Cellulolytic Enzyme System of *Trichoderma koningii*

SEPARATION OF COMPONENTS ATTACKING NATIVE COTTON

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1. Cell-free culture filtrates from *Trichoderma koningii* were concentrated by precipitation with ammonium sulphate between the limits of 20% and 80% saturation. 2. Removal of a low-molecular-weight carboxymethylcellulase (CM-cellulase) component by chromatography on Sephadex G-75 had no effect on the ability of the enzyme complex to solubilize cotton. 3. Further chromatography on DEAE-Sephadex separated a component (C_1) from the C_x (CM-cellulase) and β-glucosidase activities. Separately these components had little ability to produce soluble sugars from cotton, but when recombined in their original proportions this capacity was almost completely recovered. 4. The C_x component was further fractionated on SE-Sephadex into a fraction containing only CM-cellulase and a fraction showing CM-cellulase and β-glucosidase activities: the latter two components could be separated by heat treatment. 5. The C_1 component had no swelling factor (S-factor) activity (Marsh, Merola & Simpson, 1953; Reese & Gilligan, 1954) on its own, but it had a synergistic effect on the S-factor activity associated with the CM-cellulase and β-glucosidase components.

Though much of the work in the field of cellulose digestion by cell-free culture filtrates of micro-organisms has been confined to a study of the group of enzymes first classified as C_x by Reese, Siu & Levinson (1950), the recent publications by Mandels & Reese (1964) and Halliwell (1965), showing that cell-free preparations from *Trichoderma viride* or *Trichoderma koningii* respectively were capable of extensive solubilization of native cellulose in the form of cotton fibre, have turned attention to a study of these culture filtrates, which must contain the C_1 enzyme first postulated by Reese *et al.* (1950). This C_1 enzyme, distinct from the C_x type of enzyme, which is capable of attacking only chemically treated cellulose, possesses the capacity of attacking insoluble highly-ordered cellulose, rendering it susceptible to subsequent attack by the C_x class of enzyme. Recent efforts have centred mainly on methods of separation of the C_1 component from the rest of the cellulase complex produced by *Trichoderma*. The first of these (Mandels & Reese, 1964) established the separate identity of the C_1 enzyme, but this, like the hydrocellulase that was capable of attacking highly crystalline particles (Li, Flora & King, 1965; Flora, 1965), was still capable of producing soluble sugars from cotton cellulose in a limited way. More recently, Selby & Maitland (1967) have isolated a C_1 component that was free from C_x activities and did not solubilize cotton: in keeping with the results reported by

Mandels & Reese (1964), Li *et al.* (1965) and Flora (1965), synergistic effects were apparent with the separated components, and in this case recombination of the separated C_1, cellobiase and carboxymethylcellulase (CM-cellulase) components in their original proportions provided a mixture with the same activity towards cotton as the original unfractionated culture filtrate, thereby showing that no essential element of the cellulase system had been lost in the various fractionation procedures.

It is as yet uncertain if the C_1 component as defined by Reese *et al.* (1950) can be identified with the hydrocellulase component separated by Flora (1965) or the 'enzyme A' that is responsible for loss of tensile strength of the cotton fibre (Selby, 1963). Reports of other manifestations of an initial attack on the cotton fibre have appeared in the literature, but probably the best known of these is the early change in cotton brought about by certain culture filtrates and subsequently measured as an increase in the uptake of alkali (so-called swelling-factor or S-factor activity) (Marsh, Merola & Simpson, 1953; Reese & Gilligan, 1954). This is reported to have been detected before any loss of tensile strength, change in degree of polymerization of the cellulose or release of reducing sugar occurs. The fragmentation of cotton fibres to give short fibres, which has been reported by Halliwell (1965) and re-examined by Marsh (1966), is indicative of yet another change brought about before the appearance of significant

quantities of sugar in solution. In the work described below it is shown that the separated C_1 component of a cellulase prepared from *T. koningii* has little solubilizing action on cotton, is not solely responsible for the formation of short fibres and, though it cannot be identified with S-factor on its own, it potentiates the swelling activity associated with the C_x components.

EXPERIMENTAL

Materials

The organism used throughout the work was a culture of *T. koningii* I.M.I. 73022 obtained from the Commonwealth Mycological Institute, Kew, Surrey.

Texas cotton was obtained from the Shirley Institute, Didsbury, Manchester.

Sephadex G-75, DEAE-Sephadex (A-50) and SE-Sephadex (C-50) were purchased from Pharmacia (G.B.) Ltd., London, W. 13.

The agar used was Oxoid Agar no. 3 (Oxoid Division of Oxo Ltd., London, E.C. 4).

Sodium CM-cellulose (Cellofas B) was kindly given by Imperial Chemical Industries Ltd. Nobel Division, Steventon, Ayrshire.

o-Nitrophenyl β-D-glucoside was prepared by the method of Seidman & Link (1950). Recrystallized from ethanol it had m.p. 168–170° and $[\alpha]_D^{17} -101°$ (c 1·0 in water).

Methods

Maintenance and growth of the organism. The organism was maintained in subcultures on short potato–carrot–agar slants covered with liquid paraffin (sp.gr. 0·86–0·89; British Drug Houses Ltd., Poole, Dorset) at 1°. A stab inoculum from these master cultures was used to inoculate potato–glucose–agar slants: the thick cellular growth that resulted from incubation at 27° for 10 days was suspended in water and 1 ml. of the suspension was used to inoculate the liquid cultures (see below).

The potato–carrot–agar slants provided only very weak growths of the organism, but maintained the subsequent ability of the fungus to grow in the liquid cultures. Growth in the liquid cultures decreased after repeated subculturing on either potato–glucose–agar slants or on agar slants containing the salts medium of Saunders, Siu & Genest (1948) and covered with a filter-paper strip.

