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PURIFICATION AND SOME PROPERTIES OF A (1→4)-β-D-GLUCAN GLUCOHYDROLASE ASSOCIATED WITH THE CELLULASE FROM THE FUNGUS *Penicillium funiculosum*

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ABSTRACT

The (1→4)-β-D-glucan glucohydrolase from *Penicillium funiculosum* cellulase was purified to homogeneity by chromatography on DEAE-Sephadex and by isoelectric focusing. The purified component, which had a molecular weight of 65,000 and a pI of 4.65, showed activity on H_3PO_4-swollen cellulose, *o*-nitrophenyl β-D-glucopyranoside, cellobiose, cellotriose, cellotetraose, and cellopentaose, the K_m values being 172 mg/mL, and 0.77, 10.0, 0.44, 0.77, and 0.37mM, respectively. D-Glucono-1,5-lactone was a powerful inhibitor of the action of the enzyme on *o*-nitrophenyl β-D-glucopyranoside (K_i 2.1 μM), cellobiose (K_i 1.95 μM), and cellotriose (K_i 7.9 μM) [*cf.* D-glucose (K_i 1756 μM)]. On the basis of a Dixon plot, the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside appeared to be competitively inhibited by D-glucono-1,5-lactone. However, inhibition of hydrolysis by D-glucose was non-competitive, as was that for the gluconolactone–cellobiose and gluconolactone–cellotriose systems. Sophorose, laminaribiose, and gentiobiose were attacked at different rates, but the action on soluble *O*-(carboxymethyl)cellulose was minimal. The enzyme did not act in synergism with the endo-(1→4)-β-D-glucanase component to solubilise highly ordered cotton cellulose, a behaviour which contrasts with that of the other exo-(1→4)-β-D-glucanase found in the same cellulase, namely, the (1→4)-β-D-glucan cellobiohydrolase.

INTRODUCTION

Whereas native cellulose appears to be homogeneous chemically, it is heterogeneous physically in that it contains areas of very highly hydrogen-bonded, ordered cellulose and areas in which the cellulose is relatively disordered. The disordered ("amorphous") cellulose component, which will be partially hydrated in aqueous suspensions, is susceptible to hydrolysis by extracellular microbial cellulases, but highly ordered cellulose is attacked by few cellulases. Of the cellulases studied, those from the fungi *Trichoderma koningii*[1,2], *T. viride*[3–5], *T. reesei*[6,7], *Fusarium solani*[8], *Sporotrichum pulverulentum*[9], and *Talaromyces emersonii*[10] can hydrolyse native

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cellulose to an extent that would suggest that the more-ordered cellulose is attacked; *Penicillium funiculosum*[11] cellulase is also in this category. These micro-organisms can synthesise a specific type of exo-(1→4)-β-D-glucanase in addition to the endo-(1→4)-β-D-glucanase and β-D-glucosidases which are found in the extracellular cellulases from most of the other cellulolytic micro-organisms[12,13]. These exo- and endo-(1→4)-β-D-glucanases, when separated and purified, can solubilise amorphous and hydrated cellulose, such as that swollen in phosphoric acid, but they have little or no effect on highly ordered cellulose[13]. Solubilisation of the highly ordered celluloses (*e.g.*, cotton fibre) clearly requires the co-operative action of the two enzymes.

These exo-(1→4)-β-D-glucanases are usually (1→4)-β-D-glucan cellobiohydrolases[12,14–17], but at least one appears to be less specific and cleaves glycosidic linkages other than that broken by the cellobiohydrolases[18]. We now report on the isolation, purification, and properties of an exo-(1→4)-β-D-glucanase that apparently does not co-operate with the endo-(1→4)-β-D-glucanases to solubilise highly ordered cellulose and which appears to be a (1→4)-β-D-glucan glucohydrolase.

Part of this work has been presented in preliminary form[19].

EXPERIMENTAL

Materials. — CM-cellulose [*O*-(carboxymethyl)cellulose] sodium salt (Cellofas B) with a d.s. of ~0.5 was obtained from I.C.I. Ltd (Nobel Division), cotton fibre from the Shirley Institute (Manchester), Sephadex G-25 and DEAE-Sepharose from Pharmacia, Ultrogel AcA 44, ampholine carrier ampholytes, and isoelectric focusing columns from L.K.B. Ltd., D-glucose oxidase (Type II) from Sigma, peroxidase from Boehringer, Avicel (microcrystalline cellulose) from Honeywell and Stein Ltd., and collodion tubes from Sartorius Instruments Ltd.

Preparative methods. — (*a*) Penicillium funiculosum *cellulase.* Cell-free culture filtrates were prepared[1] from 34-day, stationary cultures (Roux bottles) of the fungus grown (28°) in a salts medium containing cotton fibre (4 g/150 mL of medium) as the sole carbon source. An enzyme concentrate (50-fold) was prepared by precipitation with $(NH_4)_2SO_4$ between 20 and 80% saturation, centrifugation (75,000*g*, 30 min), and dissolution of the precipitate in 0.1M acetic acid–NaOH buffer (pH 5.0) which was 0.02% with respect to NaN_3. The solution was stored at −18°.

