 for both H_3PO_4-swollen cellulose and CM-cellulose substrates. The capacity for solubilizing cotton cellulose was optimum at 45°C with the 2mg/7-day assay described in the Experimental section.

A 1.0ml sample of the 20–80%-satn.-$(NH_4)_2SO_4$ fraction, diluted 50-fold, solubilized 71% of a 2mg sample of cotton cellulose in 7 days (see the Experimental section), and 0.6, 0.3 and 0.1ml produced 62, 42 and 19% hydrolysis respectively in the same period.

Cotton cellulose was hydrolysed to the same extent when the incubation was allowed to proceed in an atmosphere of N_2.

Resolution of the enzyme complex

Six protein components were separated by chromatography of the concentrated partially purified [20–80%-satn.-$(NH_4)_2SO_4$] enzyme on a column of DEAE-Sephadex (Fig. 1). The first three components were eluted with 0.06M-sodium acetate buffer, pH4.8, the other three by gradient elution involving a simultaneous increase in ionic strength (0.06 to 0.1) and a decrease in pH (4.8 to 4.0).

All fractions were tested for activity to *o*-nitrophenyl β-D-glucoside, H_3PO_4-swollen cellulose, CM-cellulose, cellobiose and dextran; the distribution of activities is shown in Fig. 1.

Cellobiase activity was found only in those fractions showing β-D-glucosidase activity, and the peaks of both activities were coincident in each of the three β-glucosidase (portions A, B and F in Fig. 1) components separated. Dextranase activity, on the other hand, was found in most of the fractions collected, but the bulk of the activity appeared in fractions 71–100; the peak of activity was in fraction 85.

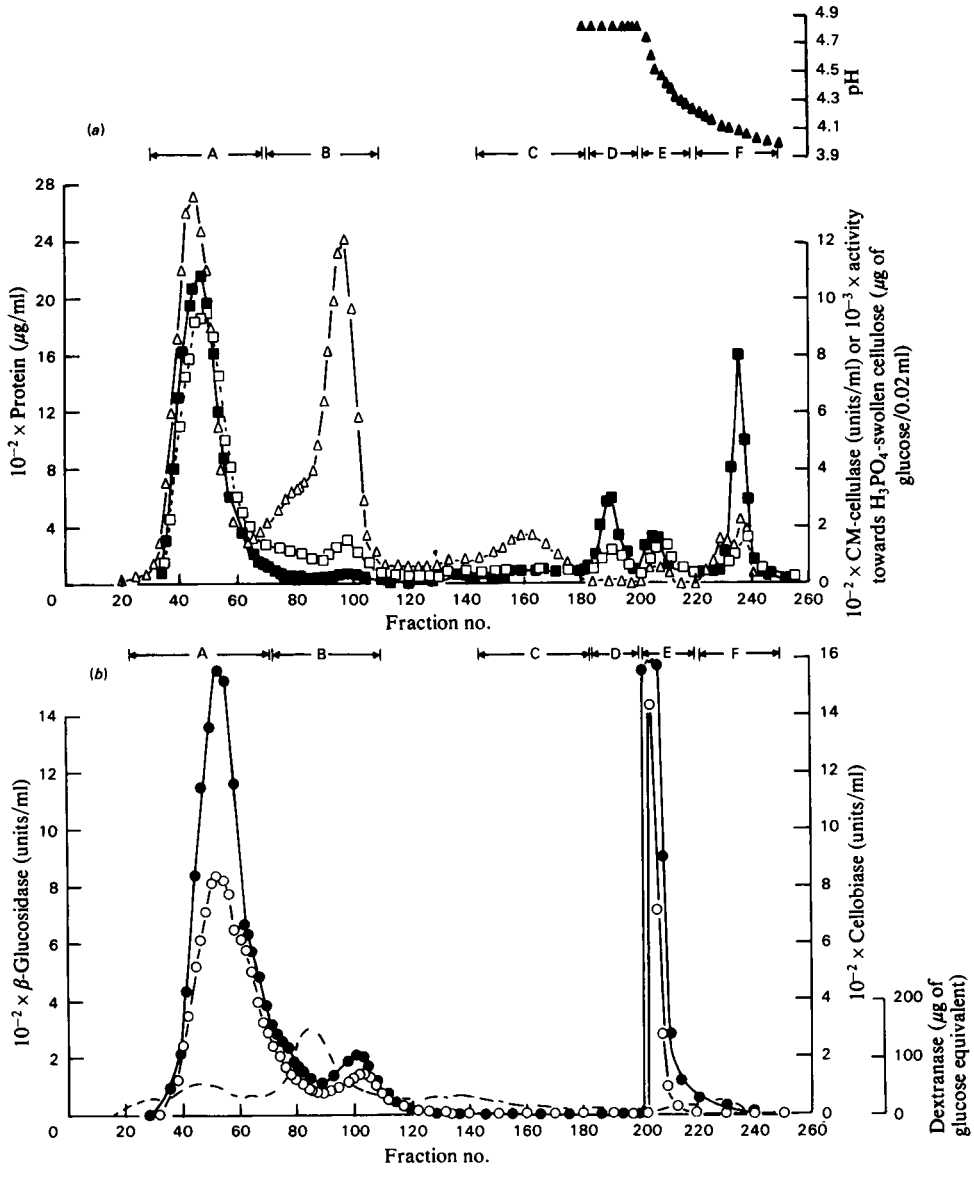

Fig. 1. *Fractionation of P. funiculosum cellulase on a column of DEAE-Sephadex*

A sample (10 ml) of concentrated partially purified enzyme [20–80%-satn.-$(NH_4)_2SO_4$ fraction] was desalted on a column of Biogel P-2 (2.5 cm × 50.1 cm) equilibrated with 10 mM-ammonium acetate buffer, pH 4.8, freeze-dried and redissolved in 15 ml of 60 mM-sodium acetate buffer, pH 4.8. A 14.75 ml portion was applied to a column (2.5 cm × 38.2 cm) of DEAE-Sephadex (acetate form) and eluted under the starting conditions at 2.5 ml/h until 180 tubes (each 2.5 ml) had been collected. The column was finally eluted with a pH gradient (▲) formed by adding 400 ml of 0.1 M-sodium acetate buffer, pH 3.8, to 400 ml of 60 mM-sodium acetate buffer, pH 4.8; fractions (9.75 ml) were collected in this stage. Assays for enzyme activity were performed as described in the Experimental section. The distribution of protein (△), CM-cellulase (■) and activity towards H_3PO_4-swollen cellulose (□) is shown in (*a*); (*b*) shows β-glucosidase (○), cellobiase (●) and dextranase (– – – –). Fractions were pooled as shown.

