The Cellulase of *Trichoderma viride*

SEPARATION OF THE COMPONENTS INVOLVED IN THE SOLUBILIZATION OF COTTON

By K. SELBY AND C. C. MAITLAND
Shirley Institute, Didsbury, Manchester 20

(Received 16 December 1966)

1. Culture filtrates from *Trichoderma viride* have been fractionated by gel filtration on Sephadex G-75 followed by ion-exchange chromatography on DEAE- and SE-Sephadex. 2. The components essential for attack on cotton are a carboxymethylcellulase, a cellobiase and a third (C_1) component which has no action on CM-cellulose, cellobiose or cotton. 3. These components, which together can completely convert cotton into water-soluble products, lose this ability when separated and regain it quantitatively when recombined in their original proportions.

The study of the mechanism of action of the enzyme systems from cellulolytic micro-organisms has now progressed to a stage where interest centres on the type of enzyme that must be produced by organisms which degrade highly ordered forms of cellulose such as unmodified fibrous cotton. This type of enzyme (C_1 -enzyme) was postulated by Reese, Siu & Levinson (1950), who recognized that it must be distinct from those 'cellulases' (C_x) capable of attacking only swollen or chemically modified forms of cellulose. Because of failure to produce culture filtrates with significant activity towards unmodified cotton most of the work in the following decade was confined to studies of C_x activity. More recently (Selby, Maitland & Thompson, 1963) culture filtrates have been prepared from *Myrothecium verrucaria* capable of extensive attack on cotton, provided that repeated applications of fresh quantities of filtrate are made. Extracts from *Trichoderma viride* are even more active (Mandels & Reese, 1964) and have recently been fractionated by these workers and by many others (Iwasaki, Hayashi & Funatsu, 1964; Shikata, 1964; Niwa, Kawamura & Nisizawa, 1965; Ogawa & Toyama, 1965; Li, Flora & King, 1965). A C_1 -type enzyme was found to be the only component capable, on its own, of solubilizing cotton (Mandels & Reese, 1964; Flora, 1965) and 'crystalline hydrocellulose' (Li *et al.* 1965; Flora, 1965), although this activity was enhanced by synergism with C_x -components. This type of synergism makes it imperative that enzyme components are rigorously purified if the mechanisms of their individual actions are to be examined. The present paper shows that, when highly purified, the C_1 -component of *T. viride* is no longer able to solubilize cotton; recombination with the C_x -components (a

cellobiase and a carboxymethylcellulase), however, restores all the activity of the original culture filtrate. Li *et al.* (1965) have stressed the desirability of thus accounting for the original activity against 'highly organized (crystalline) cellulose', but it is not clear from their published results that this was done.

MATERIALS

Organism. *Trichoderma viride* was strain 92 027 from the Commonwealth Mycological Institute, Kew, Surrey. The cotton flock on which it was grown, and the cotton yarn that was used for measurements of cellulase activity, were as described by Selby *et al.* (1963). Sephadex G-75, DEAE-Sephadex (A-50) and SE-(sulphoethyl)-Sephadex (C-50) were supplied by Pharmacia (G.B.) Ltd., London. Cellobiose was obtained from Thomas Kerfoot and Co. Ltd., Vale of Bardsley, Lancs. Yeast extract and mycological peptone were supplied by Oxo Ltd., London. Malt extract was obtained from Boots Pure Drug Co. Ltd., Nottingham. Distiller's solubles were from Hiram Walker and Sons, Dumbarton, Scotland.

METHODS

Growth of Trichoderma viride. The organism was grown in 250 ml. shake flasks (rotary shaker, 0.5 in. eccentricity, 140 rev./min.) in 75 ml. of a medium containing cotton (3%), NH_4NO_3 (0.06%), NaNO_3 (0.38%), KH_2PO_4 (0.02%), K_2HPO_4 (0.015%), NaH_2PO_4 (0.2%), Na_2HPO_4 (0.15%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.03%), urea (0.03%), asparagine (0.05%), malt (0.02%), yeast (0.02%), mycological peptone (0.02%), and distiller's solubles (0.02%). In addition, each litre of medium contained trace elements as follows: $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (1.2 mg.), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.054 mg.), H_3BO_3 (0.06 mg.), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.2 mg.), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2.0 mg.), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.08 mg.), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4 mg.), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.04 mg.). After incubation for 9 days at 25° the contents of the flasks were filtered and the filtrate (culture filtrate) was protected from infection by adding 0.005 mole of $\text{NaN}_3/\text{l.}$

