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The Cellulolytic Enzymes of *Botryodiplodia theobromae* Pat.

SEPARATION AND CHARACTERIZATION OF CELLULASES AND β-GLUCOSIDASES

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1. Filtrates from cultures of different ages of *Botryodiplodia theobromae* Pat. were fractionated by gel filtration, ion-exchange chromatography and polyacrylamide-gel electrophoresis. 2. Five cellulases (C1, C2, C3, C4 and C5) were found, and their molecular weights, estimated by gel filtration, were 46000–48000 (C1), 30000–35000 (C2), 15000–18000 (C3), 10000–11000 (C4) and 4800–5500 (C5). 3. Cellulase C5 was absent from old culture filtrates. 4. Cellulase C1 had little or no activity on CM-cellulose (viscometric assay), but degraded cotton flock and Whatman cellulose powder to give cellobiose only. 5. The other components (C2–C5) produced cellobiose and smaller amounts of glucose and cellotriose from cellulosic substrates and were more active in lowering the viscosity of CM-cellulose. 6. The ratio of activities assayed by viscometry and by the release of reducing sugars from CM-cellulose increased with decrease in the molecular weights of cellulases C2–C5. 7. Cellobiose inhibited the activities of the cellulases, but glucose stimulated at low concentrations although it inhibited at high concentrations. 8. A high-molecular-weight β-glucosidase (component B1, mol.wt. 350000–380000) predominated in filtrates from young cultures, but a low-molecular-weight enzyme (B4, mol.wt. 45000–47000) predominated in older filtrates. 9. Intermediate molecular species of β-glucosidase (B2, mol.wt. 170000–180000; B3, mol.wt. 83000–87000) were also found. 10. Cellulases C2–C5 acted in synergism with C1, particularly in the presence of β-glucosidase.

Nearly 30 years ago, Reese *et al.* (1950) proposed the C_1–C_x concept to account for the mechanism of cellulolysis by micro-organisms. According to this hypothesis, a form of cellulase designated C_1 initiates the degradation of native cellulose by disaggregating the anhydroglucose chains, and the other forms of cellulase, designated C_x, hydrolyse them into soluble sugars. In recent years, it has been shown that the C_1 enzyme is a β-1,4-glucan cellobiohydrolase which acts by successively removing cellobiose residues from the ends of cellulose chains (Wood & McCrae, 1972; Berghem & Pettersson, 1973; Halliwell & Griffin, 1973; Berghem *et al.*, 1975). The other cellulases, collectively called C_x enzymes, act on swollen or chemically modified celluloses in a random manner, and produce a greater decrease in the viscosity of CM-cellulose per unit increase in reducing sugars liberated (cf. Selby & Maitland, 1965, 1967; Wood, 1971).

It has been pointed out that the attack of cellulose by a series of enzymes, some of which act in a random fashion and others in an endwise fashion, would facilitate the degradation of cellulose chains as the random-cleaving enzymes would increase the number of cellulose chain ends available to the endwise-cleaving components (King, 1963). The obvious implication of this view has been amplified by Wood & McCrae (1972) and Berghem *et al.* (1975), who, on the basis of the endwise-cleaving properties of the C_1 enzyme, suggested that the role of initiating the attack on native cellulose can be assigned to the C_x enzymes and not the C_1 enzyme as originally proposed by Reese *et al.* (1950).

Some properties of the enzyme components found in culture filtrates of the wood-staining fungus *Botryodiplodia theobromae* have been investigated (Umezurike, 1969, 1970*a*,*b*). In the present paper, the fractionation of the enzyme components found in culture filtrates of different age has been investigated in the light of the trends discussed above.

Materials and Methods

Chemicals

o-Nitrophenyl β-D-glucopyranoside, bovine serum albumin and β-lactoglobulin were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; cytochrome *c* and glucagon were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.; pancreatic trypsin inhibitor was from Worthington Biochemical Corp., Freehold, NJ,

U.S.A.; Sephadex G-75, DEAE-Sephadex A-50 and Blue Dextran were from Pharmacia (G.B.) Ltd., Ealing, London W.5, U.K.; cotton flock, a scoured mechanically powdered flock C/8 was from Hutchinson Ltd., Ramsey Mill, Chadderton, Oldham, Lancs., U.K.; CM-cellulose (sodium salt) and the other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Methods

Growth of organism. B. theobromae Pat. (I.M.I. 115626, A.T.C.C. 26123) was grown in 200ml of 0.5% (w/v) cotton flock medium, pH6.2, at 30°C for up to 8 weeks, and culture filtrates were obtained and freeze-dried as described previously (Umezurike, 1970*a*,*b*).

Gel filtration. Gel filtration of portions (3ml) of a solution of the freeze-dried culture filtrates was carried out on a column of Sephadex G-75 as described previously (Umezurike, 1970*b*). The elution buffer was McIlvaine's phosphate/citrate buffer (McIlvaine, 1921) containing 0.1M-NaCl, pH5.0. Molecular weights were estimated by gel filtration on a column calibrated with Blue Dextran (mol.wt. 2000000), bovine serum albumin (monomer mol.wt. 65000), β-lactoglobulin (mol.wt. 35000), cytochrome *c* (mol.wt. 12270), pancreatic trypsin inhibitor (mol.wt. 6500) and glucagon (mol.wt. 3485) by the method of Andrews (1964, 1965).

Ion-exchange chromatography. A column of DEAE-Sephadex A-50 was used as described previously (Umezurike, 1970*b*) or as described by Wood (1971).

Polyacrylamide-gel electrophoresis. The method of Ornstein & Davis (1961) was used. Protein from sliced gels (1mm or 2mm slices) was extracted as described by Umezurike (1970*b*).

