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Purification and properties of the cellulases from the thermophilic fungus *Thermoascus aurantiacus*

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Three cellulases and a β-glucosidase were purified from the culture filtrate of the thermophilic fungus *Thermoascus aurantiacus*. The isolated enzymes were all homogeneous on polyacrylamide-disc-gel electrophoresis. Data from chromatography on Bio-Gel P-60 and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated mol.wts. of 87000 (β-glucosidase), 78000 (cellulase I), 49000 (cellulase II) and 34000 (cellulase III); the carbohydrate contents of the enzymes were 33.0, 5.5, 2.6 and 1.8% (w/w) respectively. Although the three purified cellulases were active towards filter paper, only cellulases I and III were active towards CM(carboxymethyl)-cellulose. Cellulase I was also active towards yeast glucan. The K_m and catalytic-centre-activity values for the enzymes were as follows; 0.52 μmol/ml and 6.5×10^4 for β-glucosidase on *p*-nitrophenyl β-D-glucoside, 3.9 mg/ml and 6.3 for cellulase I on CM-cellulose, 1.2 mg/ml and 1.1 for cellulase I on yeast glucan, 34.4 mg/ml and 0.34 for cellulase II on filter paper, and 1.9 mg/ml and 33 for cellulase III on CM-cellulose.

The current interest in the production of energy from biomass has provided fresh impetus for research in the area of cellulose degradation (Humphrey, 1978; Wang *et al.*, 1978). The cellulases from thermophiles with the ability to operate at temperatures of 55°C and higher offer the advantages of an increased rate of reaction and a stable enzyme system (Bellamy, 1977; Rosenberg, 1975). Further, the high operating temperature and acid pH required by the cellulases from thermophilic fungi restrict the growth of contaminating organisms (Eriksen & Goksöyr, 1976; Romanelli *et al.*, 1975; Rosenberg, 1975). The production of cellulases by thermophilic fungi is well documented (Fergus, 1969; Romanelli *et al.*, 1975; Seal & Eggins, 1976), although there are conflicting reports on the ability of *Thermoascus aurantiacus* to produce cellulases (Fergus, 1969; Tansey, 1971). We have examined the optimal growth conditions for cellulase production by *T. aurantiacus* and the general properties of three cellulases (C. C. Tong, A. L. Cole & M. G. Shepherd, unpublished work). There is, however, a paucity of information on the properties of purified cellulases. In the present paper we report the purification and properties of three cellulases and a β-glucosidase found in a cell-free filtrate prepared from a culture of *T. aurantiacus*.

Abbreviations used: SDS, sodium dodecyl sulphate; CM-, carboxymethyl-.

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Experimental

Materials

The thermophilic fungus *Thermoascus aurantiacus* was isolated from coastal beaches and compost heaps in Christchurch, New Zealand, by using the direct-inoculation technique (Waksman *et al.*, 1939). Whatman no. 1 filter paper and Whatman cellulose powder (CF 11) were obtained from Whatman, Maidstone, Kent ME14 2LE, U.K. CM(carboxymethyl)-cellulose 7HF (degree of substitution = 0.75) was from Hercules, Wilmington, DE, U.S.A. Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-2 and P-60 were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Glucose oxidase, *o*-dianisidine and cellobiose were from BDH Chemicals Ltd., Poole, Dorset, U.K. β-Nitrophenyl β-D-glucosidase was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals used were of Analytical-Reagent grade.

Buffers

The citrate/phosphate buffers from pH 3.0 to 8.0 were all prepared from 0.1 M-citric acid and 0.2 M-

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dibasic sodium phosphate. The buffer used for column chromatography was 0.05 M-ammonium formate, pH 5.0; 0.2 M-Tris/HCl buffer was used for pH-optimum studies.

Methods

Preparation of culture filtrate. Hyphal isolates of *T. aurantiacus* were subcultured on yeast/glucose agar and grown at 50°C. An agar/mycelium disc (0.8 cm diam.) was cut from a yeast/glucose agar slope and transferred to a Wheaton medical flat (c-16, 500 ml) containing 60 ml of Fergus (1969) medium with filter paper as a carbon source. After 21 days incubation at 50°C the filtrate was filtered through glass-fibre paper (Whatman GF/C) to remove hyphal fragments and residual insoluble cellulose. The culture filtrate (approx. 1.5 litres) was freeze-dried and then resuspended in 100 ml of 0.1 M-citrate/phosphate buffer, pH 5.0. The mixture was stirred overnight and insoluble material was removed by centrifugation (30 min at 10000 *g*). The cellulases were then purified as described below.

