The Purification and Properties of the C₁ Component of *Trichoderma koningii* Cellulase

By T. M. WOOD and SHEILA I. McCRAE
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, U.K.

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1. The C₁ component that was isolated from a *Trichoderma koningii* cellulase preparation (Wood, 1968) by chromatography on DEAE-Sephadex with a salt gradient was still associated with a trace of CM-cellulase activity (determined by reducing-sugar and viscometric methods). 2. Further chromatography on DEAE-Sephadex, with a pH gradient instead of a salt gradient, provided a C₁ component that could still produce reducing sugars from a solution of CM-cellulose (to a very limited extent), but which could no longer decrease the viscosity (i.e. under the assay conditions employed). 3. No evidence for the non-identity of C₁ component and the trace of CM-cellulase activity could be found when electrofocusing was done in a stabilized pH gradient covering three pH units (pH 3–6) or, alternatively, only 0.5 pH unit (pH 3.72–4.25). 4. The two protein peaks that were separated by electrofocusing in carrier ampholytes covering only 0.5 pH unit (isoelectric pH values of 3.80 and 3.95) were shown to be isoenzymes of the C₁ component: they differed in the extent to which they were associated with carbohydrate (9% and 33%). 5. The purified C₁ component had little ability to attack CM-cellulose or highly ordered forms of cellulose, but degraded phosphoric acid-swollen cellulose readily: cellobiose was the principal product of the hydrolysis (97%). 6. Dewaxed cotton fibre was degraded to the extent of 15% when exposed to high concentrations of C₁ component over a prolonged period: cellobiose was again the principal sugar present in the supernatant (96%). 7. Cellotetraose and cellohexaose were hydrolysed almost exclusively to cellobiose. 8. Evidence indicates that the C₁ component is a β -1,4-glucan cellobiosylhydrolase.

Certain cellulolytic fungi, when grown on media containing cotton fibres as sole carbon source, synthesize and release into solution all of the enzymes that are essential for the breakdown of native cellulose. Notable in this regard are the fungi *Trichoderma viride* (Mandels & Reese, 1964; Li *et al.*, 1965; Niwa *et al.*, 1965; Ogawa & Toyama, 1965, 1967, 1968; Selby & Maitland, 1967; Okada *et al.*, 1968), *Trichoderma koningii* (Iwasaki *et al.*, 1964; Halliwell, 1965; Wood, 1968), *Penicillium funiculosum* (Selby, 1968) and *Fusarium solani* (Wood & Phillips, 1969). Some of the fractionation studies carried out on culture filtrates prepared from these fungi (Mandels & Reese, 1964; Selby & Maitland, 1967; Wood, 1968; Selby, 1968; Wood, 1969) have shown them to contain three enzymes, or classes of enzymes, which are essential for the extensive hydrolysis of highly ordered forms of cellulose. Two of these types of enzymes have been arbitrarily called C₁ and C_x (Reese *et al.*, 1950); the third is a cellobiase or β -glucosidase.

Enzymes classified as C_x can hydrolyse swollen, soluble, or partially degraded celluloses, but are

unable to attack highly ordered substrates, such as the cotton fibre. C₁ component has little or no effect on either soluble derivatives of cellulose (such as CM-cellulose) or highly ordered substrates (Selby & Maitland, 1967; Wood, 1968, 1969, 1971), but acts synergistically with C_x enzyme(s) and cellobiase to accomplish the conversion of native cellulose into water-soluble products (Mandels & Reese, 1964; Selby & Maitland, 1967; Wood, 1968, 1969).

C_x enzyme(s) and cellobiase, then, are hydrolases, but what is the function of the C₁ component? According to the original concept of Reese *et al.* (1950), the action of C₁ component on highly ordered forms of cellulose is a prerequisite to hydrolysis by the C_x enzymes. Since then it has been tentatively suggested that this prehydrolysis step might be one involving attack on hydrogen bonds (Siu, 1963), anomalous linkages, 'weak' linkages or atypical residues in the cellulose chains (Mandels & Reese, 1964). Yet another theory envisages C₁ component to be an enzyme capable, on its own, of solubilizing 'crystalline' hydrocellulose (Li *et al.*, 1965; Flora, 1965; Ogawa & Toyama, 1967; Okada *et al.*, 1968),

although acting synergistically with the C_x components to bring about an increase in the rate of hydrolysis.

The present report describes the further purification of the C_1 component already isolated (Wood, 1968) and presents evidence which indicates that C_1 component is a β -1,4-glucan cellobiosylhydrolase.

