The Cellulase of *Fusarium solani*

PURIFICATION AND SPECIFICITY OF THE β -(1 $\rightarrow$ 4)-GLUCANASE AND THE β -D-GLUCOSIDASE COMPONENTS

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1. Cell-free culture filtrates of the fungus *Fusarium solani* were examined for homogeneity with respect to β -D-glucosidase and C_x activities. 2. *o*-Nitrophenyl β -D-glucoside and cellobiose were both used as substrates for β -D-glucosidase activity. 3. No evidence for the non-identity of nitrophenyl β -D-glucosidase and cellobiose activities could be found, either by heat treatment, gel filtration on Sephadex G-100 or by isoelectric focusing. 4. The β -D-glucosidase component was also a feeble exo- β -glucanase: it had a molecular weight of approx. 400 000. 5. The fall in viscosity of a solution of CM-cellulose, the formation of reducing sugars in a solution of CM-cellulose and the solubilization of phosphoric acid-swollen cellulose (Walseth cellulose), were all used for the measurement of C_x activity. 6. The ratio of the two types of CM-cellulase activity was not changed after gel filtration on Sephadex G-100 or after chromatography on DEAE-Sephadex. 7. Three peaks of C_x activity were obtained after electrofocusing, but all three possessed the same ratio of the two types of CM-cellulase activity as well as the same CM-cellulase/Walseth activity ratio, as the unfractionated enzyme; all three isoenzymes (isoelectric points, 4.75, 4.80–4.85 and 5.15) acted in synergism with a mixture of the C_1 and the β -D-glucosidase components to the same extent in the solubilization of cotton fibre. 8. The molecular weight of the C_x component was approx. 37 000.

Swollen or chemically modified types of cellulose are readily hydrolysed by enzymes classified as C_x (Reese, Siu & Levinson, 1950); those enzymes are β -(1 $\rightarrow$ 4)-glucanases. Attack on highly ordered forms of cellulose, however, requires the presence of an additional enzyme, called C_1 (Reese *et al.* 1950). This C_1 enzyme is absent in the culture filtrates of most cellulose-decomposing fungi, but has been isolated from cell-free filtrates of fungi such as *Trichoderma viride* (Mandels & Reese, 1964; Flora, 1965; Selby & Maitland, 1967; Ogawa & Toyama, 1968), *Trichoderma koningii* (Wood, 1968), *Fusarium solani* (Wood, 1969a) and *Penicillium funiculosum* (Selby, 1968) and has been shown to act in synergism with the C_x and cellobiase components in accomplishing the solubilization of native cellulose.

Culture filtrates that are capable of extensive solubilization of native cotton fibre would appear to be homogeneous with respect to the C_1 component. The position with regard to the C_x enzyme is, however, more complex in that, in a number of cases, a fungal culture filtrate has been shown to contain enzymes with different relative activities towards CM-cellulose and a variety of degraded celluloses

(Gilligan & Reese, 1954; Stone, 1957; Hash & King, 1958). These results have been interpreted as indicating the presence of a multiplicity of C_x enzymes. Many other demonstrations of heterogeneity of the C_x system have been claimed (Hashimoto & Nisizawa, 1963), but in most of these cases no specificity studies were carried out on the components that had been separated by electrophoretic and chromatographic techniques: the products from physical separations do not necessarily exhibit different specificities. In several instances, indeed, the multiple components that have been found have subsequently been shown to be artifacts arising from the formation of dissociable complexes between the enzyme and polysaccharides (Jermyn, 1962; Eriksson & Pettersson, 1968). It has also been suggested that other multiple components are sequential or configurational isomers (Whitaker, 1963a).

The present report is an investigation into the homogeneity of the C_x [β -(1 $\rightarrow$ 4)-glucanase] and β -D-glucosidase systems of *F. solani*: the separation of the C_1 , C_x and β -D-glucosidase components has already been reported (Wood, 1969a).

