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CELLULASE FROM *Fusarium solani*: PURIFICATION AND PROPERTIES OF THE C_1 COMPONENT*

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ABSTRACT

The C_1 component from *Fusarium solani* cellulase was purified extensively by molecular-sieve chromatography on Ultrogel AcA-54 and ion-exchange chromatography on DEAE-Sephadex. The purified component showed little capacity for hydrolysing highly ordered substrates (*e.g.*, cotton fibre), but poorly ordered substrates (*e.g.*, H_3PO_4-swollen cellulose), and the soluble cello-oligosaccharides cellotetraose and cellohexaose, were readily hydrolysed; cellobiose was the principal product in each case. Attack on *O*-(carboxymethyl)cellulose, a substrate widely used for measuring the activity of the randomly acting enzymes (C_X enzymes) of the cellulase complex, was minimal, and ceased after the removal of a few unsubstituted residues from the end of the chain. These observations, and the fact that the rate of change of degree of polymerisation of H_3PO_4-swollen cellulose was very slow compared with that effected by the randomly acting endoglucanases (C_X, CM-cellulases), indicate that C_1 is a cellobiohydrolase. Fractionation by a variety of methods gave no evidence for the non-identity of the cellobiohydrolase and the component that acted in synergism with the randomly acting C_X enzyme when solubilizing cotton fibre.

INTRODUCTION

Previous studies from these and other laboratories have shown that when such fungi as *Fusarium solani*[1–3], *Trichoderma koningii*[4], *T. viride*[5–9], *Penicillium funiculosum*[10], and *Sporotrichum pulverulentum*[11] grow on a medium containing native cellulose as the carbon source, the cellulase system that is synthesised consists of at least three enzymes, or classes of enzyme, namely[12], C_1, C_X (CM-cellulases or cellodextrinases), and β-D-glucosidases or cellobiases. These enzymes, which can solubilize cotton fibre when acting in concert, lose most of this capacity when separated, but recover it when recombined in suitable proportions.

It is now well-established that C_X enzymes are, in the main, endo-(1→4)-β-D-glucanases, but the mode of action of the C_1 component is still the subject of debate.

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

Reese *et al.*[12] suggested that C_1 is a prehydrolytic factor which causes some loosening of the cellulose chains in preparation for attack by the hydrolytic C_x-enzymes. However, there is no evidence to support this particular hypothesis, despite the fact that it has guided the experimental approach for many years. The present consensus of opinion is that C_1 is an exoglucanase[9,11,13–16]; in the case of *T. koningii*[13–15] and *T. viride*[9,16], it is a cellobiohydrolase.

We now present evidence which shows that the C_1 component of *F. solani* is also a cellobiohydrolase. Part of this work has been published as a preliminary communication[13].

EXPERIMENTAL

Materials. — The source of the materials were as follows: CM-cellulose [(*O*-carboxymethyl)cellulose], sodium salt (Cellofas B) with a degree of substitution of 0.5, I.C.I. Ltd., Nobel Division; Texas-cotton fibre, Shirley Institute, Manchester; Sephadex G-25 and DEAE-Sephadex, Pharmacia (G.B.) Ltd.; Ampholine electrofocusing equipment and Ultrogel, L.K.B. Instruments Ltd.; D-glucose oxidase (Type II), Sigma (London) Chemical Company; peroxidase, Boehringer Corporation (London) Ltd.; Avicel (microcrystalline cellulose), Honeywell and Stein Ltd.; collodion tubes (Sartorius), V. A. Howe.

Preparation of enzyme. — Cultures and cell-free filtrates were prepared from *F. solani* I.M.I. 95994 by the method previously described for *T. koningii*[4].

An enzyme concentrate (50-fold) was prepared by precipitation of the culture filtrate with $(NH_4)_2SO_4$ between the limit of 20 and 80% saturation, and then centrifuging and redissolving the precipitate in 0.1M acetic acid–NaOH buffer (pH 5.0). Enzyme assays showed that all of the cellulase (cotton-solubilization), CM-cellulase, and β-D-glucosidase activity of the original culture filtrate was recovered.

Preparation of substrates. — (*a*) *Dewaxed cotton.* Texas-cotton fibre was dewaxed as described by Corbett[36].

(*b*) *Cello-oligosaccharides.* Cello-oligosaccharides were prepared by the acetolysis of dewaxed cotton fibres, followed by deacetylation with sodium methoxide, and fractionation by gradient elution (0–35% ethanol) from a column of charcoal–Celite (1:1, w/w).

(*c*) *Reduced cellotetraose.* Cellotetraose (150 mg) was dissolved in water (2 ml), potassium borohydride (150 mg) was added, and the mixture was kept overnight at room temperature. Excess of borohydride was decomposed with glacial acetic acid, and the solution was deionised on columns of Amberlite IR-120 (H^+) and IR-45 (HO^-) resins; reduced cellotetraose was recovered by freeze-drying.

(*d*) *H_3PO_4-swollen cellulose.* A suspension of cotton (5 g) in conc. phosphoric acid (88%, w/v) was kept, with occasional stirring, for 4 h at 1°. The gelatinous product was worked up as described elsewhere[3].

Enzyme assays. — (*a*) *Activity towards CM-cellulose.* CM-cellulase activity was measured either viscometrically[3] or by a reducing-sugar method.

In the reducing-sugar method, a mixture of 1.0 ml of CM-cellulose solution (1%, w/v), 0.5 ml of 0.2M acetic acid–NaOH buffer (pH 5.4), and 0.5 ml of diluted enzyme was incubated at 37° for 1 h. The reaction was stopped by the addition of 2.0 ml of Somogyi reagent[17], and the reducing sugar determined by the method of Nelson[17]. Where only small amounts of CM-cellulase activity were present, the more sensitive, modified Park–Johnson method[14] was used to measure the reducing sugars. The reducing sugars liberated were expressed as glucose equivalent. The units of activity have already been defined[14].

(*b*) *Cellulase activity.* Dewaxed cotton fibre and the microcrystalline hydrocellulose (Avicel) were both used as substrates for measuring cellulase activity. Avicel is the substrate favoured by many other investigators working on cellulases from other fungal sources. It is more easily hydrolysed than cotton, and it is a good substrate for measuring the synergistic action between C_1 and C_X types of enzyme.