Experimental

Materials

T. koningii, culture collection no. I.M.I. 73022, was obtained from the Commonwealth Mycological Institute, Kew, Surrey, U.K. Sodium CM-cellulose (Cellofas B) with a degree of substitution of approx. 0.5 was kindly given by Imperial Chemical Industries Ltd., Nobel Division, Stevenston, Ayrshire, U.K. Texas cotton fibre was obtained from the Shirley Institute, Didsbury, Manchester, U.K. Sephadex G-25, Sephadex G-75, DEAE-Sephadex (A-50) and the glass chromatographic columns were purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. Ampholine electrofocusing equipment and the carrier ampholytes were supplied by L.K.B. Instruments Ltd., Croydon, Surrey, U.K., and Kieselgel G was from Shandon Scientific Co., London N.W.10, U.K. Glucose oxidase (type II) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and the peroxidase from Boehringer Corp. (London) Ltd., London W.5, U.K. β -1,2-Glucan (*Rhizopus japonicus*) and β -1,6-glucan (*Penicillium luteum*) were gifts from Dr. J. S. D. Bacon, Macaulay Institute for Soil Research, Aberdeen, U.K., and laminarin (from *Laminaria hyperborea*), amylopectin (from potato), amylose (from potato), glycogen (from oyster), nigeran (from *Aspergillus niger*) and dextran (source unspecified) were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Avicel (microcrystalline cellulose) was obtained from Honeywell and Stein Ltd., London W.1, U.K.

Methods

Preparation of *T. koningii* cellulase. Cultures and cell-free filtrates were prepared from *T. koningii* I.M.I. 73022 by the method previously described (Wood, 1968).

A concentrated culture filtrate (50-fold) was prepared by precipitation of the culture filtrate with $(\text{NH}_4)_2\text{SO}_4$ between the limits of 20 and 80% saturation, and then centrifuging and redissolving the precipitate in 0.1M-acetic acid-NaOH buffer, pH 5.0. Enzyme assays indicated that all of the cellulase (cotton solubilization), CM-cellulase and

cellobiase activity of the original culture filtrate was recovered.

Preparation of phosphoric acid-swollen cellulose (Walseth cellulose; Walseth, 1952). Avicel was swollen by the method detailed by Wood (1971).

Isoelectric focusing. The separations were carried out according to the method first described by Vesterberg & Svensson (1966). The L.K.B. column used had a capacity of 110ml. The density gradient (sucrose) was made manually (see the L.K.B. instruction manual) by mixing a 'light solution' (containing water and ampholyte) and a 'dense solution' (containing water, ampholytes and sucrose) in different proportions. The sample to be electro-focused was added to the column near the middle by replacing the light component of the density gradient by the sample solution. The dense electrode solution contained phosphoric acid (0.2ml), water (14.0ml) and sucrose (12.0g) and the light electrode solution contained sodium hydroxide (0.1g) and water (10.0ml). The electrodes were placed so that the anode was at the bottom of the column.

After focusing at 5°C the column was emptied at a rate of 120ml/h and fractions (approx. 2ml) were collected. The pH of each fraction was measured at 5°C with a Radiometer pH-meter fitted with a combination electrode and a temperature compensator.

Paper chromatography. Paper chromatography was carried out on Whatman no. 1 filter paper in solvent A, ethyl acetate-pyridine-water (10:4:3, by vol.). Chromatograms were sprayed, after air-drying, with silver nitrate (Trevelyan *et al.*, 1950).

Thin-layer chromatography. Plates (20cm × 20cm) were coated with a 0.25mm-thick layer of Kieselgel G, dried at 100°C and stored in a desiccator before use. Chromatograms were developed (two ascents) in solvent B, ethyl acetate-propan-2-ol-water (18:13:9, by vol.). The plate was dried at room temperature and sprayed with silver nitrate (see above) or a 2% (w/v) solution of sodium periodate in 5% (v/v) acetic acid, followed by a 0.5% solution of potassium permanganate in 4% (w/v) NaOH.

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

Determination of reducing sugars. Reducing sugars were determined either by the Somogyi-Nelson method (Nelson, 1952) or by the method of Park & Johnson (1949) modified to include $\text{FeNH}_4(\text{SO}_4)_2$ solution made up in 0.075M- H_2SO_4 instead of 0.025M- H_2SO_4 . The reducing value in each case was expressed as glucose equivalent.

Determination of glucose. Glucose was measured by glucose oxidase (see under cellobiase assay; Wood, 1969) and/or one of the reducing-sugar methods mentioned above.

Determination of total carbohydrate. Total carbo-

hydrate was measured by the phenol-H₂SO₄ method of Dubois *et al.* (1956).