MATERIALS

F. solani, culture collection no. I.M.I. 95994, 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. Sephadex G-25, Sephadex G-100 and DEAE-Sephadex (A-50) were purchased from Pharmacia (G.B.) Ltd., London W.13, U.K. *o*-Nitrophenyl β -D-glucoside was prepared by the method of Seidman & Link (1950). When recrystallized from ethanol it had m.p. 166°C and $[\alpha]_D^{16} -98^\circ$ (c 1.0 in water). Cellobiose was purchased from BDH (Chemicals) Ltd., Poole, Dorset, U.K. Glucose oxidase (type II) was supplied by Sigma (London) Chemical Co., London S.W.6, U.K., and the peroxidase was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Texas cotton fibre was obtained from the Shirley Institute, Didsbury, Manchester, U.K., and Kieselgel G from Shandon Scientific Co., London N.W.10, U.K. LKB 8100 Ampholine electrofocusing equipment and the carrier ampholytes were purchased from LKB Instruments Ltd., Croydon, Surrey, CR2 87D, U.K. Glucono-1,5-lactone [BDH (Chemicals) Ltd.] when recrystallized from 95% (v/v) ethanol had m.p. 151–153°C and $[\alpha]_D^{20} +67^\circ$ (c 1.0 in water). Cytochrome *c* (type II), α -chymotrypsinogen (type II), thyroglobulin (type II), catalase (C-40) and urease (type IV) were from Sigma Chemical Co., bovine serum albumin from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and bovine γ -globulin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Ovalbumin was gift from Dr G. A. Levvy, Rowett Research Institute, The cello-oligosaccharides were a gift from Dr R. Jeffries of the Shirley Institute.

METHODS

Preparation of *F. solani* cellulase. Cultures and cell-free filtrates were prepared from *F. solani* I.M.I. 95994 by the method previously described (Wood, 1968) for the preparation of *T. koningii* cellulase. Cultures were incubated for 25 days at 27°C.

A concentrated culture filtrate (100-fold) was prepared by dissolution (in 0.1 M-acetic acid–NaOH buffer, pH 5.5) of the fraction precipitated with $(\text{NH}_4)_2\text{SO}_4$ between the limits of 20 and 80% saturation.

Preparation of phosphoric acid-swollen cellulose (Walseth cellulose; Walseth, 1952). Avicel (10 g) was suspended in concentrated phosphoric acid (88%, w/v) and left with occasional stirring for 1 h at 1°C. The mixture was poured into ice-cold water (4 litres) and left for 30 min. The swollen Avicel was washed several times with cold water by decantation. After washing with 1% (w/v) NaHCO_3 solution, the thick suspension of swollen cellulose was dialysed at 1°C against water. After a 60 s treatment in a Waring Blender, water was added to the suspension until 1 ml contained 1 mg of cellulose (determined by the dichromate– H_2SO_4 method as described by Wood, 1969a). The suspension was made 5 mM with respect to NaN_3 and stored at 1°C.

Swollen cellulose prepared from Avicel in this way was in the form of an even suspension. The same batch of swollen cellulose was used throughout the work described

in this paper. Phosphoric acid-swollen cellulose is called 'Walseth cellulose' in the text.

Nitrophenyl β -glucosidase activity. The hydrolysis of 1.25 mM-*o*-nitrophenyl β -glucoside in 0.2 M-acetic acid–NaOH buffer, pH 4.0, was measured as described by Wood (1968). The unit of activity is defined as the amount of enzyme needed to liberate 25 μg of *o*-nitrophenol under the conditions of the assay.

Cellobiase activity. Cellobiase activity was assayed as described by Wood (1969a). The unit of activity is defined as the amount of enzyme required to liberate 20 μg of glucose under the conditions of the assay.

Where appropriate, the fractions shown to exhibit both nitrophenyl β -glucosidase and cellobiase activities are referred to as containing ' β -glucosidase' activity.

C_x activity. C_x activity was measured in three ways: (a) decrease in the viscosity of a CM-cellulose solution (Selby & Maitland, 1965), (b) appearance of reducing sugars in a solution of CM-cellulose (Wood, 1968) and (c) solubilization of Walseth cellulose (see below for details).

The viscometer was prepared from capillary tubing and was shaped like a bulb pipette. It was surrounded by a jacket through which water circulated at 30°C (Selby & Maitland, 1965). The flow time for water between marks above and below the bulb was 25 s.

A series of incubation mixtures was made, each containing 5 ml of a 1% (w/v) solution of CM-cellulose, 2.5 ml of 0.2 M-acetic acid–NaOH buffer, pH 5.4, and enzyme and water to give a total volume of 10 ml. The enzyme was added at 2 min intervals between individual test solutions, and after 30 min at 30°C the viscosity of each was determined by measuring the time required for the passage of the liquid between the two marks on the viscometer. Under these conditions the plot of rate of increase of the reciprocal of the viscosity against the enzyme concentration was linear up to a value of 0.014 min^{-1} (Thomas, 1956): this value was produced by 1 ml of the 20–80%-satd.-

Fig. 1. Effect of enzyme concentration on the solubilization of cellulose. ○, Dewaxed cotton fibre in 7 days by 20–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction; ●, H_3PO_4 -swollen cellulose ('Walseth cellulose') in 1 h by 20–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction; △, Walseth cellulose in 18 h by C_x fraction from DEAE-Sephadex column as described in the text. See the text also for standard methods of assay and for method of determination of residual cellulose.