Of the six protein components eluted, four were rich in activity to CM-cellulose (portions A, D, E and F). Two of these (portions A and E) were associated with β-glucosidase/cellobiase activity, but only in one case (portion A) was it possible to conclude, on the basis of the observed differences in the elution volumes of the peaks of the two activities, that the two activities originated in different en-

zymes. Gel electrophoresis and electrofocusing showed that each fraction contained two or more proteins.

The further purification of the CM-cellulase components is not described here, but it has been established that each is an endo-(1→4)-β-D-glucanase (T. M. Wood, unpublished work).

All four endo-(1→4)-β-D-glucanases could solubilize H_3PO_4-swollen cellulose, but so too could the large protein component in portion B, and this was virtually free from CM-cellulase activity. Since the cellobiohydrolases of *F. solani* (Wood & McCrae, 1977*b*) and *T. koningii* (Wood & McCrae, 1972) have been identified and differentiated from the other glucanases in their respective cellulase systems by the fact that they had little or no action on the soluble CM-cellulose, but could solubilize the partially degraded H_3PO_4-swollen cellulose when acting alone, it seemed likely that the cellobiohydrolase was in portion B. This was confirmed by carrying out reconstitution experiments for cellulase (cotton-solubilizing) activity in which the individual portions obtained from the DEAE-Sephadex column were recombined in their original proportions. As Table 2 shows, none of the enzymes in any of the portions could solubilize cotton fibre extensively when acting alone, but when portion B was present in admixture there was a marked synergistic response.

Synergism between the cellobiohydrolase of one cellulase system and the endo-β-(1→4)-D-glucanase of another has been demonstrated before (Wood, 1969). Further evidence that the enzyme in portion B was the cellobiohydrolase component was obtained when it was found that this was the only component capable of solubilizing cotton fibre to a

Table 2. *Cellulase activity manifested by various fractions of P. funiculosum cellulase when acting alone and in combination*

Fractions were combined as shown in Fig. 1 and assayed for cellulase (cotton-solubilizing) activity (7-day assay; see the Experimental section) after dilution to the same final volume in each case (250ml); 0.5ml was then used to assay for cellulase activity. A 0.5ml sample of the starting material that had been diluted by an equivalent amount (10ml to 250ml) was used for comparison. Experimental details are given in the text.

Portion from Fig. 1	Enzyme/protein in the portions	Solubilization of cotton cellulose (%)
A	Endoglucanase-1 + β-glucosidase-1	2.5
B	Cellobiohydrolase + β-glucosidase-2	2.1
C	Protein	Nil
D	Endoglucanase-2	Nil
E	β-Glucosidase-3 + endoglucanase-3	0.5
F	Endoglucanase-4	1.5
A + C + D + E + F	Endoglucanases-1, -2, -3 and -4 + β-glucosidases-1 and -3	24
A + B + C + D + E + F	Cellobiohydrolase + endoglucanases-1, -2, -3 and -4 + β-glucosidases-1, -2 and -3	69
Starting material		71

Table 3. *Synergism between the endo-(1→4)-β-D-glucanase fraction of T. koningii cellulase and the various fractions of P. funiculosum cellulase in solubilizing cotton cellulose*

T. koningii endoglucanase was isolated and purified as already discussed (Wood & McCrae, 1972, 1978); 1000 units of the endoglucanase (CM-cellulase) activity were added to each assay mixture, and portion A, B, C, D, E or F was present in the same proportions in which they were present in 1ml of the 20–80%-satn.-$(NH_4)_2SO_4$ fraction of *P. funiculosum* cellulase, diluted 50-fold. Experimental details are given in the text.

Enzyme in assay	Cellulase (% solubilization)
T. koningii endoglucanase + portion A (Fig. 1)	15
T. koningii endoglucanase + portion B (Fig. 1)	44
T. koningii endoglucanase + portion C (Fig. 1)	7
T. koningii endoglucanase + portion D (Fig. 1)	6
T. koningii endoglucanase + portion E (Fig. 1)	4
T. koningii endoglucanase + portion F (Fig. 1)	4
T. koningii endoglucanase	2

significant extent when acting in concert with the endo-β-(1→4)-D-glucanases from *T. koningii* cellulase (Table 3).

Of the protein applied to the column, 91% was found in the eluate: recoveries of β-D-glucosidase and CM-cellulase were 96 and 83% respectively.

Purification of the cellobiohydrolase

Analytical disc gel electrophoresis of the cellobiohydrolase (portion B in Fig. 1) showed only one protein band (Fig. 2*b*), but gel electrofocusing showed traces of contaminating proteins isoelectric at pH values higher and lower than that of the cellobiohydrolase (Fig. 2*d*). One of the contaminants was in all probability the β-D-glucosidase component that was associated with the cellobiohydrolase after chromatography on DEAE-Sephadex; the other may have been the dextranase whose peak of activity was found in fraction 85 (Fig. 1).

The cellobiohydrolase was purified by isoelectric focusing in a 110ml LKB electrofocusing column.

A portion (36ml) of cellobiohydrolase/β-D-glucosidase component (portion B in Fig. 1) was concentrated (5ml) in a collodion tube, dialysed against 10mM-ammonium acetate buffer, pH5.0, freeze-dried, redissolved in 1% (w/v) 'light ampholyte' solution (pH3–5) and applied to the column according to the method outlined in the LKB Instruction Manual. Fig. 3 shows the distribution of β-D-glucosidase, protein and activity to H_3PO_4-swollen cellulose obtained after the sample had been focused for 66h. Under these conditions the β-D-glucosidase and cellobiohydrolase were resolved.