A sample (1 mL) of the concentrated enzyme that had been diluted (× 50) to the original concentration could solubilise 71% of cotton fibre (2 mg) under the conditions of the standard assay[8].

(*b*) *H_3PO_4-Swollen cellulose.* Avicel was swollen[20] in 88% H_3PO_4 at 1°.

(*c*) *Cadoxen solution.* A mixture of ethylenediamine (280 g), water (720 mL), and cadmium oxide (100 g) was stirred at 20° for 3 h and at 4° for 18 h, and then centrifuged (2600*g* for 30 min)[21]. The clear solution was kept at 4°.

(*d*) *Cello-oligosaccharides.* Whatman cellulose powder (No. 1) was acetolysed; the products were deacetylated, and eluted from a column (105 × 3.5 cm) of charcoal (acid-washed)–Celite (1 : 1, w/w), using an ethanol gradient (0→30%) in water.

(*e*) *Reduced cellotetraose.* To a solution of cellotetraose (150 mg) in water (~5 mL) was added KBH_4 (150 mg), and the mixture was stored overnight at room temperature. Excess of borohydride was decomposed with acetic acid (glacial), and the solution was deionised on columns of Amberlite IR-120 (H^+) and IR-45 (HO^-) resins.

Isoelectric focusing. An LKB isoelectric focusing column (110 mL) was used at 5° according to the manufacturer's instructions and as detailed by Wood and McCrae[12]. The column was emptied by pumping water at 120 mL/h on to the top of the column.

Analytical methods. — (*a*) *Protein.* The method of Lowry *et al.*[22] was used with crystalline bovine serum albumin as the standard.

(*b*) *Sugars.* Reducing sugars were determined by the Nelson–Somogyi method[23], D-glucose by D-glucose oxidase[24], and total carbohydrate by the phenol–sulfuric acid method[25].

(*c*) *Enzymes.* CM-cellulase activity was measured by the reducing sugar method[15]. Activities towards *o*-nitrophenyl β-D-glucopyranoside, H_3PO_4-swollen cellulose[20], and cotton fibre (residual cellulose left after a 7-day incubation of 2 mg of dewaxed cotton[8]) were measured as already described.

Effect of the enzyme on the d.p. of H_3PO_4-swollen cellulose. — The d.p. of the cellulose left after incubation with the enzyme was determined viscometrically after dissolution in cadoxen[21].

Polyacrylamide gel electrophoresis and electrofocusing. — SDS-gel electrophoresis was performed by the method of Weber and Osborn[26] with 7.5% gels. Protein was located by staining with Coomassie Brilliant Blue R-250 (0.01%) in methanol–water–acetic acid (1:1:0.1). Destaining was effected with methanol–acetic acid–water (2:3:35).

Analytical electrofocusing was done in 7.5% gels containing 1% of ampholyte, using 2 mA/tube. The gels were washed with aqueous 5% trichloroacetic acid, stained with Coomassie Brilliant Blue R-250 (0.2%) in ethanol–water–acetic acid (5:5:1), and destained with the same mixture (3:8:1).

K_m *Values.* — The values for the purified (1→4)-β-D-glucan glucohydrolase were determined from the corresponding Lineweaver–Burk plots.

For *o*-nitrophenyl β-D-glucopyranoside, the published[1] assay was used with substrate concentrations up to 1.25mM.

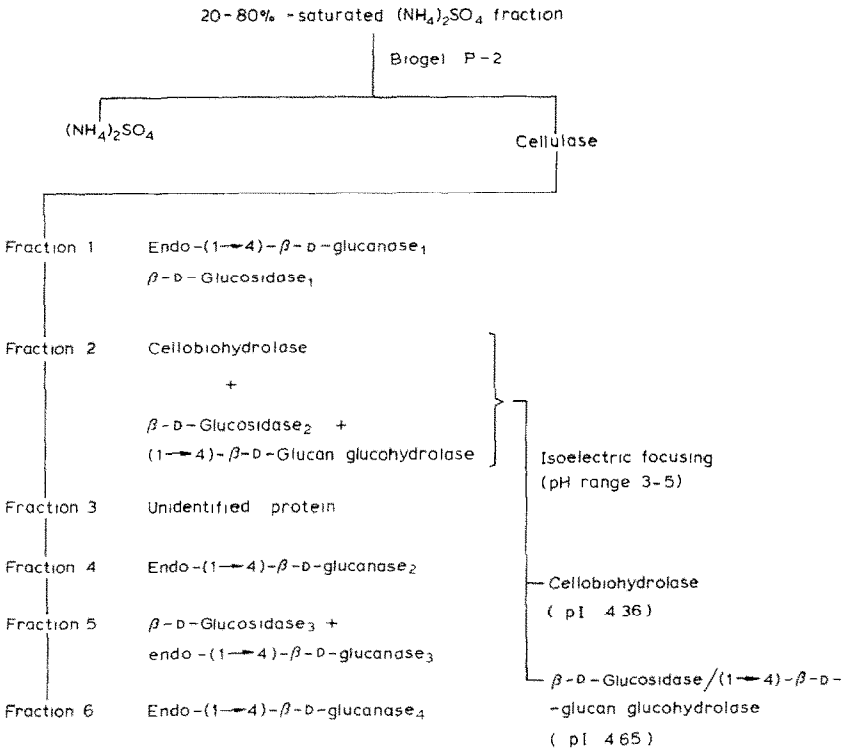
For cellobiose, cellotriose, cellotetraose, and cellopentaose, each mixture consisted of 0.5 mL of acetic acid–NaOH buffer (5 mL; 0.1mM, pH 5.0), substrate (up to 2.4mM final concentration), enzyme, and water, to give a total volume of 1 mL, and was incubated at 37° for 30 min. The D-glucose liberated was assayed by the D-glucose oxidase method[24].