Preparation of Sephadex G-75 for column chromatography. Dry Sephadex G-75 (bead form, 40–120 μ) was separated by air flotation in a vertical cylinder, the fine particles being blown out of the top of the cylinder and collected. The separation was slow, and flow at any one rate was continued for 24 hr. before increasing it to harvest the next larger fraction of particles. Particles of 50–65 μ diam. were swollen by suspension for 14 days in a culture filtrate from *T. viride* diluted fivefold with sodium phosphate eluting buffer (0.01 M in total phosphate, 0.1 M in NaCl and 0.005 M in NaN_3 , pH 6.3). They were then washed with, and resuspended in, eluting buffer.

Vertical tubes of precision-bore glass (2.5 cm. $\times$ 60 cm.) were fitted with column ends designed to give minimal hold-up of liquid. A series of closely-spaced V-shaped parallel grooves, with a single diametral V-shaped groove at right angles, was cut across the wider face of a silicone-rubber bung, chosen so that its wider end fitted the column reasonably tightly. A 1.1 mm. hypodermic tube was pushed through the centre of the bung so that one end was flush with the bottom of the groove and the other could take a narrow-bore vinyl delivery tube. The cut face of the bung was covered with a circle (2.5 cm. diam., 0.75 mm. thick) of sintered polythene (pore size 50 μ), which was made wettable by treatment with a solution of *n*-octanol in ethanol.

One of these ends was inserted into the bottom of the column and the suspension of Sephadex G-75 was siphoned in, to avoid air bubbles, and allowed to settle under gravity aided by a slow percolation of eluting buffer. A second end was then carefully introduced at the top of the column and pushed down until it reached the upper surface of the bed, with the outlet at the bottom of the column closed to avoid compression of the gel. In use the flow rate was 10 ml./hr. and a flow of buffer was maintained at all times. For use in cascade two similar columns were connected with narrow-bore vinyl tubing.

Calibration of Sephadex G-75 columns. The relation between elution volume and the logarithm of molecular weight for a number of reference materials on the two

columns in cascade was established (cf. Selby & Maitland, 1965).

Ion-exchange chromatography. Columns (1.8 cm. precision bore, with ends as described above) were packed with particles of SE- or DEAE-Sephadex of 150–600 μ diam. (fragmented gel, as supplied, with the fines removed). Buffer gradients were applied by means of a nine-chamber variable-gradient mixer (Peterson & Rowland, 1961).

Measurement of carboxymethylcellulase activity. The rapid method described by Selby & Maitland (1965) was used.

Measurement of cellobiose activity. Cellobiose (0.667 mg./ml.) was dissolved in succinate buffer solution (pH 4.5, 0.01 M in Na^+ and 0.005 M in NaN_3). A portion (100 μ l.) of the enzyme solution to be assayed, or of an appropriate dilution of it, was incubated with the cellobiose solution (3 ml.) for 3 hr. at 40°. The increase in reducing power was measured by the alkaline iodine method (Selby, 1965), the addition of the carbonate-bicarbonate buffer terminating the enzymic reaction. The reducing power of the added enzyme sample was allowed for by a blank determination. The percentage conversion of cellobiose into glucose was used as a measure of the relative cellobiose activity (Fig. 1); the accuracy was $\pm 2\%$ when the cellobiose conversion was kept within the range 20–60%. The reasons for the choice of conditions in this assay are discussed below.

Measurement of activity towards cotton. Solubilization by a culture filtrate in 7 days incubation was found to be greatest at pH 4.5 and 40° and these conditions were chosen for a standard assay. The pH of a sample of the enzyme solution (0.5 ml., unless otherwise stated) was adjusted to 4.5 by addition of a concentrated solution of either succinic acid or Na_2HPO_4 and the volume was made up to 1.5 ml. with succinate buffer (0.01 M in Na^+ , pH 4.5); a sample (5.0 mg.) of cotton yarn was added and incubated at 40° for 7 days. The residual cellulose was removed by filtration on a disk of glass-fibre filter paper (1 cm. diam., Whatman GF/A), washed briefly and successively with water, aq. 2N- NH_3 soln., 2N-acetic acid and again with water, sucked dry, transferred with the disk to a test tube and estimated by oxidation with 0.18N-potassium dichromate in 50% (v/v) H_2SO_4 (5 ml.) at 100° for 30 min. After dilution

Fig. 1. Calibration curve for cellobiose assay. Cellobiose conversion was measured, by the method given in the text, at various dilutions of the reference solution (see the text); a relative cellobiose activity of 1.0 is that of the undiluted reference solution.