Cellobiohydrolase was isoelectric at pH4.36, β-D-glucosidase at pH4.65. Much of the activity to H_3PO_4-swollen cellulose that was associated with the cellobiohydrolase after chromatography on DEAE-Sephadex (Fig. 1) was separated along with the β-D-glucosidase activity; the β-D-glucosidase peak

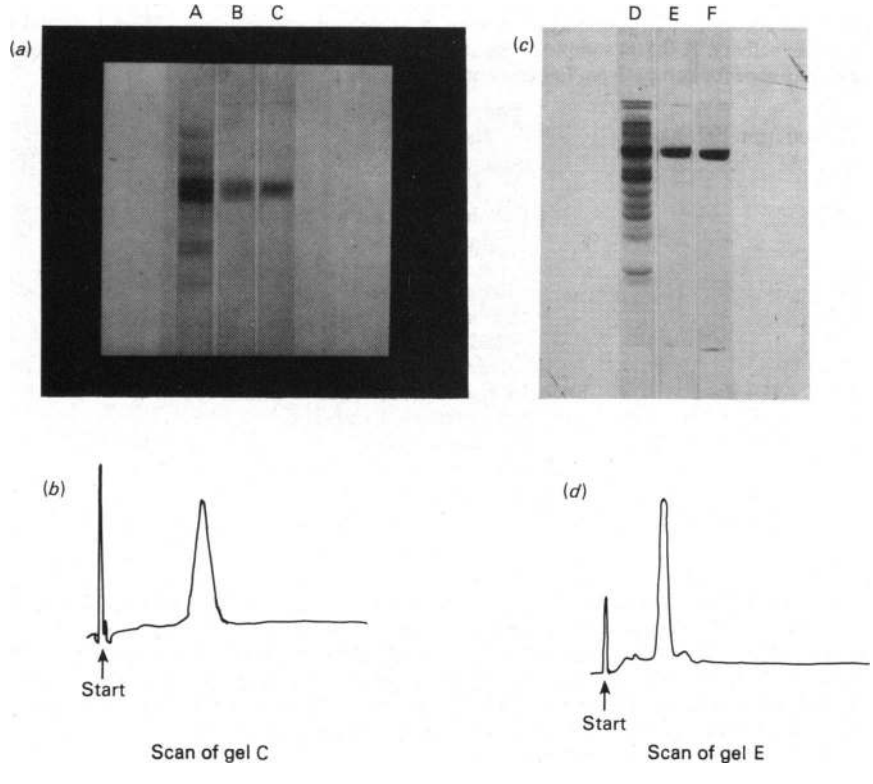

Fig. 2. *Polyacrylamide-gel electrophoresis and electrofocusing of unfractionated cellulase and the cellobiohydrolase isolated on DEAE-Sephadex (Fig. 1)*

(*a*) A, Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 20–80%-satn.-$(NH_4)_2SO_4$ fraction (142μg of protein); B and C, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of cellobiohydrolase (34μg of protein). (*b*) Scan of gel C. (*c*) D, gel electrofocusing of 20–80%-satn.-$(NH_4)_2SO_4$ fraction (34μg) in ampholyte, pH3.5–5.0; E and F, gel electrofocusing of cellobiohydrolase (34μg) in ampholyte, pH3.5–5.0. (*d*) Scan of gel E. Electrophoresis was carried out for 4h, and electrofocusing for 2h.

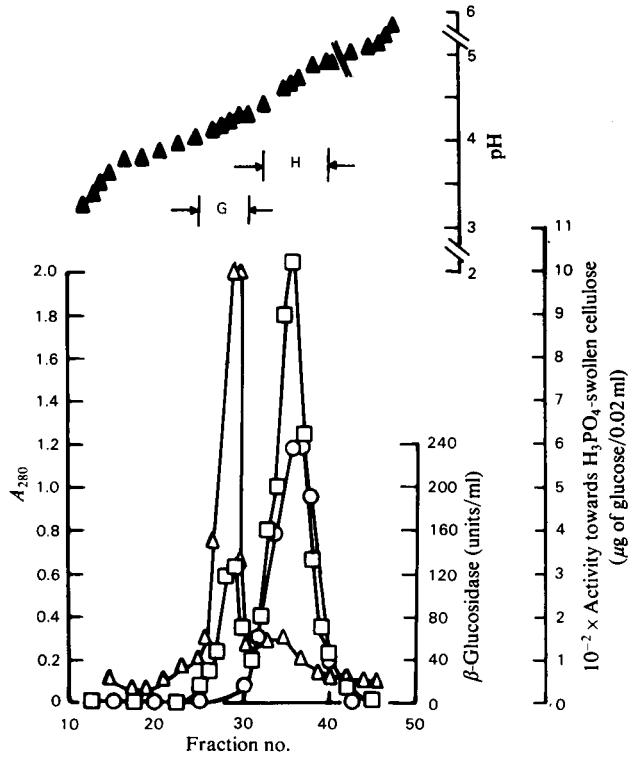

Fig. 3. *Separation of the cellobiohydrolase and β-D-glucosidase by isoelectric focusing*
Portion B (Fig. 1) was prepared for electrofocusing as described in the text. The final voltage employed during the run was 600 V; this gave a load of approx. 1.6 W. After focusing had been performed for 66 h, the contents of the column were pumped out through the bottom. Fractions (2 ml) were assayed as detailed in the Experimental section for β-D-glucosidase (○), protein (△), activity towards H_3PO_4-swollen cellulose (□) and pH (▲).

and the peak of activity to H_3PO_4-swollen cellulose were coincident in fraction 37.

Fractions 25–32 and 35–40 were pooled, and the cellobiohydrolase in fractions 25–32 was purified by a re-run in the isoelectric-focusing column under the same conditions that were used in the first run. The sucrose concentration was not taken into account in the preparation of the density gradient.

The further purified cellobiohydrolase was prepared free from ampholyte solution by precipitation with $(NH_4)_2SO_4$ (80% saturation), centrifuging, dialysing the redissolved precipitate (against 10 mM-ammonium acetate buffer, pH 5.0) in a collodion tube and freeze-drying. The enzyme was redissolved in 0.1 M-sodium acetate buffer, pH 5.0, at a concentration of 900 μg/ml. This solution, or a similar one prepared from another purification, was used for the study of the properties of the cellobiohydrolase. When purified in this way the preparation showed no detectable β-D-glucosidase or dextranase activity. Gel electrofocusing now showed only one protein band (Fig. 4).

Reconstitution experiments for cellulase (cotton solubilization) activity were again done in which the purified cellobiohydrolase and the separated endo-β-(1→4)-glucanases and β-glucosidases were mixed in their original proportions: 98% of the cellulase activity of the original cellulase complex was recovered.