For H_3PO_4-swollen cellulose, the mixture contained substrate (10 mg), 0.2M acetic acid–NaOH buffer (2 mL, pH 5.0), enzyme solution (50 μL), and water, to give a total volume of 4 mL. After incubation at 37° for 1 h, the mixture was centrifuged and the supernatant was assayed for D-glucose by the D-glucose oxidase method[24].

Products of the action of the enzyme on H_3PO_4-swollen cellulose. — A mixture similar to that described above, but with a final volume of 7 mL (adjusted with water) and containing 0.05M NaN_3 (0.1 mL), was used. Samples (1 mL) were removed at intervals, deionised with Amberlite IR-120 (H^+) and IR-45 (HO^-) resins, and freeze-dried, and the residues were analysed by t.l.c. on silica gel (two irrigations with 2-propanol–water–ethyl acetate, 6.5 : 4.5 : 9.0) and detection[27] with anisaldehyde–H_2SO_4.

RESULTS

Fractionation of P. funiculosum *cellulase.* — A sample (5 mL) of concentrated (50-fold) enzyme, partially purified by $(NH_4)_2SO_4$ precipitation, was fractionated[11] on a column (2.5 × 38.2 cm) of DEAE-Sephadex A-50 (AcO^- form) as shown in Scheme 1. Fractions 1–3 were eluted under the starting conditions [60mM acetic



Scheme 1

acid–NaOH buffer (pH 4.8)], but fractions 4–6 required a gradient formed by adding 400 mL of 0.1M acetic acid–NaOH buffer (pH 3.8) to 400 mL of the starting buffer.

Only fraction 2 was examined further; it contained both (1→4)-β-D-glucan cellobiohydrolase and (1→4)-β-D-glucan glucohydrolase activities, and these were

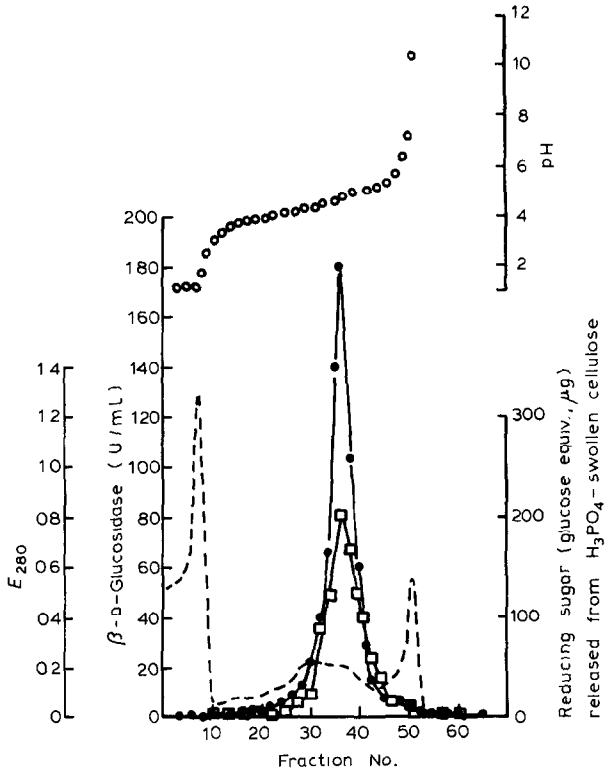


Fig. 1. Purification of (1→4)-β-D-glucan glucohydrolase by isoelectric focusing. The (1→4)-β-D-glucan glucohydrolase, which had been separated from the (1→4)-β-D-glucan cellobiohydrolase in a pH gradient in an isoelectric focusing column (see text), was collected and purified by a re-run in the electrofocusing column. Ampholyte covering the pH range 3–5 was used. The sucrose concentration in the first run was not taken into account when making up the density gradient. Partially purified enzyme was added to the "light-solution" (see LKB Instruction leaflet). The voltage at the end of the run (24 h) was 1,200 V and the current 5 mA. Fractions (2 mL) were assayed for *o*-nitrophenyl β-D-glucopyranoside (—●—) and activity to H_3PO_4-swollen cellulose (—□—). Protein (E_{280}) (---); pH gradient (—○—).

partially resolved in an isoelectric focusing column[11] using a pH gradient covering the pH range 3–5; the respective isoelectric points were at pH 4.36 and 4.65.