Fig. 2. Calibration curve for assay of activity towards cotton. Activity is expressed relative to that of the reference solution (see the text). Experimental details are given in the text.

with water (15 ml.) the excess of dichromate was titrated with 0.1N-ferrous ammonium sulphate, phenylanthranilic acid being used as an internal indicator. Although the oxidation of cotton under these conditions is stoichiometric (1.0 ml. of 0.1N-ferrous ammonium sulphate = 0.675 mg. of dry cotton), in practice, each series of determinations included a blank sample of unattacked cotton (5.0 mg.), so that frequent re-standardization of the reagents was unnecessary. Enzyme samples from gel filtration usually contained 0.1M-NaCl but this had a negligible effect on cellulase activity. The reference solution (see below) at several dilutions gave the solubilizations shown in the calibration curve (Fig. 2). Cellulase activity was most accurately measured by this method when the solubilization was between 20% and 80%.

RESULTS

Occurrence of product inhibition during solubilization of cotton. When glucose, the ultimate product of attack, was added before incubation, solubilization was inhibited. This result (Fig. 3) differs from that of Mandels & Reese (1964), who found that 1% (w/v) glucose gave a slight (8%) enhancement of this activity. Glucose retarded the solubilization of cellulose to a much greater extent than did any other polyhydric substance examined. In order of increasing inhibition at 3% (w/v) concentration these were sorbitol, glycerol, xylose, fructose, galactose and mannose; 3% mannose was only as effective as 1% glucose. Cellobiose was not included in this experiment in view of the high cellobiase activity of the enzyme preparation. Since total solubilization of the cotton used in our assay would give a 0.36%

solution of glucose the assay is unavoidably affected to some degree by product inhibition.

Solubilization of cotton by culture filtrate. The first 15–20% of the cotton was solubilized at a substantially constant rate, which thereafter fell gradually as total solubilization was approached asymptotically. First-order plots had slopes which were constant up to about 35% solubilization (Fig. 4) and from this level to total solubilization either remained constant or decreased slightly with time, depending on the enzyme preparation used. Results published by Mandels & Reese (1964) for the solubilization of cotton by *T. viride* (Q.M.6a) cellulase also give a straight first-order plot.

Fractionation of a culture filtrate from T. viride on Sephadex G-75. Culture filtrate was concentrated in a rotary evaporator, dialysed through gold-beater's skin against eluting buffer and fractionated on Sephadex G-75 under the same conditions as for the reference materials. The fractions were examined for extinction at 280 m μ , and for activity towards CM-cellulose, cotton yarn (solubilization) and cellobiose (Fig. 5). The molecular weight of the smallest (CM-cellulase) component was estimated, from its elution volume, as 12600; the components of higher molecular weight (48000–62000) were only partially resolved by a single passage through these columns. The peak of activity towards cotton fell between that of extinction at 280 m μ and those of cellobiase and CM-cellulase activity. A mixture of portions from fractions 42 and 45 had 20% more activity towards cotton than two portions from fraction 44, suggesting that this activity resulted from synergism between at least two components. The CM-cellulase of lower molecular weight had no effect on

Fig. 3. Inhibition of cellulase action by glucose. Glucose, in the quantities shown, was added to the reference cellulase preparation (see the text) before measurement of its activity towards cotton.

Fig. 4. Solubilization of cotton by a culture filtrate from *T. viride*, showing the linearity of the first-order plot up to 35% solubilization.

Fig. 5. Fractionation of a tenfold concentrate (5ml.) of a culture filtrate from *T. viride* on two columns of Sephadex G-75 in cascade at pH 6.3. Other details are given in the text. The shading shows which fractions were united.

the activity towards cotton of the enzymes in fractions 42 and 45, either alone or mixed together.