$(\text{NH}_4)_2\text{SO}_4$ fraction, diluted 5000-fold, and was chosen as the unit of activity. A plot of units of activity against efflux time from the viscometer provided a convenient calibration curve for the rapid determination of the activity of enzyme solutions.

The same CM-cellulose solution was used throughout the experimental work.

Enzyme activity towards H_2PO_4 -swollen cellulose ('Walseth activity') was measured by either a 1 h assay (Wood, 1969b) or an 18 h assay. The 18 h assay differed from the 1 h assay only in that HgCl_2 was not added to stop the enzyme action. The calibration curve of percentage solubilization against enzyme concentration is shown in Fig. 1.

Enzyme fractions containing both C_1 and CM-cellulase activities (see the Results section) produced extensive solubilization of Walseth cellulose in 1 h. Walseth activity in fractions devoid of C_1 activity was measured over 18 h.

Activity towards cotton. The degree of solubilization is referred to as 'cellulase activity' in the Results section. It is defined as the extent to which 2 mg of dewaxed cotton fibre is solubilized when incubated with enzyme for 7 days at 37°C under the conditions of the standard assay (Wood, 1969a). A 2 ml sample of the 20–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction, diluted 100-fold, produced 77% solubilization under these conditions. The graph of enzyme concentration against percentage solubilization is shown in Fig. 1.

Catalase. The substrate solution was prepared by adding 30% (w/v) H_2O_2 (0.1 ml) to $\text{m-Na}_2\text{HPO}_4$ - KH_2PO_4 buffer, pH 7.0 (50 ml). The resulting solution had E_{240} 0.540. Enzyme (0.1 ml) was added to the substrate solution (2.9 ml) in a silica cuvette (1 cm light-path) and the time required for E_{240} to decrease from 0.450 to 0.400 noted.

Determination of protein. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

Lactone inhibition. Glucono-1,5-lactone solution was used in place of water in the nitrophenyl β -glucosidase assay described by Wood (1968). The ratio of inhibitor to substrate giving 50% inhibition was estimated from a graph obtained by plotting the inhibition obtained at various concentrations of inhibitor.

Thin-layer chromatography. Plates (20 cm $\times$ 20 cm) were coated with a 0.25 mm layer of Kieselgel G, dried overnight at 100°C and stored in a desiccator before use. The sample was applied approx. 2.5 cm from the bottom edge of the plate and the chromatogram was developed in ethyl acetate-propan-2-ol-water (18:13:9, by vol.) for approx. 3 h. The plate was dried at room temperature, sprayed with a 0.5% solution of potassium permanganate in m-NaOH (Hay, Lewis & Smith, 1963) and then heated at 100°C for 3 min.

Isoelectric focusing. The separations were carried out as described by Vesterberg & Svensson (1966). The L. K. B. column used had a capacity of 110 ml. The sucrose density gradient for the column was made by the method detailed in the instruction manual. Carrier ampholytes (pH 3–6) were used at an average concentration of 1%. The final voltage employed during the run was 600 V giving a load of approximately 1 W. After focusing for 72 h, fractions (2 ml) were removed from the column. The pH of the fractions was measured with a Radiometer pH-meter.

RESULTS

Effect of heat and pH on the CM-cellulase activity. Reducing-sugar CM-cellulase activity and viscometric CM-cellulase activity in a crude preparation were compared after heating at 65°C for 30 min over the pH range 2.2–8.1 (Fig. 2a) and at 65°C for up to 120 min at pH 5.4 (Fig. 2b). The results shown in Figs. 2(a) and 2(b) indicate that the thermal stability

Fig. 2. Effect of heat on CM-cellulase activity in the absence of substrate. The enzyme used was the 20–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction diluted 100-fold. After cooling the residual activity was determined as described in the text. Residual activity is expressed relative to an unheated control. CM-cellulase was determined as reducing sugar (○) or viscometrically (●). (a) Effect of heating at 65°C in citrate-phosphate buffer (0.1 M-citric acid–0.2 M- Na_2HPO_4) for 30 min at pH 2.2–8.1. (b) Effect of heating at 65°C in 0.1 M-acetic acid– NaOH buffer, pH 5.4, for up to 120 min.

