The Cellulase of *Trichoderma koningii*

PURIFICATION AND PROPERTIES OF SOME ENDOGLUCANASE COMPONENTS WITH SPECIAL REFERENCE TO THEIR ACTION ON CELLULOSE WHEN ACTING ALONE AND IN SYNERGISM WITH THE CELLOBIOHYDROLASE

By THOMAS M. WOOD and SHEILA I. McCRAE
Rowett Research Institute, Bucksburn, Aberdeen AB29SB, Scotland, U.K.

(Received 3 August 1977)

1. Four principal endoglucanase components of *Trichoderma koningii* cellulase were separated and purified by gel filtration on Sephadex G-75, ion-exchange chromatography on DEAE- and sulphoethyl-Sephadex and isoelectric focusing. 2. All four endoglucanases hydrolysed CM-cellulose, H₃PO₄-swollen cellulose, cellotetraose and cellopentaose, but differed in the rate and mode of attack. 3. Attack on cotton fibre by the endoglucanases was minimal, but resulted in changes that were manifested by an increased capacity for the uptake of alkali, and a decrease in tensile strength. 4. All four endoglucanases acted synergistically with the exoglucanase [cellobiohydrolase; Wood & McCrae (1972) *Biochem. J.* 128, 1183–1192] of *T. koningii* during the early stages of the breakdown of cotton fibre, but only two could produce extensive solubilization of cotton cellulose when acting in admixture with the exoglucanase component. 5. The mode of action of the enzymes is discussed in relation to these synergistic effects. It is suggested that the results are compatible with the interpretation that the 'crystalline' areas of cotton cellulose are hydrolysed only by those endoglucanases capable of forming an enzyme–enzyme complex with the cellobiohydrolase on the surface of the cellulose chains.

Culture filtrates of the fungi *Trichoderma viride* (Mandels & Reese, 1964; Ogawa & Toyama, 1965; Okada *et al.*, 1968; Selby & Maitland, 1967), *Trichoderma koningii* (Toyama, 1958; Halliwell, 1965; Wood, 1968), *Fusarium solani* (Wood & Phillips, 1969; Wood, 1969), *Penicillium funiculosum* (Selby, 1968; Wood & McCrae, 1977b) and *Sporotrichum pulverulentum* (Eriksson & Rzedowski, 1969) are the best known sources of enzymes that are capable of the extensive solubilization of highly ordered forms of cellulose. Although it is now well established that the cellulases found in such culture filtrates are multi-enzyme systems, the actual mode of action of the enzymes, as well as the composition of the system, is still the subject of debate.

There is now some agreement that the system contains enzymes with endo- β -1,4-glucanase, exo- β -1,4-glucanase and β -glucosidase (or cellobiose) activities, and that only mixtures of enzyme fractions containing endo- and certain exo-glucanase activities (Wood & McCrae, 1972, 1977a,b; Halliwell & Griffin, 1973; Streamer *et al.*, 1975) can accomplish the extensive hydrolysis of highly ordered forms of cellulose. There is, however, disagreement as to whether the activity towards native cellulose can be

accounted for simply in terms of these hydrolytic enzymes, for it has been postulated by Reese and his co-workers (Reese *et al.*, 1950) that the cellulase system must contain an additional factor (so-called C₁) that carries out a localized loosening of the cellulose chains as a preliminary to hydrolysis by the hydrolytic enzymes (so-called C₂). There is as yet no evidence to support the existence of such a non-hydrolytic chain-disaggregating enzyme, and this hypothesis must give way, at least for the time being, to the more plausible argument that the hydrolysis of native cellulose is the result of the synergistic action of endo- and exo-glucanase enzymes.

In the *T. koningii* cellulase system, the exoglucanase that solubilizes highly ordered cellulose when acting in concert with the endoglucanase components is a cellobiohydrolase (Wood & McCrae, 1972; Halliwell & Griffin, 1973). To account for the synergistic activity manifested by this enzyme and the endoglucanases, we have suggested that the process of solubilization is initiated by the endoglucanases, and that the new chain ends generated by the endoglucanases are subsequently attacked by the cellobiohydrolase (Wood & McCrae, 1972). This conclusion was based on experiments with a highly

purified cellobiohydrolase and an enzyme fraction known to contain several endoglucanases. Three components have now been separated from the endoglucanase fraction, and these, along with the low-molecular-weight endoglucanase component previously isolated (Wood, 1968), account for most of the endoglucanase activity found in *T. koningii* cellulase. We now report on the properties of all four endoglucanases, and, in particular, their capacities for solubilizing highly ordered cellulose when acting in concert with the cellobiohydrolase component. The discovery that the cellobiohydrolase can produce extensive hydrolysis of cotton cellulose only when acting in concert with two of the four endoglucanases shows that our previous hypothesis is an oversimplification. We therefore suggest that these results are compatible with the interpretation that the cellobiohydrolase can act synergistically only with those endoglucanases with which it can form an enzyme-enzyme complex on the surface of the cellulose chain.

Part of this work has been presented in preliminary form (Wood & McCrae, 1975, 1977b).

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 ICI, Nobel Division, Stevenston, Ayrshire, Scotland, U.K. Texas cotton fibre was purchased from the Shirley Institute, Didsbury, Manchester, U.K. Ampholine electrofocusing equipment and the carrier ampholytes were supplied by LKB Instruments, Croydon, Surrey, U.K. Cotton yarn was kindly given by J. and P. Coates, Paisley, Renfrewshire, Scotland, U.K. Cytochrome *c* (type II), α -chymotrypsinogen (type II) and thyroglobulin (type II) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., bovine serum albumin was from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and bovine γ -globulin from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Ovalbumin was a gift from Dr. J. Conchie, Rowett Research Institute. β -D-Glucopyranosylamine (m.p. 125°C) was prepared by the method of Isbell & Frush (1958). Collodion bags (Sartorius-Membranfilter G.m.b.H., Göttingen, Germany) were purchased from V. A. Howe and Co., London SW6 3EP, U.K., and methylcellulose was from BDH Chemicals, Poole, Dorset, U.K. Avicel was from Honeywell and Stein, London W.1, U.K. SE-Sephadex C-50 (sulphoethyl-Sephadex) was purchased from Pharmacia (G.B.), London W5 5SS, U.K.