(*i*) Dewaxed cotton (2 mg) was incubated with enzyme for 7 days at 37° as previously described[2]. A 1-ml sample of the 20–80%-saturated-$(NH_4)_2SO_4$ fraction, diluted 50-fold, produced 71% solubilization under these conditions.

(*ii*) The assay contained 0.5 ml of a 1% aqueous suspension of Avicel, 0.25 ml of 0.2M acetic acid–NaOH buffer (pH 5.0), and enzyme and water to give a total volume of 1 ml. 0.05M Sodium azide (0.02 ml) was added, the mixture was incubated for 18 h at 37°, and the soluble sugars liberated were determined either by the phenol–H_2SO_4 method[18] or by the method of Nelson[17]. The sugars liberated were expressed as glucose equivalent.

(*c*) *Activity towards H_3PO_4-swollen cellulose.* A 5-ml sample of an aqueous suspension (4% w/v) of H_3PO_4-swollen cellulose was pipetted into a centrifuge tube. After centrifugation, and careful withdrawal of 3.4 ml of the supernatant with an automatic pipette, the residue was mixed with 0.04 ml of 0.05M sodium azide, 0.2 ml of acetic acid–NaOH buffer (pH 5.0), and enzyme and water to give a total volume of 2 ml. The mixture was incubated at 37° for 18 h, and centrifuged, and the soluble sugars liberated were determined as in (*b*)-(*ii*).

(*d*) *Activity towards reduced cellotetraose.* The incubation mixture, containing 1 ml of an aqueous solution (0.15% w/v) of reduced cellotetraose, 0.5 ml of NaOH–acetic acid buffer (pH 5.0), and 0.5 ml of enzyme and water to give a total volume of 2 ml, was heated at 37° for 2 h, and the reducing sugars liberated were measured by the method of Nelson[17].

(*e*) *β-D-Glucosidase.* β-D-Glucosidase was measured with *o*-nitrophenyl β-D-glucopyranoside as substrate, by the method already described[4].

Other assays. — Reducing sugars were measured by the method of Nelson[17], or by the modified method of Park and Johnson[14]. Total carbohydrate was determined by the phenol–H_2SO_4 method[18], and D-glucose by a modified D-glucose oxidase method[19]. In each case, the reagents were calibrated against D-glucose.

Protein was determined by the method of Lowry *et al.*[20], calibrated against

crystalline, bovine serum albumin. In some cases, the protein content of column fractions was determined from the extinction at 280 nm.

Separation methods. — (*a*) Descending paper chromatograms on Whatman No. 1 paper were developed with ethyl acetate–pyridine–water (10:4:3) (solvent *A*), and sprayed with alkaline silver nitrate[15]. Thin-layer plates (Kieselgel G) were developed (two ascents) with ethyl acetate–propan-2-ol–water (18:13:9) (solvent *B*), and sprayed with silver nitrate or with anisaldehyde–sulphuric acid[22].

(*b*) Separations by isoelectric focusing were performed[14] in a 110-ml LKB electrofocusing column. After focusing at 5°, the column was emptied at a rate of 120 ml/h. Fractions (1.0 ml) were collected, and the pH of each was measured at 5° with a Corning–Eel pH meter fitted with a combination electrode.

RESULTS

Fractionation and purification of F. solani *cellulase.* — (*a*) *Separation of β-D-glucosidase from C_1 and C_X (CM-cellulase) activities by molecular-sieve chromatography.* The β-D-glucosidase component associated with *F. solani* cellulase can be readily and reproducibly separated from the rest of the cellulase complex (C_1 and C_X) by molecular-sieve chromatography.

Fig. 1 shows a typical elution profile of a sample (5 ml) of concentrated, cell-free filtrate [20–80%-saturated-$(NH_4)_2SO_4$ fraction] on a column of Ultrogel AcA-54 equilibrated with 0.1M acetic acid–NaOH buffer (pH 5.0), using a flow rate of 30 ml/h. Many such separations were performed with no alteration in the elution volume observed.

Recoveries of β-D-glucosidase, CM-cellulase (C_X), and protein were 120, 90, and 110%, respectively.

Assays for cellulase (cotton-solubilizing) activity were done on portions *A* and *B* after adjustment to the same final volume as the 20–80%-saturated-$(NH_4)_2SO_4$ fraction diluted 50-fold. Under the conditions of the standard assay, 1.0 ml of portion *A* (*i.e.*, β-D-glucosidase) produced only 1% solubilization of cotton fibre, and 1 ml of portion *B* (*i.e.*, C_1 and C_X) only 58%. However, an assay containing 1.0 ml of each showed the same capacity for solubilizing cotton fibres as 1.0 ml of the starting material [20–80%-saturated-$(NH_4)_2SO_4$ fraction, diluted 50-fold], namely 71%.

(*b*) *Separation of C_1 and C_X components by chromatography on DEAE-Sephadex.* Portion *B* (Fig. 1) was concentrated in a collodion tube, and dialysed in the same tube against 0.05M acetic acid–NaOH buffer (pH 5.5) for 3 days. The sample (5 ml) was applied to a column (27.5 × 1.5 cm) of DEAE-Sephadex (acetate form) and eluted with 0.05M acetic acid–NaOH buffer (pH 5.5). Under the starting conditions, 97% of the CM-cellulase (C_X) activity and 42% of the protein were eluted as one peak (portion *C*); the remainder of the protein (C_1) and the CM-cellulase activity were eluted together (portion *D*) by the application of a pH-gradient [200 ml of acetate buffer (pH 5.5)–200 ml of acetate buffer (pH 3.8)].