Fig. 3. Effect of heat on nitrophenyl β -glucosidase and cellobiase activities. The enzyme [20–80%-satd.-(NH₄)₂SO₄ fraction diluted 100-fold] was heated at 60°C for 30 min in the absence of substrate, cooled and the residual activity in a sample determined as described in the text. The residual activities are expressed relative to an unheated control. ●, Cellobiase; ○, nitrophenyl β -glucosidase. (a) Effect of heating at 60°C in citrate-phosphate buffer (0.1 M-citric acid–0.2 M-Na₂HPO₄); (b) effect of heating at 60°C in 0.1 M-acetic acid–NaOH buffer, pH 5.0, for periods up to 60 min.

Fig. 4. Gel filtration of a 4 ml sample of *F. solani* cellulase [20–80%-satd.-(NH₄)₂SO₄ fraction] on a column of Sephadex G-100. The gel (93.0 cm $\times$ 2.5 cm) was equilibrated with 0.01 M-acetic acid–ammonium acetate buffer, pH 5.5. Fractions (4.4 ml) were collected. Assays were carried out as described in the text. The eluate was examined for nitrophenyl β -glucosidase (---), cellobiase (----), CM-cellulase by reducing sugar (—), and CM-cellulase by viscosity (····) and these are shown in (a); (b) shows protein (····) and Walseth activity (—). Portion A is fractions 37–41 and portion B is fractions 45–75.

of both types of activity was very similar under all conditions. It was remarkable that the enzyme was completely inactivated when heated at 65°C and pH 3.4 for 30 min, but only partially destroyed at lower pH values: at pH 2.2, 60% of the activity of the control sample was recovered (Fig. 2a). Results

similar to those shown in Fig. 2(a) for citrate-phosphate buffer were obtained with acetic acid–sodium hydroxide buffer, pH 3.5–5.8, and acetic acid–hydrochloric acid buffer, pH 2.0–3.6.

Heating experiments carried out on the purified C_x component (purified by chromatography on

Table 1. *Fractionation of Fusarium solani cellulase on Sephadex G-100*

Assays were carried out as described in the text. The 1 h assay for Walseth activity was used (cf. Fig. 1). Fractions 37–41 and 45–75 (Fig. 4) were combined to give portion A and portion B respectively. Totals are shown in each column.

Source of enzyme	Protein (mg)	Nitrophenyl β -glucosidase (units)	Cellobiase (units)	CM-cellulase (units)		Walseth activity (units)
				Viscometric assay	Reducing-sugar assay	
Portion A, Fig. 4	2	3360	4700	0.2×10^3	2.2×10^3	Trace
Portion B, Fig. 4	158	0	0	16×10^3	215×10^3	755
20–80%-satd.-(NH ₄) ₂ SO ₄ fraction (4 ml)	172	3376	4750	20×10^3	268×10^3	800

Table 2. *Relative activities of portions separated on Sephadex G-100 towards dewaxed cotton and Walseth cellulose*

See Fig. 4 for details of fractionation on Sephadex G-100. The volumes of portion A and portion B were adjusted so that their volumes were equivalent in terms of the starting material [20–80%-satd.-(NH₄)₂SO₄ fraction, diluted 100-fold]. For assay of Walseth activity, 0.5 ml of diluted fractions was used, and 2 ml samples were used for assay of cellulase (cotton-solubilization) activity. The 1 h assay for Walseth activity was employed (cf. Fig. 1).

Enzyme	Portion	Solubilization of Walseth cellulose (%)	Walseth activity (unit)	Recovery of Walseth activity	Solubilization of cotton (%)	Recovery of cellulase (cotton- solubilizing activity, %)
β -Glucosidase	A	2	Trace	Trace	2	1
C ₁ + C ₂	B	74	0.95	95	58	53
C ₁ + C ₂ + β -glucosidase	A + B	76	1	100	77	100
20–80%-satd.-(NH ₄) ₂ SO ₄ fraction		76	1	100	77	100

DEAE-Sephadex, see Table 3) produced similar results to those in Fig. 2. In addition, the behaviour of the Walseth activity of the purified C₂ component was found to be similar to that of the CM-cellulase activity over the pH range 2.2–8.1 (cf. Fig. 2a) when heated at 65°C for 30 min.

Effect of heat on the nitrophenyl β -glucosidase and cellobiase activities. The heat stabilities of the nitrophenyl β -glucosidase and cellobiase activities were similar when heated at 60°C for 10 min over the pH range 3.5–7.5 (Fig. 3a) and at 60°C at pH 5.0 for up to 60 min (Fig. 3b).

Fractionation on Sephadex G-100. The concentrated culture filtrate (100-fold) obtained by ammonium sulphate precipitation was fractionated on a Sephadex G-100 column. A typical elution profile is shown in Fig. 4.