Attempts to resolve the synergistic components further by recycling chromatography on Sephadex G-75. The method used was that described by Porath & Bennich (1962). Several portions of a tenfold concentrate of a culture filtrate were fractionated as described above and fractions 40–49 were combined, reconstituted and dialysed to give a partially purified culture filtrate concentrated approximately 80-fold. A portion (10ml.) of this concentrate was cycled four times through the same Sephadex G-75 columns; the component detected as a peak of extinction was clearly separated from

Fig. 6. Separation of C_1 and C_x components from *T. viride* (fractions 40–49 from Fig. 5) by recycling chromatography on Sephadex G-75 at pH 5.0. Details are given in the text. The arrow shows the position of the protease.

the cellobiase, but the cellobiase and CM-cellulase still overlapped. Attempts to demonstrate synergism between these components failed; very small solubilizations of cotton, indicating 93% loss of activity, were obtained and, moreover, a second peak of CM-cellulase had appeared. These troubles were probably caused by a protease, which was active at pH 6.3 and which had an elution volume at this pH similar to that of the enzymes being fractionated. Since the protease was less active at pH 5.0 the separation was repeated at this pH (0.01M-sodium acetate buffer, 0.1M in sodium chloride and 0.005M in sodium azide) (Fig. 6). The component detected as a peak of extinction was again adequately separated from the remainder and the protease (arrowed) was separated from the β -glucosidases (the cellobiase and CM-cellulase),

which had changed their order of emergence but were still not separated from each other. Maximum synergism was obtained between fractions 14 and 30, suggesting that the CM-cellulase was more effective than the cellobiase in synergizing with the component detected by its extinction, which was presumably C_1 . The recovery of activity towards cotton (33%) was better at pH 5.0 than at pH 6.3.

Removal of protease from partially fractionated culture filtrate. After partly successful attempts to remove the protease by specific adsorption on solid proteins, it was found to be adsorbed on SE-Sephadex (C-50) at pH 5.0 and, if necessary, recovered by elution at pH 7.0. A large sample of culture filtrate was therefore purified preparatory to further attempts to separate the synergistic components.

Culture filtrate (11.) was concentrated tenfold, dialysed against eluting buffer (pH 6.3) and fractionated (in two parts; 60 ml.) on a wide column (5.0 cm. $\times$ 30 cm.) of Sephadex G-75. The fractions were batched to include all components that would have appeared in fractions 38–51 in Fig. 5. This crude fractionation removed materials of low molecular weight, including the CM-cellulase that appears between fractions 66 and 72 in Fig. 5. In a similar fractionation it had been shown that activity against cotton was not lost; in fact, equivalent dilutions of the cellulase before and after removal of the material of low molecular weight gave solubilizations of 58.0% and 56.8% respectively. The batched fractions were concentrated to 100 ml., dialysed against sodium succinate buffer (0.01 M in Na^+ and 0.005 M in sodium azide, pH 5.0) and passed through a column (1 cm. $\times$ 8 cm.) of Sephadex C-50 equilibrated with the same buffer, followed by 10 ml. of the buffer. The resulting protease-free partially purified culture filtrate was used for the further fractionation studies described below and is called the 'reference solution'. Its activities towards cellobiose and cotton can be seen in the calibration curves (Figs. 1 and 2); its CM-cellulase activity was 58×10^{-2} unit.

Further fractionation of partially purified culture filtrate by ion-exchange chromatography. In view of the small differences between the elution volumes of the synergistic components on Sephadex G-75, the behaviour of the system on ion-exchange forms of Sephadex was studied. On DEAE-Sephadex neither cellobiase nor CM-cellulase was strongly adsorbed from 0.01 M buffer solutions with pH values below 7.0, and at higher pH values activity was lost. However, the C_1 -component was adsorbed from buffer solutions with pH values between 5.5 and 6.5 and could be recovered without loss by elution at pH 3.7. When the C_1 -component was obtained by elution in a pH gradient (Fig. 7), it had little action on cotton and neither cellobiase

Fig. 7. Separation of the C_1 - and C_x -components from *T. viride* by chromatography on a DEAE-Sephadex column (1.8 cm. $\times$ 12.5 cm.; sodium succinate buffers 0.01 M in Na^+ and 0.005 M in NaN_3). A sample (10 ml.) of the reference solution (see text) slightly diluted (CM-cellulase activity 51.5×10^{-2} unit, relative cellobiase activity 0.88) was applied to the column at pH 5.35, followed by a steep pH gradient (30 ml. each of buffer solutions at pH 5.35 and 4.6). The C_x -component had emerged before the pH of the eluate began to change. When the pH had fallen to about 5.0, the gradual pH gradient shown in the Figure was applied [buffer solutions at pH 4.6, 3.8, then four at 3.6 (30 ml. in each compartment)] to elute the C_1 -component. The rate of flow was 10 ml./hr. The shading shows which fractions were united.

nor CM-cellulase activity; it is believed to be the most homogeneous specimen of a C_1 -component yet reported.