Assays for cellulase (cotton-solubilizing) activity were again performed after

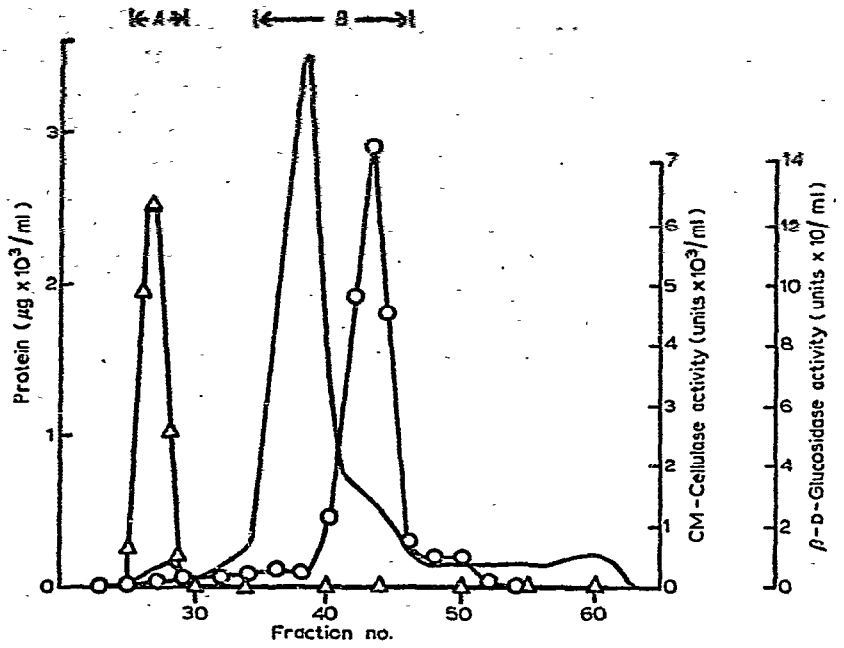


Fig. 1. Molecular-sieve chromatography on Ultrogel AcA-54. A sample (5 ml) of partially purified, concentrated 20–80%-saturated-$(NH_4)_2SO_4$ fraction was applied to a column of Ultrogel AcA-54 (86.5 × 2.5 cm) equilibrated with 0.1M acetic acid–NaOH buffer (pH 5.0). Fractions (7 ml) were collected and assayed for β-D-glucosidase (—△—), protein (——), and CM-cellulase (—○—) by the reducing-sugar method. Portion *A* comprises fractions 25–29; and portion *B*, fractions 34–46.

diluting portions *C* (*i.e.*, C_X) and *D* (*i.e.*, C_1) so that the volumes were equivalent in terms of the unfractionated 20–80%-saturated-$(NH_4)_2SO_4$ fraction, diluted 50-fold. Under the conditions of the standard assay, 1.0 ml of diluted portion *D* (*i.e.*, C_1) solubilized cotton fibres to the extent of 2% and 1%, respectively, whereas an assay mixture containing 1.0 ml of each gave 58% solubilization. Since, under the same conditions, 1.0 ml of diluted portion *B*, Fig. 1 (*i.e.*, C_1 and C_X) also produced 58% solubilization of cotton (see above), it was obvious that no factor essential for the hydrolysis of cotton had been lost during the fractionation on DEAE-Sephadex.

Recovery of the various enzyme activities from this fractionation and that shown in Fig. 1 was in excess of 90%. It was possible, therefore, to calculate the proportions in which the various components had existed in the original, unfractionated 20–80%-saturated-$(NH_4)_2SO_4$ fraction. It was found that 1.0 ml of this fraction diluted 50-fold contained ~500 μg of protein, 1110 units of CM-cellulase activity (determined by reducing sugar), and 10 units of β-D-glucosidase. In each of the subsequent purification procedures described in this report, the recovery of the C_1 activity was determined by measuring the cellulase (cotton-solubilizing) activity of a reconstituted mixture in which 500 μg of protein was mixed with 1110 units of CM-cellulase (C_X), and 10 units of β-D-glucosidase.

The C_1 component, when purified on Ultrogel and then on DEAE-Sephadex, showed little hydrolytic activity towards cotton, Avicel, or CM-cellulose, but cellulose previously swollen in H_3PO_4, and cellotetraose that had been reduced with KBH_4, were extensively degraded. In each of the subsequent purification stages, therefore, activity towards each of these substrates was measured in order to test for possible heterogeneity. In essence, we were investigating the possibility that the enzyme that acted in synergism with the C_X and β-D-glucosidase enzymes to solubilize cotton fibre or Avicel (*i.e.*, C_1) did not originate in the same enzyme protein as the hydrolytic enzyme that could attack H_3PO_4-swollen cellulose or reduced cellotetraose. Reese *et al.*[12], after all, had envisaged that C_1 would have a non-hydrolytic action.

(*c*) *Further purification of the C_1 component on Ultrogel AcA-54.* Clear evidence of contamination of the C_1 component with CM-cellulase components of different kinds was obtained by gel filtration on a column of Ultrogel AcA-54, equilibrated with 0.01M ammonium acetate (Fig. 2). Fractions 120–130 (Fig. 2a) contained a small amount of a CM-cellulase of molecular weight lower than that of the C_1 component, while fractions 90–100 contained a CM-cellulase of higher molecular weight. The C_X component of lower molecular weight differed from C_X of higher molecular weight in that it had apparently no capacity for hydrolysing reduced cellotetraose (*cf.* Figs. 2a and 2b).

The peak of activity towards H_3PO_4-swollen cellulose, cotton, or Avicel [both with added C_X (portion *C*) and β-D-glucosidase (portion *A* Fig. 1)] coincided in fraction 107: the results with cotton are not shown in Fig. 2.

Fractions 100–118 were combined, concentrated in a collodion tube, and purified further by rechromatography on the same column of Ultrogel.

(*d*) *Further purification of the C_1 component on DEAE-Sephadex.* The C_1 component (purified by molecular-sieve chromatography on Ultrogel) was freeze dried, redissolved in 0.05M acetic acid–NaOH buffer (pH 5.65), and applied to another column of DEAE-Sephadex equilibrated with the same buffer.