(1→4)-β-D-Glucan glucohydrolase. — (*a*) *Purification.* The partially purified enzyme obtained by isoelectric focusing as described above[11] was subjected to a second isoelectric focusing using carrier ampholyte covering the pH range 3–5. A single cellulase component (fractions 30–40, Fig. 1) was obtained, which attacked both *o*-nitrophenyl β-D-glucopyranoside and H_3PO_4-swollen cellulose. Fractions 30–40 from several similar runs were combined (69 mL), concentrated (to 9 mL) in a collodion membrane, and dialysed at 1° against 0.1M acetic acid–NaOH buffer (pH 5.0) for 4 days.

(*b*) *Homogeneity.* The enzyme preparation obtained in (*a*) migrated as a single

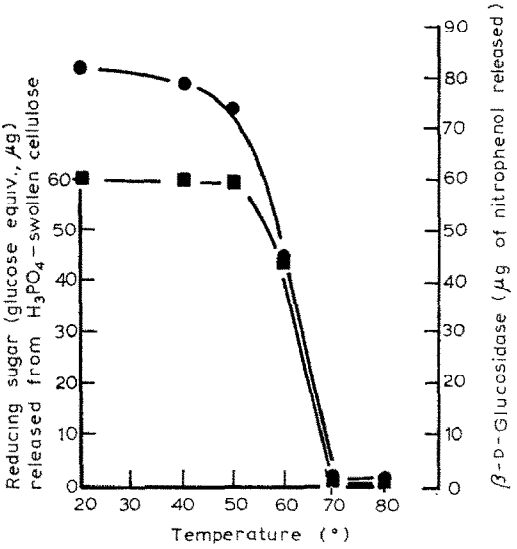


Fig. 2. Effect of heat on the (1→4)-β-D-glucan glucohydrolase. Purified enzyme (0.02 mL containing 112 μg of protein) was diluted with acetic acid–NaOH buffer (1.98 mL, pH 5.0) and heated for 10 min at the temperatures shown. An aliquot (0.25 mL) was used to measure residual *o*-nitrophenyl β-D-glucosidase activity (—●—) and another (1.5 mL) to measure activity to H_3PO_4-swollen cellulose (—■—).

protein component on Ultrogel AcA 44; peaks of activity towards *o*-nitrophenyl β-D-glucopyranoside, cellobiose, and H_3PO_4-swollen cellulose were coincident. On polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, and on polyacrylamide-gel electrofocusing using ampholyte covering the pH range 3.5–5.0, only one protein band was observed.

(*c*) *Molecular weight.* The mol. wt. of the purified enzyme was determined by using a calibrated column (80 × 1.5 cm) of Ultrogel AcA 44. A linear relationship was obtained when the elution volumes of the standard proteins were plotted against the log mol. wt., and the mol. wt. of the enzyme was estimated from this graph to be

TABLE I

KINETIC CONSTANTS OF THE (1→4)-β-D-GLUCAN GLUCOHYDROLASE FROM *P. funiculosum* CELLULASE

Substrate	K_m (mM)	K_i (μM) D-*Glucono-1,5-lactone*	K_i (μM) D-*Glucose*
o-Nitrophenyl β-D-glucopyranoside	0.77	2.1	1756
Cellobiose	10.0	1.95	1528
Cellotriose	0.44	7.9	N.d.
Cellotetraose	0.77	N.d.[a]	N.d.
Cellopentaose	0.37	N.d.	N.d.
H_3PO_4-Swollen cellulose	172[b]	1.9	N.d.

[a]Not determined. [b]Mg/mL.