The cellobiase activities of dilutions of the batched fractions (4–10) were compared with those of the reference solution at corresponding dilutions chosen to allow for the fact that, in chromatography and batching, the C_x -components had been diluted 2.55 times; there had been negligible loss of activity. The CM-cellulase activity in fractions 4–10 was 23×10^{-2} unit, which also represents complete recovery. The C_1 -component had been diluted 1.6 times by chromatography and was therefore 1.8 times as dilute as in the reference solution. Before assessing the effect of recombination a sample (1.8 ml.) of the fractionated C_1 -component was diluted to 2.55 ml. so that it had the same concentration relative to the reference solution as C_x . The

Table 1. Activities of the C_1 - and C_x -fractions from *Trichoderma viride* towards cotton

Component(s) (0.5 ml. of each component, total volume made up to 1.5 ml. with buffer solution)	Solubilization (%)	Relative cellulase activity	Recovery of cellulase activity (%)
Reference solution diluted 2.55 times	59.0	0.39 ₅	100
C_1	2.0	< 0.01	1
C_x	3.8	0.02	5
$C_1 + C_x$	59.5	0.40 ₅	102

activities towards cotton of these diluted components were compared with that of the reference solution, diluted 2.55 times (Table 1) to confirm that no activity had been lost during separation of the C_1 - and C_x -components.

Fractionation of the C_x -component. The view that the cellobiase and CM-cellulase activities of C_x were due to different enzymes, suggested by their partial separation on Sephadex G-75, was strengthened by the results of selective destruction. At pH 2.5 and 40° 99.7% of the cellobiase activity of C_x was destroyed in 18 hr., and 54% of the original CM-cellulase activity remained. Conversely, after 18 hr. at pH 7.5 and 40°, 99.6% of the CM-cellulase activity was destroyed and 51% of the cellobiase activity remained. However, these treatments also reduced the ability of the two enzymes to act synergistically with the C_1 -component; whereas intact C_x , added to C_1 , gave 69% solubilization of cotton, the heat-treated cellobiase and CM-cellulase together, added to C_1 , gave only 32% solubilization, although their concentrations had been chosen to give undiminished cellobiase and CM-cellulase activities.

Li *et al.* (1965) have described a method for separating the exoglucanase (cellobiase?) and endoglucanase of *T. viride*, in which the endoglucanase was adsorbed on alkali-swollen cellulose from 0.05 M-phosphate buffer at pH 5.5 and 2° and was then eluted with water. When this method was applied to our C_x -fraction the cellobiase, which was not adsorbed, was quantitatively recovered free from CM-cellulase, but elution of the adsorbed CM-cellulase, whether with water, 1% CM-cellulose, aq. 30% ethanol or 0.01 M-EDTA, was slow and incomplete.

A better method of separation was devised with SE-Sephadex, with a long column to compensate for weak retention. The behaviour of these β -glucosidases on SE-Sephadex was studied in the region pH 4.5–5.5. pH proved to be critical in this separation; too low a pH led to loss of cellobiase but at higher pH values the separation was inadequate. The best compromise was achieved at pH 5.1 (Fig. 8) and the recoveries of both CM-cellulase (fractions 12–20) and cellobiase (fractions 23–29) were 96%.

Fig. 8. Separation, on a SE-Sephadex column (1.8 cm. $\times$ 63.5 cm.; sodium succinate buffer, pH 5.1, 0.01 M in Na^+ and 0.005 M in NaN_3), of the CM-cellulase and cellobiase from a sample (3.2 ml., CM-cellulase activity 45.0×10^{-2} unit, relative cellobiase activity 0.63) of C_x from *T. viride*. This sample was obtained from a fractionation of C_1 and C_x similar to that shown in Fig. 7, from which, to avoid excessive dilution, two fractions only (corresponding to fractions 6 and 7 in Fig. 7) were taken from the peak of C_x , giving a somewhat altered ratio of CM-cellulase to cellobiase activity. The rate of flow was 10 ml./hr. The shading shows which fractions were united.