C_x activity. C_x activity was measured in two ways: (a) the decrease in the viscosity of a CM-cellulose solution (Wood, 1971); (b) the appearance of reducing sugars in a solution of CM-cellulose. In the viscometric method it was found that, under the conditions of the assay, the plot of rate of increase of the reciprocal of the viscosity against the enzyme concentration was linear up to at least a value of 0.027 min⁻¹ (Thomas, 1956). A value of 0.020 min⁻¹ was found for 1.0 ml of the 20–80% satd.-(NH₄)₂SO₄ fraction diluted 2500-fold, and this was chosen as the unit of activity.

In the reducing-sugar method a mixture of 2.5 ml of the 1% solution of CM-cellulose, 1.5 ml of 0.2 M-acetic acid-NaOH buffer, pH 5.4, and 1.0 ml of diluted enzyme and water was incubated at 37°C for 1 h. A sample (1.0 ml) of this reaction mixture was taken and the reducing sugars present were measured by the method of Park & Johnson (1949) modified as stated above. The unit of activity is defined as the amount of enzyme needed to liberate reducing sugar equivalent to 5 µg of glucose.

A sample (1.0 ml) of the 20–80% satd.-(NH₄)₂SO₄ fraction, diluted 50-fold, contained 1820 units of CM-cellulase activity when assayed by the reducing-sugar method [hereafter called CM-cellulase activity (reducing sugar)] and 50 units of CM-cellulase activity when measured by the viscometric method [hereafter called CM-cellulase activity (viscometric)].

Cellobiase activity. Cellobiase was determined by the method described by Wood (1969).

Activity towards dewaxed cotton. The degree of solubilization (referred to as 'cellulase activity' in the Results section) is defined as the extent to which 2 mg of dewaxed cotton fibre is solubilized in 7 days when incubated with enzyme at 37°C under the conditions of the standard assay (Wood, 1969).

Activity towards phosphoric acid-swollen cellulose, Avicel and Whatman cellulose powder (no. 1). Each substrate was suspended in water so that 1.0 ml contained approx. 1 mg of cellulose (determined by the dichromate-H₂SO₄ method described by Wood, 1969). NaN₃ (final concn. 0.02%) was added as preservative.

Assays for enzymic activity towards these substrates consisted of 2 ml of substrate suspension, 2.5 ml of 0.2 M-acetic acid-NaOH buffer, pH 4.8, and enzyme and water to give a total volume of 5 ml. After incubation at 37°C for the times stated, the residual cellulose was assayed by the dichromate-H₂SO₄ method described previously (Wood, 1969).

Preparation of cello-oligosaccharides. Cello-oligosaccharides were prepared by the acetolysis of dewaxed cotton fibres and then were separated by gradient elution (0–35% ethanol) from a charcoal-Celite (1:1, w/w) column.

Results

Separation of the C₁ component from C_x and cellobiase activities

It has been demonstrated that the removal of a low-molecular-weight C_x component from culture filtrates from *Trichoderma* species (Selby & Maitland, 1967; Wood, 1968) does not affect the kinetics of solubilization of cotton fibre. The low-molecular-weight component isolated in one case (Wood, 1968) had little capacity for solubilizing cotton fibre and was unable to form 'short-fibres' (Halliwell, 1965). All of the 'short-fibre'-forming activity as well as all of the solubilization activity shown by the unfractionated culture filtrate was associated with the high-molecular-weight component. This behaviour contrasts with that shown by the low-molecular-weight component that was incompletely separated from the higher-molecular-weight material by Halliwell & Riaz (1970).

In the present investigation relatively large quantities of C₁ component were prepared from *T. koningii* cellulase as follows: the low-molecular-weight component was first removed from a sample (10 ml) of the concentrated cell-free filtrate [20–80% satd.-(NH₄)₂SO₄ fraction] on a column (2.5 cm × 93.0 cm) of Sephadex G-75 equilibrated with 0.01 M-acetic acid-ammonium acetate buffer, pH 5.0. The high-molecular-weight material was combined and freeze-dried. Three such fractionations were done and the high-molecular-weight components were combined and applied to a column (5.0 cm × 30.2 cm) of DEAE-Sephadex (acetate form). The C_x and cellobiase components were eluted under the starting conditions (0.1 M-acetic acid-NaOH buffer, pH 5.0) as previously described (Wood, 1968), and the C₁ component was finally desorbed by the application of an ionic-strength gradient (0–0.5 M-NaCl). The separated components were precipitated with (NH₄)₂SO₄ (80% saturation), centrifuged, and redissolved in 0.1 M-acetic acid-NaOH buffer, pH 5.0 (30 ml).

The C₁ component was still associated with a trace of CM-cellulase activity, but this only amounted to 0.03% of the CM-cellulase activity (viscometric) and 0.6% of the CM-cellulase activity (reducing-sugar) of the CM-cellulase activity applied to the column.