Preparation of the cellulase. Native Texas-cotton fibre (4 g.) was shaken with 150 ml. of the salts medium of Saunders *et al.* (1948) until saturated. The cotton was teased out and placed inside a 1 l. Roux flask together with the solution not absorbed by the fibre. This method resulted in an even distribution of the saturated fibre over the available surface area of the flask. The flasks were then inoculated with cells from the potato–glucose–agar slants (see above) and incubated at 27°. Growth appeared in 36 hr. and in 7 days the whole surface area of the cotton exposed to the air was covered with a thick growth, which was green in some areas and bright yellow in others.

After 28 days incubation, the culture medium was filtered, centrifuged at 66000g for 20 min. at 3° and precipitated with (NH$_4$)$_2$SO$_4$ between the limits of 20% and 80% saturation. The precipitate was redissolved in 0·1 M-acetate buffer (acetic acid–NaOH), pH 5·0, to give a 50-fold concentration of the original culture filtrate. The solution was finally filtered through a sintered glass bacteriological filter (porosity 5/3), made 5 mM with respect to sodium azide and stored at 1°.

This 20–80%-saturated (NH$_4$)$_2$SO$_4$ fractionated enzyme, when diluted 50-fold, had all the activity of the original crude cell-free filtrate towards cotton. One ml. of this diluted solution contained 1650 CM-cellulase units, 10 S-factor units, 99 β-glucosidase units (see below for definition of units) and produced 43·5% solubilization of native cotton fibre under the conditions of the standard assay (see below). Only a trace of protease (Nomoto & Narahashi, 1959) could be found.

Enzyme assays

β-*Glucosidase.* The incubation mixture contained 1·0 ml. of 0·2 M-acetate buffer (acetic acid–NaOH), pH 4·0 (Fig. 4), 1·0 ml. of 5 mM-o-nitrophenyl β-D-glucoside, 1·0 ml. of diluted enzyme preparation and 1·0 ml. of water. After 1 hr. at 37°, 4·0 ml. of 0·4 M-glycine–NaOH buffer, pH 10·8, was added and the liberated nitrophenol measured on the Spekker photoelectric absorptiometer with Ilford no. 601 violet filters (peak transmission 430 mμ). The usual enzyme and substrate controls were incubated.

The unit of activity was defined as the amount of enzyme needed to liberate 25 μg. of o-nitrophenol under the conditions of the assay.

CM-cellulase. A mixture of 5 ml. of a 1% solution of CM-cellulose, 2·5 ml. of 0·2 M-acetate buffer (acetic acid–NaOH), pH 5·4 (Fig. 4), and 2·5 ml. of diluted enzyme and water was incubated at 37° for 1 hr. A sample (2 ml.) of this solution was pipetted directly into 2 ml. of Somogyi (1952) reagent to terminate the reaction. The mixture was boiled for 15 min. and 2 ml. of Nelson (1944) reagent added to the cooled tubes. The mixture was diluted to 10 ml. with water, and the precipitated unhydrolysed CM-cellulose was sedimented by centrifugation. The supernatant was read against a water blank on the Spekker absorptiometer with Ilford no. 604 green filters (peak transmission 515 mμ).

Under these conditions the amount of hydrolysis was proportional to the enzyme concentration. The unit of activity is defined as the amount of enzyme in 2 ml. of the reaction system required to liberate reducing sugar equivalent to 20 μg. of glucose.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) by using the Spekker absorptiometer with Ilford no. 608 red filters (peak transmission 680 mμ). Crystalline bovine plasma albumin was used as reference protein.

Activity towards cotton

Degree of solubilization, S-factor activity and short-fibre formation were all used as criteria of activity towards cotton.

Solubilization. In the standard assay 2 mg. of native cotton fibre was incubated in round-bottomed tubes with 1–3 ml. of enzyme, 5 ml. of 0·2 M-acetate buffer (acetic acid–NaOH), pH 4·8 (Fig. 4), and water to give a final volume of 10 ml. Then M-sodium azide (0·05 ml.) was added to each tube and the incubation carried out for 1 week at
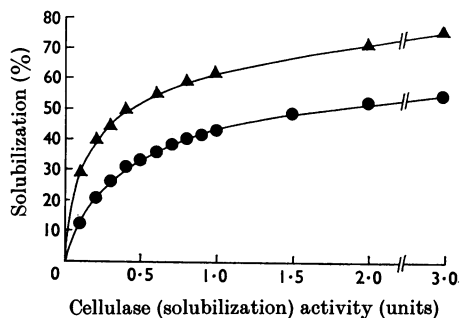
Fig. 1. Effect of enzyme concentration on the solubilization of cotton. Incubation was for 7 days at 37°. The enzyme used was the 20–80%-saturated (NH₄)₂SO₄ fraction diluted 50-fold. The portion of the curve given by volumes up to 1 ml. of enzyme was used as the calibration curve for the assay of activity towards native cotton fibre. ●, Native cotton; ▲, dewaxed cotton (Halliwell, 1965). For conditions of assay see the text.

Fig. 2. Effect of enzyme concentration on S-factor activity. The enzyme used was the 20–80%-saturated (NH₄)₂SO₄ fraction diluted 50-fold. See the text for assay conditions.

37°. The residual cellulose was separated on filter sticks and estimated by oxidation with dichromate–H_2SO_4 reagent (Halliwell, 1958). The graph of enzyme concentration plotted against solubilization is shown in Fig. 1. Dewaxed cotton was completely solubilized in 14 days by 3 ml. of the 20–80%-saturated (NH₄)₂SO₄ fraction diluted 50-fold, whereas native cotton was solubilized to 82% in the same period. Three ml. of the undiluted 20–80%-saturated (NH₄)₂SO₄ fraction solubilized native cotton completely in 6 days.

If hydrolysis of cotton went to completion the calculated glucose concentration produced would be 0·02%: it was found that adding glucose at this concentration to a mixture of enzyme and cotton had no effect on the hydrolysis of the latter.