Properties of the cellobiohydrolase

General properties. Purified cellobiohydrolase showed a bimodal pH–activity profile with H_3PO_4-swollen cellulose as substrate: there was a sharp peak of activity at the remarkably low pH of 2.5 and a plateau of activity at pH 4–5.5 showing 75% of the activity shown at pH 2.5. With Avicel as substrate only a small amount of reducing sugar was liberated in an 18 h incubation; a single peak of activity with an optimum at pH 3.5 was observed on this more highly ordered substrate.

The bimodal nature of the pH–activity profile observed with H_3PO_4-swollen cellulose, although unusual, is not unique. We have found the cellobiohydrolase isolated from *T. koningii* cellulase (T. M. Wood & S. I. McCrae, unpublished work)

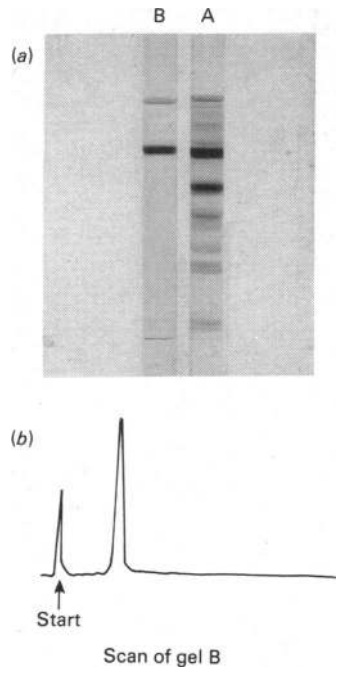

Fig. 4. *Polyacrylamide-gel electrofocusing of unfractionated cellulase and the cellobiohydrolase purified by electrofocusing (Fig. 3)*

(*a*) A, 20–80%-satn.-$(NH_4)_2SO_4$ fraction (42 μg of protein); B, cellobiohydrolase (43 μg of protein). (*b*) Scan of gel B. Conditions: ampholyte, pH 3–5; duration 2 h; voltage 320 V; 2 mA/tube.

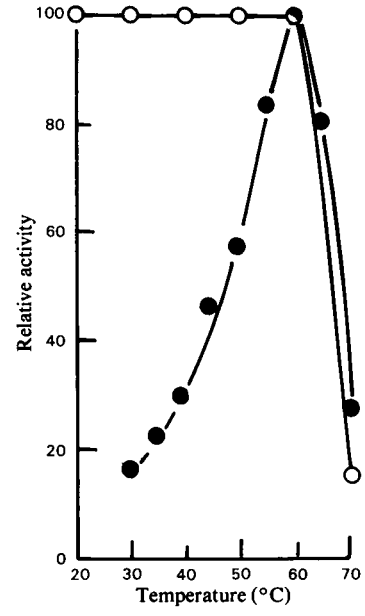

Fig. 5. *Effect of heat on the cellobiohydrolase activity*

H_3PO_4-swollen cellulose was the substrate. ●, Temperature–activity profile; the activity was measured as described in the Experimental section. ○, Thermal stability; cellobiohydrolase [25 μg of protein (Folin–Lowry method) in 0.025 ml of 0.1 M-sodium acetate buffer, pH 5.0] was heated with 0.1 M-sodium acetate buffer, pH 5.0 (1.25 ml), for 10 min. A 1.0 ml portion was removed and assayed for activity towards H_3PO_4-swollen cellulose by the method described in the Experimental section.

to exhibit a similar curve on both H_3PO_4-swollen cellulose and Avicel with optima at pH 2.5 and 5.0 in each case; *F. solani* cellobiohydrolase had pH optima at 4.0 and 5.5 on Avicel (T. M. Wood & S. I. McCrae, unpublished work). A cellobiohydrolase isolated from *T. viride* cellulase, in contrast, is reported to have a sharp pH–activity curve with an optimum of 4.8 on Avicel (Berghem *et al.*, 1975).

Like the cellobiohydrolase of *T. koningii* (Wood, 1968) and *F. solani* (Wood, 1969) cellulases, *P. funiculosum* cellobiohydrolase showed no capacity for effecting changes in cotton fibre resulting in an increase in the uptake of 18% alkali relative to a control (so-called S-factor; Marsh *et al.*, 1953).

The purified enzyme was stable at room temperature and was not affected by freezing at −20°C. Repeated freezing and thawing did not appear to produce a significant loss in activity. With H_3PO_4-swollen cellulose as substrate, optimum activity was at 60°C (Fig. 5), which is remarkable considering that the assay period was 18 h; even at 70°C an appreciable amount of activity was apparent. In the absence of substrate the enzyme was much more labile, and heating at 70°C for 10 min destroyed 90% of the activity (Fig. 5).

The enzyme was completely adsorbed on a column of concanavalin A–Sepharose from 0.1 M-potassium phosphate buffer, pH 6.5: it was eluted unchanged by a 0.5% solution of α-methyl mannoside in the same buffer.

The molecular weight of the purified enzyme, when determined by molecular-sieve chromatography on a column of Ultrogel, was 46 300.

Specificity of the cellobiohydrolase

The action of the purified cellobiohydrolase on a variety of glucans was tested by incubating substrate (5 mg) with 0.1 M-sodium acetate buffer, pH 5.0 (0.05 ml), enzyme solution [0.46 ml containing 900 μg of protein (Folin–Lowry)] and 50 mM-NaN_3 solution (1 μl) for 18 h at 37°C. After incubation, the solution was deionized with Amberlite resins IR-120 (H^+ form) and IR-45 (OH^- form), and the hydrolysis products were separated by t.l.c. The following substrates were not attacked by the enzyme: dextran [(1→6)-α-D-glucan], amylose

[(1→4)-α-D-glucan], glycogen and amylopectin [mixed (1→4)- and (1→6)-α-D-glucan], nigeran [mixed (1→4)- and (1→3)-α-D-glucan], lutean [(1→6)-β-D-glucan], CM-pachyman and a sample of (1→2)-β-D-glucan. Laminarin [(1→3)-β-D-glucan] (water-soluble) gave a small amount of glucose observable only on heavily spotted chromatograms. Lichenin and barley β-D-glucan [mixed (1→4)- and (1→3)-β-D-glucans] were hydrolysed to a small extent, and glucose (trace), cellobiose (R_{Glc} 0.56) and another spot with R_{Glc} 0.87 were found in the solution.

Xylan from oat straw was not attacked by the purified enzyme.