The very shallow pH-gradient used to elute the C_1 component (Fig. 3) was constructed and automatically programmed by an LKB Ultrograd gradient mixer connected to an LKB Uvicord II and LKB Level Sensor. The Level Sensor, which was connected in parallel with the Uvicord, was programmed to instruct the Ultrograd to feed a fixed ratio of the two buffers used to construct the pH gradient [0.05M acetic acid–NaOH buffer (pH 4.8) and 0.05M acetic acid–NaOH buffer (pH 4.0)] as soon as the input signal from the Uvicord fell below 95% of maximum (*i.e.*, 95% transmission at 280 nm). Fig. 3 shows a typical purification on DEAE-Sephadex using these facilities.

The peak of activity towards reduced cellotetraose, H_3PO_4-swollen cellulose, and Avicel [*i.e.*, with added C_X from portion *C* [see (*b*) above], and β-D-glucosidase from *A*, Fig. 1] were coincident in fraction 165 (*cf.* Figs. 3a and 3b). Activity towards cotton (again with added C_X and β-D-glucosidase) also reached an optimum in fraction 165 (not shown in Fig. 3). Fractions (120–158) contained only small amounts of CM-cellulase activity and were discarded. Recovery of protein was 92%.

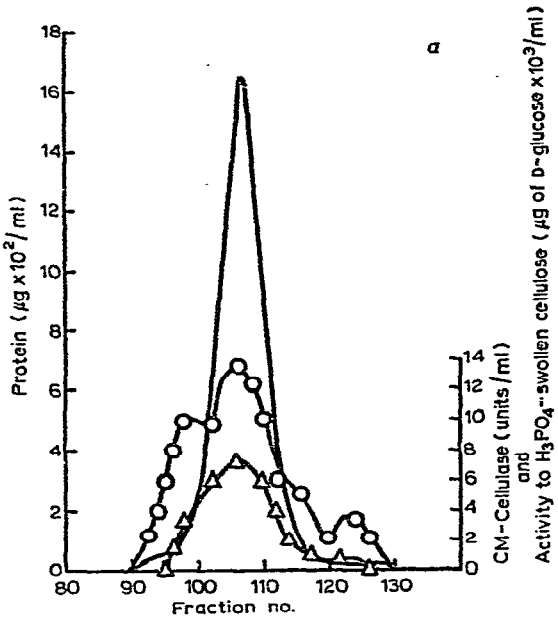
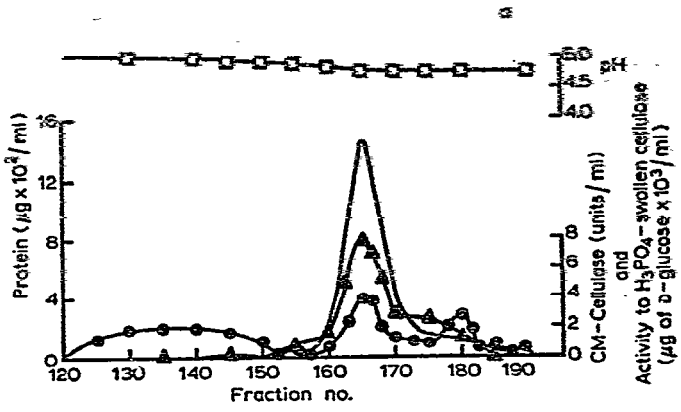


Fig. 2. Purification of the C_1 component on Ultrogel AcA-54. C_1, which was separated from C_x (CM-cellulase) on DEAE-Sephadex (see text), was concentrated in a collodion tube to 5.5 ml, and 5.4 ml of this solution was applied to a column (86.5 × 2.5 cm) of Ultrogel equilibrated with 0.1M ammonium acetate. The column was eluted at 15 ml/h and the eluate was monitored at 280 nm (LKB Uvicord II). Fractions (2.5 ml) were collected and assayed for CM-cellulase (—○—), protein (——), and activity to H_3PO_4-swollen cellulose (—△—), and these are shown in (*a*); (*b*) shows activity to Avicel (—○—) with added C_x and β-D-glucosidase, and activity to reduced cellotetraose (—□—). Note the very large scale used for CM-cellulase activity in this Figure, compared with that used in Fig. 1.



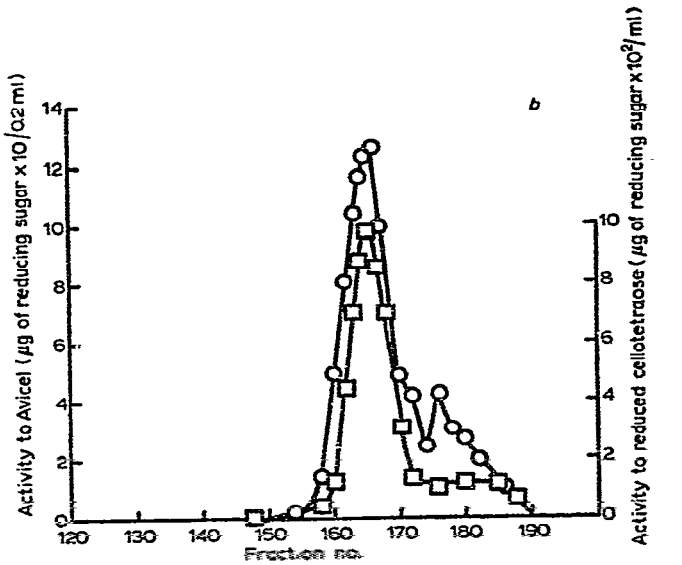


Fig. 3. Purification of the C_1 component on a column of DEAE-Sephadex. The C_1 component purified on Ultrogel was prepared for ion-exchange chromatography as described in the text, applied to the column (27.0 × 1.5 cm) of DEAE-Sephadex, and washed with 40 ml of 0.05M acetic acid–NaOH buffer (pH 5.65). The column was eluted at 10 ml/h with a pH gradient formed by adding a solution of 0.05M acetic acid–NaOH buffer (pH 4.8) to the starting buffer (see text). After 123 fractions (2.5 ml) had been collected, the column was eluted with another pH gradient constructed with the same buffer (pH 4.0 added to pH 4.8); pH, —□— in *3a*. Fractions were assayed for protein (——), CM-cellulase (—●—) by reducing sugar, and activity to H_3PO_4-swollen cellulose (—▲—), and these are shown in (*a*); (*b*) shows activity to reduced cellotetraose (—□—), and activity to Avicel (—○—) after adding C_X and β-D-glucosidase.