Preparative 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).

Preparation of H_3PO_4 -swollen cellulose. Avicel was swollen by the method detailed elsewhere (Wood, 1971). Dewaxed cotton fibre (Corbett, 1963) was swollen in a similar manner except that the swelling period was extended to 4 h and the treatment in the Waring Blendor was only 30 s. This substrate was used to study the effect of the various enzymes on the degree of polymerization of cellulose.

Preparation of Cadoxen solution. A mixture of ethylenediamine (99% pure; BDH) (280 g), water (720 ml) and cadmium oxide (100 g) was stirred at room temperature (20°C) for 3 h, at 4°C for 18 h, and then centrifuged (2600g for 30 min). The clear solution was kept at 4°C.

Preparation of cello-oligosaccharides. Cello-oligosaccharides were prepared by the acetolysis of dewaxed cotton fibres, and separated by gradient elution (0–35% ethanol) from a column (90 cm $\times$ 3.5 cm) of charcoal (BDH; acid-washed)/Celite (1:1, w/w).

Isoelectric focusing. The separations were performed in an LKB isoelectric-focusing column (100 ml) by the method described by Wood & McCrae (1972).

After focusing at 5°C, the column was emptied by pumping water (120 ml/h) into the top of the column. The pH of each fraction was measured at 5°C by using a combination electrode fitted to a Corning EEL pH-meter.

Analytical methods

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 by the Somogyi-Nelson method (Nelson, 1952) or by the method of Park & Johnson (1949) modified to include $FeNH_4(SO_4)_2$ solution made up in 0.075 M- H_2SO_4 instead of 0.025 M- H_2SO_4 , and expressed as glucose equivalent.

Determination of glucose. Glucose was determined by the glucose oxidase method of Lloyd & Whelan (1969).

Determination of total carbohydrate. Total carbohydrate was measured by the phenol/ H_2SO_4 method of Dubois *et al.* (1956).

Activity towards CM-cellulose. CM-cellulose activity was measured either viscometrically (Wood & McCrae, 1972) or by the reducing-sugar method (Wood & McCrae, 1972) and is expressed as CM-cellulase (viscometric) or CM-cellulase (reducing sugar) in the Results section.

Activity towards H_3PO_4 -swollen cellulose. Assays for this activity consisted of 2 ml of a 0.1% suspension of H_3PO_4 -swollen Avicel, 2.5 ml of 0.2M-acetic acid/NaOH buffer, pH 4.8, which was 0.02% with respect to NaN_3 , enzyme solution and water to give a total volume of 5 ml. After incubation at 37°C for 18 h, the residual cellulose was determined by the dichromate/ H_2SO_4 method (Wood, 1969).

Cellulase (cotton-solubilization) activity. Cellulase activity was determined by measuring either (a) the residual cellulose left after 7 days' incubation of 2 mg of cotton (Wood, 1969) or (b) the soluble sugars released in 18 h from 20 mg of cotton.

In the 18 h assay, 20 mg of cotton was incubated at 37°C with 0.2 ml of 1M-acetic acid/NaOH buffer, pH 4.8, 0.04 ml of 0.05M- NaN_3 , and enzyme and water to give a total volume of 2.0 ml. After centri-

fuging (200g/10 min), the soluble carbohydrate in the clear supernatant was assayed by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956).

Effect of enzymes on degree of polymerization of cellulose. The assay contained 10 ml of a suspension of H_3PO_4 -swollen cellulose (containing 4 mg of cellulose/ml), 1.0 ml of 1M-acetic acid/NaOH buffer, pH 4.8, 0.1 ml of 0.05M- NaN_3 , and enzyme in a volume of 5–20 μ l. The mixture was incubated at 37°C, filtered on a porosity-3 sintered-glass crucible, washed several times with the above buffer, several times with water, and freeze-dried. A sample (approx. 25 mg) was moistened with water (0.5 ml) and dissolved with stirring in Cadoxen solution (10 ml). The clear solution was diluted with water (9.5 ml) and the viscosity determined with a Cannon-Ubbelohde viscometer (constant 0.008) at 25°C. The

Scheme 1. Fractionation of the cellulase complex

For further details see Wood (1968) and Wood & McCrae (1972, 1975). Abbreviation: SE-Sephadex, sulphoethyl-Sephadex.

degree of polymerization of the cellulose was calculated from the data by the method of Henley (1961).

Effect of enzymes on tensile strength of cotton yarn. The assay contained a 52 cm length (approx. 65 mg) of cotton yarn [Menoufi, Egypt; treated consecutively at room temperature with Lissapol ND (2 g/litre), 0.02 M-HCl and 9 mM- Na_2HPO_4], 4.0 ml of 0.2 M-acetic acid/NaOH buffer, pH 4.8, 0.1 ml of 0.05 M- NaN_3 solution, and enzyme and water to give a total volume of 5 ml. The mixture was incubated at 37°C for the required time, and the tensile strength of the yarn measured (without drying) on a laboratory-constructed tensile tester similar to that used by Evans *et al.* (1974), but modified to include a spring balance in place of a load scale.

Swelling-factor (S-factor) activity. This was assayed as previously described (Wood, 1968).

Enzyme activity on cello-oligosaccharides. Equimolar amounts of purified cello-oligosaccharides in 0.1 M-acetic acid/NaOH buffer, pH 5.0, were incubated with enzyme at 37°C for 4 h and the change in the reducing power was assayed by the method of Park & Johnson (1949), modified as described above.

Paper chromatography. Paper chromatography was carried out on Whatman no. 1 filter paper in ethyl acetate/pyridine/water (10:4:3, by vol.). Chromatograms were sprayed, after air-drying, with AgNO_3 (Trevelyan *et al.*, 1950).

Thin-layer chromatography. Plates (20 cm $\times$ 20 cm) were coated with a layer (0.25 mm thick) of Kieselgel G. Chromatograms were developed (two ascents) in ethyl acetate/propan-2-ol/water (18:13:9, by vol.), dried at room temperature, and sprayed with anisaldehyde/ H_2SO_4 (Stahl & Kaltenbach, 1961).