Action of the cellobiohydrolase on insoluble and soluble cellulose substrates

Hydrolysis of highly ordered substrates such as cotton fibre, Whatman cellulose powder (prepared from cotton) and Avicel was minimal, but the more disordered substrates such as the α-cellulose from oat straw and H_3PO_4-swollen cellulose were more highly degraded (Table 4). Qualitative analysis of the products of the reactions by t.l.c. showed that cellobiose was the principal product in each case, and this was confirmed by quantitative analysis by using the phenol/H_2SO_4 method to measure total sugar and glucose oxidase to assay glucose. Cellobiose was released exclusively from cotton.

All the assays whose results are given in Table 4 were performed with 2.0mg of substrate in a 5ml reaction mixture. The effect of substrate concentration on reaction velocity was measured in detail only for H_3PO_4-swollen cellulose. By use of the reductimetric procedure at a series of concentrations of the substrate ranging from 0.25 to 0.025%, the K_m was 217mg/100ml from a Lineweaver–Burk plot. Since the H_3PO_4-swollen cellulose sample had d.p. 1998 (determined viscometrically after dissolution in Cadoxen; Wood & McCrae, 1978), the mol.wt. was approx. 324000, and the K_m value therefore 6.7μM.

The action of the cellobiohydrolase on the soluble CM-cellulose was minimal. Indeed reducing sugars equivalent to only 10μg of glucose were released after 2h incubation of 200μg (protein) of cellobiohydrolase in the standard assay (see the Experimental section), and this did not increase further on more prolonged incubation. In a separate experiment the cellobiohydrolase (200μg of protein) showed no capacity for producing a detectable change in the viscosity (Wood, 1971) of a solution of CM-cellulose. This result is consistent with endwise attack, and is similar to the results obtained with the

Table 4. *Action of the cellobiohydrolase of P. funiculosum on some insoluble cellulose substrates*

The substrates (20mg) were incubated for 24h in a 5ml reaction mixture consisting of 1ml of 0.1M-sodium acetate buffer, pH 5.0, 50μg of enzyme protein and water to give a total volume of 2ml. The products of hydrolysis were separated by t.l.c. Quantitative analysis was done with Somogyi–Nelson, phenol/H_2SO_4 and glucose oxidase reagents. Experimental details are given in the text. Abbreviations: G_1, D-glucose; G_2, cellobiose.

Substrate	Reducing sugar liberated (μg of glucose equivalent)	Products of hydrolysis
Cotton	88	G_2
Whatman cellulose powder	151	G_2 (96%) + G_1 (4%)
Avicel	278	G_2 (94%) + G_1 (6%)
Oat straw α-cellulose	643	G_2 (96%) + G_1 (4%)
H_3PO_4-swollen cellulose	1199	G_2 (96%) + G_1 (4%)

Table 5. *Action of the cellobiohydrolase on cello-oligosaccharides*

Equimolar amounts of the cello-oligosaccharides were incubated at 37°C with 0.5ml of 0.1M-sodium acetate buffer, pH 5.0, enzyme (50μg of protein) and water to give a total volume of 1ml. After 4h a sample was assayed for reducing sugar by the modified Park–Johnson method (Wood & McCrae, 1978). The products of hydrolysis were separated by t.l.c. Experimental details are given in the text. Abbreviations: G_1, D-glucose; G_2, cellobiose; G_3, cellotriose.

Substrate	Reducing sugars liberated (μg of glucose equivalent)	Products of hydrolysis
Cellobiose	Nil	Nil
Cellotriose	67	$G_2 + G_1$
Cellotetraose	145	G_3 (trace) + G_2 + G_1 (trace)
Cellopentaose	249	$G_3 + G_2$

cellobiohydrolases of both *F. solani* (Wood & McCrae, 1977*b*) and *T. koningii* (Wood & McCrae, 1972).

Action of cellobiohydrolase on cello-oligosaccharides

Cellobiose was not a substrate for purified cellobiohydrolase, but the cello-oligosaccharides cellotriose, cellotetraose and cellopentaose were readily hydrolysed, the rate increasing as the d.p. increased (Table 5).

With cellotriose the K_m was found to be 0.85 mM from a Lineweaver–Burk plot and by the use of glucose oxidase method to measure the liberated glucose.

Effect of the cellobiohydrolase on the d.p. of H_3PO_4-swollen cellulose

Purified cellobiohydrolase decreased the d.p. of H_3PO_4-swollen cellulose slowly, as would be expected from an endwise-acting enzyme; indeed, when 7% of the substrate had been solubilized the chain length had only decreased from 1998 to approx. 1400 (Table 6). Even this fall in d.p. is greater than expected after 7% hydrolysis; the reason is not clear.

The effect of a mixture of the endo-(1→4)-β-D-glucanases and β-D-glucosidase (portions A, D, E and F in Fig. 1) components on the d.p. was more dramatic than the effect shown by the cellobiohydrolase; at approximately 10% solubilization the substrate had d.p. 220. These results are similar to those reported for the cellobiohydrolases and endo-(1→4)-β-D-glucanases of *F. solani* (Wood & McCrae, 1977*b*) and *T. viride* (Pettersson *et al.*, 1972).

Effects of various additives on the activity of purified cellobiohydrolase of P. funiculosum (Table 7)

Sugar solutions were added directly to the H_3PO_4-swollen cellulose digest (see the Experimental section) and the effect of enzyme (50 μg of protein) was measured after 18 h, by using the reductimetric method. Under these conditions neither glucose nor cellobiose nor gluconolactone was inhibitory at 10 mM concentration; methylcellulose at a concentration of 0.1% caused 61% inhibition.

The effects of metal ions and other additives were tested by preincubating an aqueous solution of the additive, enzyme (50 μg of protein in 0.05 ml of 0.1 M-sodium acetate buffer, pH 5.0) and water to give a total volume of 0.25 ml, for 30 min at 25°C, and then testing the activity of a 0.2 ml sample of the solution with H_3PO_4-swollen cellulose as substrate (see the Experimental section); the sugar that was solubilized was assayed by the phenol/H_2SO_4 method. 2-Hydroxy-5-nitrobenzyl bromide was dissolved in aq. 5% (v/v) acetone and then preincubated with enzyme as above. The results, which are presented in Table 7, show clearly that only Ca^{2+} stimulated the enzyme significantly; *N*-bromosuccinimide was a potent inhibitor.