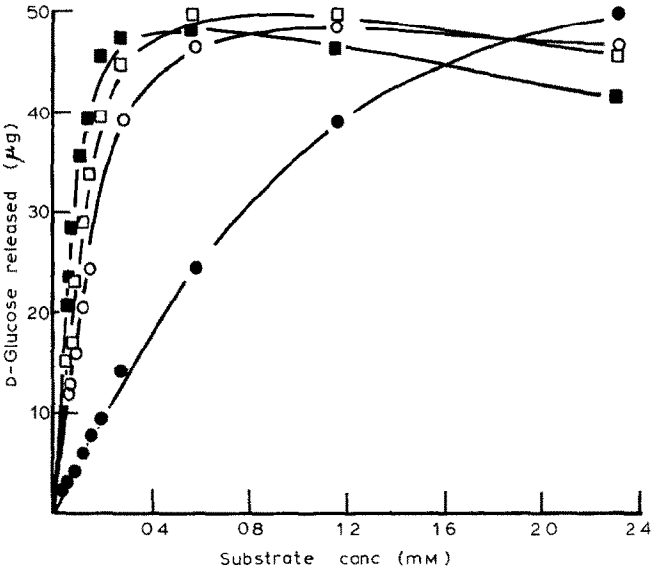


Fig. 3. Effect of substrate concentration on (1→4)-β-D-glucan glucohydrolase activity. The D-glucose liberated was measured by the D-glucose oxidase method; cellobiose (—●—), cellotriose (—○—), cellotetraose (—□—), and cellopentaose (—■—).

65,000. The enzyme and bovine serum albumin (mol. wt. 67,000) had elution volumes of 94 and 95 mL, respectively.

(*d*) *pH Optimum.* The activity of the enzyme towards *o*-nitrophenyl β-D-glucopyranoside and H_3PO_4-swollen cellulose was optimal at pH 5.0.

(*e*) *Heat stability.* The thermostability of the enzyme using *o*-nitrophenyl β-D-glucopyranoside and H_3PO_4-swollen cellulose substrates is shown in Fig. 2. At pH 5.0 and 50°, the enzyme was stable, but there was a significant loss in activity at 60° and complete inactivation at 70°.

(*f*) K_m *and* K_i *values.* *o*-Nitrophenyl β-D-glucopyranoside, cellobiose, cellotriose, cellotetraose, cellopentaose, and H_3PO_4-swollen cellulose were used as substrates for the determination of K_m values (Table I). Each value was obtained from a Lineweaver–Burk plot of the data presented in Fig. 3, which shows the effect of substrate concentration on the activity. Inhibition occurred with higher substrate concentrations of cellotriose, cellotetraose, and cellopentaose. Enzyme action was inhibited by cellobiose, and substantially at 12mM (not shown in Fig. 3).

Enzyme activity–substrate concentration curves for the hydrolysis of each of the substrates conformed to the Michaelis–Menten pattern. The derived K_m values show that the enzyme possessed a much lower affinity for cellobiose than for cellotriose, cellotetraose, and cellopentaose; the affinity for cellopentaose was the greatest.

On the basis of a Dixon plot, the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside appeared to be competitively inhibited by D-glucono-1,5-lactone. However, the inhibition by D-glucose was non-competitive as was that in the gluconolactone–

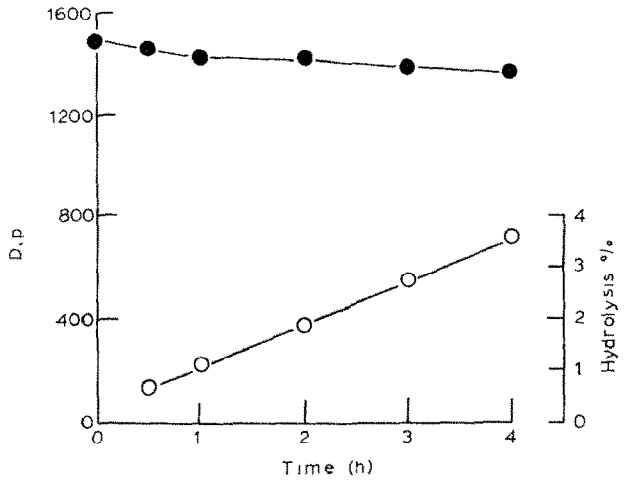


Fig. 4. The effect of (1→4)-β-D-glucan glucohydrolase action on the d.p. of H_3PO_4-swollen cellulose. The substrate (44 mg) was incubated with enzyme (56 μg of protein) for the times shown, and the d.p. of the residual cellulose was measured after dissolution in Cadoxen[21]; d.p. (—●—); % hydrolysis calculated from the reducing sugar (Nelson–Somogyi) liberated (glucose equivalent) (—○—).

cellobiose and gluconolactone–cellotriose systems. D-Glucono-1,5-lactone was a much more powerful inhibitor (K_i 1.95–7.9 μM) (Table I) than was D-glucose (K_i 1756 μM). D-Glucose was a competitive inhibitor of enzyme action on cellobiose.

(*g*) *Action on H_3PO_4-swollen cellulose.* H_3PO_4-swollen cellulose (10 mg) was hydrolysed to D-glucose (920 μg) and a trace of cellobiose (which appeared to remain constant) by the glucohydrolase (10 μg of protein) when incubated in a 5-mL reaction mixture at 37° for 18 h. More-extensive hydrolysis (26%) occurred when the incubation was allowed to proceed for 4 days. A smaller sample (2 mg) of H_3PO_4-swollen cellulose was 69% solubilised in 7 days.