The assays carried out on the fractions obtained from the Sephadex G-75 column were modified slightly in procedure: cellulose digestions, as above, were carried out in conical centrifuge tubes (15 ml.) and the insoluble cellulose was sedimented by centrifugation. Supernatants were withdrawn until only 1·8 ml. of liquid was left in the tube. Repeated washing and centrifugation removed all soluble sugars before the addition of the dichromate–H_2SO_4 reagent.

Degree of solubilization is referred to as 'cellulase activity' in the text.

S-factor activity. The incubation mixture contained 100 mg. of native cotton fibre, 2·0 ml. of 0·2 M-acetate buffer (acetic acid–NaOH), pH 5·0, and enzyme and water to give a total volume of 10 ml. The cotton was removed after 2 hr. at 27°, partially dried on filter paper and placed in 20 ml. of 18% (w/v) NaOH solution. The mixture was shaken by hand for 30 sec. and replaced in the incubator. After 1 hr. the swollen sample was removed, placed in a sintered-glass micro filter funnel (BTL type K832H, porosity 2) and centrifuged for 30 min. at 340 g in a 15 ml. centrifuge tube (8 × 15 ml. Trunnion carrier) on an MSE Super-Medium centrifuge. Radius of centrifugation was 13·6 cm. The sample was transferred to a weighing bottle and weighed. Controls were included in each experiment.
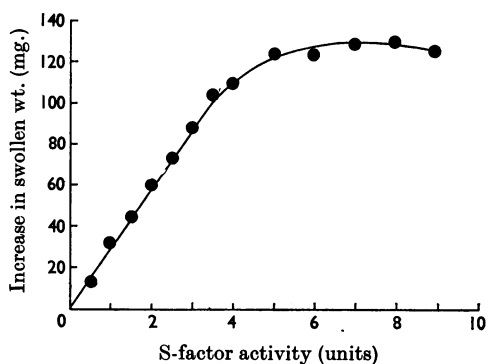
The graph of increase in swollen weight plotted against enzyme concentration was linear at low enzyme concentrations (Fig. 2) and the unit of activity is defined as the amount of enzyme required to produce an increase in swollen weight of 15 mg. beyond the control value.

Because of its marked inhibitory effect on S-factor activity, care was taken to remove all the NaCl used in the various fractionation procedures by dialysis before S-factor assay.

Formation of short fibres. Short-fibre-forming activity was measured by the method detailed by Halliwell (1965). Native cotton fibres were used in the standard assay. The graph of extinction of the short fibres plotted against enzyme concentration was linear and the unit of activity is defined as the amount of enzyme needed to produce an extinction of 0·2 under the conditions of the standard assay.

Column chromatography

Column chromatography was carried out at room temperature and fractions were collected with a Beaumaris fraction collector (Beaumaris Co., Anglesey).

Preparation of Sephadex G-75 column. The glass tube (112 cm. × 3 cm.) used for the construction of the column had a sintered-glass disk (porosity 3) 1 cm. from one end. A rubber bung carrying a fine capillary tube was inserted into the bottom of the glass tube until it was almost touching the sinter. The sinter was covered with a 1 cm. layer of sand (acid-purified; British Drug Houses Ltd. laboratory reagent), and the gel, which had been swollen in 0·1 M-acetate buffer (acetic acid–NaOH), pH 5·0, was poured into the column half-full with buffer. Packing was done with a liquid head of 20 cm. When the gel had settled completely, a layer of Sephadex G-25 (1 cm. thick) was placed on top of the Sephadex G-75 gel between two disks of Whatman glass paper. The final dimensions of the Sephadex G-75 gel were 100 cm. × 3 cm. and the void volume (with blue dextran as a test substance) was 210 ml. Under a pressure of 20 cm. the flow rate was 7 ml./hr.

Preparation of Sephadex ion-exchangers. DEAE-Sephadex (A-50) was prepared in the acetate form and equilibrated with 0·1 M-acetate buffer (acetic acid–NaOH), pH 5·0.
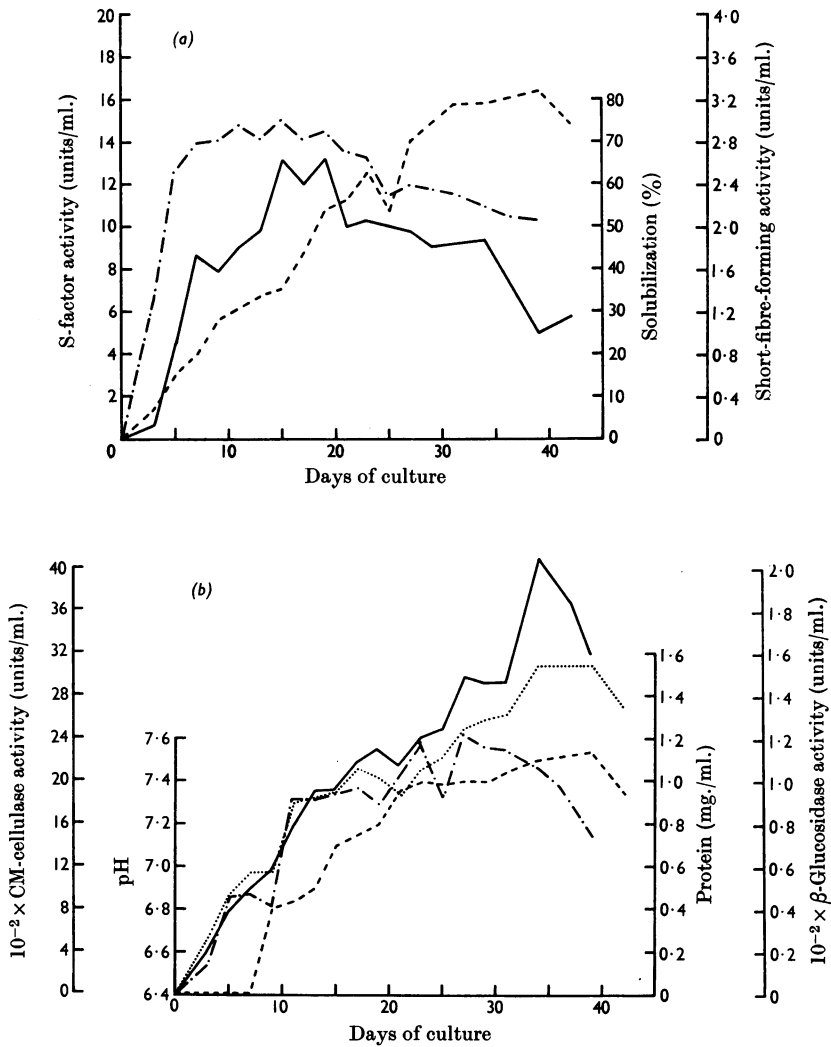
Fig. 3. Rate of development of activity towards cotton, CM-cellulose, and o-nitrophenyl β-D-glucoside in cultures of *T. koningii*. At intervals five flasks were removed from various positions in the incubator and the culture filtrates isolated, after combination, as described in the text. Assays of activity towards native cotton (see the text for conditions of assay) included short-fibre formation (– – – –), solubilization (– · – · –) and S-factor (———), and the results are shown in (a). Other assays were for CM-cellulase (– · · – · –), β-glucosidase (———), protein (·······) and pH (– – – –), and these are shown in (b).