Table 6. *Comparison of the rate of change in degree of polymerization of H_3PO_4-swollen cellulose effected by P. funiculosum cellobiohydrolase and other enzymes of the complex*

The enzymes were incubated with approx. 40 mg of H_3PO_4-swollen cellulose for the times shown, and the d.p. of the residual cellulose was measured after dissolution in Cadoxen (Wood & McCrae, 1978). The assay mixtures contained approx. 50 μg of protein. Endo-(1→4)-β-D-glucanase/β-glucosidase was made up of equal volumes of portions A, D, E and F in Fig. 1. Experimental details are given in the text.

	D.p. of H_3PO_4-swollen cellulose after enzymic hydrolysis	
Time (h)	Cellobiohydrolase	Endoglucanase/ β-glucosidase
½	1854	500
1	1905	310
2	1824	220
4	1884	160
24	1419	—

Table 7. *Effects of various additives on the activity of the cellobiohydrolase of P. funiculosum*

See the text for details of the assays.

Additive	Concentration mixed with enzyme (mM)	Relative activity (%)
Ba^{2+}	50	103
Ca^{2+}	50	130
Zn^{2+}	50	100
Mg^{2+}	50	90
Co^{2+}	50	110
Mn^{2+}	50	110
Mg^{2+}	50	104
Cu^{2+}	5	73
	50	73
Gluconolactone	10	100
Glucose	10	100
Cellobiose	10	100
Methylcellulose	0.1%	39
EDTA	50	118
N-Bromosuccinimide	5	0
2-Hydroxy-5-nitrobenzyl bromide	10	98
N-Acetylimidazole	50	120

Synergistic effects between the cellobiohydrolase and the endo-(1→4)-β-D-glucanase/β-glucosidase in solubilizing H_3PO_4-swollen cellulose

The actions of the cellobiohydrolase (50μg of protein, determined by the Folin–Lowry method) when acting alone and in concert with a mixture of the endo-(1→4)-β-D-glucanases (5 units of CM-cellulase) and β-D-glucosidases (3.5 units) were compared. It was found that the reducing sugars liberated by the mixture of enzymes in 24h (3.25mg) from 22mg of substrate (see the Experimental section) was greater than that released by the cellobiohydrolase (0.63mg) and endoglucanase/β-glucosidases (1.28mg) acting separately (Fig. 6). Similar results have been reported for cellobiohydrolase and endo-β-(1→4)-D-glucanase of *F. solani* cellulase (Wood & McCrae, 1979). Streamer *et al.* (1975), in contrast, could find no synergism between the exo-β-D-glucanase and endo-(1→4)-β-D-glucanases of *S. pulverulentum* cellulase with H_3PO_4-swollen cellulose as substrate.

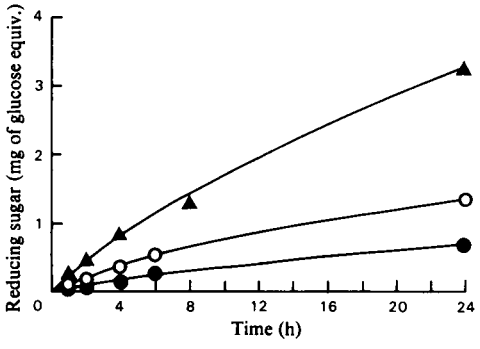

Fig. 6. *Synergistic effects between the cellobiohydrolase and the other components of the cellulase complex in solubilizing H_3PO_4-swollen cellulose*

●, Cellobiohydrolase (50μg of protein; Folin–Lowry method); O, endo-(1→4)-β-D-glucanase (5 units of CM-cellulase) and β-D-glucosidase (3.5 units); ▲, cellobiohydrolase (50μg of protein) and endo-(1→4)-β-D-glucanase (5 units of CM-cellulase) and β-D-glucosidase (3.5 units). Endo-(1→4)-β-D-glucanase/β-glucosidase was from portion A in Fig. 1.

Synergism between the cellobiohydrolase of P. funiculosum and the endo-(1→4)-β-D-glucanases of other fungi

Synergism between the cellobiohydrolase of *P. funiculosum* and the endo-(1→4)-β-D-glucanases elaborated by two types of cellulolytic fungi was tested: those fungi that always release a cellobiohydrolase in active form into the culture medium along with the endo-(1→4)-β-D-glucanase (*T. koningii* and *F. solani*), and those that release only the endo-(1→4)-β-D-glucanases (e.g. *Myrothecium verrucaria*, *Stachybotrys atra* and *Memnoniella echinata*). As Table 8 shows, synergism in solubilizing cotton cellulose was apparent in all cases, but it was more marked when *P. funiculosum* cellobiohydrolase was acting in concert with the endo-(1→4)-β-D-glucanases of *T. koningii* or *F. solani*.

Chemical composition of the cellobiohydrolase of P. funiculosum and a comparison with the compositions of F. solani and T. koningii cellobiohydrolases

The neutral-sugar composition of the cellobiohydrolases of *P. funiculosum* as analysed by g.l.c. of the alditol acetates is shown in Table 9; the

Table 8. *Cotton-solubilizing activity shown by mixtures of the cellobiohydrolase of P. funiculosum and the endo-β-(1→4)-D-glucanases of miscellaneous cellulolytic fungi*

Assay mixtures (Wood, 1969) contained the same number of units (1000) of CM-cellulase [endo-(1→4)-β-D-glucanase] and β-D-glucosidase activities; β-D-glucosidase from *F. solani* was added to make all assay mixtures equivalent in terms of this activity. Cell-free-culture filtrates were prepared from 28-day cultures by using the method described for the preparation of *P. funiculosum* cellulase (see the Experimental section). *T. koningii* and *F. solani* endo-β-(1→4)-D-glucanases were prepared free from their respective cellobiohydrolases by chromatography on DEAE-Sephadex (Wood, 1968, 1969). *P. funiculosum* cellobiohydrolase (200μg) had little capacity for solubilizing cotton fibre when acting alone. Experimental details are given in the text.

	Solubilization of cotton cellulose (%)	
Source of endoglucanase	Endoglucanase acting alone	Endoglucanase with *P. funiculosum* cellobiohydrolase added
T. koningii I.M.I. 73022	1	51
F. solani I.M.I. 95994	1	45
Myro. verrucaria I.M.I. 45541	6	20
Stach. atra I.M.I. 82021	4	11
Memn. echinata I.M.I. 16201	5	18

carbohydrate analyses of the cellobiohydrolases of *T. koningii* and *F. solani* have not been published previously, and are provided for comparison.