β-Glucosidase assay. This was done as described by Umezurike (1971*a*), with *o*-nitrophenyl β-D-glucopyranoside in 0.05M-sodium acetate buffer, pH5.0, as substrate.

CM-cellulase assay. As a routine this was done viscometrically as described previously (Umezurike, 1970*a*) with CM-cellulose as substrate, or sometimes by the release of reducing sugars or glucose.

Assay of cellulase activity with insoluble substrates. Cellulase activity towards cotton flock or Whatman cellulose powder (standard grade) was measured by incubating 4mg of substrate, 1ml of 0.05M-sodium acetate buffer, pH5.0, 5mM-NaN_3 or a drop of toluene to inhibit microbial growth, and 1ml of enzyme solution for 4 days at 40°C. Unhydrolysed solids were then removed by filtration through a sintered-glass crucible (porosity 2) followed by centrifugation of the filtrate at 20000*g* for 15min. The clear solution was then assayed for reducing sugars or glucose.

Assay of short-fibre-forming activity. This was determined with 2mg of cotton fibres as described by Umezurike (1970*b*).

Estimation of fine cellulose particles. Fine cellulose particles in reaction mixtures containing cotton flock (4mg) were estimated by filtration through a sintered-glass crucible (porosity 2), followed by centrifugation of the filtrate at 20000*g* for 15min. A suspension of the precipitated particles in 3ml of 0.05M-sodium acetate buffer, pH5.0, was used for measurements of A_{650} in a Unicam SP.500 spectrophotometer.

Carbohydrate determination. Reducing sugars (as glucose equivalent) were determined by Somogyi's (1952) method. Glucose was determined with the glucose oxidase colorimetric reagents of Boehringer und Soehne G.m.b.H., Mannheim, Germany, according to the manufacturer's instruction.

Paper chromatography of carbohydrates. The method used has been described (Umezurike, 1971*b*).

Results

Gel filtration on Sephadex G-75

Freeze-dried filtrates from 2-week-old cultures that still contained some unsolubilized cellulose, and those from 8-week-old cultures of *B. theobromae* in which the cellulose had been completely solubilized, were suspended in the same buffer as used for elution and centrifuged at 20000*g* for 15min at 4°C. The precipitated particles obtained from the 2-week-old culture sample were retained. The 8-week-old culture sample gave little or no precipitate. Fig. 1 shows the results obtained when the centrifuged 2-week-old culture sample was used for gel filtration on Sephadex G-75. The major β-glucosidase was completely excluded from the gel. The cellulases in the sample are labelled C1, C2, C3, C4 and C5 in Fig. 1. Coloured material in the sample was eluted last, with a peak in fraction 103. The results for the 8-week-old culture sample are presented in Fig. 2. This sample contained an extra β-glucosidase (marked B4 in Fig. 2) of lower molecular weight than enzyme BX. More C1 and C2 activities were found in the 8-week-old culture than in the 2-week-old culture. Cellulase C1 had only a little activity on CM-cellulose when assayed by viscometry, but was more active in producing reducing sugars from cotton flock or cellulose powder. Cellulase C5 was absent from older cultures.

When a suspension of the fine cellulose particles was used for gel filtration on Sephadex G-75, these were completely excluded. The fractions containing these particles showed some cellulase activities on cotton flock and CM-cellulose. When a suspension of the particles in 0.05M-sodium acetate buffer, pH5.0, was incubated at 37°C for 4 days, the par-