SE-Sephadex (C-50) was allowed to swell in a large excess of 5 mM-succinate buffer (sodium succinate–sodium hydrogen succinate), pH 5·0, the supernatant being removed and replaced by fresh buffer solution three times over a period of 24 hr.

The gradient apparatus employed consisted of two small beakers of equal dimensions and connected by a siphon. The liquid in the mixing beaker was agitated by a magnetic stirrer: this arrangement provided a linear gradient.

RESULTS

Rate of development of activity towards cotton, CM-cellulose and o-nitrophenyl β-glucoside in cultures

The rate of development of activity towards these substrates in culture filtrates prepared in stationary culture is shown in Fig. 3. Short-fibre formation,
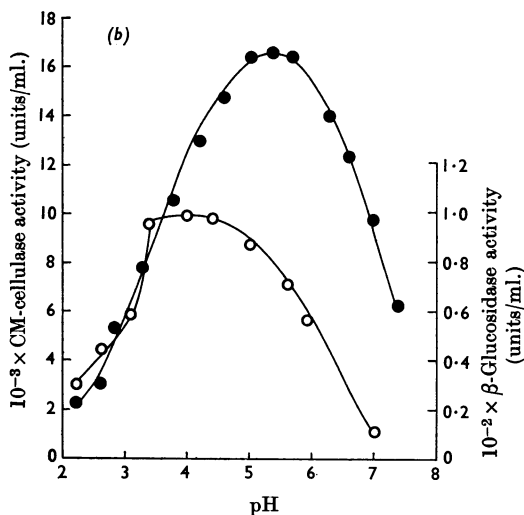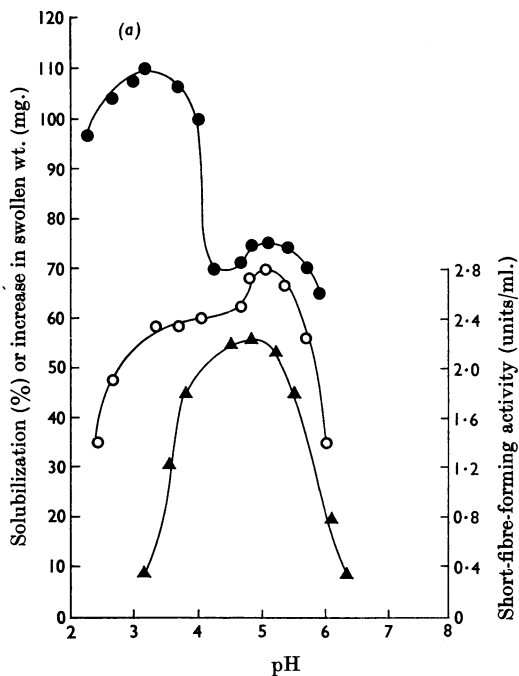
solubilization and uptake of alkali (S-factor) were all used as criteria of cotton attack.

Optimum cellulase activity in the culture filtrate was reached in only 11 days, whereas S-factor activity, CM-cellulase and β-glucosidase required 15 days, 21 days and 34 days respectively for maximum potency to be achieved. Short-fibre-forming activity maintained a steady increase until 31 days of incubation.

Culture filtrates used in this work were harvested after 28 days of incubation, at which time cellulase (solubilization) activity was on the decline (see the Discussion section).

Effect of pH *on the various enzyme activities.* The variation in S-factor activity with pH over the range pH 2·2–5·8, in acetate buffer, is shown in Fig. 4. Optimum activities were recorded at pH 3·2–3·4 and pH 4·8–5·8, with appreciable activity still evident at pH 2·2. In citrate buffer two optima were observed at pH 3·2–3·4 and pH 5·5; the acid peak was 18% higher than that produced in acetate buffer. Bimodal pH–swelling-factor activity curves of this type have been reported before for culture filtrates of *Myrothecium verrucaria* (Marsh *et al.* 1953), *T. viride* and *Penicillium pusillum* (Reese & Gilligan, 1954).

Single-peak pH–activity curves were obtained for β-glucosidase, CM-cellulase and cellulase (solubilization), but the curve for short-fibre-forming activity showed an optimum at pH 5·0 and a plateau at pH 3·1–3·5 (Fig. 4). For the last activity Halliwell (1965) found pH optima at pH 3·6 and pH 5·0 in his work on *T. koningii.*

Fractionation of cell-free filtrate on Sephadex G-75. A column load of 5ml. of the dark-brown concentrated 20–80%-saturated ammonium sulphate fraction, eluted with 0·1M-acetate buffer, pH 5·0, gave the distribution of protein and enzymic activities shown in Fig. 5. Extinction measurements of each fraction at 280mμ (not shown) revealed a sharp peak of absorption in fractions 44–49. There was no corresponding peak with the Lowry method of determination of protein and the fractions did not show any of the enzymic activities mentioned in this paper. The very high background in the u.v. absorption made interpretation difficult and protein was measured throughout by the method of Lowry *et al.* (1951).