Separation of products of hydrolysis on Bio-Gel P-2. The supernatants from the enzymic digests were deionized on columns (1.5 cm $\times$ 30 cm) of Amberlite resins IR-120 (H^+) and IR-45 (OH^-) and evaporated to dryness. The residue was dissolved in the minimum amount of water, and applied to a column (0.95 cm $\times$ 142.5 cm) of Bio-Gel P-2. The glucose and low-molecular-weight cello-oligosaccharides were eluted with water and measured by the phenol/ H_2SO_4 method of Dubois *et al.* (1956).

Fractionation of the cellulase complex. A concentrated (50-fold) partially purified [20–80% satd.-(NH_4) $_2\text{SO}_4$ fraction] cell-free culture filtrate was fractionated as shown in Scheme 1 into a cellobiohydrolase component, a low-molecular-weight endoglucanase component (E_1), a fraction containing higher-molecular-weight endoglucanase components (E_3 , E_4) and a fraction containing β -glucosidase plus a trace of CM-cellulase activity (E_5) (Wood, 1968; Wood & McCrae, 1972, 1975).

The cellobiohydrolase was further purified by repeated chromatography on columns of DEAE-

Sephadex equilibrated with buffers of different ionic strength (Wood & McCrae, 1972).

None of the separated components could solubilize cotton fibre to any significant extent when acting alone, but a reconstituted mixture containing the cellobiohydrolase, the β -glucosidase and the endoglucanase components E_3 and E_4 , all in their original proportions, showed the same cellulase (cotton-solubilizing) activity as the original unfractionated starting material [20–80% satd.-(NH_4) $_2\text{SO}_4$ fraction]. As shown previously (Wood, 1968), the removal of the low-molecular-weight component (E_1) did not affect the kinetics of solubilization of the rest of the cellulase complex.

A 1 ml portion of the 20–80% satd.-(NH_4) $_2\text{SO}_4$ fraction, diluted 50-fold, solubilized cotton to the extent of 71% in 7 days (see above for details of assay).

Results

Separation and purification of components

Purification of the low-molecular-weight endoglucanase component (E_1). All traces of high-molecular-weight material were removed from the low-molecular-weight endoglucanase component (E_1 ; Scheme 1) by rechromatography on a column (95.0 cm $\times$ 2.5 cm) of Sephadex G-75 equilibrated with 0.01 M-ammonium acetate/acetic acid buffer, pH 5.0. Fractions containing the low-molecular-weight CM-cellulase component were pooled, freeze-dried, re-dissolved in 2 ml of 0.05 M-acetic acid/NaOH buffer, pH 5.6, and added to a column (1.5 cm $\times$ 28 cm) of DEAE-Sephadex equilibrated with the same acetic acid/NaOH buffer. The low-molecular-weight CM-cellulase component appeared in the eluate as a single peak when the column was eluted under the starting conditions. Fractions containing CM-cellulase activity were pooled, concentrated (5 ml) in a collodion tube and dialysed against 0.01 M-ammonium acetate. The dialysis residue was freeze-dried, redissolved in a mixture of sucrose and ampholyte solution (pH 3–5), and subjected to isoelectric focusing. The low-molecular-weight endoglucanase component (E_1) focused as one peak at pH 4.73.

Isoelectric focusing of endoglucanase fraction E_3 , E_4 (Scheme 1). The higher-molecular-weight endoglucanase fraction (E_3 , E_4), which had been separated from the β -glucosidase activity on SE-Sephadex (Scheme 1), was prepared for electrofocusing as detailed in the legend to Fig. 1. Fig. 1 shows a typical elution pattern after electrofocusing for 40 h in a pH gradient over the pH range 4–6. Components E_3 (fractions 15–20) and E_4 (fractions 27–35) had pI 4.32 and 5.09 respectively. Recovery of CM-cellulase activity was 91%, and components E_3 and E_4 were present in the ratio of 11:9.

Fig. 1. Isoelectric focusing of endoglucanase fraction E_3, E_4 (Scheme 1)

The high-molecular-weight endoglucanase fraction (E_3, E_4), separated from the β -glucosidase activity on SE-Sephadex (Scheme 1), was concentrated in an Amicon cell by using a PM-10 membrane, and mixed with ampholyte solution (LKB) of pH range 4–6. A sample containing approx. 28000 units of CM-cellulase (Wood & McCrae, 1972) and 9 mg of protein was added to an isoelectric-focusing column of 110 ml capacity. The voltage at the end of the run (40 h) was 700 V, and the current 1 mA. Fractions (2 ml) were assayed for CM-cellulase activity (reducing sugar) (●) and protein (Δ). The pH of the fractions is shown as (○). Combined fractions (15–20) and (27–35) are designated E_3 and E_4 respectively in the text.

Fig. 2. Chromatography of component E_3 on SE-Sephadex

Two lots of fraction E_3 (i.e. tubes 15–20) obtained by isoelectric focusing (Fig. 1) were 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, pH 5.0, and freeze-dried. The freeze-dried material was dissolved in 0.01 M-succinate buffer (succinate/NaOH), pH 4.4 (1.5 ml), and a sample (1.4 ml) applied to a column (70 cm $\times$ 1.6 cm) of SE-Sephadex. The column was eluted under the starting conditions at 9 ml/h. Fractions (2 ml) were assayed for CM-cellulase activity (reducing sugar) (●) and protein (Δ). Assays are described in the text. Component E_{3a} is pooled fractions 16–31 and component E_{3b} is pooled fractions 32–47.

In one or two of the electrofocusing experiments, there was some evidence of heterogeneity of peak I (i.e. fractions 15–20), when another CM-cellulase component was partially separated. However, all attempts to obtain complete resolution of these two CM-cellulase components by isoelectric focusing were unsuccessful.