Portions of the C₁- and the C_x-cellobiase components were diluted so that the volumes were equivalent in terms of the unfractionated 20–80% satd.-(NH₄)₂SO₄ material, diluted 50-fold. Under these conditions, 1.0 ml of the diluted C₁ component and 1.0 ml of the diluted C_x-cellobiase component solubilized cotton fibres to the extent of 4 and 6% respectively (see the Experimental section for the standard assay), whereas an assay mixture containing 1.0 ml of each gave 69% solubilization. Since, under the same conditions, 1.0 ml of the original culture filtrate also gave 69% solubilization of cotton, it was

clear that there had been no loss in cellulase activity as a result of various manipulations.

It was found that 1.0 ml of the C_1 component, diluted as described above, contained 180 μ g of protein, and 1 ml of the diluted C_x -cellulase component contained 35 units of CM-cellulase activity (viscometric), 1510 units of CM-cellulase activity (reducing sugar) and 32 units of cellobiase activity. In each of the subsequent fractionation procedures reported in the present paper, the recovery of C_1 activity was determined by doing reconstitution experiments for the solubilization of cotton in which 180 μ g of C_1 component was mixed with 1.0 ml of the diluted C_x -cellobiase fraction just described.

Further purification of the C_1 component on DEAE-Sephadex

Further chromatography of the partially purified C_1 component on DEAE-Sephadex, with a pH

gradient (pH 4.8–3.6) with formic acid–NaOH buffer of ionic strength 0.1, resulted in the removal of another C_x component (Fig. 1). The major protein component (C_1), which was eluted at pH 3.95, still possessed a very limited ability to produce reducing sugars from a solution of CM-cellulose, but now showed no capacity for decreasing the viscosity. Similar results were obtained by the gradient (pH) elution of C_1 component from three other DEAE-Sephadex columns in which the ionic strength of the formate buffers were 0.01, 0.05 or 0.15: under these conditions the C_1 component was eluted at pH 3.54, 3.72 and 4.2, respectively.

Recovery of protein (Fig. 1) was 93%.

Reconstitution experiments for cellulase (solubilization) activity were again done, in which the highly purified C_1 component and the separated C_x -cellobiase fraction were mixed in their original proportions (see above): 95% of the cotton-solubilization activity of the original cellulase complex was re-

Fig. 1. Further purification of the C_1 component on a column (21.6 cm $\times$ 1.5 cm) of DEAE-Sephadex equilibrated with 0.1 M-formic acid–NaOH buffer, pH 4.8

A sample (2.5 ml) of the C_1 component that had been eluted from the DEAE-Sephadex (acetate form) column with a NaCl gradient (see the text) was desalted on a column of Sephadex G-25 (43.2 cm $\times$ 2.6 cm; equilibrated with 0.01 M-acetic acid–ammonium acetate buffer, pH 5.0), freeze-dried, redissolved in 0.1 M-formic acid–NaOH buffer, pH 4.9 (6 ml), and applied to the DEAE-Sephadex column. The column was eluted at a rate of 15 ml/h with a pH gradient formed by adding 150 ml of 0.1 M-formic acid–NaOH buffer, pH 3.5, to 150 ml of 0.1 M-formic acid–NaOH buffer, pH 4.8. Fractions (4.2 ml) were collected. Assays were done as described in the text and the results are shown as CM-cellulase activity (reducing sugar) (---) and CM-cellulase activity (viscometric) (····). The pH of the fractions is shown as $\circ$ and the concentration of protein as —.

Fig. 2. Recycling chromatography of C₁ component on Sephadex G-75

A sample (5.0 ml) of the C₁ component that had been eluted from the DEAE-Sephadex (acetate form) column with a NaCl gradient (see the text) was desalted on a column of Sephadex G-25 (43.2 cm × 2.6 cm; equilibrated with 0.01 M-acetic acid-ammonium acetate buffer, pH 5.0), freeze-dried, redissolved in 0.1 M-acetic acid-NaOH buffer, pH 5.0, (5.0 ml) and applied to a column (2.5 cm × 69 cm) of Sephadex G-75. The column was fitted with two Pharmacia (see the Experimental section) end units. The column was eluted (upward flow) at 4 ml/h and the eluate was monitored at 280 nm (LKB Uvicord II). The component that was detected as a peak of extinction was cycled four times through the same column before being eluted. Fractions (3.0 ml) were collected. CM-cellulase activity (reducing sugar) is shown as ----, CM-cellulase activity (viscometric) as and concentration of protein as —.

covered. C₁ component acting in isolation still gave 4% solubilization under the conditions of the standard assay.