The low-molecular-weight CM-cellulase component appearing in part Ab (Fig. 5) solubilized cotton only to a very limited extent (Table 1), and it did not appear to be an essential component of the cellulase complex in that, when part Aa was diluted to 250ml., the mixture provided a solution with the same cellulase activity as the original 20–80%-saturated ammonium sulphate fraction diluted 50-fold. The low-molecular-weight CM-cellulase component (part Ab) did, however, make some
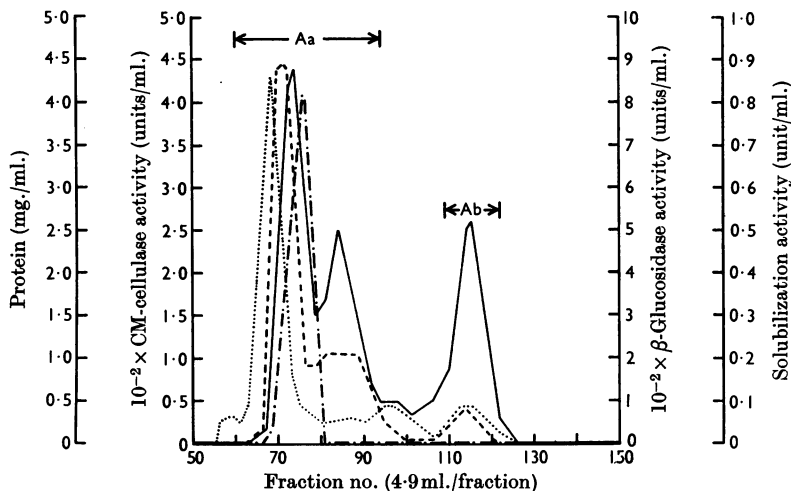
Fig. 4. Effect of pH on enzymic activity towards cotton, CM-cellulose and *o*-nitrophenyl β-D-glucoside. Activity towards cotton, shown in (*a*), was measured as S-factor (●), solubilization (▲) and short-fibre formation (○). Assays were carried out in 0·2M-acetate buffer (HCl–acetic acid for pH values 2·2–3·2 and acetic acid–NaOH for the remainder). Measurements of CM-cellulase (●) and β-glucosidase (○) activities, shown in (*b*), were carried out in phosphate–citrate buffer (0·1M-citric acid–0·2M-Na$_2$HPO$_4$). CM-cellulase and β-glucosidase showed the same pH optima when assayed in 0·2M-acetate buffer (acetic acid–NaOH).

Fig. 5. Separation of a sample (5 ml.) of the concentrated culture filtrate [20–80% saturated $(NH_4)_2SO_4$ fraction redissolved to give a 50-fold concentration] on a column (100 cm. × 3 cm.) of Sephadex G-75, the eluent being 0·1 M-acetate buffer (acetic acid-NaOH), pH 5·0. ——, CM-cellulase; – · – · –, β-glucosidase; ·······, protein; – – – –, solubilization. Assays were done as stated in the text. In a similar fractionation, fractions 60–94 (designation portion Aa, in the text) were combined as well as fractions 109–122 (portion Ab), and assays for recovery of cellulase (solubilization) and S-factor activity were carried out on these portions. Results are shown in Table 1.

Table 1. *Activities towards cotton of the components separated on Sephadex G-75*

A second 5 ml. sample of the concentrated (50-fold) 20–80%-saturated $(NH_4)_2SO_4$ fraction was diluted to 250 ml. and this was used as the standard reference solution. Portions Aa and Ab (Fig. 5) were diluted to 250 ml.; 1 ml. of each was used for the cellulase assay and 0·5 ml. for the S-factor assay.

	Solubilization			S-factor		
	%	Units/ml.	Recovery of cellulase activity (%)	Increase in swollen wt. (mg.)	Units/0·5 ml.	Recovery of S-factor activity (%)
Aa	44·1	1·05	102	68	4·7	90
Ab	6·1	0·04	4	6	0·4	8
Aa + Ab	44·3	1·06	103	78	5·4	104
20–80%-satd. $(NH_4)_2SO_4$ fraction	43·8	1·03	100	75	5·2	100

contribution towards the overall S-factor activity, for, though part Aa could account for only 90% of the original activity, when recombined with the low-molecular-weight component in their original proportions recovery was 104% (Table 1).

All of the short-fibre-forming activity (not shown in Fig. 5) lay in part Aa, the peak of activity coinciding with the peak of activity towards cotton (solubilization).

The overall recoveries of CM-cellulase and β-glucosidase were 91% and 100% respectively.

Separation of the C_1 component on DEAE-Sephadex. Part Aa from the Sephadex G-75 column

was dialysed at 1° in Visking tubing for 48 hr. against several changes of distilled water. A fresh dialysis bag was used every 12 hr. throughout the period. The non-diffusible material was freeze-dried and redissolved in 0·1 M-acetate buffer, pH 5·0. There was no loss in any of the enzymic activities during dialysis or on freeze-drying.