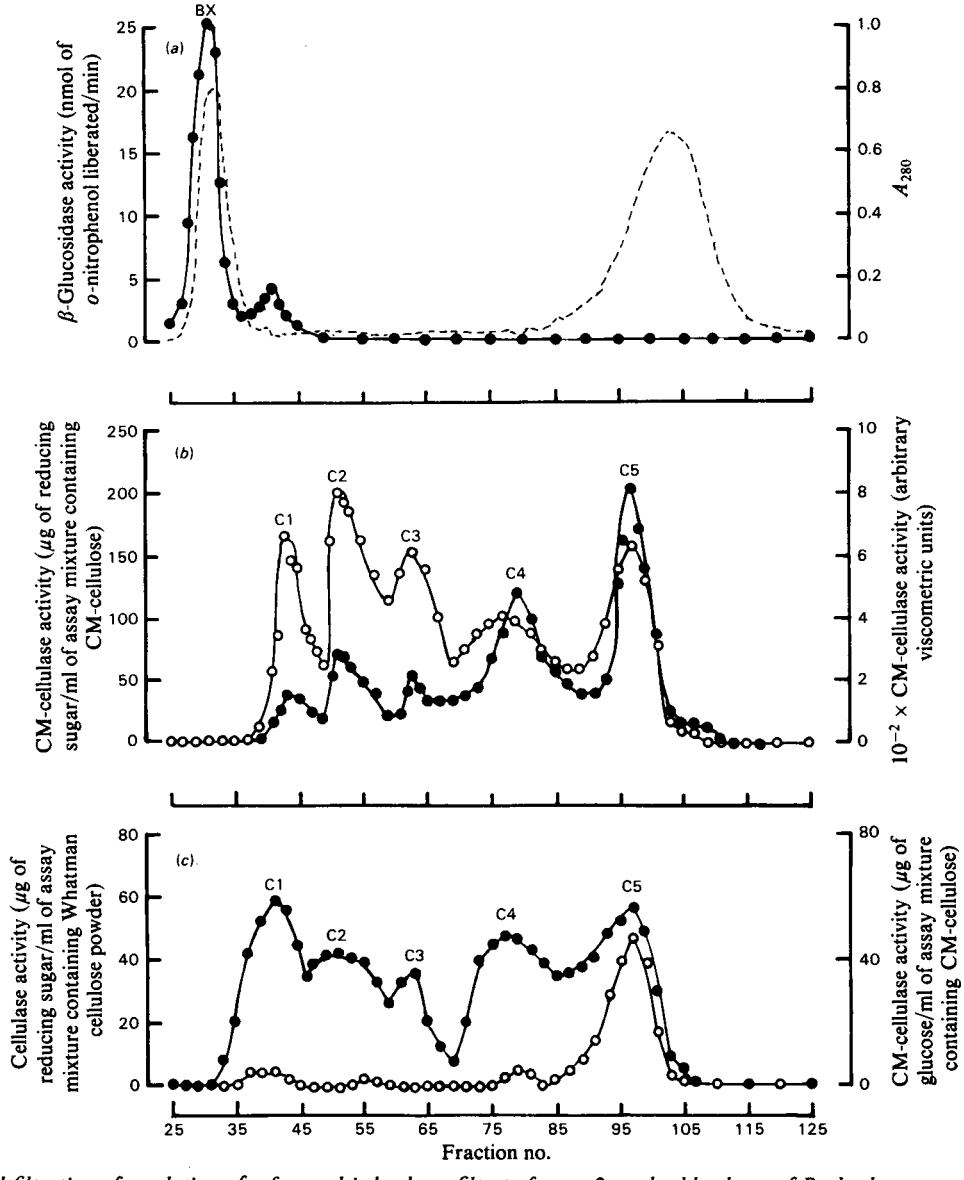

Fig. 1. *Gel filtration of a solution of a freeze-dried culture filtrate from a 2-week-old culture of B. theobromae on a column (100 cm × 2.5 cm) of Sephadex G-75*

(*a*) Protein distribution (----) was measured as A_{280} and β-glucosidase activity was determined with *o*-nitrophenyl β-D-glucopyranoside as substrate (●); (*b*) cellulase activity was measured by the release of reducing sugars (as glucose equivalent) from CM-cellulose (○) or as decrease in viscosity of CM-cellulose solutions (●); (*c*) cellulase activity was determined by the release of reducing sugars from Whatman cellulose powder (standard grade) (●) or of glucose from CM-cellulose (○). The elution buffer was McIlvaine's phosphate/citrate buffer containing 0.1 M-NaCl, pH 5.0. The fraction size was 3.0 ml. See the text for further details.

ticles were solubilized and reducing sugars released, suggesting that the particles contained adsorbed enzymes. Gel filtration of the reaction mixture showed a measurable increase in free cellulases C1, C2 and C5. These were also released from the particles by shaking a suspension in 0.01 M-sodium

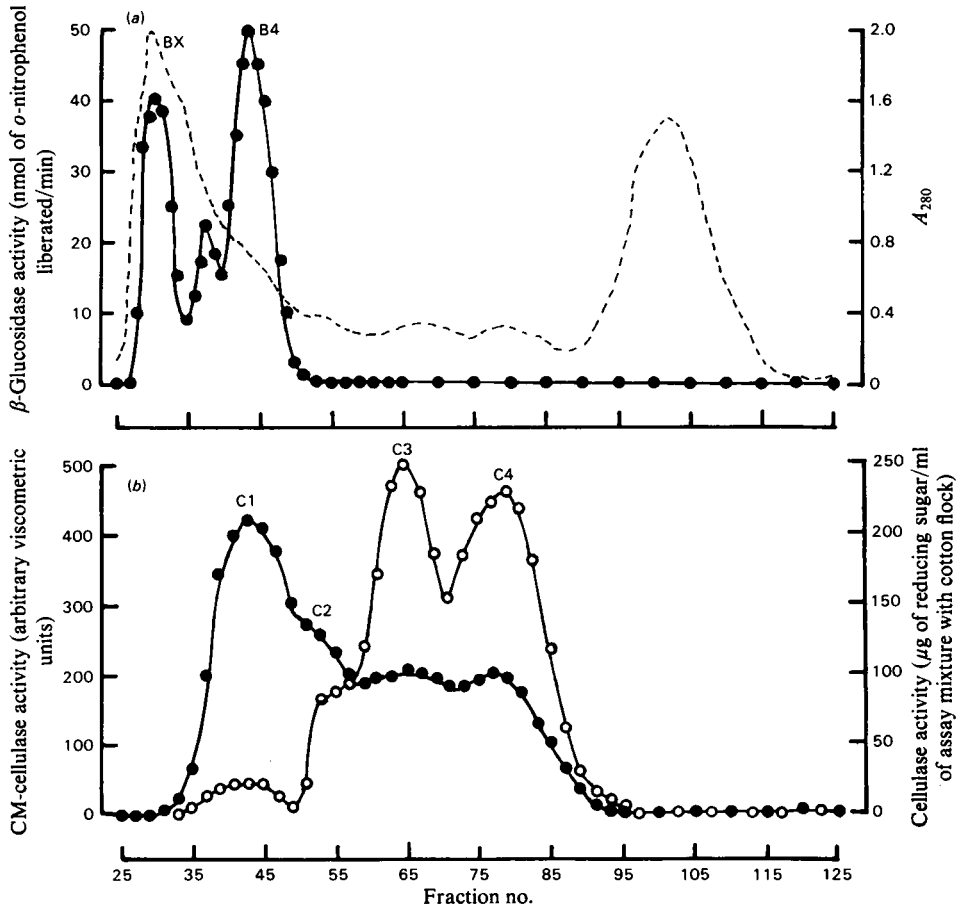

Fig. 2. *Gel filtration of a solution of a freeze-dried culture filtrate from an 8-week-old culture of B. theobromae on a column (100 cm × 2.5 cm) of Sephadex G-75*

(*a*) Protein distribution (----) was measured as A_{280}, and β-glucosidase activity was determined with *o*-nitrophenyl β-D-glucopyranoside as substrate (●); (*b*) cellulase activity was measured by the release of reducing sugars from cotton flock (●) or a decrease in viscosity of CM-cellulose solutions (○). Other conditions are as shown in the legend to Fig. 1.

tetraborate buffer, pH9.0 (cf. Selby *et al.*, 1963), for 15min at 4°C. The supernatant solution obtained after centrifugation of the suspension at 20000*g* for 15min was immediately adjusted to pH5.0 with 0.05M-HCl and freeze-dried. Gel filtration showed that by this procedure cellulases C1, C2 and particularly C5 were released from the cellulose particles. Thus it appears that the decreased activities of cellulases C1 and C2 in concentrated filtrates from young cultures are partly due to adsorption on the cellulose particles.