Chromatography of component E_3 (Fig. 1) on SE-Sephadex. Both CM-cellulase components in fraction E_3 (Fig. 1) could be adsorbed on SE-Sephadex from buffer solutions of pH values below 3.5, provided the ionic strength was no greater than 0.01. Partial resolution was obtained by using a pH gradient covering the pH range 3.5–5.0, but the best separation was obtained on a long column equilibrated with pH 4.5 buffer (Fig. 2). Since both components were isoelectric in the region 4.5–4.6, the separation on SE-Sephadex was probably dependent to a large extent on non-specific differential adsorption, or gel filtration, rather than ion exchange.

Pooled fractions (16–31 and 32–47) from Fig. 2 (now called E_{3a} and E_{3b} respectively) were assayed for

activity towards both CM-cellulose and H_3PO_4 -swollen cellulose. Although component E_{3b} was a relatively minor CM-cellulase component, it was a major component (62%) in terms of the total activity towards H_3PO_4 -swollen cellulose that was eluted from the column.

Molecular weights of the endoglucanase components. The molecular weights of components E_1 , E_{3a} and E_4 were estimated by comparing the elution volumes from a column of Sephadex G-100 (40 cm $\times$ 2.5 cm) with those of proteins of known molecular weight (given in parentheses), by the method used by Whitaker (1963). Cytochrome *c* (12400), chymotrypsinogen (24800), ovalbumin (45000), bovine serum albumin (67000), bovine γ -globulin (160000) and thyroglobulin (670000) were used to prepare the calibration curve.

The molecular weights of the respective components were: E_1 , 13000; E_{3a} , 48000; E_4 , 31000.

The molecular weight of component E_{3b} was not determined, but since a mixture of components E_{3b} and E_{3a} chromatographed as a single component on Sephadex G-75, it is clear that they have very similar molecular weights.

Action of endoglucanases on dewaxed cotton

Synergistic effects with the cellobiohydrolase. The synergistic action of mixtures of the cellobiohydrolase with components E₁, E_{3a}, E_{3b} and E₄ was studied by using both a short-term 18h assay and a long-term 7-day assay. In the short-term 18h assay, where a small amount of soluble sugars is released from a relatively large amount of cotton, the results showed clearly that the cellobiohydrolase acted synergistically with all four endoglucanase components (Table 1). Synergism was highest between a mixture of the cellobiohydrolase and component E₄, although components E₁, E_{3a} and E_{3b} were all reasonably efficient in this respect.

Quite different results were obtained in the long-term solubilization assays, where extensive changes are involved, and where the residual cellulose left after a 7-day exposure to enzyme is measured (Table 2). In this case, little or no synergism was shown by reconstituted mixtures or the cellobiohydrolase with component E_{3b}, or with the low-molecular-weight component E₁.

Inhibition of synergistic action of endoglucanases and cellobiohydrolase. Both glucose and cellobiose had a strong inhibitory effect on the synergistic activity shown by a reconstituted mixture of the cellobiohydrolase and the various endoglucanases (Table 3).

Table 1. Total sugar produced by the components of the cellulase complex acting alone and in combination on dewaxed cotton for 18h

See the Experimental section for details of 18h assay. All assays contained the same amount of that enzyme present in 0.1ml of 20–80%-satd.-(NH₄)₂SO₄ fraction, diluted 50-fold. Cellobiohydrolase is as in Scheme 1; component E₁ (Scheme 1) was purified as detailed in the Results section. For components E_{3a} and E_{3b} see Fig. 2, and for E₄ see Fig. 1.

Enzyme	Total carbohydrate by phenol/H ₂ SO ₄ method (μg of glucose equivalent)
Cellobiohydrolase	44
E ₁	41
E _{3a}	38
E _{3b}	31
E ₄	79
Cellobiohydrolase+E ₁	290
Cellobiohydrolase+E _{3a}	254
Cellobiohydrolase+E _{3b}	282
Cellobiohydrolase+E ₄	329

Table 2. Solubilization of dewaxed cotton by reconstituted mixtures of the components of the cellulase complex in 7 days

See the Experimental section for details of 7-day assay. All components were recombined in the same proportions in which they were present in 1.0ml of the 20–80%-satd.-(NH₄)₂SO₄ fraction, diluted 50-fold. None of the components acting alone solubilized cotton fibre to a greater extent than 2%. Cellobiohydrolase is as in Scheme 1; component E₁ (Fig. 1) was purified as detailed in the Results section. For components E_{3a} and E_{3b} see Fig. 2, and for E₁ see Fig. 1.

Enzyme	Solubilization of cotton fibre (%)
Cellobiohydrolase+E ₁	2
Cellobiohydrolase+E _{3a}	34
Cellobiohydrolase+E _{3b}	2
Cellobiohydrolase+E ₄	51
Cellobiohydrolase+E _{3a} +E ₄	53
Cellobiohydrolase+E ₁ +E _{3a} +E _{3b} +E ₄	53
Cellobiohydrolase+E ₁ +E _{3a} +E _{3b} +E ₄ +β-glucosidase	72
20–80%-satd.-(NH ₄) ₂ SO ₄ fraction (i.e. unfractionated complex)	71

Table 3. Inhibition of cellulase (cotton-solubilization) activity by glucose and cellobiose

The cellulase used was a reconstituted mixture of the cellobiohydrolase and components E_{3a} and E₄ in their original proportions. Cotton fibre was incubated with the enzymes for 7 days and the residual cellulose assayed as detailed in the Results section.

Concn. of cellobiose (mM)	Inhibition (%)	Concn. of glucose (mM)	Inhibition (%)
0.29	2	0.29	9
2.9	43	2.9	17
29.0	79	29.0	45

Effect of endoglucanases on tensile strength of cotton yarn. Tensile-strength measurements were made after 24, 48, 96 and 190h, and the results are shown in Fig. 3. After 96h, by which time the percentage change in wet strength was approximately constant in each case, the respective losses in tensile strength were 44%, 17%, 8% and 21% for components E₁, E_{3a}, E_{3b} and E₄.

The cellobiohydrolase component had little effect on the tensile strength of cotton fibre (Wood, 1975).