These small elution volumes (column volume = 50 fractions) show that this separation depended on exclusion by ionic repulsion and not on retention by ionic attraction. The CM-cellulase had no activity on cellobiose, and the cellobiase had very little on CM-cellulose.

Behaviour of the separated components on Sephadex G-75. Since they had unchanged elution volumes when chromatographed on the calibrated

Table 2. *Solubilization of cotton by components of Trichoderma viride cellulase, alone and in combination*

Component(s) (0.5 ml. of each component, total volume made up to 1.5 ml. with buffer solution, pH 4.5)	Solubilization (%)	Relative cellulase activity	Recovery of cellulase activity (%)
Reference solution diluted 4 times	46.0	0.24	100
C ₁ + C _x	47.7	0.25	104
C ₁	1.0	< 0.01	1
CM-cellulase	3.2	0.01	4
Cellobiase	0.8	< 0.01	< 1
CM-cellulase + cellobiase	1.8	< 0.01	2
C ₁ + CM-cellulase	21.0	0.08 ₅	35
C ₁ + cellobiase	14.0	0.05	20
C ₁ + cellobiase + CM-cellulase	47.2	0.25	104

pair of columns of Sephadex G-75, each of the separated components had survived the whole purification procedure without detectable change of molecular weight.

Synergism between components. The cellobiase and CM-cellulase had been somewhat diluted during separation on SE-Sephadex and were therefore concentrated and dialysed against buffer (pH 4.5) to give activities corresponding to a fourfold dilution of the reference solution (CM-cellulase = 14.5×10^{-2} unit, cellobiose conversion = 40%). The solution containing C₁ was diluted to a similar equivalent concentration (1.8 ml. of solution + 2.2 ml. of pH 4.5 buffer) as was that containing C_x (2.55 ml. of solution + 1.45 ml. of buffer). The activities towards cotton of combinations of these solutions are shown in Table 2. On CM-cellulose or cellobiose there was no synergism between any of the three components. Activity towards cotton, lost by separation of the components, was recovered completely on recombining all three in the proportions present in the reference solution. There was little synergism between CM-cellulase and cellobiase. Neither component alone could restore all the lost activity of C₁ and the CM-cellulase was more effective than the cellobiase, as had been tentatively deduced from the results of recycling chromatography on Sephadex G-75 at pH 5.0.

Effect of dialysis on the solubilization of cotton by C₁ and C_x. Each enzyme preparation (0.5 ml.), diluted with succinate buffer solution (1.0 ml., 0.01 M in Na⁺ and 0.005 M in sodium azide, pH 4.5), was applied to a sample (5 mg.) of cotton and continuously dialysed against the same buffer (150 ml.) during incubation for 7 days at 40° in a glass cell (1.8 cm. diam.), closed at the bottom with goldbeaters' skin. The activity of C_x was not enhanced but that of C₁ was increased by 50% (solubilization rose from 5.6% to 8.0%). This effect, though small, is reproducible and has been repeated with other preparations of C₁.

DISCUSSION

Determination of relative cellobiase activities. The assay for cellobiase departed from ideal in two ways, which led to the use of non-absolute units. First, the substrate concentration (1.9 mM) was below that needed to saturate the enzyme. Li *et al.* (1965) report for their exoglucanase acting on cellobiose a K_m 2.2 mM and apparent inhibition by the substrate at concentrations above 0.05 M; we have found, measuring conversions of between 5% and 15%, that the rate of conversion shows a sharp maximum at cellobiose concentrations near 5 mM. It seemed preferable to work at a lower concentration, although the rate would be influenced by availability of substrate, rather than at a higher concentration where inhibition predominates. Secondly, substantial degrees of substrate conversion were measured; at lower degrees of conversion the possible effects of product inhibition would be lessened and the conversion would be more nearly proportional to enzyme concentration but the measurement of conversion by this method, or by any other depending on reducing power, would become less accurate because of the initial reducing power of the cellobiose.