In the assay containing the reconstituted mixture of enzymes, the CM-cellulase activity (reducing sugar) associated with the C₁ component amounted to only 1.5 units; this compares with the total of 1510 units of CM-cellulase activity (reducing sugar) in the assay.

Recycling on Sephadex G-75

Recycling chromatography on a long column of Sephadex G-75 (Fig. 2) was found to be as effective as DEAE-Sephadex (Fig. 1) in removing the small CM-cellulase component from C₁ component. The large protein peak was surprisingly sharp considering that it had been cycled four times through the column.

Isoelectric focusing of the C₁ component in a pH gradient in the range pH 3–6

The purified C₁ component (Fig. 1) prepared for electrofocusing as detailed in the legend to Fig. 3, was focused in a stabilized pH gradient covering the range pH 3–6. The fractions that were collected were worked up by precipitating the enzyme with (NH₄)₂SO₄ (80% saturation), centrifuging, dialysing and freeze-drying. This procedure was adopted only after several attempts at removing the ampholytes by dialysis through Visking tubing were unsuccessful; the complete removal of ampholytes was found to be necessary, as even traces were found to interfere with the protein and CM-cellulase assays.

Fig. 3 shows a typical elution pattern after electrofocusing for 66 h. As with ion-exchange chromatography (Fig. 1) and gel filtration (Fig. 2), no evidence for the non-identity of the C₁ component and the

Fig. 3. Isoelectric focusing of the C_1 component with ampholyte covering range pH3–6

The C_1 component from the DEAE-Sephadex column (Fig. 1) was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation), desalted on a column (43.2 cm $\times$ 2.6 cm) of Sephadex G-25 equilibrated with 0.01 M-acetic acid–ammonium acetate buffer, pH 5.0, and freeze-dried. The freeze-dried C_1 components from two such fractionations were combined (20 mg), dissolved in 2% ampholyte solution, pH 3–6 (10 ml), and incorporated into the sucrose density gradient (see the Experimental section). The final voltage employed during the electrofocusing was 600 V; this gave a load of approx. 1 W. After focusing for 66 h the contents of the column were pumped out through the bottom. Fractions (2.0 ml) were collected. Each fraction was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation), centrifuged, redissolved in 0.01 M-acetic acid–ammonium acetate buffer, pH 5.0, dialysed against 0.01 M-acetic acid–ammonium acetate buffer at 4°C for 6 days, freeze-dried and redissolved in 0.1 M-acetic acid–NaOH buffer, pH 4.8 (5.0 ml). Fractions were assayed as detailed in the Experimental section. CM-cellulase activity (reducing sugar) is shown as $\cdots$, concn. of protein as — and the pH of the fractions as $- - -$.

trace of CM-cellulase activity (reducing sugar) could be found: both were isoelectric at pH 3.95.

Protein recovery was 89%.

Reconstitution experiments for cellulase (solubilization) activity done as described above indicated that 90% of the cellulase activity of the 20–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction was recovered.

Although only one protein peak was observed (Fig. 3), the leading edge was always characterized by a slight shoulder. For this reason an ampholyte solution covering only 0.5 pH unit was prepared.

Preparation of narrow-pH-range ampholyte solution

A density gradient was prepared in the usual way (see the Experimental section), but the concentration of the ampholytes (pH 3–5) was increased to 8%. No enzyme was added at this stage. During electrofocusing the load was kept below 3 W. After 24 h a white precipitate was formed at the bottom of the column at the interface of the dense electrode solution (anode) and the ampholyte solution. At the end of the run (142 h) the voltage was steady at 460 V and the current at 6 mA. To avoid mixing the white precipitate with the ampholyte pH gradient, the contents of the column were removed through the top of the

column by way of a capillary tube and a pump, the capillary tube being kept just below the surface of the liquid during the operation. Despite this departure from the normal emptying procedure, the pH gradient that was formed was subsequently found to be remarkably linear. Fractions covering the pH range 3.72–4.25 were combined to give an approx. 8% ampholyte–sucrose solution.

Isoelectric focusing of the C_1 component in a pH gradient covering only 0.5 pH unit

The approx. 8% ampholyte–sucrose solution covering the pH range 3.72–4.25 was used to prepare the column for a normal electrofocusing run (see the Experimental section): the final ampholyte concentration in the column was approx. 1.5%. The sucrose concentration in the pre-run was not taken into account when making up the new density gradient. Enzyme (C_1 from Fig. 1) was prepared for electrofocusing as detailed in the legend to Fig. 3 and dissolved in narrow-range ampholyte solution (10 ml) prepared as described above.