Unlike the cellobiohydrolase of *P. funiculosum*, which appears to be homogeneous after isoelectric focusing, the cellobiohydrolases of *T. koningii* (Wood & McCrae, 1972) and *F. solani* (Wood & McCrae, 1977*b*) exist in multiple forms that are separable by isoelectric focusing provided that carrier ampholyte covering a very narrow pH range (0.5 pH unit) is used. The analyses shown in Table 9 were performed on cellobiohydrolases purified by ion-exchange chromatography and isoelectric focusing covering 2 pH units, and under these conditions separation of the various isoenzymes is incomplete. In the case of *F. solani*, then, the value for the neutral sugar composition is the mean of the carbohydrate component of four cellobiohydrolases, and in the case of *T. koningii* the mean of two.

Table 9 shows that the neutral sugar composition of the cellobiohydrolases from the three fungi consisted largely of mannose. Mannose has already been shown to be the predominant neutral sugar of the four cellobiohydrolases isolated from *T. viride* (Gum & Brown, 1977).

The cellobiohydrolase of *P. funiculosum* consisted of 9% of neutral sugar (determined by g.l.c.); the amount of carbohydrate associated with the cellobiohydrolases of *F. solani* (Wood & McCrae, 1977*b*) and *T. koningii* (Wood & McCrae, 1972) has already been discussed.

The amino acid composition of the cellobiohydrolases from the three different cellulases is presented in Table 10. The enzymes were high in acidic amino acid content and low in basic amino acid content, a factor that explains the acidic isoelectric pH recorded in each case (Wood & McCrae, 1972, 1977*b*). *F. solani* cellobiohydrolase contained the lowest percentage of threonine and the highest percentage of lysine.

Table 9. *Neutral carbohydrate composition of the cellobiohydrolases from P. funiculosum, F. solani and T. koningii*
The monosaccharides were identified and quantified by g.l.c. (see the Experimental section). The isolation of the cellobiohydrolase of *F. solani* (Wood & McCrae, 1977*b*) and of *T. koningii* (Wood & McCrae, 1972) has already been described.

	Neutral sugar composition (% of total sugars)		
Sugar	*P. funiculosum* cellobiohydrolase	*F. solani* cellobiohydrolase	*T. koningii* cellobiohydrolase
Mannose	88	83	73
Xylose	7	—	Trace
Glucose	4	—	27
Galactose	1	17	—

Table 10. *Amino acid analyses of the cellobiohydrolases of P. funiculosum, F. solani and T. koningii cellulases*
Experimental details are given in the text.

	Composition (mol/100 mol)		
	P. funiculosum cellobiohydrolase	*F. solani* cellobiohydrolase	*T. koningii* cellobiohydrolase
Aspartic acid	12.2	14.8	13.0
Threonine	13.6	7.5	12.3
Serine	10.6	8.9	11.0
Glutamic acid	5.5	6.9	10.0
Proline	4.1	5.4	5.8
Glycine	12.6	11.0	13.4
Alanine	7.9	5.8	6.7
Half-cystine	4.7	4.7	4.9
Valine	6.1	6.2	2.5
Methionine	1.5	1.8	0.8
Isoleucine	3.1	2.9	2.0
Leucine	4.6	5.5	5.0
Tyrosine	4.6	5.2	4.4
Phenylalanine	3.0	4.3	2.5
Lysine	2.1	6.1	2.9
Histidine	1.6	1.1	1.1
Arginine	2.2	1.9	1.7

During the amino acid analysis the hydrolysates were found to contain very small amounts of hexosamine. Glucosamine was found in the hydrolysates of all three cellobiohydrolases, but galactosamine was found in hydrolysates from the cellobiohydrolases of *T. koningii* and *P. funiculosum* only. More accurate hexosamine values obtained by using alternative methods of analysis would have to be obtained before any suggestion with regard to the structural significance of these sugars could be made.

Discussion

The extracellular cellulase elaborated by the fungus *P. funiculosum* is a highly active cellulase in terms of its capacity for hydrolysing highly ordered cellulose. Indeed, it is comparable with the cellulases of *T. koningii* and *F. solani* (Wood & McCrae, 1977*b*) in this regard, and therefore belongs to a select band of enzymes that can solubilize all forms of cellulose.

Like the cellulases of *T. koningii* and *F. solani* prepared in this laboratory, and the cellulases of *T. viride* (Mandels & Reese, 1964; Emert *et al.*, 1974) and *S. pulverulentum* (Eriksson & Pettersson, 1975) prepared in others, *P. funiculosum* cellulase comprises a complex of enzymes. Fractionation studies on DEAE-Sephadex showed that the *P. funiculosum* complex contains a cellobiohydrolase, three or more endo-(1→4)-β-D-glucanases and two or three β-D-glucosidases. These enzymes acting in isolation had little capacity for solubilizing cotton cellulose, but, when recombined in their original proportions, the activity to cotton cellulose was recovered quantitatively.

The question of the involvement of other enzymes in the breakdown of cellulose has been raised from time to time; oxidative enzymes have been shown to be a feature of the cellulase of *S. pulverulentum* (Eriksson *et al.*, 1974). It seems likely, however, on the basis of fractionation and other studies reported in the present paper, that the cellulase of *P. funiculosum* can be described completely in terms of hydrolytic activities. The activity of the cellulase of *S. pulverulentum* was diminished markedly when the hydrolysis of cotton cellulose was allowed to proceed in an atmosphere of N_2; the activity of *P. funiculosum* cellulase, in contrast, was unaffected by N_2.

The cellobiohydrolase of *P. funiculosum* was purified to a state of homogeneity as judged by gel electrophoresis and gel electrofocusing. In this purified form, the enzyme had a substrate specificity and mode of action identical with those reported earlier for the cellobiohydrolases of *F. solani* (Wood & McCrae, 1977*b*) and *T. koningii* (Wood & McCrae, 1972). It had, for example, little capacity for solubilizing highly ordered celluloses such as cotton, Avicel or Whatman cellulose powder, but a partially disordered and highly hydrated cellulose such as that swollen in H_3PO_4 was more readily degraded; in each case the principal product of the hydrolysis was cellobiose.