Determination of protein. Protein was determined by a modification (Eggstein & Kreutz, 1967) of the method of Lowry *et al.* (1951), with sodium citrate being used instead of sodium tartrate. Crystalline bovine serum albumin was used as a standard. The A_{280} was used for monitoring protein in column effluents.

Determination of reducing sugars. The number of reducing-sugar groups created by hydrolysis of the cellulosic substrates were measured spectrophotometrically by using the Nelson–Somogyi procedure (Nelson, 1944; Somogyi, 1952).

Assay of filter-paper-degrading activity. An indication of total cellulolytic activity was obtained by the determination of filter-paper-degrading activity. The standard reaction mixture containing 20 mg of filter paper (Whatman no. 1), 0.9 ml of citrate/phosphate buffer, pH 5.0, 0.1 ml of enzyme solution of appropriate dilution and one drop (10 μl) of toluene were incubated at 60°C for 24 h. The mixture was then analysed for the production of reducing sugar. The toluene added to prevent bacterial growth was found to have no effect on enzyme activity. Reaction mixtures were checked for contamination by withdrawing samples and streaking on to nutrient-agar plates and incubated at 37 and 50°C.

Assay of CM-cellulase activity. Cellulase activity towards CM-cellulose was also measured by the appearance of reducing end groups in a solution of CM-cellulose. The assay conditions were 0.9 ml of 0.75% (w/v) CM-cellulose in citrate/phosphate buffer, pH 4.5, and 0.1 ml of enzyme solution incubated for 30 min at 70°C, and the rate of production of reducing sugars was determined.

An absolute definition of a unit of cellulase activity is difficult. This is because, in the substrate such as CM-cellulose, the glucose molecules are substituted with carboxymethyl groups, and the products of the enzyme reaction on filter paper and CM-cellulose are heterogenous polymers; the effect of this on the absorption coefficient of reducing end groups is not known. It is not, therefore, valid to use a glucose standard to determine the quantity (μmol) of reducing end groups. In addition, there is little to be gained by expressing the activity in terms of glucose equivalents, since glucose is not the only product of the enzyme reaction (Hurst *et al.*, 1977). A unit of filter-paper degrading activity or CM-cellulase activity is defined as that amount of enzyme that produces an increase in absorbance of 0.10 at 560 nm under the conditions defined. A change of 0.1 *A* is equivalent to 95 μg of glucose under the conditions given, and thus cellulase preparations with units quoted in glucose equivalents can be compared.

Assay of β-glucosidase. β-Glucosidase activity was assayed by a modification of the method of Umezurike (1969) using *p*-nitrophenyl β-D-glucoside as substrate. Enzyme solution (0.1 ml) and 0.4 ml of 1 mM-β-nitrophenyl glucoside in citrate/phosphate buffer, pH 5.0, were incubated for 30 min at 70°C. After incubation, 1.0 ml of 1 M-sodium carbonate solution was added to 0.5 ml of the assay mixture, diluted with 10 ml of distilled water and the nitrophenol released was measured from the A_{420}. One unit of β-glucosidase activity is defined as that amount of enzyme needed to liberate 1 μmol of *p*-nitrophenol/min under the conditions of the assay.

Determination of carbohydrate. Total carbohydrate was measured by the anthrone/H_2SO_4 method of Herbert *et al.* (1971), with glucose as standard.

Preparation of alkali-swollen cellulose. This was prepared by the method of Hash & King (1958).

Column chromatography. Unless otherwise stated, column chromatography was carried out at room temperature. Each gel was swollen in the appropriate buffer and active fractions corresponding to the enzyme activity were combined and concentrated by freeze-drying before the next purification step.

For the determination of molecular weights, a column of Bio-Gel P-60 (2.2 cm × 47 cm) was calibrated by the technique described by Andrews (1964), with 0.05 M-Tris/HCl, pH 7.5, containing 0.1 M-KCl. Samples (2–6 mg) were applied in a volume of 1 ml and a constant flow rate of 15 ml/h was used for elution. Fractions of 2.5 ml were collected for analysis. Marker proteins were located by their A_{280}.

Polyacrylamide-gel electrophoresis. Disc-gel electrophoresis was performed at 4°C by the

method of Ornstein & Davis (1964) in a 7.5% (w/v) polyacrylamide gel with Bromophenol Blue as tracking dye. The gels were prepared in glass tubes (0.5 cm × 9.0 cm) with citrate/phosphate buffer, pH 5.5. The citrate/phosphate buffer in both the upper and the lower tank was five times more concentrated than that used for gel preparation. The gels were run at 3.0 mA/tube for 30 min and then at 4.5 mA/tube for 8 h. The staining technique of Reinsner *et al.* (1975) was used. For preparative purposes, several disc gels (0.7 cm × 12.0 cm) were run at 4.0 mA/tube for 1 h and then at 7.0 mA/tube for a further 23 h. SDS/polyacrylamide gels were performed and stained by the method of Weber *et al.* (1972).