All of the nitrophenyl β -glucosidase and cellobiase activities (portion A) were excluded from the gel and were completely separated from the C₂ activities of different kinds (portion B). The peaks of nitrophenyl β -glucosidase and cellobiase activity were coincident in fraction 39 where the ratio of cellobiase to nitrophenyl glucosidase was 1.41 (cf. ratio of these activities in the unfractionated 20–

80%-satd.-ammonium sulphate fraction = 1.4; Table 1). Only a small amount of CM-cellulase activity was associated with the nitrophenyl glucosidase–cellobiase component.

The Walseth activities in portions A and B were compared with the starting material (20–80%-satd. ammonium sulphate fraction) after dilution for assay in each case to the same final extent (Table 2). Assays showed that whereas only traces of Walseth activity were associated with the nitrophenyl glucosidase–cellobiase component, portion B accounted for 95.5% of the initial value. It is notable that only a total of 24% of the Walseth activity of the starting material was found when the fractions in portion B (fractions 45–75) were assayed individually. It would appear therefore that some synergistic action that was present in portion B was no longer so effective in the separated fractions.

Portion A did not solubilize Walseth cellulose to any significant extent and it increased the Walseth activity shown by portion B only very slightly (Table 2). This behaviour contrasts, however, with that shown when cotton was used as substrate. In this case although only 53% of the original cellulase (cotton-solubilizing) activity of the starting material

was found in portion B, all of the original cellulase activity was recovered with portion A in admixture with portion B (Table 2).

Further fractionation of *F. solani* cellulase on DEAE-Sephadex. The nitrophenyl β -glucosidase-cellulase component was removed as described in Fig. 4 from two 4 ml portions of the 20–80%-satd.-ammonium sulphate fraction on Sephadex G-100. Portion B from both runs was pooled and freeze-dried. The enzyme was dissolved in 0.1 M-acetic acid-sodium hydroxide buffer, pH 5.5 (10 ml), and loaded on to a column (34.5 cm $\times$ 2.5 cm) of DEAE-Sephadex (acetate form). The column was eluted initially with starting buffer (21) and then with a linear sodium chloride gradient (750 ml of 0.1 M-acetic acid-sodium hydroxide buffer, pH 5.5: 750 ml of 0.1 M-acetic acid-sodium hydroxide buffer, pH 5.5, 0.1 M with respect to sodium chloride).

The bulk of the CM-cellulase activity (assayed by both viscosity and reducing-sugar methods; see the Methods section) was not adsorbed on the DEAE-Sephadex from the 0.1 M-acetic acid-sodium hydroxide buffer solution at pH 5.5. The peaks of activity located by the viscosity and reducing-sugar methods were coincident. Recovery of CM-cellulase activity was of the order of 90% by both methods of assay. The C_1 component, which was readily adsorbed under the starting conditions, was easily removed by the application of the salt gradient (Wood, 1969a). Only a trace of CM-cellulase activity was associated with the C_1 component (Table 3).

Of the 1510 units of Walseth activity applied to the column, 770 units were associated with the CM-cellulase component and 182 units with the C_1 component. This represented a recovery of only 63%. The apparently poor recovery was not real, however, for 93% of the Walseth activity of the material put on the column was accounted for when the separated components were recombined in their

original proportions. It is clear that C_1 synergized with CM-cellulase in accelerating the solubilization of Walseth cellulose. The C_1 component was not ultimately essential for the solubilization of Walseth cellulose, however, for it was found that very small amounts of the purified C_x component (5 viscosity units of CM-cellulase activity) could accomplish 95% solubilization of 2 mg of Walseth cellulose when the assay under the standard conditions was extended to 7 days.

Fractionation of the C_x component by isoelectric focusing. Fig. 5 shows a typical elution pattern obtained after electrofocusing of the C_x component from the DEAE-Sephadex column. A pH gradient with the shallow part lying between 3 and 6 was used. The three peaks of CM-cellulase activity obtained showed approximately the same reducing-sugar CM-cellulase/viscometric CM-cellulase ratio (Table 4). Further, this ratio was approximately the same as that shown by the starting material (C_x component eluted from the DEAE-Sephadex column). The three components thus separated had isoelectric points at pH 4.75, 4.80–4.85 and 5.15.