As shown in Fig. 4, the d.p. of another sample of H_3PO_4-swollen cellulose had

TABLE II

EFFECT OF (1→4)-β-D-GLUCAN GLUCOHYDROLASE ON β-D-GLUCOSE DISACCHARIDES[a]

Substrate	*Linkage*	*Activity (μg of D-glucose liberated in 5 h)*
Sophorose	(1→2)	194
Laminaribiose	(1→3)	224
Cellobiose	(1→4)	179
Gentiobiose	(1→6)	37

[a]The reaction mixture consisted of substrate (17 mg), 0.1M acetic acid–NaOH buffer (pH 5.0, 2.5 mL), enzyme (1.5 mL, 42 μg of protein), and water to give a total volume of 5 mL. After incubation at 37° for 5 h, the D-glucose liberated was assayed by the D-glucose oxidase method.

fallen to only 1370 from the original 1480 during incubation for 4 h, despite the fact that ~3.5% of the substrate had been hydrolysed.

(*h*) *Action on other cellulosic substrates.* When Whatman No. 1 cellulose, cotton fibre, Avicel, or Solka Floc was incubated with enzyme (10 μg of protein) as in (*g*), only traces of reducing sugar were released. Solka Floc was the most susceptible to hydrolysis, 103 μg of glucose equivalent being released from 10 mg of substrate during 18 h; there was no further hydrolysis. Under the same conditions, only 25 μg of glucose equivalent was released from 10 mg of CM-cellulose during 1 h and there was no further release after 2 h.

(*i*) *Action on β-D-glucose disaccharides.* The (1→2)- and (1→3)-linked disaccharides were better substrates than the (1→4)-linked disaccharide (Table II), and the activity on the (1→6)-linked disaccharide was comparatively low.

Release of soluble sugars by the components of P. funiculosum *cellulase, acting alone and in concert.* — (*a*) *On H_3PO_4-swollen cellulose.* The (1→4)-β-D-glucan cellobiohydrolase and the endo-(1→4)-β-D-glucanase/β-D-glucosidase were separated from partially purified, 20–80% $(NH_4)_2SO_4$ fraction as indicated in Scheme 1. (1→4)-β-D-Glucan cellobiohydrolase[11] and (1→4)-β-D-glucan glucohydrolase were purified by chromatography on DEAE-Sepharose and by isoelectric focusing, desalted by using Sephadex G-25, and isolated by freeze-drying. The endo-(1→4)-β-D-glucanase/β-D-glucosidase fraction was reconstituted by combining fractions 1 and 4–6 in the proportions in which they were eluted from the column.

The purified (1→4)-β-D-glucanglucohydrolase was more active against H_3PO_4-swollen cellulose than was purified (1→4)-β-D-glucan cellobiohydrolase (Table III), but much less active than the endo-(1→4)-β-D-glucanase/β-D-glucosidase fraction.

TABLE III

RELEASE OF SOLUBLE SUGARS BY COMPONENTS OF *P. funiculosum* ACTING ALONE AND IN COMBINATION ON H_3PO_4-SWOLLEN CELLULOSE[a]

Enzyme	*Sugar released*		
	(a) Reducing sugar (glucose equivalent) (μg)	*(b) Total sugar (glucose equivalent) (μg)*	*(c) Glucose (μg)*
Cellobiohydrolase (CBH)	254	485	12
Glucohydrolase (GH)	920	931	898
Endo-glucanase + β-D-glucosidase	2830	2955	2350
CBH + GH	1184	1403	988
GH + endo-glucanase + β-D-glucosidase	4526	4538	4255
CBH + endo-glucanase + β-D-glucosidase	3723	3810	2862

[a]The cellobiohydrolase (10 μg of protein), glucohydrolase (10 μg of protein), and endo-glucanase and β-D-glucosidase (10 μg of protein) added were purified as discussed in the text. The assay used was as detailed elsewhere[15].

TABLE IV

SYNERGISM BETWEEN ENDO- AND EXO-(1→4)-β-D-GLUCANASE COMPONENTS OF *P. funiculosum* CELLULASE IN SOLUBILISING COTTON CELLULOSE[a]

Enzyme	*Relative cellulase activity (%)*
Cellobiohydrolase (CBH)	1
Glucohydrolase (GH)	1
Endo-glucanase + β-D-glucosidase	15
CBH + GH	2
GH + endo-glucanase + β-D-glucosidase	15
CBH + endo-glucanase + β-D-glucosidase	98
CBH + GH + endo-glucanase + β-D-glucosidase	98
Original unfractioned enzyme	100

[a]Assays were made with dewaxed cotton fibre[8] (2 mg). Partially purified, cell-free enzyme (1 mL) solubilised 71% of the cotton during 7 days. Relative cellulase activities were obtained by plotting the degree of solubilisation against enzyme concentration. Enzymes were purified as shown in the text, and recombined in the proportions in which they were eluted from the column of DEAE-Sephadex[11].