Gel electrophoresis

The results of electrophoresis on polyacrylamide gel using the same 2-week-old culture sample is shown in Fig. 3. β-Glucosidase BX, which was excluded from Sephadex G-75 on gel filtration, was resolved into three components (B1, B2 and B3), of which B1 was the predominant form. These results agree with those previously reported (Umezurike, 1975). The results for the 8-week-old culture sample are presented in Fig. 4. The predominant form of β-glucosidase was B4; form B1 was absent. Some cellulase-C1 activity was found on the gel near β-glucosidase B4; cellulases C2, C3 and C4 were also present. A diagram of one of the gels that was stained for protein with Amido Schwartz is also shown in Fig. 4. The distribution of protein bands correlates with that of enzymic activities on sliced gels and with the results of gel filtration. The same

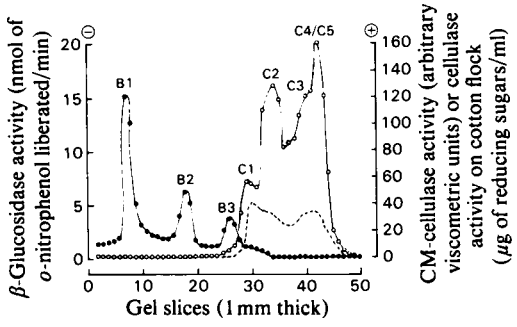

Fig. 3. *Distribution of β-glucosidase and cellulase activities in gel slices after polyacrylamide-gel electrophoresis of a solution of a freeze-dried culture filtrate from a 2-week-old culture of B. theobromae*

After electrophoresis, the gel was sliced into 1 mm pieces starting from the top of the small-pore gel. Each slice was extracted with 2 ml of 0.05 M-sodium acetate buffer, pH 5.0, and portions of the extracts were assayed for β-glucosidase activity with *o*-nitrophenyl β-D-glucopyranoside (●), CM-cellulase activity by viscometry (○) and cellulase activity by the liberation of reducing sugars from cotton flock (----). See the text for details. The positive and negative electrode ends of the gel are indicated as ⊕ and ⊖ respectively.

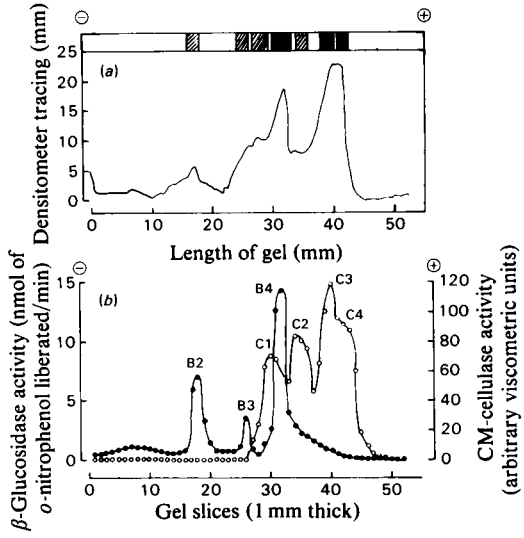

Fig. 4. *Distribution of β-glucosidase and cellulase activities in gel slices after polyacrylamide-gel electrophoresis of a solution of freeze-dried culture filtrate from an 8-week-old culture of B. theobromae*

(*a*) A diagram of a gel that was stained for protein with 1% (w/v) Amido Schwartz in 7% (v/v) acetic acid and its densitometer tracing determined on a Joyce–Loebl Chromoscan fitted with a 595 nm filter; (*b*) slices (1 mm) of an unstained small-pore gel were extracted with 2 ml of 0.05 M-sodium acetate buffer, pH 5.0, and portions of the extracts were assayed for β-glucosidase activity with *o*-nitrophenyl β-D-glucopyranoside as substrate (●) and CM-cellulase activity by viscometry (○). See the text for details. The positive and negative electrode ends of the gels are indicated as ⊕ and ⊖ respectively.

numbering (B4, C1, C2, C3, C4/C5) was used for the fractions found after electrophoresis as those separated by gel filtration migrated to corresponding positions after electrophoresis. However, cellulases C4 and C5 could not be resolved by electrophoresis on 7.5% (w/v) polyacrylamide gels, which are suitable only for the resolution of proteins in the mol.wt. range 10000–400000 (see Umezurike, 1970*b*). The resolution of *B. theobromae* β-glucosidase into four components has been reported (Umezurike, 1971*a*, 1975).

Estimation of molecular weight

The approximate molecular weights of the various enzymes were estimated by gel filtration (cf. Andrews, 1964, 1965) on a calibrated column of Sephadex G-75. The approximate molecular weights of the cellulases were estimated to be 46000–48000 (C1), 30000–35000 (C2), 15000–18000 (C3), 10000–11000 (C4) and 4800–5500 (C5). That for β-glucosidase B4 was found to be 45000–47000, in agreement with previous results (Umezurike, 1975). The molecular weights of the larger β-glucosidases have been reported (Umezurike, 1975) to be 350000–380000 (B1), 170000–180000 (B2) and 83000–87000 (B3) on the basis of gel filtration on Sephadex G-200.