Assays for Walseth activity were carried out on the fractions showing the peak CM-cellulase activity (fractions 30, 33 and 38), and also on the starting material. Samples were taken from the fractions so that each assay contained a total of 3 viscometric units of CM-cellulase activity. The arrangement was either 3 units from one fraction, 1.5 units from each of two fractions or 1.0 unit from each of three fractions. Under these conditions all assays produced approximately the same degree of solubilization, as did the assay containing the starting material. All three fractions, clearly, contained approximately the same CM-cellulase activity/Walseth activity ratio and this was very similar to that shown by the starting material (Table 4).

A further indication that the contents of fractions

Table 3. *Distribution of enzymic activities after the separation of C_1 and C_x components of *F. solani* on DEAE-Sephadex*

See text for details of separation and methods of assay. The 1 h assay for Walseth activity was used (cf. Fig. 1). Totals are shown in each column.

Enzyme source	Protein		CM-cellulase (by reducing sugar)		CM-cellulase (by viscosity)		Walseth activity	
	(mg)	(% recovery)	(units)	(% recovery)	(units)	(% recovery)	(units)	(% recovery)
C_x (not adsorbed on DEAE-Sephadex)	77	24	388×10^3	91	29.5×10^3	92	770	51
C_1 (adsorbed on DEAE-Sephadex)	182	57	Trace only	—	880	0.18	182	12
20–80%-satd.- (NH_4) ₂ SO ₄ fraction (8 ml)	320	100	430×10^3	100	32×10^3	100	1520	100

30, 33 and 38 were in fact isoenzymes of identical specificity was obtained from the observation that 50 viscosity units of CM-cellulase activity (taken from fraction 30, fraction 33 or fraction 38) when in admixture with a sample of the C₁ component (from the DEAE-Sephadex column) and the β-glucosidase component (portion A, Fig. 4), produced the same degree of solubilization of cotton fibre in each case. Similarly 50 units of CM-cellulase activity made up from 25 units from each of two fractions, or, alternatively, 17.5 units from each of the three fractions, synergized with the C₁ and β-glucosidase to produce identical cellulase activity in each case.

In another electrofocusing experiment similar to that shown in Fig. 5, all fractions were examined for Walseth activity. It was found that fractions devoid of CM-cellulase activity were also lacking in Walseth activity. Peaks of Walseth activity were coincident with peaks of activity for CM-cellulase.

Isoelectric focusing of the nitrophenyl β-glucosidase-cellobiase component. Further evidence that the nitrophenyl β-glucosidase and the cellobiase activities were associated with the same protein component was provided by the isoelectric-focusing technique. Both activities were found in optimum amount in fraction 14, the pH of which was 3.15 (Fig. 6). In addition, the cellobiase/nitrophenyl

Fig. 5. Isoelectric focusing of CM-cellulase activities. The C_x fraction from the DEAE-Sephadex column was precipitated with (NH₄)₂SO₄ (80% satd.), desalted on a column of Sephadex G-25 (50.0 cm × 2.5 cm), dissolved in 0.01M-acetic acid-ammonium acetate buffer, pH 5.0 (10ml), and freeze-dried. The solid material was dissolved in 0.5% ampholyte solution, pH 3-6 (10ml), and a fraction (3.5ml) was mixed with the sucrose-ampholyte solution (see the Methods section). After isoelectric focusing, the contents of the column were collected in 2ml fractions. Each fraction was precipitated with (NH₄)₂SO₄ (80% satd.) and CM-cellulase was determined by reducing sugar (—) and viscometrically (.....). The pH of the fractions is shown as O.

Table 4. Isoelectric focusing of C_x fraction isolated on DEAE-Sephadex

See Fig. 5 for the separation. Assays were carried out as described in the text. The 18h assay for Walseth activity was used (cf. Fig. 1). See the text for details of starting material (C_x component from DEAE-Sephadex column). A single fraction was taken from the first and third peaks (Fig. 5) and two from the broad second peak. Totals are shown in each column.

Source of enzyme	Protein (mg)	CM-cellulase (by reducing sugar) (units)	CM-cellulase (by viscosity) (units)	Ratio of CM-cellulase (by reducing sugar) to CM-cellulase (by viscosity)	Walseth activity (units)	Ratio of CM-cellulase (by viscosity) to Walseth activity
Fraction 30	1.67	9.1 × 10 ³	700	13.0	255	2.74
Fraction 33	1.82	13.0 × 10 ³	960	13.5	335	2.86
Fraction 34	1.85	12.4 × 10 ³	965	13.0	350	2.74
Fraction 38	2.35	19.5 × 10 ³	1425	13.5	545	2.62
C _x component from DEAE-Sephadex column	27	124 × 10 ³	9.25 × 10 ³	13.4	3400	2.72

β -glucosidase ratio (1.4) was unchanged by isoelectric focusing. The ratio of these activities was thus preserved throughout the purification procedures.