Fig. 4 shows that the minor protein component that had appeared as a shoulder to the main peak when C_1 component was electrofocused in a pH

Fig. 4. Isoelectric focusing of the C₁ component in ampholyte pH 3.72–4.25

The narrow-pH-range ampholyte was prepared from an ampholyte mixture covering the range pH 3–5 as described in the text. The partially purified C₁ component (20 mg), prepared for isoelectric focusing as detailed in the legend to Fig. 3, was dissolved in the prepared ampholyte solution covering the pH range 3.72–4.25 and was added to the column as described in the Experimental section. The voltage at the end of the run (90.5 h) was 720 V and the current 3 mA. The column was emptied by pumping water into the top of the column at a rate of 120 ml/h. Fractions (2.4 ml) were worked up as before (see legend to Fig. 3) and assayed for protein (—) by the method of Lowry *et al.* (1951), and for CM-cellulase activity (reducing sugar) (---). The pH of the fractions is shown as o.

gradient covering the range pH 3–6 (Fig. 3) was now clearly separated in ampholyte pH range 3.72–4.25. The minor peak accounted for 24% of the protein recovered from the column. Another shoulder had also appeared on the main peak (fraction 29).

Both minor (isoelectric pH 3.8) and major (isoelectric pH 3.95) protein peaks were still associated with traces of CM-cellulase activity (reducing sugar) and both were equally effective in solubilizing cotton fibres when acting in synergism with C_x component and cellobiase (Table 1). When acting in isolation the minor protein component solubilized cotton to the extent of 2%, whereas the major protein component produced 4% solubilization.

The minor and major protein components were found to be associated with 33 and 9% of carbohydrate respectively [carbohydrate was determined as glucose equivalent by the phenol-H₂SO₄ method and protein was determined by the method of Lowry *et al.* (1951); see the Experimental section].

Activity of the C₁ component towards other glucans

The specificity of the C₁ component (Fig. 3) was studied by incubating polysaccharide (0.5 mg), enzyme (50 µg), NaN₃ (1 µg) and 0.01 M-acetic acid-NaOH buffer, pH 5.0 (0.05 ml), for 18 h at 37°C. A sample (10 µl) was spotted on paper chromatograms: these were subsequently run in solvent A (see the

Experimental section) and sprayed with AgNO₃-NaOH solution. No degradation products were found when the following glucans were tested as substrates: β-1,2-glucan, β-1,6-glucan (lutean), β-1,3-glucan (laminarin), mixed α-1,4- and α-1,3-glucan (nigeran), mixed α-1,4- and α-1,6-glucan (glycogen and amylopectin), α-1,6-glucan (dextran) and α-1,4-glucan (amylose). Lichenin was slightly degraded; chromatography of the products of hydrolysis showed a spot with the same mobility as cellobiose in solvent A and a trace of glucose.

C₁ activity towards cello-oligosaccharides

Activity towards cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose was studied by using the same amount of enzyme, buffer etc. as used for the glucans (see above). The reaction mixtures were incubated at 37°C for 2 h and 18 h. Both C₁ components that had been separated by isoelectric focusing (Fig. 4) were used. Chromatographic examination of the products [on paper (solvent A) or t.l.c. plates (solvent B)] showed that whereas cellobiose was not attacked, cellotriose was broken down very slowly to produce cellobiose and glucose. Cellotetraose and cellohexaose were rapidly hydrolysed and yielded mainly cellobiose; traces of cellotriose and glucose were also present, but these were

only apparent on heavily spotted chromatograms. Cellopentase yielded cellobiose and cellotriose with only traces of glucose.

Table 1. *Cellulase activity of the C₁ components separated by isoelectric focusing when recombined with C_x and cellobiase*

The isoelectric focusing of the C₁ component is described in Fig. 4. The recovery of C₁ activity in the two protein peaks shown in Fig. 4 (i.e. fractions 18–24 and 27–33) was determined by recombining each of these pooled fractions with the C_x-cellobiase component (separated from C₁ component by chromatography on DEAE-Sephadex; see the text) and then measuring the extent to which 2mg of cotton fibre was solubilized in 7 days (see the Experimental section for standard cellulase assay). In these reconstitution experiments all components were recombined in the same proportions in which they were present in 1 ml of 20–80%-satd.-(NH₄)₂SO₄ fraction, diluted 50-fold (see the text for details).

	Amount of C ₁ protein recombined with C _x -cellobiase component (μg)	Solubiliza- tion (%)
C ₁ component		
Fractions 18–24	137*	59
Fractions 18–24	43	9
Fractions 27–33	137	60
Fractions 18–24 + fractions 27–33	43 + 137 } 180	59
C ₁ component (Fig. 1)	180	66

* The correct proportion of C₁ component in fractions 18–24 is 43 μg; 137 μg was added to compare the cellulase activity of that part of the C₁ component in fractions 18–24 with that part in fractions 27–33.