Ion-exchange chromatography

On columns of DEAE-Sephadex swollen and equilibrated in a solution of 0.01 M-Tris containing 5 mM-NaN_3 that had been adjusted to pH 5.2 with 0.2 M-HCl, cellulases and β-glucosidases were strongly adsorbed. When the centrifuged and dialysed 2-week-old culture sample was so adsorbed, only CM-cellulase and β-glucosidase activities were eluted by stepwise elution with 0.01 M-Tris/5 mM-NaN_3 solution that had been adjusted to pH 2.5 with 0.2 M-HCl. Only a small amount of cellulase-C1 activity was eluted from the column when it was further washed with the eluting solution (pH 2.5) containing 0.2 M-NaCl (Fig. 5). The elution profile obtained when the 8-week-old culture sample was fractionated under the same conditions was similar to that shown in Fig. 5. When fractions 35–60 of a separation on Sephadex G-75 similar to that shown in Fig. 2 were

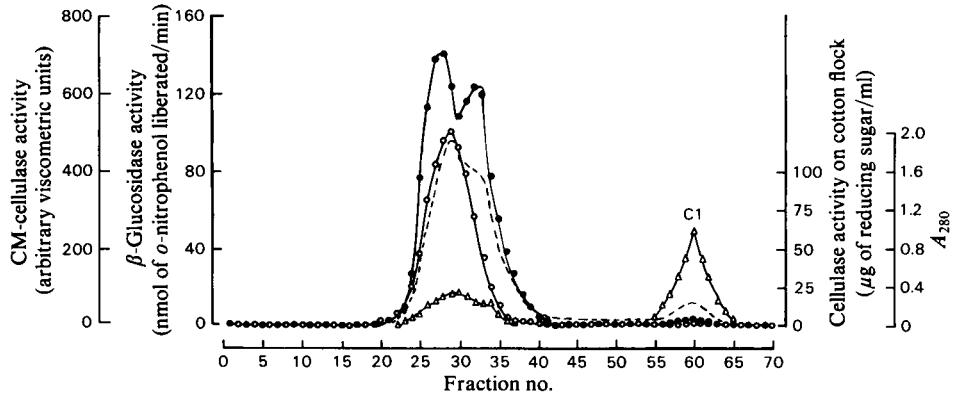

Fig. 5. *Fractionation of a solution of a freeze-dried culture filtrate from a 2-week-old culture of B. theobromae on a column (10.0 cm × 1.5 cm) of DEAE-Sephadex A-50*

The freeze-dried sample was dissolved in 5ml of 0.01 M-Tris/5mM-NaN_3 solution adjusted to pH5.2 with 0.2M-HCl, and applied to the gel column. The column was washed with 100ml of the same solution and subjected to stepwise elution with a solution of 0.01 M-Tris/5mM-NaN_3 that had been adjusted to pH2.5 with 0.2M-HCl until 53 fractions were collected and then with a solution of 0.01 M-Tris/HCl/5mM-NaN_3 containing 0.2M-NaCl (pH2.5). The size of the fractions was 3.0ml. Protein distribution (----) was measured as A_{280}; β-glucosidase activity was determined with *o*-nitrophenyl β-D-glucopyranoside as substrate (○); CM-cellulase activity was determined by viscometry (●); and cellulase activity was determined by the release of reducing sugars from cotton flock (△). See the text for details.

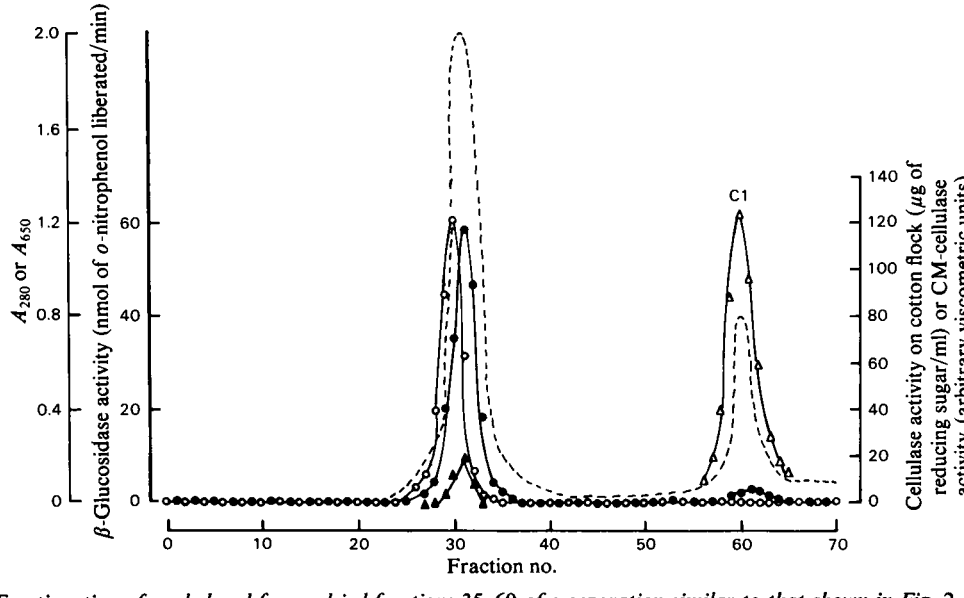

Fig. 6. *Fractionation of pooled and freeze-dried fractions 35–60 of a separation similar to that shown in Fig. 2 on a column (10.5 cm × 1.5 cm) of DEAE-Sephadex A-50*