Recovery of β -glucosidase and cellobiase was 80% and 75% respectively. No nitrophenyl β -glucosidase active fraction was devoid of cellobiase activity and conversely. Reducing-sugar CM-cellulase activity was still associated with the glucosidase activity, but only traces of viscometric CM-cellulase were detected.

Activity of the β -glucosidase component on Walseth cellulose and on cello-oligosaccharides. The sample of β -glucosidase used was that obtained after the purification by electrofocusing (Fig. 6) of portion A, Fig. 4. In general, the rate of attack on the cello-oligosaccharides was less than on cellobiase. Cello-oligosaccharides DP (degree of polymerization) 2–6 were used and the glucose that was released from the substrate (4mg) in 2h was determined by the modification of the glucose oxidase method described by Wood (1969a). Under these conditions the respective rates of attack on cellotriose, cello-tetraose, cello-pentaose and cellohexaose were found to be 0.72, 0.60, 0.66 and 0.56 of that on

cellobiose. Walseth cellulose was also attacked, but only at one-tenth of the rate of attack on cellobiose. In the latter case the data may be misleading, however, for the possibility exists that the low-molecular-weight fragments which must be present in the Walseth cellulose may be preferentially attacked: cellooligosaccharides DP > 10 would be insoluble.

Examination by t.l.c. of the products of hydrolysis of the cello-oligosaccharides revealed that, even at a substrate concentration of only 1%, transferase activity was evident. Cellobiose ($R_{\text{glucose}} 0.77$) provided only one transfer product ($R_{\text{glucose}} 0.52$), whereas cellotriose ($R_{\text{glucose}} 0.52$) formed two ($R_{\text{glucose}} 0.34$ and 0.22). Authentic cellotetraose and cello-pentaose had R_{glucose} values of 0.34 and 0.22 respectively.

Gluconolactone inhibition of nitrophenyl β -glucosidase activity. From the curve relating lactone concentration to percentage inhibition, nitrophenyl β -glucosidase activity was found to be inhibited to the extent of 70%, 52% and 34% by 0.1mM, 0.05mM and 0.025mM concentrations of gluconolactone respectively. The molar ratio of inhibitor to substrate at 50% was 0.04.

Molecular-weight determination of (a) C_x component, (b) β -glucosidase component. The method used was similar to that used by Whitaker (1963b). Sephadex G-100 or Sephadex G-200 was equilibrated with 0.1M-acetic acid-sodium hydroxide buffer, pH 5.0, which was also 0.1M in sodium chloride and 0.02% with respect to sodium azide. The column was 2.5cm in diameter and the gel height 37cm. A calibration curve was prepared for each gel by using proteins of known molecular weight. The elution volumes of the molecular-weight markers (cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, catalase, γ -globulin, urease and thyroglobulin) were determined spectrophotometrically at 280nm or 412nm (cytochrome c) or by enzyme assay (for urease, see Weatherburn, 1967; for catalase, see the Methods section).

The molecular weight of the C_x components from the DEAE-Sephadex column, determined from the calibration curve on Sephadex G-100, was approx. 37 000.

The β -glucosidase component (portion A, Fig. 4) was excluded from the Sephadex G-100 column and also from a column of Sephadex G-150: its elution volume from the column of Sephadex G-200 suggested that it had a molecular weight of approx. 400 000.

DISCUSSION

Two modes of enzymic attack on polysaccharides are recognized. In one, attack is completely random (endo-enzymes), whereas in the other only the

Fig. 6. Isoelectric focusing of nitrophenyl β -glucosidase-cellobiase component. The enzyme in portion A from the Sephadex G-100 column (Fig. 4) was freeze-dried, dissolved in 0.5% ampholyte solution, pH 3–6 (9ml), and a portion (6ml) of this was used for the electrofocusing experiment. Cellobiase (---); nitrophenyl β -glucosidase (-.-.-); CM-cellulase by reducing sugar (—); CM-cellulase by viscosity (....). The pH gradient is shown as O. Fractions (2ml) were collected. The sucrose in each fraction was removed by dialysis against 0.1M-acetic acid-ammonium acetate buffer, pH 5.0. Enzyme activities in the sucrose-free fractions were determined as indicated in the text.

terminal bonds of the chains are cleaved (exo-enzymes). The fact that cellulose hydrolases (C_x enzymes) are endo- β -glucanases is now well established, but additional enzymes with exo- β -glucanase activity have been reported in *T. viride* (Li, Flora & King, 1965; Okada, Nisizawa & Suzuki, 1968) and *Stachybotrys atra* (Youatt & Jermyn, 1959) culture filtrates.