Effect of endoglucanase on the uptake of alkali by cotton fibre. One of the more rapid and sensitive measures of activity of cellulolytic enzymes is provided by the alkali-swelling centrifuge test (Marsh *et al.*, 1953). This activity has been called swelling factor or S-factor by Marsh *et al.* (1953), who found that if cotton fibres were immersed first in culture

Fig. 3. Loss in wet strength of cotton fibre as a result of enzyme action

All assays contained 30 units (Wood & McCrae, 1972) of CM-cellulase (reducing sugar) activity. $\circ$, E_1 ; $\bullet$, E_{3a} ; $\blacktriangle$, E_{3b} ; $\triangle$, E_4 . See Scheme 1 for component E_1 , Fig. 2 for components E_{3a} and E_{3b} , and Fig. 1 for E_4 .

Table 4. Swelling-factor activities of the various components, alone and in combination

The assay was carried out as described by Wood (1968). Component E_1 (Scheme 1) was purified by chromatography on DEAE-Sephadex and isoelectric focusing as detailed in the Results section. For components E_{3a} and E_{3b} see Fig. 3, and for E_4 see Fig. 1. Cellobiohydrolase is the component isolated as shown in Scheme 1: acting alone it showed no swelling-factor activity (Wood, 1968). All assays contained the same CM-cellulase activity (reducing sugar).

Enzyme	Increase in swollen weight (mg)
E_1	46
E_{3a}	28
E_{3b}	24
E_4	28
E_1 + Cellobiohydrolase	68
E_{3a} + Cellobiohydrolase	64
E_{3b} + Cellobiohydrolase	54
E_4 + Cellobiohydrolase	57

filtrates from cellulolytic organisms, and then in 18% (w/v) NaOH, there was an increase in the uptake of alkali relative to the control. S-factor activity is not associated with the cellobiohydrolase component (Wood, 1968), and we now report on the swelling-factor activity manifested by the various endoglucanase components.

The results (Table 4) show clearly that all endoglucanase components produced some degree of swelling, and that the low-molecular-weight component E_1 was the most efficient in this regard. The latter result was unexpected, since a similar component previously isolated from the rest of the cellulase complex by gel filtration on Sephadex G-75

Fig. 4. Relationship between fluidity and reducing power during the hydrolysis of CM-cellulose by components E_1 , E_{3a} , E_{3b} and E_4

$\circ$, Component E_1 ; $\bullet$, component E_{3a} ; $\blacktriangle$, component E_{3b} ; $\triangle$, component E_4 . Component E_1 (Scheme 1) was purified by chromatography on DEAE-Sephadex and isoelectric focusing. See Fig. 2 for components E_{3a} and E_{3b} , and Fig. 1 for E_4 .

Table 5. Solubilization of H_3PO_4 -swollen cellulose by the various enzyme components

All assays contained 500 units of CM-cellulase activity (reducing sugar). Component E_1 (Scheme 1) was purified as detailed in the text. For components E_{3a} and E_{3b} see Fig. 2, and for E_4 see Fig. 1.

Enzyme	Solubilization (%)		
	1 h	2 h	4 h
E_1	25	28	34
E_{3a}	15	20	29
E_{3b}	62	65	78
E_4	64	70	83

was not particularly rich in S-factor activity (Wood, 1968). This new result, however, could be reproduced, and it was only after purification on DEAE-Sephadex and gel filtration that the low-molecular-weight component showed its full S-factor potential.

Action of enzymes on H_3PO_4 -swollen cellulose

Solubilization and effect on degree of polymerization. All the endoglucanase components isolated during the various fractionation procedures were capable of hydrolysing H_3PO_4 -swollen cellulose; components E_{3b} and E_4 were the most effective (Table 5).

The low-molecular-weight CM-cellulose (component E_1), which appeared to produce the greatest decrease in viscosity (a parameter related to chain length) of a solution of CM-cellulose per unit increase

Fig. 5. Effect of enzyme components on the degree of polymerization of H_3PO_4 -swollen cellulose

The degree of polymerization was determined by viscometry after dissolution in Cadoxen. $\circ$, E_1 ; $\bullet$, E_{3a} ; $\blacktriangle$, E_{3b} ; $\triangle$, E_4 . All assays contained 30 units (Wood & McCrae, 1972) of CM-cellulase activity (reducing sugar). For component E_1 see Scheme 1. See Fig. 2 for components E_{3a} and E_{3b} , and Fig. 1 for E_4 .

in reducing power (Fig. 4), also produced the most rapid change in degree of polymerization of H_3PO_4 -swollen cellulose; after 4 h incubation, indeed, the average degree of polymerization was decreased from 1990 glucose residues to 160 (Fig. 5).

Of the four endoglucanase components tested, component E_{3b} gave the slowest change in degree of polymerization.

The soluble breakdown products from the hydrolysis of H_3PO_4 -swollen cellulose by the various enzymes are shown in Table 6. Component E_{3b} did not produce detectable amounts of glucose, but glucose was the principal product of the reaction with E_4 . Only traces of higher cello-oligosaccharides were found in the soluble products resulting from the actions of components E_1 , E_{3a} and E_4 ; this contrasts with the considerable quantity of cellotriose found in the reaction products of component E_{3b} .

Inhibition of enzyme action on H_3PO_4 -swollen cellulose by the products of the reaction. Concentrations of glucose up to 29 mM had no effect on the capacity of any of the endoglucanase components for solubilizing H_3PO_4 -swollen cellulose. The activities of the components E_{3a} and E_{3b} and the low-molecular-weight component E_1 were similarly unaffected by concentrations of cellobiose up to 29 mM, but there

Table 6. Soluble sugars produced by the action of the various endoglucanase components on H_3PO_4 -swollen cellulose

The sugars were separated on a column of Bio-Gel P-2 as described in the Experimental section. Component E_1 (Scheme 1) was purified as detailed in the Results section. For components E_{3a} and E_{3b} see Fig. 2, and for E_4 see Fig. 1.

Enzyme	Yield of soluble sugars (glucose equivalent)			
	Glucose	Cellobiose	Cellotriose	Cellotetraose
E_1	1	3	Trace	Nil
E_{3a}	1	4	Trace	Trace
E_{3b}	Nil	6	1	Trace
E_4	3	1	Nil	Trace

was a slight inhibitory effect (12%) on component E_4 with a cellobiose concentration of only 2.9 mM; inhibition was unchanged, however, on increasing the concentration of cellobiose to 29 mM.