Elution of enzymic activities was carried out as shown in the legend to Fig. 5. Protein distribution (----) was measured as A_{280}; β-glucosidase activity was determined with *o*-nitrophenyl β-D-glucopyranoside as substrate (○); CM-cellulase activity was determined by viscometry (●); cellulase activity was determined by the release of reducing sugars from cotton flock (△); short-fibre-forming activity was determined on cotton fibres as A_{650} (▲). See the text for details.

pooled, freeze-dried and then fractionated on DEAE-Sephadex, the results presented in Fig. 6 were obtained. The separated cellulase C1 had no effect on the viscosity of CM-cellulose, but produced reducing sugars from cotton flock. Short-fibre-forming activities were associated with the CM-cellulase fractions and not with the C1 fractions. Recoveries of β-glucosidase and CM-cellulase activities were 98 and 93% respectively. In another experiment by the method of Wood (1971), β-glucosidase and CM-cellulase activities were not adsorbed on a column of DEAE-Sephadex swollen and equilibrated with 0.1M-acetic acid/NaOH buffer, pH5.5. Cellulase C1 was readily adsorbed and could be eluted with a linear gradient of NaCl prepared from 500ml of the buffer and 500ml of the buffer containing 0.2M-NaCl, pH5.5 (cf. Wood, 1971). Cellulase C1 so obtained had similar properties to that isolated by the method described above.

When the fine cellulose particles obtained from the 2-week-old culture were applied to a column of DEAE-Sephadex in the 0.01M-Tris/HCl/5mM-NaN_3, pH5.2, system the particles were not adsorbed, but, in passing through the column, lost all their β-glucosidase activity and 95% of the CM-cellulase activity.

Synergism between separated components

In these studies, the unfractionated but dialysed solution (1ml) of the 8-week-old culture sample in 0.05M-sodium acetate buffer, pH5.0, was diluted 10-fold and used as reference solution. The solution containing β-glucosidase BX (fraction 32 in Fig. 1) and that containing a mixture of cellulases C3–C5 (a mixture of equal volumes of fractions 63, 77 and 98 in Fig. 1) were adjusted with 0.05M-sodium acetate buffer, pH5.0, to give β-glucosidase and CM-cellulase activities corresponding to those in the reference solution. A solution containing only cellulase C2 and β-glucosidase B4 activities (fraction 32 in Fig. 6) was diluted with the same buffer to give the same β-glucosidase activity as that in the reference solution. A solution of cellulase C1 obtained after fractionation on DEAE-Sephadex (fraction 60 in Fig. 6) was diluted with the same buffer to give the same activity as fraction 52 in Fig. 2 after the latter had been diluted by the same factor as the mixture of cellulases C3–C5 as indicated above. The activities towards cotton flock of combinations of these solutions are shown in Table 1. The CM-cellulases C3–C5 were more effective in producing fine cellulose particles than was cellulase C1. The particle content appeared to increase in the presence of cellulase C1 and the CM-cellulases. Cellulase C1, however, appeared to be more effective than the CM-cellulases in liberating reducing sugars from cotton flock. The higher cellulase activity in the presence of cellulase C1 and β-glucosidase B4 than that observed in the presence of cellulase C1 alone suggested that β-glucosidase was hydrolysing some inhibitory product (cellobiose). A reconstituted mixture that did not contain cellulase C5 appeared to be less effective than one that contained it.

Table 1. *Relative activities of separated cellulolytic components of B. theobromae on cotton flock, alone and in combination*
Solutions containing the various enzyme components were adjusted to give corresponding enzymic activities to the diluted starting material (a solution of freeze-dried culture filtrate). Portions (1ml) of these solutions, alone and in combination, were incubated with 4mg of cotton flock and one drop of toluene in a final volume of 3ml in 0.05M-sodium acetate buffer, pH5.0, for 3 days at 37°C. After removal of unsolubilized cotton flock by filtration through a sintered-glass crucible (porosity 2) the filtrates were read at 650nm and then centrifuged at 20000*g* for 15min. The clear supernatant solutions were then assayed for reducing sugars. See the text for details.

Component(s)	Content of fine cellulose particles (A_{650})	Cellulase activity (μg of reducing sugars liberated)	Recovery of cellulase activity (%)	Saccharification (concn. of reducing sugars as percentage of substrate concn.)
Reference solution	0.20	1200	100	30.0
Cellulase C1	0.02	325	17.1	8.1
β-Glucosidase BX	0.01	10	0.8	0.3
CM-cellulases (C3 + C4 + C5)	0.10	105	8.8	2.6
CM-cellulase C2 + β-glucosidase B4	0.11	165	13.8	4.1
Cellulase C1 + CM-cellulases (C3 + C4 + C5)	0.14	825	68.8	20.6
Cellulase C1 + β-glucosidase B4	0.12	575	47.9	14.4
Cellulase C1 + CM-cellulases (C3 + C4 + C5) + β-glucosidase BX	0.18	1175	97.9	29.4
Cellulase C1 + CM-cellulases (C3 + C4) + β-glucosidase BX	0.16	1105	92.1	27.6
Cellulase C1 + CM-cellulase C2 + β-glucosidase B4	0.16	1190	99.2	29.8

Table 2. *Relative activities of CM-cellulase components of B. theobromae on CM-cellulose assayed by different methods*
The activities of the various CM-cellulase components (C2–C5) obtained by gel filtration on Sephadex G-75 were determined by decrease in viscosity, liberation of reducing sugars and liberation of glucose with CM-cellulose as substrate. See the text for further details.