Enzymes acting on cellulose in a random manner will produce a greater decrease in the viscosity of a polymer solution (e.g. CM-cellulose) per unit increase in reducing power than will an enzyme acting from the end of the chain. A change in the ratio of these activities during fractionation studies would be suggestive of heterogeneity. In the work reported in this paper, therefore, CM-cellulase activity has been assayed throughout by both viscometric and reductimetric methods.

CM-cellulose is normally used for the assay of C_x activity. The use of this substrate, however, could lead to the omission during fractionation studies of a hypothetical enzyme which is an important part of the cellulase complex. In this regard it is noteworthy that Gilligan & Reese (1954) separated an enzyme from a culture filtrate of *Myrothecium verrucaria* which possessed little CM-cellulase activity, but exhibited a high capacity for attacking Walseth cellulose; Wood (1969b) has reported that certain fungal preparations rich in CM-cellulase activity were devoid of Walseth activity. Walseth cellulose has therefore been used, in addition to CM-cellulose, for the assay of C_x activity in *F. solani* preparations.

No evidence for heterogeneity of the C_x system of *F. solani* could be found. The ratio of reductimetric CM-cellulase activity to viscometric CM-cellulase activity that was shown by the unfractionated culture filtrate was maintained throughout chromatography on Sephadex G-100 and DEAE-Sephadex. Further, although three protein peaks were separated by the isoelectric-focusing technique, each exhibited the same ratio of the two CM-cellulase activities as the unfractionated enzyme complex. The CM-cellulase/Walseth activity ratio was also identical in the three peaks, and there was no synergism between the three fractions when Walseth cellulose was used as substrate.

That the three peaks were identical in nature was supported by the observation that a mixture of the C_1 and β -glucosidase enzymes synergized equally effectively each of these three components to produce the same degree of solubilization of cotton fibre in each case.

The enzymes in the peaks from the electrofocusing experiment must differ in some aspect of structure, but no further examination has been made. Complex-formation of a single enzyme with carbohydrate has been found to account for the apparent hetero-

geneity of the CM-cellulase component in *Stereum sanguinolentum* (Eriksson & Pettersson, 1968) and also in the β -glucosidase present in *Stach. atra* filtrates (Jermyn, 1962).

It would appear that the cellobiase and the aryl β -glucosidase activities in fungal culture filtrates do not always originate from the same enzyme protein. In this regard Jermyn (1955a,b) and Hash & King (1958) have isolated an aryl β -glucosidase from *Stach. atra* and *M. verrucaria* respectively which had no activity towards cellobiose, and Bucht & Eriksson (1969) report the isolation of a cellobiase from *S. sanguinolentum* which showed very little aryl β -glucosidase activity. *F. solani* nitrophenyl β -glucosidase and cellobiase activities, in contrast, appear to have their origin in the same enzyme protein. This conclusion is supported by the fact that (a) the heat stabilities were remarkably similar, (b) the isoelectric points were identical and (c) the ratio of these activities was maintained throughout the various purification procedures.

Exoglucanases remove mono- and di-saccharides from the non-reducing ends of polysaccharides. Their action, however, is not restricted to polymers, but extends also to dimers, trimers, tetramers, etc. It is here that a certain amount of confusion exists in that the lower oligosaccharides are also substrates for glucosidase action. Reese, Maguire & Parrish (1968) have studied a number of properties of exoglucanases and glucosidases and have drawn up criteria for the characterization and differentiation of these two alleged groups of activities. The nitrophenyl β -glucosidase-cellobiase component of *F. solani* would appear to satisfy some of the conditions for classification as a β -glucosidase, rather than an exoglucanase, in that (a) it was inhibited by relatively low concentrations of gluconolactone, (b) it possessed the ability of transferring glucosyl groups to cellobiose, and (c) it attacked cellobiose faster than higher oligosaccharides in the homologous series. Nevertheless, the enzyme does exhibit some exoglucanase activity in that it can slowly produce reducing sugars from CM-cellulose and from Walseth cellulose.

It is concluded that the cellulase system of *F. solani*, when grown in stationary culture on cotton fibre as stated in the Methods section, consists of three enzymes that are essential for the complete breakdown of cotton cellulose. These are (a) a C_1 component whose action is still obscure, (b) a single C_x enzyme that is capable of hydrolysing insoluble degraded cellulose, as well as soluble cellulose oligosaccharides, and (c) a β -glucosidase component which is also a feeble exo- β -glucanase.

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