C₁ activity towards cellulose substrates

Although C₁ component showed little capacity for solubilizing Avicel, Whatman cellulose powder or dewaxed cotton (Table 2), cellulose that had been previously swollen in phosphoric acid was readily hydrolysed. When the supernatant from the digestion of phosphoric acid-swollen cellulose was deionized [Amberlite resins IR-45 (OH⁻) and IR-120 (H⁺)] and examined on paper chromatograms run in solvent A, only cellobiose was detected in any quantity; the small amount of glucose that was also present was shown to account for only 3% of the total carbohydrate present (Table 2).

Dewaxed cotton was attacked only slowly in an assay mixture containing the same amount of C₁ component as was present in 1 ml of unfractionated cell-free filtrate. When the period of incubation was extended to 28 days, solubilization was increased from 4 to 7%. By using double and treble the original concentration of enzyme, the respective degrees of solubilization were increased to 9 and 15% in 28 days. Paper chromatography (solvent A) of the supernatant isolated from the assay showing the highest degree of solubilization (i.e. 15%, with a fourfold concentration of C₁ component for 28 days) again showed cellobiose to be the main product of C₁ action; as with phosphoric acid-swollen cellulose only small amounts of glucose were present. Table 3 shows in fact that the glucose represented only 4% of the total carbohydrate present in the supernatant.

Both the C₁ components that had been separated by isoelectric focusing in narrow-pH-range ampholyte solution (Fig. 4) readily hydrolysed phosphoric acid-swollen cellulose; in each case the products of the hydrolysis were cellobiose and a trace of glucose.

The products of hydrolysis of phosphoric acid-swollen cellulose were cellobiose and a trace of glucose regardless of the extent of the degradation of the substrate.

Discussion

C_x activity is normally determined by measuring either the decrease in viscosity or the increase in

Table 2. *Degree of solubilization of miscellaneous substrates exposed to the C₁ component for various times*

The assay is described in the Experimental section. The C₁ component used was that isolated after electrofocusing (Fig. 3): a sample containing 180 μg of C₁ protein was added to each assay mixture (see the text for explanation of the amount used).

Time (h)	Solubilization of phosphoric acid-swollen cellulose (%)	Solubilization of Avicel (%)	Solubilization of Whatman cellulose (%)	Solubilization of dewaxed cotton (%)
2	19	Nil	Nil	Nil
19	52	2	1	1
44	72	5	1	2
168	86	7	2	4

Table 3. *Digestion of dewaxed and phosphoric acid-swollen cellulose with C₁ component*

The phosphoric acid-swollen cellulose was prepared as described previously (Wood, 1971) and was digested with C₁ enzyme (180 µg; Fig. 3) for 3 days in the assay described in the Experimental section. The dewaxed cotton was digested with the C₁ component for 28 days with a threefold concentration of enzyme (540 µg; Fig. 3) and the residual cellulose was measured by the dichromate-H₂SO₄ method as described previously (Wood, 1969).

Substrate	Total carbohydrate by phenol-H ₂ SO ₄ method (glucose equivalent, µg)	Glucose by glucose oxidase (µg)	Solubilization (%)
Phosphoric acid-swollen cellulose	1220	40	87
Dewaxed cotton	320	12	15

reducing power of a solution of CM-cellulose. In actual fact these two methods of assay cannot always be regarded as alternatives. On the one hand the reducing-sugar method would favour the detection of a C_x type of enzyme that would attack the CM-cellulose from the chain ends, whereas on the other hand, the viscometric method would favour the detection of a C_x type of enzyme attacking the long CM-cellulose chains at random. In this regard it is noteworthy that both endoglucanase and exoglucanase activities have been shown to exist in *Trichoderma* cellulase preparations (Li *et al.*, 1965; Okada *et al.*, 1968).

In the main, individual reports in the literature about the separation of the C₁ and C_x components present in the culture filtrates of certain cellulolytic fungi have involved only one of these methods of assaying C_x activity (Selby & Maitland, 1967; Eriksson, 1969). By using both methods of assay we have shown that the C₁ component of *T. koningii*, which was earlier reported to contain a trace of CM-cellulase activity (reducing sugar) after displacement from DEAE-Sephadex with a salt gradient (Wood, 1968), in fact also contained CM-cellulase activity (viscometric). However, further chromatography of the purified C₁ component on DEAE-Sephadex, with a pH gradient, has now resulted in the separation of another small peak of C_x activity. This peak contained (under the assay conditions used) all of the CM-cellulase activity (viscometric) as well as part of the CM-cellulase activity (reducing sugar) of the C₁ component that was put on the column. No evidence for the non-identity of the C₁ component and the residual CM-cellulase activity (reducing sugar) could be found by further chromatography on DEAE-Sephadex (under a variety of conditions), by recycling chromatography on Sephadex G-75 or by isoelectric focusing in a stabilized gradient covering the pH range 3-6. Further, although isoelectric focusing in an ampholyte solution covering only 0.5 pH unit resolved C₁ enzyme into a major and a minor component, both of these still possessed the capacity of producing reducing sugars from CM-