	CM-cellulase activity on CM-cellulose			
Component	Viscometric assay (arbitrary units) (*a*)	Reducing sugars liberated (μg/ml of assay mixture) (*b*)	Glucose liberated (μg/ml of assay mixture) (*c*)	*a*/*b* ratio
C2	210	160	4	1.31
C3	495	184	6	2.69
C4	440	108	5	4.07
C5	810	156	50	5.19

Products of cellulose hydrolysis

A portion (5ml) of a dialysed solution containing cellulases C3–C5 (a mixture of equal volumes of fractions 63, 77 and 98 in Fig. 1) was incubated with 4mg of cotton flock in 0.05M-sodium acetate buffer, pH5.0, in the presence of a drop of toluene for 4 days at 40°C. The solids were then removed by filtration and centrifugation at 20000*g*, and the solution was concentrated under reduced pressure. The predominant product found by paper chromatography was cellobiose, but glucose and a trace of cellotriose were also found. Similar results were obtained when only C3 or C4 or C5 was used, although C5 was more effective than the other CM-cellulases in producing glucose from cotton flock. A similar experiment in which a solution of cellulase C1 obtained by fractionation on DEAE-Sephadex was used gave cellobiose as the only product of hydrolysis of cotton flock. It thus appears that cellulase C1 is an exocellulase that successively removes cellobiose residues from the ends of the cellulose fibres, whereas cellulases C3–C5 are endocellulases (random-splitting). A fraction containing cellulase C2 behaved like CM-cellulases C3 and C4.

Relative activities of CM-cellulases

The results of a comparison of the CM-cellulase activities of components C2–C5 measured by decrease in viscosity and liberation of reducing sugars and of glucose with CM-cellulose as substrate are shown in Table 2. The ratio of CM-cellulase activity measured by viscometry to that measured by the release of reducing sugars appears to increase with decrease in the molecular weights of the enzyme components. Cellulase C5 appears to be more effective than the others (C2–C4) in liberating glucose as one of the products of CM-cellulose breakdown (Table 2). This enzyme may therefore be the most active random-cleaving component.

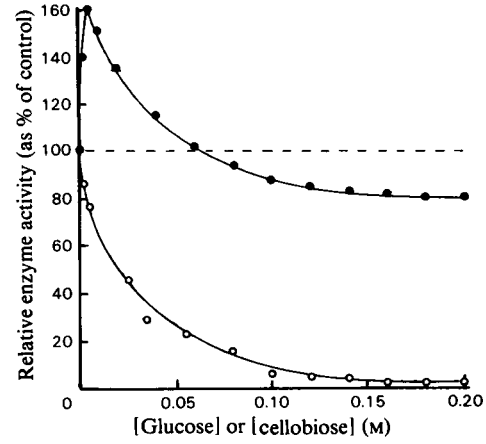

Fig. 7. *Effect of glucose* (●) *or cellobiose* (○) *on the CM-cellulase activity of cellulase component C4*
CM-cellulase activity was determined by the viscometric technique in the absence or the presence of glucose or cellobiose at the final concentrations indicated on the abscissa.

Effect of glucose or cellobiose on enzymic activity

The effect of added glucose on the activities of the dialysed CM-cellulases (assayed by viscometry) depended on the concentration of glucose added to the assay mixtures: a typical result is shown in Fig. 7. The activity of component C4 increased with an increase in glucose concentration up to about 4mM-glucose, when there was about 60% stimulation. The enhancement in activity was progressively diminished as glucose concentration was increased, and in the presence of 0.2M-glucose there was about 20% inhibition. These results agree with those for unfractionated culture filtrates (Umezurike, 1970*a*). Cellobiose was a potent inhibitor of the activity of each of the CM-cellulases: a typical result is shown

in Fig. 7 for the inhibition of CM-cellulase C4. The pattern of inhibition suggests that cellobiose is a competitive inhibitor.

In another experiment, increasing concentrations of cellobiose progressively increased the inhibition of the activity of cellulase C1 (obtained by chromatography on DEAE-Sephadex). Again the pattern of inhibition by cellobiose was suggestive of competitive inhibition. The effect of glucose on cellulase C1 activity was somewhat similar to that on CM-cellulases, except that the maximum activation observed was only about 10%, and there was about 5% inhibition with 0.2M-glucose. Cellulase C1 is therefore relatively less sensitive to added glucose than are the CM-cellulases.

Glucose has been reported to be a potent competitive inhibitor of β-glucosidase from *B. theobromae* (Umezurike, 1970*a*, 1971*b*), and cellobiose is a substrate for the enzyme (Umezurike, 1971*a*).

Discussion

This investigation has shown that the wood-staining fungus *B. theobromae* produces a series of enzymes involved in cellulose degradation when grown on cotton flock. The predominant molecular forms of β-glucosidase and cellulase in culture filtrates of the fungus appear to be affected by the age of the cultures. Small amounts of cellulases C1, C2 and C5 are found adsorbed on fine cellulose particles recovered from young culture filtrates. These particles had previously been considered to be a distinct enzyme of high molecular weight because of the cellulase activity associated with them (Umezurike, 1970*b*). The present results show that this was incorrect, as cellulase C1 and CM-cellulase activities, particularly C5, could be released from the particles under alkaline conditions. In addition these particles became completely soluble on incubation in the absence of added enzymes, thus freeing the adsorbed enzyme components.

Cellulase components (fractions I and III with mol.wt. 55000 and 5300 respectively) found in culture filtrates of *Myrothecium verrucaria* and corresponding in molecular weight to cellulase C1 and CM-cellulase C5 of *B. theobromae* have been shown to be more readily adsorbed by cellulose than a third component (fraction II, mol.wt. 30000) also found in culture filtrates from the same organism (Selby & Maitland, 1965).