Reconstitution experiments for cellulase (cotton-solubilizing) activity were also performed; the purified C_1 component (combined fractions 159–174) and the separated C_X and β-D-glucosidase components were mixed in their original proportions (see above): 96% of the cotton-solubilizing activity of the original 20–80%-saturated-$(NH_4)_2SO_4$ fraction was recovered. The same recovery of cellulase activity was obtained when the enzyme(s) in fractions 175–190 were included in the assays: these fractions were therefore not considered further.

The purified C_1 component was still associated with a trace of CM-cellulase activity.

(*e*) *Isoelectric focusing of the C_1 component.* An ampholyte solution of narrow pH-range (4.6–5.1) was prepared from an ampholyte solution covering the pH-range 4–6 by electrofocusing an 8% solution of the (pH 4–6)-ampholytes (without added enzyme) for 7 days using the normal procedure (see Methods Section). During the

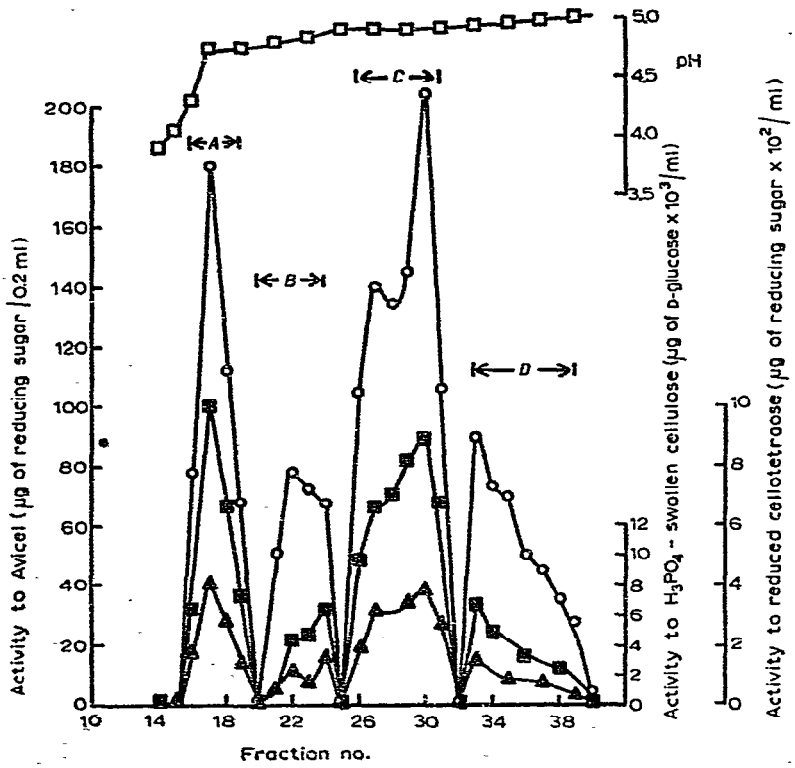


Fig. 4. Isoelectric focusing of the C_1 component. The narrow-pH-range ampholyte solution (pH 4.6–5.1, see text) was used at a final concentration of 1%. The C_1 component (Fig. 3) was electrofocused for 92 h. The voltage at the end of the run was 1080 V and the current 1 mA. The column was emptied by pumping water into the top of the column at 120 ml/h. The fractions (1 ml) were worked-up as described in the text, and assayed for activity to H_3PO_4-swollen cellulose (—▲—) and reduced cellotetraose (—■—), and acitivity to Avicel when acting synergistically with added C_X and β-D-glucosidase (—○—): pH (—□—).

electrofocusing, the load was kept below three watts. At the end of the run, the voltage was steady at 420 V and the current at 2 mA.

The enzyme in fractions 159–174 (Fig. 3) was prepared for electrofocusing in the ampholyte solution of narrow pH-range by dialysis in a collodion tube against 0.01M acetic acid–NaOH buffer (pH 5.0) for 2 days. The enzyme was concentrated (5.9 ml) and added to the electrofocusing column as described in the Methods section. The concentration of the pre-run was not taken into account when making up the new density gradient.

Fig. 4 shows that the C_1 component was resolved into two major (pI 4.75 and 4.90) and two minor components (pI 4.82 and 4.95). However, from the shape of the various peaks, it is clear that some of these components, particularly the major component with pI 4.90, were not homogeneous.

Each of the four components was associated with carbohydrate: component *A* (Fig. 4) contained 21%, and components *B*, *C*, and *D* contained 10, 12, and 1%, respectively (carbohydrate determined by phenol–H_2SO_4[18], and expressed as glucose equivalent; protein determined by the method of Lowry *et al.*[20]).

Each of the four components acted synergistically with added C_X and β-D-glucosidase in solubilizing Avicel, and also hydrolysed reduced cellotetraose and H_3PO_4-swollen cellulose when acting alone. Cotton fibre was extensively degraded by each of the four components when acting in synergism with C_X and β-D-glucosidase (Table I).

TABLE I

CELLULASE (COTTON-SOLUBILIZING) ACTIVITY OF THE C_1 COMPONENTS (FIG. 4) WHEN RECOMBINED WITH C_X AND β-D-GLUCOSIDASE[a]

C_1 components	*Solubilization* (%)
A (fractions 16–19)	59
B (fractions 21–24)	60
C (fractions 26–31)	67
D (fractions 33–39)	68
C_1 component (Fig. 3)	71

[a]All assays contained the same amount of protein, CM-cellulase (C_X), and β-D-glucosidase activity. C_X was from portion *C* (see text), and β-D-glucosidase was from portion *A* (Fig. 1).