cellulose, albeit to a very limited extent. This latter evidence is particularly convincing and there now seems little doubt that C₁ activity and the trace of CM-cellulase activity (reducing sugar) originate in the same enzyme protein.

Both major and minor components isolated by electrofocusing were the C₁ type of enzymes. This was clearly established when it was observed that either component acted synergistically with the C_x-cellobiase component to the same extent in the solubilization of cotton. In fact these components appeared to be isoenzymes, for no function of one component was observed that was not equally a function of the other. The components did differ, however, in the extent of their association with carbohydrate (9 and 33%), and this was, in all probability, the cause of the apparent heterogeneity observed on electrofocusing. The nature of the association of the protein and the carbohydrate is not yet known, but it is noteworthy that protein-carbohydrate complexes have already been shown to be responsible for the existence of multiple components in other 'cellulase' (C_x) systems (Jermyn, 1955; Eriksson & Pettersson, 1968).

The purified C₁ component of *T. koningii* showed minimal activity towards highly ordered substrates, such as dewaxed cotton fibre, Whatman cellulose powder or Avicel, when assayed over 7 days. However, in the same period, a highly amorphous substrate, phosphoric acid-swollen cellulose, was extensively degraded; the hydrolysate consisted of 97% cellobiose and 3% glucose. Cellobiose was also the primary product (96%) in the hydrolysate of dewaxed cotton that had been 15% degraded by exposure to a high concentration of C₁ component for 28 days. No traces of higher cello-oligosaccharides were detected in either case.

These findings, when considered together with the observation that cellobiose was produced almost exclusively when cellotetraose and cellohexaose were exposed to C₁ component, are clearly compatible with the interpretation that C₁ enzyme is a β-1,4-glucan cellobiosylhydrolase.

C₁ enzyme can therefore be regarded as a specific

type of C_x component that is unable to attack CM-cellulose or highly ordered substrates to any significant extent, but which is still capable of degrading more accessible forms of cellulose by removing successive units of cellobiose from the chain ends. Highly ordered ('crystalline') cellulose substrates would have relatively few chain ends accessible to the C_1 enzyme, whereas highly hydrated swollen celluloses (with relatively low degrees of polymerization) would have many. Presumably the chain ends on CM-cellulose are too highly substituted for enzyme binding to occur: C_1 action would therefore be effectively blocked once the unsubstituted cellobiose residues were removed.

The observation that cellobiose is virtually the sole product of the hydrolysis of a cellulose substrate by a C_1 -type enzyme has also been reported by Li *et al.* (1965). However, the 'hydrocellulase' that was isolated from a *T. viride* cellulase preparation differed from the C_1 component of *T. koningii* in still retaining the capacity for producing extensive hydrolysis of 'crystalline' cellulose. In one other report (Gilligan & Reese, 1954), the C_1 -type component that was isolated (from another *T. viride* cellulase preparation) by chromatography on a calcium phosphate-gel column resembled *T. koningii* C_1 component in several respects: it displayed very little CM-cellulase activity, but hydrolysed phosphoric acid-swollen cellulose to cellobiose. The ability to solubilize cotton was not tested.

Highly ordered cellulose substrates are converted rapidly into soluble sugars only when C_1 and C_x enzymes are present in the solution simultaneously (Selby & Maitland, 1967; Wood, 1968). We consider, therefore, that the part played by C_1 component in the degradation of these substrates will be concerned with the hydrolysis of the new chain ends that will be formed by the randomly acting endoglucanases. The function of the endoglucanases (C_x) will be to increase the effective concentration of accessible end groups for the endwise-acting cellobiohydrolase. C_x component, and not C_1 component, as originally suggested by Reese *et al.* (1950), can therefore be assigned the role of initiating the attack on native cellulose.

This mechanism, although supplying one possible explanation for the synergism displayed when C_1 and C_x enzymes are present in admixture, still does not explain why the C_1 and C_x enzymes are individually both capable of degrading swollen cellulose, but not highly ordered forms of cellulose, yet when acting in concert can solubilize highly ordered forms of cellulose with comparative ease. The precise mechanism of synergistic action between the C_1 and C_x components at the site of attack still requires elucidation.

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