The C_1 component was strongly adsorbed from the 0·1 M-acetate buffer, pH 5·0, on the DEAE-Sephadex column, whereas the bulk of the CM-cellulase and all of the β-glucosidase passed through rapidly (Fig. 6). Final elution of the C_1 component (fractions 77–90 and called part Bb in the text) was
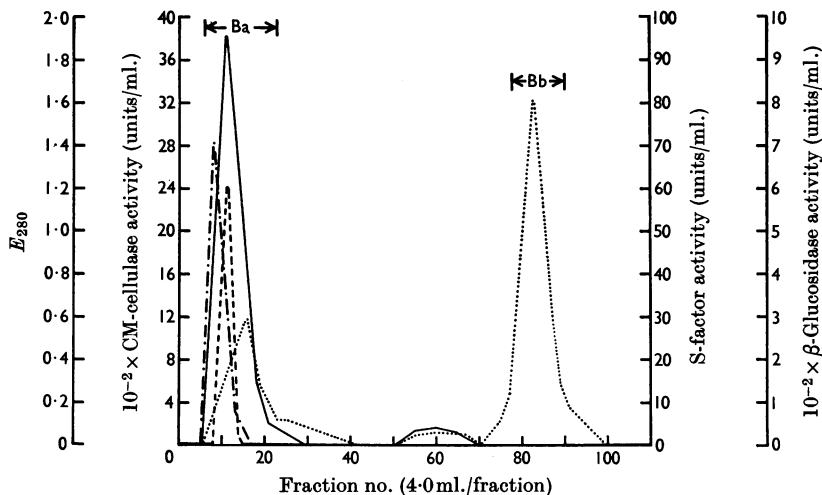
Fig. 6. Separation of C_1 and C_x components on a column (28 cm. × 1·6 cm.) of DEAE-Sephadex (acetate form). The cellulase component (portion Aa) from the Sephadex G-75 column (Fig. 5) was dialysed, freeze-dried and redissolved in 20 ml. of 0·1 M-acetate buffer (acetic acid–NaOH), pH 5·0. Half of this was applied to the column and eluted initially with 0·1 M-acetate buffer, pH 5·0. After 39 fractions had been collected the column was eluted with a linear salt gradient (150 ml. of acetate buffer, pH 5·0, and 150 ml. of acetate buffer, pH 5·0, 0·5 M with respect to NaCl). Fractions 6–23 were combined (called portion Ba in the text) and fractions 77–90 (portion Bb). Portion Bb contained the C_1 component. Assays were carried out as stated in the text. The eluate was examined for E_{280} (·······) and β-glucosidase (– · – · –), CM-cellulase (——) and S-factor (————) activities.

Table 2. *Activities towards cotton of components separated on DEAE-Sephadex*

See legend to Fig. 6 for details of separation. Fractions were combined as shown in Fig. 6 and assayed for cellulase (solubilization) and S-factor activities after dilution to make all portions equivalent in terms of the original 20–80%-saturated $(NH_4)_2SO_4$ fraction; 1 ml. was used in the solubilization assay and 0·5 ml. in the S-factor assay.

Sample	Solubilization			S-factor		
	%	Unit/ml.	Recovery of cellulase activity (%)	Increase in swollen wt. (mg.)	Units/0·5 ml.	Recovery of S-factor activity (%)
Ba	5·1	0·03	3	51	3·5	69
Bb	6·1	0·04	6	Nil	Nil	Nil
Ba+Bb	42·2	0·96	96	68	4·7	92
20–80%-satd. $(NH_4)_2SO_4$ fraction	43·5	1·0	100	74	5·1	100

effected with a salt gradient, desorption occurring when the solution was approx. 0·25 M with respect to sodium chloride. C_1 component was always associated with a trace of CM-cellulase activity and it has not been possible, thus far, with a variety of salt or pH gradients, to remove the CM-cellulase activity completely. Nevertheless, the partially purified C_1 component (part Bb), like the fraction containing the bulk of the CM-cellulase and the β-glucosidase component (Ba), could only solubilize cotton to a very limited extent (Table 2). In the

latter case, probably only the more accessible regions of the cotton fibre were being attacked, for, when 2 mg. of short fibres (derived from native cotton fibres by shaking under the conditions of the standard assay with the 20–80%-saturated ammonium sulphate fraction; see the Experimental section) were substituted for the 2 mg. of native cotton fibres in the standard solubilization assay, no loss in weight was observed with part Ba, whereas part Bb produced only 2% solubilization. Recombination of parts Ba and Bb, in the same

proportions as they were present in the 20–80%-saturated ammonium sulphate fraction, showed that 96% of the cellulase (solubilization) activity had been recovered.

Recoveries of the CM-cellulase and the β-glucosidase activities were 101% and 97% respectively.

The column effluent was colourless, in contrast with the highly pigmented unfractionated enzyme solution.

Short-fibre-forming activity was shown by both part Ba and part Bb, but appeared to be more closely associated with part Ba. This part had a specific activity of 64units/mg. of protein, whereas that of part Bb was only 11. All of the original activity could be accounted for in the two parts and there appeared to be no synergism between them.

As far as the S-factor was concerned, 69% of the activity put on the column was found in part Ba, whereas the C_1 component (part Bb), after dialysis to remove the sodium chloride, showed none of this activity. Synergistic effects were apparent here, for recombination of the components in their original proportions accounted for 92% of the original S-factor activity (Table 2).

Further fractionation of C_x component on SE-Sephadex. The conditions described by Selby & Maitland (1967) for the separation of the cellobiase and the CM-cellulase of *T. viride* on a column of SE-Sephadex were not suitable for the separation of the β-glucosidase and the CM-cellulase of *T. koningii*. However, a further fractionation of the CM-cellulase of part Ba (Fig. 6) was achieved on a short column of SE-Sephadex at pH 5·0, when 18% of the CM-cellulase and all of the β-glucosidase was adsorbed from a solution 5mM with respect to sodium succinate buffer: these were finally eluted with a salt gradient, desorption occurring when the eluent was approx. 0·06M with respect to sodium chloride (Fig. 7). Recoveries of CM-cellulase and β-glucosidase were 92% and 93% respectively.

The first 39 fractions were examined for S-factor directly, and combined fractions 40–50, 51–60, 61–62 (part Cb, Fig. 7) and 63–70, after dialysis to remove the sodium chloride: activity was found only in fractions showing CM-cellulase activity. Of the S-factor activity put on the column 7·4% lay in part Ca (Table 3), and part Cb could only account for a further 14·6%. Recombination of these two parts in the proportions in which they were applied to the column restored 51% of the original activity.