Properties of the C_1 component. — (*a*) *Effect of C_1 on cellulose substrates.* The rate of attack of the cellulose swollen in H_3PO_4 was much higher than that on such highly ordered substrates as cotton, Avicel, or Whatman cellulose powder (Fig. 5). Cellobiose was the sole sugar found in the supernatants of the digests of the three highly ordered substrates, but 4% of the total carbohydrate liberated by enzymic hydrolysis of H_3PO_4-swollen cellulose was glucose. No traces of higher cello-oligosaccharides were detected by t.l.c. or by chromatography of the concentrated supernatants (of several digests) on a column of Bio-gel P-2 previously calibrated with

standard cello-oligosaccharides. No traces of higher cello-oligosaccharides were obtained regardless of the extent of the degradation of the substrate.

Both of the major C_1-components isolated by isoelectric focusing (Fig. 4) digested H_3PO_4-swollen cellulose to cellobiose and a trace of glucose.

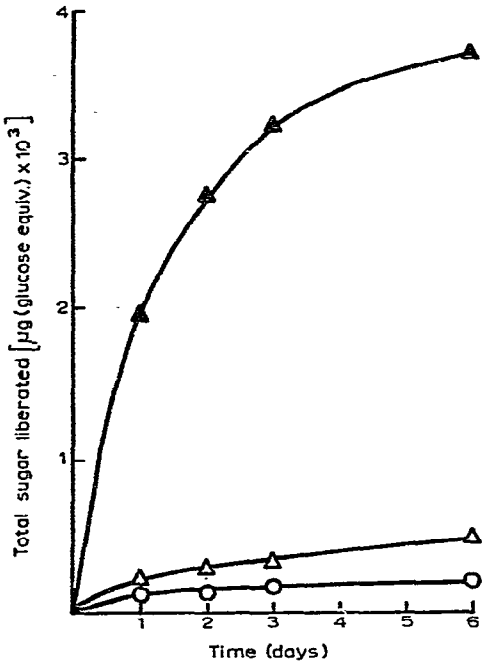


Fig. 5. Rate of attack of C_1 on various cellulose substrates. The substrates (~20 mg) were incubated with 50 μl of C_1 (Fig. 3) by the procedure described for assaying activity to H_3PO_4-swollen cellulose (Methods section). The reducing sugars liberated were estimated by the method of Nelson[17]. Activity to H_3PO_4-swollen cellulose, —▲—; cotton, —○—; and Avicel, —△—.

(*b*) *Effect of C_1 on cello-oligosaccharides.* The rate of attack of the C_1 component (Fig. 3) on the soluble cello-oligosaccharides, cellotriose, cellotetraose, and cellopentaose increased with increasing d.p. of the substrate (Fig. 6); cellobiose was not attacked. T.l.c. examination of the products showed that cellotetraose was hydrolysed principally to cellobiose, and cellopentaose to a mixture of cellobiose and cellotriose: these results were confirmed by gel filtration of the concentrated supernatants on a column of Biogel P-2 which had been previously calibrated with well-characterized cello-oligosaccharides.

Cellohexaose was hydrolysed principally to cellobiose.

Both of the major C_1-components isolated by isoelectric focusing (Fig. 4) gave similar results.

(*c*) *Comparison of the effect of C_1 and C_X on the d.p. of H_3PO_4-swollen cellulose.* A mixture containing 10 ml of a suspension (4% w/v) of H_3PO_4-swollen cellulose in

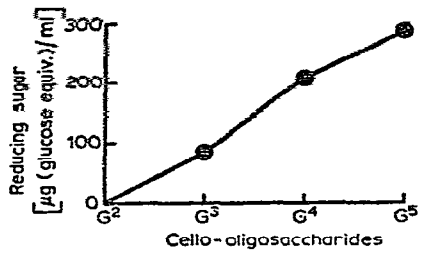


Fig. 6. Relative activity of C_1 on cello-oligosaccharides. Equimolar amounts of the cello-oligosaccharides were incubated at 37° with 0.5 ml of 0.1M acetic acid–NaOH buffer (pH 5.0), and enzyme and water to give a total volume of 1 ml. After 4 h, a sample (0.1 ml) was assayed for reducing sugar by a modified Park–Johnson ferricyanide method[14].

acetate buffer (pH 5.0) and 0.1 ml of 0.05M sodium azide was pre-incubated for 30 min at 37° before enzyme (0.15 ml) was added. The mixture was incubated for 0.5, 1, 2, or 4 h, filtered quickly through a sintered-glass crucible (porosity 2), washed with water, and freeze-dried. A sample, (~25 mg) was moistened with water (0.5 ml) and dissolved in cadoxen[23]. The solution was diluted with water (10.0 ml) and transferred to a Cannon–Ubbelohde dilution viscometer (size 75), and the viscosity was measured at 25°. The d.p. of the sample was calculated by using the formula of Schulz-Blaschke[24] with a value[25] of $k = 0.28$. The controls for each incubation time contained no enzyme.

The rate of change of d.p. shown by the C_1 component and the randomly acting C_X component is shown in Fig. 7.

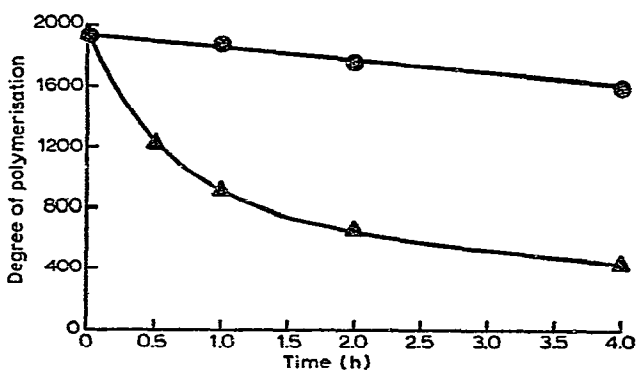


Fig. 7. Comparison of the rate of change in degree of polymerisation of H_3PO_4-swollen cellulose by C_1 and C_X enzymes. C_1 was from Fig. 3, and C_X was portion *C* (see text). See text for details of assay. Change in d.p. as a result of C_1 action, —●—; C_X action, —▲—.