Action of endoglucanase components on CM-cellulose

Decrease in viscosity in relation to the increase in reducing power. A dramatic decrease in the viscosity of a solution of CM-cellulose can be caused by the cleavage of the long polymer chains at sites remote from the end of the chain. Clearly, enzymes acting in a random manner will produce a greater decrease in the viscosity of a solution per unit increase in reducing power than will an enzyme attacking from the end of the chain, and this has been used to distinguish 'cellulases' (CM-cellulases) that differ in the pattern of their attack (Gilligan & Reese, 1954).

Fig. 4 shows the slopes of the lines obtained by plotting fluidity against reducing power for the various endoglucanases of *T. koningii* cellulase.

Inhibition of CM-cellulase activity. The results of viscometric tests for CM-cellulase activity show that cellobiose had a marked effect on the CM-cellulase activity of components E_1 , E_{3a} , E_{3b} and E_4 (Table 7). Components E_{3a} , E_{3b} and E_4 were inhibited by all concentrations of cellobiose tested, but the low-molecular-weight component E_1 , in contrast, was stimulated (115%) by concentrations as low as 0.29 mM.

Glucosylamine, which has been shown to be a potent inhibitor of β -glucosidase at low concentrations (Lai & Axelrod, 1973), was an effective inhibitor of the CM-cellulase activity shown by components E_1 , E_{3a} , E_{3b} and E_4 , but only with relatively high concentrations of inhibitor (Table 8); gluconolactone was a less effective inhibitor at similar concentrations.

Methylcellulose was a poor inhibitor of the CM-cellulase activity (16% inhibition at a concentration of 0.1%) in the crude culture filtrate, and also in the separated fractions E_{3a} , E_{3b} and E_4 ; this contrasts

Table 7. *Effect of cellobiose on the CM-cellulase activity of the various enzyme components*

CM-cellulase activity was determined by the viscosity method (see the Experimental section). Component E₁ (Scheme 1) was purified as detailed in the Results section. For components E_{3a} and E_{3b} see Fig. 2, and for E₄ see Fig. 1. + indicates stimulation; – indicates inhibition.

Enzyme	Cellobiose concn. (mM)	Inhibition of CM-cellulase activity (%)		
		... 29	2.9	0.29
E ₁		+310	+190	+115
E _{3a}		–84	–41	–16
E _{3b}		–40	–22	–5
E ₄		–83	–47	–1

Table 8. *Effect of glucosylamine on CM-cellulase activity of the various enzyme components*

CM-cellulase activity was determined by the viscosity method (see the Experimental section). Component E₁ (Scheme 1) was purified as detailed in the Results section. For components E_{3a} and E_{3b} see Fig. 2, and for E₄ see Fig. 1. + indicates stimulation; – indicates inhibition.

Enzyme	Glucosylamine concn. (mM)	Inhibition of CM-cellulase activity (%)		
		... 100	10	
E ₁		–69	+22	
E _{3a}		–91	–26	
E _{3b}		–81	–15	
E ₄		–93	–31	

with the results reported by Gilligan & Reese (1954) for the individual CM-cellulase components isolated from a *T. viride* culture filtrate.

The CM-cellulase activity of the low-molecular-weight component E₁ was stimulated (20%) by methylcellulose concentrations of 0.1%.

Action of endoglucanases on short-chain cello-oligosaccharides

Cellobiose was not attacked by any of the enzyme components, and attack on cellotriose was relatively slow by components E_{3a}, E_{3b} and E₄; the low-molecular-weight component E₁ did not attack cellotriose. Cellotetraose was a substrate for all the components, but examination of the products of the reactions by t.l.c. showed that there were differences in the relative proportions of the products produced: glucose, cellobiose and cellotriose in the approximate ratios of 1:1:1, 2:1:2, and 3:2:3 were released by components E_{3a}, E_{3b} and E₄ respectively. Under the conditions used, only glucose and cellobiose were found in the products of the action of the low-molecu-

lar-weight component E₁ on cellotetraose, which is remarkable, for no glucose should be produced without a corresponding amount of cellotriose. As we have not found any evidence that component E₁ can synthesize higher-molecular-weight material from various mixtures of glucose and cellotriose, the reason for this anomalous behaviour is not known.

Discussion

Nomenclature

In previous papers on *T. koningii* cellulase we have used the C₁–C_x terminology first advanced to describe the enzymes of the cellulase system by Reese *et al.* (1950). According to them, C_x enzymes hydrolysed β -1,4-bonds in cellulose molecules that had first been 'activated' by the hypothetical non-hydrolytic factor (so-called C₁). The designation C_x covered randomly acting endo- β -1,4-glucanases and endwise-acting exo- β -1,4-glucanases. However, the recognition that the component that we (Wood, 1968; Wood & McCrae, 1972), and others (Selby, 1968; Halliwell & Griffin, 1973), have previously tentatively identified as C₁ (cellulase preparations from which it has been removed showed little capacity for rendering cellulose soluble) is an exo- β -1,4-glucanase that removes successive units of cellobiose from the non-reducing end of the chain clearly introduces an element of confusion. In the present paper we no longer use the C₁–C_x nomenclature, and the enzymes are described in terms of their established activities, namely cellobiohydrolase and endoglucanase. In so doing, however, it is not our intention to imply that the precise mechanism of synergistic action at the site of attack can be discussed purely in these terms.

Specificity and mode of action

By using CM-cellulose, H₃PO₄-swollen cellulose and short-chain cello-oligosaccharides as substrates, we have shown that the culture filtrate of *T. koningii* contains four principal endo- β -1,4-glucanase components (designated E₁, E_{3a}, E_{3b} and E₄). These four endo- β -1,4-glucanases have broad substrate specificities, but are distinct enzymes with different modes of action.

Where the specificity is concerned, all four components have in common the ability to attack, with comparative ease, H₃PO₄-swollen cellulose, CM-cellulose, cellotetraose and cellopentaose. They differ, however, in that their specific activities towards these substrates differ greatly. Cellobiose is not hydrolysed by any of the components, whereas cellotriose is a substrate for all but the low-molecular-weight component (E₁).