Part Ca, with its peaks of activity in tubes 9 and 15, obviously contains at least two CM-cellulase components, and the result of selective heat destruction suggest that β-glucosidase is not identical with CM-cellulase in part Cb. At pH 4·0 and 60° for 20min. all of the β-glucosidase and only 40% of the CM-cellulase was destroyed.
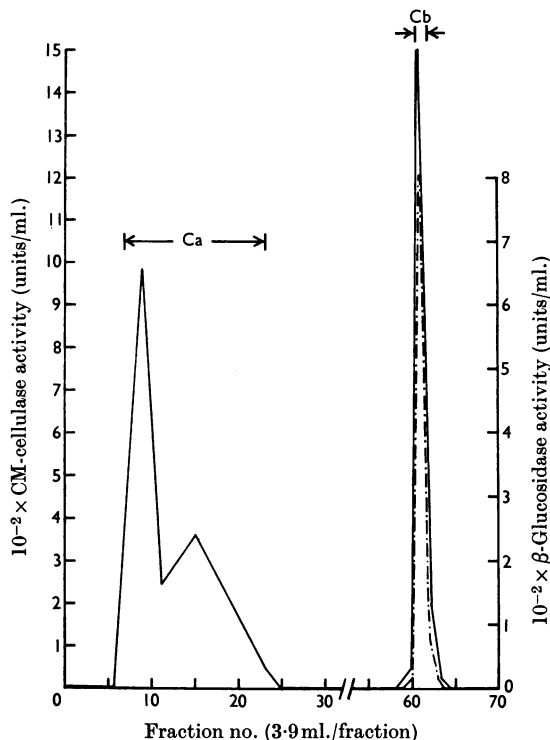

Fig. 7. Separation of C_x components on a column of SE-Sephadex. Portion Ba from the DEAE-Sephadex column (Fig. 6) was dialysed and concentrated by freeze-drying. The concentrate was made up to a volume of 7ml. with succinate buffer (sodium succinate–sodium hydrogen succinate), pH 5·0, and 2ml. was applied to the column (32cm. × 1·6cm.). Elution was initially with 5mM-succinate buffer, pH 5·0, and then after the passage of 152ml. with a salt gradient (125ml. of 5mM-succinate buffer, pH 5·0, and 125ml. of 5mM-succinate buffer, pH 5·0, 0·2M with respect to NaCl). The eluate was examined for CM-cellulase (——) and β-glucosidase (–·–·–) activities. Fractions were combined as shown in the Figure under Ca and Cb. Assays were carried out as stated in the text.

Synergistic effects between fractions. Reconstitution experiments were carried out for solubilization of cotton and S-factor activity in which fractions set aside in the first two stages of chromatography were combined with fractions obtained in the last stage. Dilutions were made so as to make all fractions equivalent in terms of starting material.

A comparison of the results shown in Tables 3 and 5 shows that the C_1 component (part Bb from the DEAE-Sephadex column) had a potentiating effect on the S-factor activity that was associated with parts Ca and Cb (isolated from the SE-Sephadex column). Recombination of parts Bb, Ca and Cb in their original proportions gave an overall

Table 3. *S-factor activity of the components separated on SE-Sephadex*

See legend to Fig. 7 for details of separation and sample used. Portions Ca and Cb were dialysed, concentrated and diluted with acetate buffer (acetic acid–NaOH), pH 5·0, so that the 0·5 ml. of each solution used in the S-factor assay was equivalent in terms of enzyme concentration to 0·5 ml. of the starting material [20–80%-saturated $(NH_4)_2SO_4$ fraction diluted 50-fold]. Assays were done as stated in the text. The S-factor activity applied to the column was 304 units.

Sample	Increase in swollen wt. (mg.)	Units/ 0·5 ml.	Total units in sample	Recovery of S-factor activity (%)
Ca	4	0·25	17·9	7·4
Cb	8	0·5	35·8	14·6
Ca+Cb	29	2·0	155	51

Table 4. *Effect of the components separated on SE-Sephadex on the solubilization of cotton, alone and in combination with C_1 component*

See Fig. 7 for details of the separation. The volumes of portions Bb (Fig. 6), Ca and Cb (Fig. 7) were adjusted, after dialysis and concentration by freeze-drying, so that the volumes were equivalent in terms of the starting material [20–80%-saturated $(NH_4)_2SO_4$ fraction diluted 50-fold]. Assays were as detailed in the text.

Sample	Solubilization (%)	Unit/ml.	Recovery of cellulase activity (%)
Ca	4·1	0·02	2
Cb	4·8	0·03	3
Bb+Ca	32·5	0·48	48
Bb+Cb	25·1	0·30	30
Bb+Ca+Cb	39	0·77	77
20–80%-satd. $(NH_4)_2SO_4$ fraction	43·6	1·0	100

Table 5. *Synergistic effects on S-factor activity obtained by recombination of components separated on the various Sephadex gels in their correct proportions*

The final volumes of the portions from the various columns were adjusted so that when 0·5 ml. of each was mixed the assay contained the same proportion of that component that was present in the original 20–80%-saturated $(NH_4)_2SO_4$ fraction. Assays were carried out as detailed in the text.

Sample	Increase in swollen wt. (mg.)	Units/ml.	Recovery of S-factor activity (%)
Bb+Ca	8	0·55	11
Bb+Cb	15	1·0	20
Bb+Ca+Cb	52	3·6	72
Bb+Ca+Cb+Ab	58	4·0	80
20–80%-satd. $(NH_4)_2SO_4$ fraction	73	5	100

recovery of 72% of the original S-factor activity, and this was increased to 80% by the addition of the low-molecular-weight CM-cellulose that had been separated on Sephadex G-75 (part Ab).

In the assay for solubilization of cotton, component C_1 synergized more effectively part Ca from the SE-Sephadex column than it did part Cb from the same separation (Table 4). A combination of all three fractions in the same proportions in which they were present in the original 20–80%-saturated ammonium sulphate fraction accounted for 77% of that activity.