(*d*) *Determination of the molecular weight of C_1 by molecular-sieve chromatography.* Chromatography was performed on a column (78.5 × 1.6 cm) of Ultrogel AcA-44, which was calibrated with the proteins, cytochrome C, chymotrypsinogen,

ovalbumin, bovine serum albumin, γ-globulin, and thyroglobulin. The molecular weight (C_1, Fig. 3) calculated from a plot of V/V_0 against the log molecular weight was 41,000. This value is lower than an estimate of 45,000 obtained[12] on Sephadex G-75.

(*e*) *Effect of C_1 on CM-cellulose.* The C_1 component, when purified by chromatography on DEAE-Sephadex (Fig. 3), still possessed a very limited ability to produce reducing sugars from a solution of CM-cellulose, but showed no capacity for producing a measurable decrease in the viscosity of a similar solution: clearly, attack was confined to the end of the CM-cellulose chain. Under the conditions of the standard assay, the production of reducing sugars increased slowly for 2 h, until the reducing value from 500 μg of C_1-protein had reached the equivalent of that produced by 10 μg of D-glucose; extending the incubation period showed no increase in reducing power. Cellobiose was the only sugar found in the hydrolysate.

(*f*) *Specificity of the C_1 component.* The action of the C_1 component (Fig. 3) was investigated by incubating the substrate (0.5 mg) with 0.01M acetic acid–NaOH buffer (pH 5.0, 0.5 ml), enzyme (50 μg of protein), and sodium azide (1 μg) for 18 h at 37°. A sample (100 μl was analysed (without deionisation) by paper chromatography (solvent *A*; detection with $AgNO_3$[21]). No degradation products were found with (1→2)-β-D-glucan, lutean [(1→6)-β-D-glucan], laminarin [(1→3)-β-D-glucan], nigeran [mixed (1→4)- and (1→3)-α-D-glucan], glycogen and amylopectin [mixed (1→4)- and (1→6)-α-D-glucan], dextran [(1→6)-α-D-glucan], and amylose [(1→4)-α-D-glucan]. Barley β-D-glucan [mixed (1→4)- and (1→3)-β-D-glucan] was hydrolysed to some extent, and glucose, cellobiose (R_{GLC} 0.57), and another spot with R_{GLC} 0.16 were found in the solution; authentic cellotriose and cellotetraose had R_{GLC} values of 0.24 and 0.08, respectively, in solvent *A*.

TABLE II

EFFECT OF VARIOUS ADDITIVES ON THE ACTION OF C_1 ON H_3PO_4-SWOLLEN CELLULOSE

Additive[a]	*Concentration used in assay (mM)*	*Inhibition (%)*	*Stimulation (%)*
Ba^{2+} (p)	100	23	—
Ca^{2+} (p)	100	16	—
Mg^{2+} (p)	100	16	—
Co^{2+} (p)	100	16	—
Mn^{2+} (p)	100	11	—
Zn^{2+} (p)	100	21	—
D-Glucose	10	Nil	Nil
Cellobiose	10	56	—
N-Bromosuccinimide (p)[b]	5	100	—
N-Acetylimidazole (p)	50	—	7
2-Hydroxy-5-nitrobenzyl bromide (p)	50	27	—
EDTA	50	—	26
Bovine serum albumin	200	Nil	Nil

[a]Preincubation indicated by (p). [b]Dissolved in aqueous acetone (20:1) before incubation.

(*g*) *Effect of various additives on C_1 activity.* Enzyme solutions containing 62 μg of C_1 protein were incubated with additives by substituting the additive for water in the normal assay for determining activity on H_3PO_4-swollen celluloses (see Methods); the sugars that were liberated were determined by the method of Nelson[17].

In those cases where the enzyme was preincubated with additive (Table II), the enzyme–additive mixtures (total volume, 0.25 ml) were heated at 25° for 30 min before the activity in a sample (0.2 ml) was determined by the standard assay for activity on H_3PO_4-swollen cellulose.

D-Glucono-1,5-lactone is not particularly stable at pH 5.0, and is not, in consequence, suitable for inhibition studies involving overnight incubation. The effect of this compound on C_1 activity was measured, therefore, using reduced cellotetraose as substrate (1.5 mg in 1 ml) in an assay containing 0.25 ml of 0.2M acetic acid–NaOH buffer (pH 5.0), enzyme (50 μg of protein), water, and inhibitor solution in a total volume of 2 ml. Under these conditions, the reducing sugars liberated in 2 h were inhibited by 59 and 19% by 100mM and 50mM concentrations of D-glucono-1,5-lactone, respectively.

DISCUSSION

It is now well-established that the C_1 and C_X components found in certain fungal-cellulase preparations act synergistically to effect the solubilization of highly ordered cellulose. Clearly, because of this synergism, meaningful studies on the mode of action of the C_1 component can only be carried out after the removal of all traces of contaminating C_X-activity. In the present investigation, molecular-sieve chromatography on Ultrogel, followed by ion-exchange chromatography on DEAE-Sephadex with a pH gradient, provided a more highly purified C_1-component than the method previously reported[2]. Purified by this new procedure, the C_1 component, although still associated with a small amount of CM-cellulase activity, had little or no capacity for solubilizing cotton fibre when acting alone, and this behaviour contrasts with the results previously recorded[2] (7% solubilization). Moreover, as a result of this further purification, C_1 lost none of its capacity for acting synergistically with the separated C_X and β-D-glucosidase components, for a reconstituted mixture containing C_1, C_X, and β-D-glucosidase in their original proportions showed the same cellulase (cotton-solubilizing) activity as the unfractionated culture filtrate; this, too, was an improvement in the recovery reported previously[2], and it demonstrates clearly that all factors essential to the hydrolysis of cotton fibres are present in the purified fractions.

Rechromatography of the C_1 component on Ultrogel and DEAE-Sephadex resulted in the removal of further amounts of C_X activity, but these were traces only, and it was necessary to use the very sensitive Park–Johnson reagent[14] for measuring the reducing sugar liberated from the CM-cellulose; this level of activity would not have been detected with the dinitrosalicylic acid reagent[9].

In spite of the rigorous purification, the C_1 component was still associated with a trace of CM-cellulase activity. However, it seems likely, particularly in view of the

fact that similar results were obtained with a highly purified C_1 from *T. koningii*[14], that C_1 and the trace of CM-cellulase of *F. solani* reside in the same enzyme protein. As with *T. koningii* C_1, the associated CM-cellulase activity could produce reducing sugars from a solution of CM-cellulose, but could not change the viscosity; this result is consistent with endwise attack.