There was no synergism when the first two enzymes acted together, but this phenomenon was observed with a mixture of the purified enzyme and the randomly acting endo-(1→4)-β-D-glucanase/β-D-glucosidase fraction.

(*b*) *On the cellulose in cotton fibre.* The synergism observed in (*a*) was not observed with the highly ordered cellulose in cotton fibre (see Table IV). However, when (1→4)-β-D-glucan cellobiohydrolase and the endo-(1→4)-β-D-glucanase/β-D-glucosidase acted together, there was a high degree of synergism which accounted for 98% of the cellulase activity of the original cellulase system.

Transglucosidase action. — When the purified enzyme (56 μg of protein was incubated with cellobiose (5%) at 37° in a reaction volume of 0.5 mL buffered at pH 5.0, cellotriose and cellotetraose, which appeared after incubation for 2 h, reached maximum concentration after 6 h.

Changes in optical rotation during the hydrolysis of cellotetraitol by (1→4)-β-D-glucan glucohydrolase. — When a reaction mixture containing substrate (5 mg), (1→4)-β-D-glucan glucohydrolase (1 mg of protein), and 100 mmol of potassium phosphate (pH 5.0) in 2 mL of water was filtered through a 0.45-micron filter, the α_D value of a portion (1 mL) increased from +0.01 to +0.18 during 60 min. When the contents of the polarimeter tube were mixed with the remainder of the reaction mixture and 150 μL of conc. NH_4OH were added, the α_D value of 0.18 fell to 0.04 within 2 min.

DISCUSSION

The (1→4)-β-D-glucan glucohydrolase described herein was purified to homo-

geneity as judged by polyacrylamide gel electrophoresis, polyacrylamide gel electrofocusing, and molecular sieve chromatography on Ultrogel AcA 44. The single protein band obtained in each of these procedures exhibited the same ratio of activity to *o*-nitrophenyl β-D-glucopyranoside, cellobiose, and H_3PO_4-swollen cellulose. These properties caused doubts as to the purity as well as to the classification of the enzyme.

Criteria for distinguishing β-D-glucosidases and exo-glucanases have been drawn up for enzymes from several fungal sources[28]. It was concluded that β-D-glucosidases differ from exo-glucanases in transferase activity, in having greater activity on dimers than on higher cello-oligosaccharides, in retaining anomeric configuration during hydrolysis, and in the high sensitivity to inhibition by D-glucono-1,5-lactone. However, these definitions have become blurred, because an exo-(1→4) β-D-glucanase that was 83% inhibited in its action on Avicel by μM D-glucono-1,5-lactone[18] and an exo-(1→4)-β-D-glucanase that acted with retention of anomeric configuration[29] have been found. Transferase activity, which is a feature of β-D-glucosidases in general, is also a property of endo-(1→4)-β-D-glucanase activity of fungal origin[30,31].

Therefore, the enzyme described herein is not unique in exhibiting what might be regarded as "anomalous" results. It possesses some properties (transferase activity, sensitivity to D-glucono-1,5-lactone, and high activity towards *o*-nitrophenyl β-D-glucopyranoside) normally associated with D-glucosidase activity, but the relative activity towards cellobiose, cellosaccharides, and cellulose, and the apparent inversion of anomeric configuration during hydrolysis, are typical of some exo-glucanases[32]. The slow change in the d.p. of H_3PO_4-swollen cellulose during hydrolysis and the inability to hydrolyse CM-cellulose are observations that would be expected from a depolymerase that acts end-wise.

The inhibition of the enzyme by D-glucono-1,5-lactone and D-glucose warrants further comment. On the basis of Dixon plots, it appeared that inhibition by the lactone of the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside was competitive and that of cellobiose and cellotriose was non-competitive. One interpretation of these results is that the enzyme possesses two active sites, one of which (site A) is concerned with the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside and the other (site B) with the hydrolysis of cellobiose, cellotriose, and probably higher saccharides. Since D-glucose was a non-competitive inhibitor of the action of the enzyme on *o*-nitrophenyl β-D-glucopyranoside, but a competitive inhibitor of the action on cellobiose, presumably D-glucose is also involved with site B.

Alternatively, the results could also reflect contamination of the (1→4)-β-D-glucan glucohydrolase with a β-D-glucosidase, but no evidence for heterogeneity was obtained using the chromatographic and electrophoretic procedures that are widely accepted criteria of homogeneity.

The (1→4)-β-D-glucan glucohydrolase was active against H_3PO_4-swollen cellulose, which is a model substrate for the amorphous cellulose component of native fibres, but not against cotton fibre, which comprises highly ordered cellulose. Thus, the enzyme was similar to such other exo-glucanases as the (1→4)-β-D-glucan

cellobiohydrolase found in the multi-component cellulase system elaborated by *P. funiculosum*, but differed from the cellobiohydrolase in its capacity to act synergistically with the randomly acting endo-(1→4)-β-D-glucanase in solubilising the cellulose in cotton fibre. Whereas mixtures of endo-glucanase and cellobiohydrolase showed a high degree of co-operation, resulting in extensive solubilisation of cotton fibre, mixtures of endo-glucanase and glucohydrolase did not.