Other points of similarity between the cellobiohydrolases of *P. funiculosum* and the cellobiohydrolases of *T. koningii* (Wood & McCrae, 1972, 1977*a*) and *F. solani* (Wood & McCrae, 1977*b*) were (*a*) the rate of change of the degree of polymerization of H_3PO_4-swollen cellulose was low compared with the rate shown by the endo-(1→4)-β-D-glucanases, (*b*) it was not inhibited by low concentrations of D-glucono-(1→5)-lactone, (*c*) it showed no transferase activity, (*d*) it was highly specific for the (1→4)-β-linkage, and (*e*) it attacked soluble cello-oligosaccharides at a rate that increased as the degree of polymerization increased. All of these observations are compatible with the interpretation that the enzyme is an exo-(1→4)-β-D-glucanase (Reese *et al.*, 1968); the fact that cellobiose was the principal product of hydrolysis of highly ordered and partially degraded celluloses suggests that the enzyme is a cellobiohydrolase.

That the cellobiohydrolase existed in the cellulase in only one form is noteworthy, for, in the main, the cellobiohydrolases isolated from other sources exist in multiple forms that are separable by isoelectric focusing and gel electrophoresis. *F. solani* cellobiohydrolase (Wood & McCrae, 1977*b*) and *T. viride* cellobiohydrolase (Gum & Brown, 1977), for example, have been isolated in four forms, and *T. koningii* cellobiohydrolase (Wood & McCrae, 1972) in two. The various forms of the cellobiohydrolases found in any one enzyme system differ in respect of their carbohydrate composition (Wood & McCrae, 1972, 1977*b*; Gum & Brown, 1977) and they have minor differences in their amino acid composition (Gum & Brown, 1977) and in pI, but they have the same substrate specificity and mode of action (Wood & McCrae, 1972, 1977*b*). The cellobiohydrolases from *F. solani* (Wood & McCrae, 1977*b*) and *T. koningii* (Wood & McCrae, 1972) have isoelectric pH values in the region pH 3.8–4.95; *P. funiculosum* cellobiohydrolase was isoelectric at pH 4.36.

P. funiculosum cellobiohydrolase had a mol.wt. of 46000 when determined by molecular-sieve chromatography on Ultrogel (LKB), and this compares with a value of 45000 for *F. solani* cellobiohydrolase (Wood & McCrae, 1977*b*) evaluated in the same way. Mol.wts. of 62000 for *T. koningii* cellobiohydrolase (Wood, 1972) and 48000 for *T. viride* cellobiohydrolase (Gum & Brown, 1977; Berghem *et al.*, 1975) have been reported.

All of these properties clearly are important for enzyme characterization, but of particular interest in

terms of the mode of action is the synergism that exists between the cellobiohydrolase and the endo-(1→4)-β-D-glucanases in solubilizing highly ordered celluloses. The fact that the enzyme isolated from *P. funiculosum* cellulase that has the special property of acting synergistically with the other components of the complex is a cellobiohydrolase is important, but it takes on a special significance in mechanistic terms since the same type of enzyme has been previously found to exist in only two different fungal genera (*Fusarium* and *Trichoderma*).

The enzyme isolated from *S. pulverulentum* cellulase with synergistic properties similar to those displayed by the cellobiohydrolases discussed above is also reported to be an exo-(1→4)-β-D-glucanase. However, in liberating glucose, cellobiose, cellotriose and cellotetraose it is certainly fundamentally different from the endwise-acting synergists from the other species of fungus, which are highly specific for the penultimate linkage on the non-reducing end of the cellulose chain. But the exo-(1→4)-β-D-glucanase of *S. pulverulentum* is remarkable in other respects: its action on Avicel is strongly inhibited by D-glucono-(1→5)-lactone, a concentration of 1 μM being sufficient to cause 83% inhibition. As a rule, D-glucono-(1→5)-lactone is a poor inhibitor of exoglucanases (Reese *et al.*, 1968); it certainly was with *P. funiculosum* cellobiohydrolase (nil at 10 μM), as it was with *F. solani* cellobiohydrolase (Wood & McCrae, 1977*b*).

In the context of the present discussion another observation made during the present investigation is of importance. That is the fact that the β-glucosidase separated from the cellobiohydrolase by isoelectric focusing was also capable of hydrolysing H_3PO_4-swollen cellulose. An enzyme with a capacity for attacking both a nitrophenyl glucoside and a long-chain polymer such as that found in H_3PO_4-swollen cellulose is, according to definition (Reese *et al.*, 1968), more likely to be an exo-(1→4)-β-D-glucanase than a β-D-glucosidase or cellobiase. If it is an exoglucanase then an interesting point is raised, for we have found (Wood & McCrae, 1979) that this enzyme, unlike the cellobiohydrolase, is unable to act synergistically with the endo-(1→4)-β-D-glucanases to solubilize cotton fibre.

Why is it that an enzyme that can remove only one glucose residue at a time from the cellulose chain cannot act synergistically with the endo-(1→4)-β-D-glucanases, whereas another enzyme that removes glucose residues two at a time (cellobiohydrolase) can? Clearly there are several possible explanations. The contention (Reese, 1975) that the cellobiohydrolase (defined as the enzyme that liberates cellobiose from H_3PO_4-swollen cellulose) is associated with a C_1-type component [defined by Reese (1975) as the component that acts in synergism with the endoglucanases to solubilize highly ordered celluloses] can almost certainly be discounted, since there is no evidence for the non-identity of the two activities (cellobiohydrolase and C_1) in the present or any other study (Wood & McCrae, 1972, 1977*a*). An alternative speculation, which recognizes that the steric rigidity and conformation of the anhydroglucose unit in the cellulose crystallite are important factors in a cellulase context, is more appealing. Since cellobiose is the repeating unit in the crystallite, clearly one could visualize that for stereochemical reasons only a cellobiohydrolase would be a suitable catalyst. The cellobiohydrolase would be capable of attacking the new chain ends in the crystallite that were created by the endo-(1→4)-β-D-glucanases, but the glucohydrolase would not; the glucohydrolase would be capable of attacking only the chain ends of substrates that were fully hydrated, e.g. H_3PO_4-swollen cellulose.

But the requirement for attack on the cellulose crystallite may be even more demanding, and attack may be effected only by the formation of an enzyme–enzyme complex of cellobiohydrolase and certain endo-(1→4)-β-D-glucanases on the surface of the crystallite (Wood & McCrae, 1978). The observation that the cellobiohydrolase of *P. funiculosum* can act synergistically in solubilizing cotton fibre with the endoglucanases of some fungi (e.g. *T. koningii* and *F. solani*) rather better than others (e.g. *Myro. verrucaria* and *Stach. atra*) could be interpreted as supporting such a hypothesis.

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