The use of CM-cellulose for the measurement of (1→4)-β-D-glucanase activity warrents further comment, particularly as it has been used almost exclusively for this purpose in fractionation studies involving C_1 and C_X. CM-cellulose has been widely used because of its high reactivity, its ready availability, and the simplicity of the assay methods used for detecting even small, enzyme-catalyzed changes. However, there are several disadvantages. It suffers to some extent because it is negatively charged[26], and from the fact that the carboxymethyl substituents are randomly arranged[27], but its most serious disadvantage, in the present context, is that it would seem to be more suited to the measurement of the activity of enzymes that attack the chain at random, rather than enzymes that attack from the end of the chain. It is unlikely that the bulky carboxymethyl substituents would be involved in enzyme binding, and clearly enzyme action involving attack from the end of the chain will cease after the removal of a limited number of residues; this number is determined by the specificity and mode of action of the enzyme, as well as by the relative positions of the carboxymethyl substituents. Measurement of exoglucanase action will require, with certain enzymes, an easily accessible substrate having none of the constraints mentioned for CM-cellulose; H_3PO_4-swollen cellulose is such a substrate.

The C_1 component of *T. koningii* is an exoglucanase which has little action on CM-cellulose, but which degrades H_3PO_4-swollen cellulose by removing successive units of cellobiose from the end of the chain[14]. We found the C_1 component of *F. solani*, after rigorous purification, to be similar to the C_1 of *T. koningii* in both respects. Action of *F. solani* C_1 on CM-cellulose ceased after the removal of a few cellobiose units, and in this behaviour, it resembled C_1 of *T. koningii*[28,29]. These findings, considered together with the observations that (*a*) cellobiose was the sole product of the hydrolysis of cellotetraose and cellohexaose, and (*b*) the rate of change of d.p. of H_3PO_4-swollen cellulose was low compared with that shown by the randomly acting C_X-enzymes, are clearly compatible with the interpretation that *F. solani* C_1 is a cellobiohydrolase.

Reese has suggested that C_1 and cellobiohydrolase may be two different protein components having similar net charge and molecular weight[30]. To test this possibility, we have measured C_1 (defined as the enzyme that acts in synergism with the C_X enzymes to solubilize cotton fibre or other highly ordered celluloses) and cellobiohydrolase activities in each column fraction that was collected during the various purification procedures. However, no evidence for the non-identity of C_1 and cellobiohydrolase peaks of activity was found on Ultrogel or on DEAE-Sephadex, using cotton and Avicel to detect synergism of C_1 with C_X, and H_3PO_4-swollen cellulose and reduced cellotetraose to detect cellobiohydrolase. But data which put the answer beyond dispute were obtained by isoelectric focusing of the C_1 component in a

stabilized pH-gradient covering only 0.5 of a pH unit. Under these conditions, C_1 was resolved into four components, each having identical hydrolytic properties (cellobiohydrolase) and similar capacities for acting in synergism with the reconstituted C_X–β-D-glucosidase mixture. Moreover, the peaks of both types of activity were coincident in each of the four components isolated.

The C_1 component of *F. solani* is highly specific for (1→4)-β-D-glucans. The (1→3)-β-D-glucan, laminarin, was not a substrate, but barley glucan, which contains mixed (1→3)- and (1→4)-β-D linkages, yielded glucose, cellobiose, and an unidentified trisaccharide. The appearance of this trisaccharide is of special interest with respect to the specificity of the C_1 component, and requires further study. Parrish, Perlin, and Reese[31] found 4-*O*-β-D-laminaribiosyl-D-glucose in the products of hydrolysis of oat glucan with a crude cellulase preparation from *T. viride*.

Reese, McGuire, and Parrish[32] have studied the properties of a number of exoglucanases and have drawn up criteria for their characterization. The C_1 component of *F. solani* appears to satisfy some of these criteria, in that (*a*) it was not inhibited by low concentrations of D-glucono-1,5-lactone, (*b*) it was highly specific for the (1→4)-β-D linkage, (*c*) it was free from transferase activity, and (*d*) its rate of attack on short-chain cello-oligosaccharides was cellotriose<cellotetraose<cellopentaose. However, we have been unable to find any evidence to suggest that C_1 from *F. solani* acts by inversion of configuration, and in this respect it differs from the exoglucanase of *S. pulverulentum*[11] and the cellobiohydrolase of *Cellvibrio gilvus*[33].

The effects of various additives on C_1 action on H_3PO-swollen cellulose have provided additional information on the enzyme. Metal ions such as Ba^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} were inhibitory at relatively high concentrations; the stimulation of activity with the chelating agent EDTA was consistent with this inhibition. Cellobiose was inhibitory, and this supports the observations made with the C_1 component of *T. koningii*[15,29].

There is little information available on the inhibition of cellulases by group-specific reagents, but it has been shown that tryptophan residues are important for the activity of a "cellulase" (C_X) from *Penicillium notatum*[34]. The possibility that tryptophan residues are required for activity of *F. solani* C_1 is shown by the complete inhibition by *N*-bromosuccinimide, but it is difficult to reconcile this observation with the relatively high concentration of 2-hydroxy-5-nitrobenzyl bromide required to effect even a small degree of inhibition.

The hypothesis that the attack on highly ordered cellulose is initiated by a chain-separating enzyme is attractive, but difficult to prove or disprove. It has some support from the observation that the cellulose substrates that are refractory to enzymes classified as C_X are readily attacked after they have been rendered more accessible by ball-milling, swelling, or reprecipitation from solvents. As C_1 enzymes are not required for the hydrolysis of these highly hydrated substrates, the argument that these treatments have simulated C_1-action clearly has some appeal. However, until a chain-disaggregating, prehydrolytic factor has been isolated, this hypothesis must give way, for the present, to the more plausible argument that hydrolysis of

"crystalline" cellulose must be described in terms of endo- and exo-glucanase activities.

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