DISCUSSION

The importance of culture conditions for microbial cellulase production has been examined many times in the past and in particular for *Trichoderma*

cellulase by Norkrans & Wahlström (1961) and Toyama (1958). Toyama (1958), in his experiment conducted to determine the most favourable conditions for the production of *T. koningii* cellulase, found that greater activity was always associated with surface cultures with a good oxygen supply than with stationary submerged cultures. My findings are in keeping with these results, for attempts to prepare large volumes of enzyme (10 l.) by stationary bubble-aerated submerged cultures gave variable results. In only a few cases were active filtrates produced and these did not compare favourably with filtrates obtained from the surface cultures.

Culture filtrates used in the present work were harvested after 28 days of incubation. These conditions were obviously not ideal for optimum cellulase activity, which seemed to increase in potency for 10 days and to decline slightly after 20 days, but this work was initially orientated towards obtaining an enzyme that was capable of producing the short fibres first reported by Toyama (1960) and later studied by Halliwell (1965) and Marsh (1966): short-fibre-producing activity increased until 39 days of incubation. It was my intention to employ the short-fibre assay detailed by Halliwell (1965) as a rapid measurement of cellulase action and to investigate the possible association of the enzyme responsible for the production of these short fibres with one of the other enzyme species. However, separation of the C_1 enzyme from the C_x components on DEAE-Sephadex showed that the short-fibre-forming activity was associated with both these components. The bulk of the activity lay in the C_x component, which had a specific activity of 64 units/mg. of

protein as against a value of 11 for the C_1 component. The two components were additive in this respect.

Cotton fibre that had not been treated chemically has been used as the substrate for the assay of true cellulase action and hence for the evaluation of C_1 component (no direct measure of C_1 component is yet available because its mode of action is still not understood). Hydrolysis over and above that which could conceivably be accounted for in terms of the amorphous content of the cotton fibre has been used as the criterion for true cellulase action.

The cellulase system elaborated by *T. koningii* differed from *T. viride* cellulase examined by Selby & Maitland (1967) in that it contained very little proteolytic activity, but resembled the latter in that removal of the low-molecular-weight CM-cellulase component did not result in any decrease in the effectiveness of the cellulase component in degrading cotton. The various CM-cellulase components from the two sources appeared to differ only in the relative proportions of each present and, though this could perhaps be accounted for in terms of the differing methods of preparation of the enzyme, it could also be a consequence of choice of method for estimation; whereas Selby & Maitland (1967) used a viscosimetric method, I favoured a reducing-sugar method. Another point of experimental difference between myself and Selby & Maitland (1967) lies in my use of nitrophenyl glucoside instead of cellobiose for the assay of oligosaccharase activity. The relative activity towards these two substrates may vary considerably in passing from one type of β-glucosidase preparation to another according to source. Preliminary experiments with a *T. koningii* preparation, however, indicate that no nitrophenyl β-glucosidase (or cellobiase) active fraction was lacking in the other type of activity.

Unlike Selby & Maitland (1967) I have not been able to prepare C_1 component completely free from CM-cellulase activity, but it may be that this is again merely a question of the measurement of different parameters. The reducing-sugar method of assay would favour the detection of a C_x type of enzyme that attacked CM-cellulose from the chain ends: both endoglucanase and exoglucanase activity have been reported to be associated with a *Trichoderma* cellulase preparation (Li *et al.* 1965). Mandels & Reese (1964), too, have found difficulty in removing the traces of CM-cellulase activity (measured by reducing-sugar production) from the C_1 component (*T. viride* cellulase), but they did succeed in obtaining a portion of the C_1 component pure after electrophoresis. Despite the trace of CM-cellulase activity, however, my C_1 component had very little solubilizing action on native cotton, but when combined with the C_x components in their original proportions 77% of the original solubilizing activity was recovered. Similar synergistic effects were observed by Mandels & Reese (1964), Flora (1965) and Selby & Maitland (1967) in their studies of *T. viride* cellulase: Selby & Maitland (1967) obtained complete recovery of activity towards a cotton yarn that had been autoclaved at 140° in 1% sodium hydroxide for 6 hr. to remove impurities.

Alkali swelling factor (S-factor) was found in all fractions showing C_x activity. Of these components isolated by the various fractionation procedures only the C_1 component, with its trace of CM-cellulase activity, was devoid of S-factor activity. However, C_1 component did make some contribution to the overall S-factor activity shown by the unfractionated complex in that it showed synergistic effects when mixed with all other fractions showing C_x activity. Recovery of S-factor in the fractionation procedure involving SE-Sephadex was only 51%, though recoveries of the CM-cellulase and β-glucosidase components were 92% and 93% respectively. Perhaps another component showing only S-factor activity has been lost at this stage; no report of the isolation of such an enzyme has, however, appeared in the literature thus far. Reese & Gilligan (1954) were inclined to the opinion that S-factor was a C_x type of enzyme. That this C_x type of enzyme need not necessarily be identified with CM-cellulase activity was suggested by Youatt (1962) in his studies on culture filtrates from *Aspergillus terreus* and *Stachybotrys atra*. Whether or not S-factor exists as a separate enzyme, the bulk of this activity, at least in culture filtrates from *T. koningii*, seems to be closely associated with enzymes possessing C_x activity, and it is possible that the swelling factor results from the synergistic effect of several enzymes acting in concert.

It would appear from the present results that the cellulase complex is not an adventitious aggregate of enzymes, for in spite of certain procedural differences the system examined by me resembles that of Selby & Maitland (1967) and that of Mandels & Reese (1964) very closely; these similarities seem to suggest one fundamental mechanism rather than a random mixture of enzymes. Moreover, it is evident that for future studies of cellulase action it will be adequate to concentrate on C_1 component and CM-cellulase with the primary objective of reducing that action to meaningful chemical terms by sequential action of these two enzymes.

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