With regard to the mode of action, the different relationship between the increase in fluidity of a

solution of CM-cellulose and the concomitant release of reducing sugars is suggestive of differences in the pattern of attack. A similar conclusion can be reached from the different proportions of glucose and short-chain cello-oligosaccharides found in the products of hydrolysis of H_3PO_4 -swollen cellulose by the various components, and from the differences in the rate of change of the average degree of polymerization of H_3PO_4 -swollen cellulose. All of these observations are consistent with the conclusion that the components are endoglucanases.

None of the endoglucanases could solubilize cotton cellulose to any significant extent when acting in isolation, but all could produce changes in the cotton fibre manifested by an increase in the uptake of alkali (S-factor, swelling-factor activity) or a decrease in the tensile strength; of the four endoglucanases tested, the low-molecular-weight component, E_1 , effected the greatest changes of this kind. This, perhaps, is not altogether surprising, for it seems reasonable to expect that an enzyme of this size would be capable of attacking sites in the cotton fibre not accessible to the higher-molecular-weight components, assuming always that all components had similar shapes. However, the possibility cannot be excluded that factors other than accessibility may be involved, for the low-molecular-weight endoglucanase component differed in other respects. In particular, its action on CM-cellulose was strongly stimulated by added cellobiose, whereas enzymes E_{3a} , E_{3b} and E_4 were strongly inhibited.

With respect to its apparent stimulation by added cellobiose and its high S-factor activity (after extensive purification), component E_1 was very similar to an endoglucanase isolated from a *T. viride* cellulase preparation by chromatography on a column of hydroxyapatite (Gilligan & Reese, 1954). The molecular weight of this component was not specified. Endoglucanases similar in molecular weight to component E_1 have been isolated from culture filtrates of *T. viride* (Selby & Maitland, 1967; Berghem *et al.*, 1976), but the effect of these components on cotton fibre, as shown by changes in tensile strength and alkali-swelling, was not reported.

Synergism between endoglucanases and cellobiohydrolase when solubilizing cotton cellulose

Although the substrates CM-cellulose, H_3PO_4 -swollen cellulose and cello-oligosaccharides are useful for classifying enzymes of the cellulase system, it is clear from the results given here and elsewhere that they are of limited value in providing information on the solubilization of the most 'crystalline' portions of native cellulosic substrates. Cotton fibre is an ideal substrate to provide such information, and it has been extensively used, in this respect, ever since Mandels & Reese (1964) used it to demonstrate

the synergism existing between the various components separated from *T. viride* cellulase. Cotton fibre, however, is a heterogeneous substrate in that it contains highly ordered 'crystalline' as well as more 'amorphous' areas, and interpretation of the results of cellulase (cotton-solubilizing) action must take account of this fact.

Enzymic attack on cotton fibre is characterized by an initial high rate of hydrolysis, followed by a reaction that is rather slower (Mandels & Reese, 1964). The rapid digestion in the first phase of the reaction has been shown to correspond to the hydrolysis of the more easily accessible portions of the fibre (Betarbet *et al.*, 1974). Conclusions on the mechanism of cellulase action that are based entirely on changes in the first phase are unlikely to apply to the digestion of the inaccessible poorly hydrated highly hydrogen-bonded regions of the fibre, and this is a factor not always recognized. Indeed, there is a regrettable tendency to extrapolate and make generalizations on the mechanism of cellulase action on the basis of observations involving very small amounts of hydrolysis. In the present context, it is only to be expected that hydrolysis of easily hydrated areas of the fibre by an enzyme system containing both random-acting and endwise-acting enzymes must occur at a higher rate than that shown by enzymes acting independently, for, clearly, attack by the random-acting enzymes will generate new chain ends for the endwise-acting cellobiohydrolase. To illustrate this point we have used both an 18 h and a 7-day assay to test for synergistic action between the cellobiohydrolase component and the various endoglucanases. In the 18 h assay we are obviously measuring attack on a very small portion of the more accessible part of the substrate, whereas in the 7-day assay we are concerned with extensive changes involving both ordered and less ordered areas. In the 18 h assay, a synergistic action between the cellobiohydrolase and each of the endoglucanases was apparent, but in the 7-day assay only reconstituted mixtures of the cellobiohydrolase and component E_{3a} or E_4 showed a significant synergistic response. Clearly, tests involving extensive hydrolysis of cotton cellulose are necessary if the results are to be considered meaningful in terms of attack on the more crystalline regions.

One other factor must be considered in measuring cellulase (cotton-solubilizing) activity, namely the inhibition by the products of the reaction. Cellobiose has been shown to inhibit the action of the cellobiohydrolase from a *T. koningii* culture filtrate on bacterial cellulose (Halliwell & Griffin, 1973) and on H_3PO_4 -swollen cellulose (Wood & McCrae, 1975). It was not surprising, therefore, that cellobiose, which was a major product of cellulase action, had a significant effect on the action of the reconstituted mixture of cellobiohydrolase and endoglucanase

activities on cotton. What was remarkable, however, was the fact that glucose, the other major product of cellulase action, was also a potent inhibitor of the synergism shown by the reconstituted enzyme mixture, for glucose was found to have no effect on the capacities of the individual components for hydrolysing H_3PO_4 -swollen cellulose, or indeed CM-cellulose, even when the concentrations were as high as 1%.

The fact that the cellobiohydrolase can effect extensive solubilization of cotton fibre when acting in combination with only two (E_{3a} and E_4) of the four endoglucanase components is very interesting, especially when all four endoglucanase components have similar substrate specificities when acting independently. It was perhaps particularly surprising that the low-molecular-weight component E_1 showed so little capacity for acting synergistically with the cellobiohydrolase component. Component E_1 , after all, was the 'most random' acting of the four endoglucanases tested (as judged by its action on CM-cellulose and on H_3PO_4 -swollen cellulose), and it showed the highest capacity for producing changes in the cotton fibre leading to an increase in the uptake of alkali (S-factor) or to a decrease in the tensile strength.