These observations raise the question as to why an enzyme that removes cellobiose from the cellulose chain can co-operate with the endo-glucanases in solubilising cotton cellulose, whereas an enzyme that removes only one D-glucose residue cannot. Stereochemical considerations must be important in this context[33,34]. It seems likely that, since cellobiose is the repeating unit in the cellulose crystallite and is rigidly held in position by hydrogen bonds, only a cellobiohydrolase can effect the removal.

(1→4)-β-D-Glucan glucohydrolases have been isolated from cellulase preparations from *Trichoderma viride*, but they differed from *P. funiculosum* (1→4)-β-D-glucan glucohydrolase in that they showed only minimal action on H_3PO_4-swollen cellulose and relatively high activity on CM-cellulose[29,32].

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REFERENCES

1 T. M. WOOD, *Biochem. J.*, 109 (1968) 217–227.
2 G. HALLIWELL, *Biochem. J.*, 95 (1965) 270–281.
3 M. GRITZALI AND R. D. BROWN, *Adv. Chem. Ser.*, 181 (1979) 237.
4 K. OGAWA AND N. TOYAMA, *J. Ferment. Technol.*, 50 (1972) 236–243.
5 G. OKADA, K. NISIZAWA, AND H. SUZUKI, *J. Biochem (Tokyo)*, 63 (1968) 591–607.
6 M. MANDELS AND E. T. REESE, *Dev. Ind. Microbiol.*, 5 (1964) 5–20.
7 M. MANDELS, *Biotechnol. Bioeng. Symp.*, 5 (1975) 81–105.
8 T. M. WOOD, *Biochem. J.*, 115 (1969) 457–464.
9 K.-E. ERIKSSON, B. PETTERSSON, AND U. WESTERMARK, *FEBS Lett.*, 49 (1974) 282–285.
10 M. A. FOLAN AND M. P. COUGHLAN, *Int. J. Biochem.*, 9 (1978) 717–722.
11 T. M. WOOD, S. I. McCRAE, AND C. M. MACFARLANE, *Biochem. J.*, 189 (1980) 51–65.
12 T. M. WOOD AND S. I. McCRAE, *Biochem. J.*, 128 (1972) 1183–1192.
13 T. M. WOOD, *Biotechnol. Bioeng. Symp.*, 5 (1975) 111–137.
14 E. K. GUM AND R. D. BROWN, *Biochim. Biophys. Acta*, 492 (1977) 225–231.
15 T. M. WOOD AND S. I. McCRAE, *Carbohydr. Res.*, 57 (1977) 117–133.
16 L. E. R. BERGHEM, L. G. PETTERSSON, AND U.-B. AXIÖ-FREDRIKSSON, *Eur. J. Biochem.*, 53 (1975) 55–62.
17 L. G. FÄGERSTAM AND L. G. PETTERSSON, *FEBS Lett.*, 119 (1980) 97–100.
18 K.-E. ERIKSSON AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1975) 213–218.
19 T. M. WOOD AND S. I. McCRAE, *Adv. Chem. Ser.*, 181 (1979) 181.
20 T. M. WOOD, *Biochem. J.*, 121 (1971) 353–362.
21 T. M. WOOD AND S. I. McCRAE, *Biochem. J.*, 171 (1978) 61–72.
22 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 376–380.

23 N. NELSON, *J. Biol. Chem.*, 153 (1952) 376–380.
24 J. B. LLOYD AND W. J. WHELAN, *Anal. Biochem.*, 30 (1969) 467–469.
25 M. DUBOIS, K. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
26 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406–4412.
27 E. STAHL AND U. KALTENBACH, *J. Chromatogr.*, 5 (1961) 351–352.
28 E. T. REESE, A. H. MAGUIRE, AND F. W. PARRISH, *Can. J. Biochem.*, 46 (1968) 25–34.
29 K. NISIZAWA, T. KANDA, S. SHIKATA, AND K. WAKABAYASHI, *J. Biochem. (Tokyo)*, 83 (1978) 1625–1630.
30 S. P. SHOEMAKER AND R. D. BROWN, *Biochim. Biophys. Acta*, 523 (1978) 133–146.
31 M. STREAMER, K.-E. ERIKSSON, AND B. PETTERSSON, *Eur. J. Biochem.*, 59 (1975) 607–613.
32 L. H. LI, R. M. FLORA, AND K. W. KING, *Arch. Biochem. Biophys.*, 111 (1965) 439–447.
33 T. M. WOOD, *Colloque Cellulolyse Microbienne*, CNRS Marseille, 1980, pp. 167–176.
34 T. M. WOOD, *Proc. Int. Symp. Wood Pulping Chem.*, 3 (1981) 31–38.