Solubilization of cotton by culture filtrate. In the assay of activity towards cotton high levels of conversion were employed in case the earliest stages of cellulase attack were on some particularly susceptible part of the cotton structure but, in view of the nearly first-order course of the solubilization reaction (see below), this fear may have been unfounded. Comparisons between the attack of the cellulase of *Myrothecium verrucaria* and of mineral acid on cotton have already been made (Selby, 1961); others can now be made between acid and the cellulase of *T. viride*. Attack by mineral acid follows first-order kinetics except in the initial stages when a part of the cotton (about 10%) is solubilized at a rate much higher than the

remainder. This reactive part has been termed amorphous, though in the fringe-micellar theory the amorphous part was often considered to be as high as 40% to account for other properties of the cotton (Howsmon & Sisson, 1954; but for a more recent theory see Warwicker, Jeffries, Colbran & Robinson, 1966). A supposed analogy of its structure to that of certain reprecipitated celluloses that are very susceptible to cellulases may have led several workers (Norkrans, 1950; Walseth, 1952; Reese, Segal & Tripp, 1957) to believe that these 'amorphous' regions must also be the first to be attacked by cellulases. The absence of any marked fall in rate during the early stages of solubilization by the cellulase of *T. viride* (Fig. 4) shows that these acid-labile regions are not especially susceptible to these enzymes. This may be because the enzyme molecules are confined by their size to larger openings in the cotton structure and so, for example, may be able to act only on the surfaces of the elementary fibrils of which cotton is composed. In the solubilization of a solid substrate by superficial erosion, first-order kinetics would not be expected unless the erodible area were, throughout the process, proportional to the amount of substrate remaining. Foster & Wardrop (1951) and Sharples (1957) have shown that this requirement is fulfilled by a model in which the erodible dimension of the substrate particles is exponentially distributed (in the statistical sense) and have so explained the first-order erosion of cotton by acid. The first-order erosion by cellulases may be explained by a similar model but, if our present views on cellulase action are correct, the erodible dimension of the fibrils is their width, whereas Sharples (1957) suggests that the dimension erodible by acids is the length of fibrils lying between the acid-susceptible regions.

Properties of the separated components. The C_1 -component is a glycoprotein with a carbohydrate:protein ratio approx. 1:1 [carbohydrate determined as equivalent glucose by orcinol method (Weimer & Moshin, 1952), protein by micro-Kjeldahl digestion followed by determination of ammonia on an amino acid analyser]. Its molecular weight, estimated from its behaviour during gel filtration, is approx. 61 000; it is heat-labile, losing 50% of its potential activity towards cotton on heating in solution at pH 4.5 and 100° for about 2.5 min. It is homogeneous on Sephadex G-75, DEAE-Sephadex and SE-Sephadex and gives a single protein band by disk electrophoresis at pH 6.7 (method of Davis, 1964, as modified by Broome, 1963). The lack of activity in the purified C_1 -component raised the possibility that its role when acting synergistically with the C_x -components might be a non-specific protein effect. However, attempts to enhance the activity of the mixed C_x -components towards cotton by adding serum albumin (0.7 mg./ml.)

failed completely; the already low activity of the C_x preparation was further reduced.

Synergistic action of two enzymes in solubilizing a solid substrate may be of two kinds. It may be either on the substrate, when the first enzyme makes the substrate susceptible to solubilization by the second, or in solution, when the first solubilizes the substrate but a second is needed to remove inhibitory products formed. To test for synergism on the substrate, C_1 and C_x were applied to cotton either simultaneously or in sequence (Table 3). Clearly neither component can complete its action in the absence of the other. The limited synergism found in the sequential treatments could have been due to incomplete removal of one component from the cotton before application of the other. Because continuous dialysis enhanced the solubilization of cotton by C_1 but not by C_x , it seems likely that the synergism may be in solution, C_1 being strongly inhibited by a soluble, dialysable product which C_x is able to remove.

The most significant feature of these results is that the C_1 -component of *T. viride*, when freed from the C_x -components, has little or no action on cellobiose, CM-cellulose or cotton. Mandels & Reese (1964) have reported a similar separation of a culture filtrate from *T. viride* on DEAE-Sephadex, but without prior removal of protease or CM-cellulase of low molecular weight, and the relative positions of their β -, A- and C-components were similar to those of our cellobiase, CM-cellulase and C_1 -component respectively. The major difference was that their C-component had lost less activity towards cotton than had our C_1 -component, so that the synergistic effect was less marked. Flora (1965) also separated a C_1 -component from *T. viride*, by adsorption on Avicel, but found that it still degraded cotton; he obtained only a threefold enhancement of activity on recombining it with his C_x -fraction.