That fungal cellulase may exist in multiple forms has been reported by many workers, although the number of such forms differs even in culture filtrates of the same organism (cf. Whitaker *et al.*, 1954; Selby & Maitland, 1965). This observation may sometimes partly reflect differences in cultural conditions and methods of enzyme assay. Table 3 shows the number of multiple forms of cellulase found in culture filtrates of some fungi, in cases where molecular weights have been quoted. The molecular weights of the cellulases of *B. theobromae* are similar to those of some of these cellulases.

The CM-cellulases (C2–C5) produced by *B. theobromae* appear to be random-cleaving enzymes, which produce cellobiose, glucose and a trace amount of cellotriose. Cellulase C1 produces only cellobiose, and is therefore an endwise-cleaving enzyme. Many workers have reported the presence of

Table 3. *Multiple forms of cellulase in culture filtrates of some fungi*
The values in parentheses after the names of the fungi indicate the number of cellulase components found.

Organism		Mol.wt.	Sedimentation coefficient (S)	Reference
Myrothecium verrucaria (3)	I	55000	—	Selby & Maitland (1965)
	II	30000	—	
	III	5300	—	
Myrothecium verrucaria (1)	—	63000	—	Whitaker *et al.* (1954)
Coriolus versicolor (5)	B_1	51000	4.21	Pettersson & Porath (1963),
(formerly *Polyporus versicolor*)	B_2	—	4.26	Pettersson *et al.* (1963)
	C_1	—	2.00	
	C_2	—	1.90	
	D	11400	1.53	
Trichoderma viride (2)	C_1	—	—	Li *et al.* (1965)
	C_x	52000	—	
Verticillium albo-atrum (5)	A	75000	—	Whitney *et al.* (1969)
	B	32000	—	
	C	16000	—	
	D	6500	—	
	E	2200	—	
Fusarium solani (2)	C_1	—	—	Wood (1971)
	C_x	37000	—	

endwise-cleaving and random-cleaving enzymes in culture filtrates of many micro-organisms (Whitaker, 1953; Kooiman *et al.*, 1953; Gilligan & Reese, 1954; Storvick & King, 1960; King, 1963). More recently, the C_1 enzyme of *Trichoderma viride* or of *Trichoderma koningii* has been found to be an endwise-cleaving enzyme and is therefore a β-1,4-glucan cellobiohydrolase (Wood & McCrae, 1972; Berghem & Pettersson, 1973; Halliwell & Griffin, 1973; Berghem *et al.*, 1975). A mol.wt. of 46000 (by gel filtration) or 41800 (by sedimentation-equilibrium analysis) has been quoted for the C_1 enzyme of *T. viride* (Berghem & Pettersson, 1973; Berghem *et al.*, 1975). On the basis of molecular weight and catalytic properties, the corresponding enzyme of *B. theobromae* is cellulase C1.

The enhancement in the activities of cellulases in the presence of β-glucosidase (cf. Table 1) supports the observation that the activity of each cellulase is subject to inhibition by the major product (cellobiose) of cellulose degradation. Halliwell & Griffin (1973) have shown that the inhibition of the C_1 enzyme of *T. koningii* by cellobiose is competitive. Similarly, it has been shown that the inhibition of β-glucosidase of *B. theobromae* by glucose is competitive (Umezurike, 1970*a*, 1971*b*). These factors apparently contribute to the difficulties encountered by various workers in achieving complete solubilization of cellulose by isolated enzymes from cellulolytic fungi. In culture, where the fungus rapidly utilizes the glucose liberated by the action of β-glucosidase, the cellobiohydrolase (C_1) acting alone could effect a considerable solubilization of cellulose, as has been reported (Berghem *et al.*, 1975). However, the rate of solubilization of cellulose will be influenced by the action of the random-cleaving cellulases. This appears to be the explanation for the synergistic effect observed in the presence of cellulase C1 (cellobiohydrolase) and the CM-cellulases, particularly in the presence of β-glucosidase (cf. Table 1).

The suggestion that the C_x enzymes and not the C_1 enzyme should be assigned the function of initiating the degradation of native cellulose (Wood & McCrae, 1972; Berghem *et al.*, 1975) requires some comments. Loss of tensile strength of cotton is better achieved in the presence of the lowest-molecular-weight C_x component (mol.wt. 5300) of *M. verrucaria* (Selby & Maitland, 1965). Swelling or pulping of native cellulose partially enhances the action of component C_1, and alkali 'swelling factor' is closely associated with the C_x components (cf. Mandels & Reese, 1964). The present results and those of Halliwell & Riaz (1970) show that short-fibre-forming activities are associated with CM-cellulases. The lowest-molecular-weight CM-cellulase (C5) of *B. theobromae* is the predominant cellulolytic enzyme found in young cultures, but is absent from older cultures. This enzyme is similar in many respects to the lowest-molecular-weight (5300) 'exhaustible' component of *M. verrucaria* (Selby & Maitland, 1965). Its presence in reconstituted mixtures appears to cause a slightly higher synergistic effect than in its absence (cf. Table 1), and it differs from the other random-cleaving components in being more active in producing a decrease in the viscosity of CM-cellulose per unit increase in reducing sugars liberated (Table 2). The random-cleaving enzymes can be visualized as being involved primarily in the fragmentation of cellulose chains, without causing much solubilization, thus increasing the number of cellulose chain ends available to the endwise-cleaving cellobiohydrolase. These low-molecular-weight enzymes (CM-cellulases) produced by many fungi are important enough to warrant further studies, particularly in view of the observed effects of products of cellulose degradation on their activities. In Nature, as for example in the cell wall of wood, they may be contributing to the control of the rate of cellulose degradation, more so as the ease with which cellulase components can penetrate naturally occurring cellulose chains in plant cell walls would be inversely related to their molecular size.

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