Clearly, there are several ways of interpreting the results of the experiments designed to show synergistic action. It is possible, for example, that there is an additional factor, as yet unidentified, that has to be present in reaction mixtures containing endo- and exo-glucanases, if highly ordered substrates are to be solubilized. For *T. koningii* cellulase, this factor would be associated with components E_{3a} and E_4 , but not with components E_1 or E_{3b} . It would, however, have had to be present in minute amounts to have escaped detection as protein during the separation components of E_{3a} and E_4 by the isoelectric-focusing technique, particularly as the separation was so complete, and this must therefore be considered unlikely.

An alternative hypothesis for the mechanism of cellulase action has been put forward by Leatherwood (1969), and must be reconsidered in the context of the present observations. Leatherwood (1969) interpreted his studies on roll-tube cultures of different variants of the rumen bacterium *Ruminococcus albus* to indicate the formation of a single cellulase complex that could degrade cellulose; he discussed his hypothesis in terms of an affinity factor, i.e. C_1 , and a hydrolytic factor, i.e. C_x , forming an active cellulase complex. We would suggest that the formation of a complete cellulase complex in the enzyme system produced by *T. koningii* is a possibility that must be considered. However, with *T. koningii* cellulase, the complex would be formed purely from hydrolytic enzymes, there being no evidence for the involvement of a non-hydrolytic

affinity factor (i.e. C_1) of the type envisaged by Leatherwood (1969).

In essence, then, we consider the breakdown of cellulose by *T. koningii* to involve sequential action, in which the randomly acting endoglucanase initiates the attack and the new chain ends generated are then removed by the endwise-acting cellobiohydrolase (Wood & McCrae, 1972). We now suggest, however, that, where attack is on the more highly crystalline portions of the cellulosic material, the second stage must follow the first instantly to prevent re-formation of the glucosidic linkage between two glucose residues held firmly in position by intra- and inter-molecular hydrogen bonds. In view of the observations recorded in the present paper, it seems reasonable to suggest therefore that the breakdown of these highly ordered areas of the cellulosic substrate can best be accomplished by those enzymes (cellobiohydrolase and endoglucanase E_{3a} , or cellobiohydrolase and endoglucanase E_4) capable of forming a loose complex on the surface of the cellulose chain (Wood & McCrae, 1977b).

References

- Berghem, L. E. R., Pettersson, L. G. & Åxiö-Fredriksson, U.-B. (1976) *Eur. J. Biochem.* **61**, 621–630
- Betrabet, S. M., Khandeparkar, V. G. & Patil, N. B. (1974) *Cellulose Chemistry and Technology* **8**, 339–344
- Corbett, W. M. (1963) *Methods Carbohydr. Chem.* **3**, 3–4
- Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Eriksson, K.-E. & Rzedowski, W. (1969) *Arch. Biochem. Biophys.* **129**, 683–688
- Evans, E. W., Burnett, J. & Bines, J. A. (1974) *Br. J. Nutr.* **31**, 273–284
- Gilligan, W. & Reese, E. T. (1954) *Can. J. Microbiol.* **1**, 90–106
- Halliwell, G. (1965) *Biochem. J.* **95**, 270–281
- Halliwell, G. & Griffin, M. (1973) *Biochem. J.* **135**, 587–594
- Henley, D. (1961) *Ark. Kemi* **18**, 327–391
- Isbell, H. S. & Frush, H. L. (1958) *J. Org. Chem.* **23**, 1309–1319
- Lai, H. L. & Axelrod, B. (1973) *Biochem. Biophys. Res. Commun.* **54**, 463–468
- Leatherwood, J. M. (1969) in *Cellulases and their Applications* (Gould, R. F., ed.), pp. 53–59, American Chemical Society, Washington
- Lloyd, J. B. & Whelan, W. J. (1969) *Anal. Biochem.* **30**, 467–469
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mandels, M. & Reese, E. T. (1964) *Dev. Ind. Microbiol.* **5**, 5–20
- Marsh, C. B., Merola, G. V. & Simpson, M. E. (1953) *Text. Res. J.* **23**, 831–841
- Nelson, N. (1952) *J. Biol. Chem.* **153**, 376–380
- Ogawa, K. & Toyama, N. (1965) *Proc. Cellulases Relat. Enzymes 5th* 85–92
- Okada, G., Nisizawa, K. & Suzuki, H. (1968) *J. Biochem. (Tokyo)* **63**, 591–607

- Park, J. T. & Johnson, M. J. (1949) *J. Biol. Chem.* **181**, 149–151
- Reese, E. T., Siu, R. G. H. & Levinson, H. S. (1950) *J. Bacteriol.* **59**, 485–497
- Selby, K. (1968) *Proc. Int. Biodeterioration Symp.* **1st** 62–78
- Selby, K. & Maitland, C. C. (1967) *Biochem. J.* **104**, 716–724
- Stahl, E. & Kaltenbach, U. (1961) *J. Chromatogr.* **5**, 351–352
- Streamar, M., Eriksson, K.-E. & Pettersson, B. (1975) *Eur. J. Biochem.* **59**, 607–613
- Toyama, N. (1958) *Bull. Fac. Agric. Miyazaki Univ.* **4**, 40–58
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Whitaker, J. R. (1963) *Anal. Chem.* **35**, 1950–1953
- Wood, T. M. (1968) *Biochem. J.* **109**, 217–227
- Wood, T. M. (1969) *Biochem. J.* **115**, 457–464
- Wood, T. M. (1971) *Biochem. J.* **121**, 353–362
- Wood, T. M. (1975) *Biotechnol. Bioeng. Symp.* **5**, 111–137
- Wood, T. M. & McCrae, S. I. (1972) *Biochem. J.* **128**, 1183–1192
- Wood, T. M. & McCrae, S. I. (1975) *Proc. Symp. Enzymatic Hydrolysis Cellulose*, 231–254
- Wood, T. M. & McCrae, S. I. (1977a) *Carbohydr. Res.* **57**, 117–133
- Wood, T. M. & McCrae, S. I. (1977b) *Proc. Symp. Bioconversion Cellulosic Substances Energy, Chem. and Microb. Protein* in the press
- Wood, T. M. & Phillips, D. R. (1969) *Nature (London)* **222**, 986–987