Table 3. *Relative activities of C_1 and C_x applied to cotton separately, together, and in sequence*

These samples of C_1 and C_x were obtained by recycling chromatography (Fig. 6, C_1 comprised fractions 13, 14, 15; C_x comprised fraction 29). C_1 (1.0 ml.) and C_x (0.5 ml.) were either mixed together or diluted to 1.5 ml. Each treatment was for 1 week; when two were applied in sequence, each was for 1 week with thorough washing of the cotton sample with buffer solution (pH 4.5) after the first. The activities are expressed relative to the mixture of C_1 and C_x , which gave a solubilization of 78%.

Treatment	Relative activity (%)
$C_1 + C_x$	100
C_1	8
C_x	2
C_1 then C_x	15
C_x then C_1	28

The fact that the activity lost during the fractionation procedure described here was regained completely on recombining the fractions in their original proportions (Table 2) proved that the C₁-component, though inactive alone, was essential for extensive solubilization of cotton, but its contribution to the synergistic action of the whole cellulase system is still not understood.

We are indebted to our colleague Mrs K. V. A. Thompson, who supplied culture filtrates from *T. viride*, and to Dr N. J. King of Forest Products Research Laboratory, Princes Risborough, who examined the C₁-component by disk electrophoresis. We are grateful to Glaxo Laboratories Ltd. for their sponsorship of this work.

REFERENCES

- Broome, J. (1963). *Nature, Lond.*, **199**, 179.
- Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.
- Flora, R. M. (1965). Ph.D. Thesis: Virginia Polytechnic Institute; Ann Arbor, Mich.: University Microfilms (65-2041).
- Foster, D. H. & Wardrop, A. B. (1951). *Aust. J. sci. Res. A*, **4**, 412.
- Howsmon, J. A. & Sisson, W. A. (1954). In *Cellulose and Cellulose Derivatives*, 2nd ed., p. 231. Ed. by Ott, E., Spurlin, H. M. & Grafflin, M. W. New York: Interscience Publishers Inc.
- Iwasaki, T., Hayashi, K. & Funatsu, M. (1964). *J. Biochem., Tokyo*, **55**, 209.
- Li, L. H., Flora, R. M. & King, K. W. (1965). *Arch. Biochem. Biophys.* **111**, 439.
- Mandels, M. & Reese, E. T. (1964). In *Developments in Industrial Mycology*, vol. 5, p. 5. Washington, D.C.: American Institute of Biological Sciences.
- Niwa, T., Kawamura, K. & Nisizawa, K. (1965). *Proc. 5th Symp. Cellulase and Related Enzymes*, p. 44. Osaka University, Japan: Cellulase Association.
- Norkrans, B. (1950). *Physiol. Plant.* **3**, 75.
- Ogawa, K. & Toyama, N. (1965). *Proc. 5th Symp. Cellulase and Related Enzymes*, p. 85. Osaka University, Japan: Cellulase Association.
- Peterson, E. A. & Rowland, J. (1961). *J. Chromat.* **5**, 330.
- Porath, J. & Bennich, H. (1962). *Arch. Biochem. Biophys. Suppl.* **1**, p. 152.
- Reese, E. T., Segal, L. & Tripp, V. W. (1957). *Text. Res. (J.)*, **27**, 626.
- Reese, E. T., Siu, R. G. H. & Levinson, H. S. (1950). *J. Bact.* **59**, 485.
- Selby, K. (1961). *Biochem. J.* **79**, 562.
- Selby, K. (1965). *J. chem. Soc. p.* 1554.
- Selby, K. & Maitland, C. C. (1965). *Biochem. J.* **94**, 578.
- Selby, K., Maitland, C. C. & Thompson, K. V. A. (1963). *Biochem. J.* **88**, 288.
- Sharples, A. (1957). *Trans. Faraday Soc.* **53**, 1003.
- Shikata, S. (1964). *J. Biochem., Tokyo*, **56**, 1.
- Walseth, C. S. (1952). *T.A.P.P.I.* **35**, 233.
- Warwicker, J. O., Jeffries, R., Colbran, R. L. & Robinson, R. N. (1966). In *Shirley Institute Pamphlet no. 93: A Review of the Literature on the Effect of Caustic Soda and other Swelling Agents on the Fine Structure of Cotton*. Manchester: Cotton Silk and Man-Made Fibres Research Association.
- Weimer, H. E. & Moshin, J. R. (1952). *Amer. Rev. Tuberc. pulm. Dis.* **68**, 594.