Kinetic analysis. The enzyme-kinetic parameters K_m and $V_{max.}$ were derived from data analysed by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), by using a program developed for a Hewlett–Packard 9821A calculator. Velocities were expressed as units/μg of protein and K_m values as mg of substrate/ml or μmol of *p*-nitrophenol in the case of β-glucosidase.

Purification of cellulases. Apart from the column chromatography, which was carried out at room temperature, all operations were conducted at 0–4°C. Concentration of the enzyme fractions was achieved by freeze-drying; in this procedure the salt concentration did not increase because of the volatile nature of the buffer.

Solid $(NH_4)_2SO_4$ was slowly added to 100 ml of stirred crude culture filtrate to give 10% increments in $(NH_4)_2SO_4$ saturation as indicated in Table 1. Stirring was continued for a further 30 min after the addition of the $(NH_4)_2SO_4$. The solution was centrifuged (10000 *g* for 30 min) and the precipitate resuspended in 20 ml of formate buffer. Insoluble material was removed and discarded by centrifugation (10000 *g* for 30 min). Fractions containing the various $(NH_4)_2SO_4$ concentrations were treated separately by using the following purification steps.

Fractions from the ammonium sulphate precipitation were desalted on a column (2.7 cm × 30 cm) of Bio-Gel P-2 resin (100–200 mesh) equilibrated with the ammonium formate buffer (flow rate approx. 100 ml/h) with a void volume of 50 ml. Fractions (5 ml) were collected. The eluate (approx. 60 ml) was freeze-dried and the residue resuspended in 5 ml of the formate buffer. Any undissolved material was removed and discarded by centrifugation (10000 *g* for 30 min).

The desalted samples were layered on to a column (2.75 cm × 91.8 cm) of Sephadex G-100 equilibrated with ammonium formate buffer. At a flow rate of approx. 45 ml/h, fractions (3.5 ml) were collected, and those fractions with more than 30% of the activity of the peak fraction were pooled and freeze-dried. The residue was suspended in formate buffer and centrifuged (10000 *g* for 30 min) to remove undissolved material. Recycling through the same column of Sephadex G-100 was necessary in order to separate completely the β-glucosidase from the cellulases. The purified β-glucosidase was desalted on a Bio-Gel P-2 column and the cellulases purified by disc-gel electrophoresis.

In the purification of cellulases by disc-gel electrophoresis 0.1 ml of the partially purified cellulase enzyme solution was mixed with 40 μl of aq. 80% (v/v) glycerol and 10 μl of Bromophenol Blue (0.2%, w/v) before it was layered on top of each gel tube. After electrophoresis, unstained gels were scanned at 280 nm on a Joyce–Loebl u.v. scanner and then sliced. Protein was eluted in formate buffer, pH 5.0, by grinding the gels in uniform poly(tetrafluoroethylene) ('PTFE')-pestle/glass-body homogenizers with repeated washings. The gelatinous material was removed by centrifuging at 10000 *g* for 30 min. The supernatant obtained was filtered through a Millipore filter (0.45 μm pore size) and the filtrate concentrated by freeze-drying. The dried material was dissolved in 3.0 ml of formate buffer and desalted on a Bio-Gel P-2 column. The active fractions were pooled and the resulting purified cellulases were used for subsequent studies.

Results

Purification of cellulases

An important feature of the purification scheme was the $(NH_4)_2SO_4$ fractionation. Between 10–60% of $(NH_4)_2SO_4$ saturation, 95% of the enzyme activity was recovered, whereas 61% of the protein was removed. Table 1 shows that 96% of the CM-cellulase activity was associated with the 30–50%-satd.-$(NH_4)_2SO_4$ pellet; 80% of the β-glucosidase was with the 20–40% pellet, whereas the filter-paper-degrading enzyme was spread between 30 and 60% $(NH_4)_2SO_4$ saturation. Pure β-glucosidase was obtained from chromatography of the 10–40% pellet on Sephadex G-100 (Figs. 1 and 2). The 10–30% and the 30–40% fractions only contained three other proteins after the β-glucosidase was removed (Fig. 2); the 40–50% $(NH_4)_2SO_4$ pellet contained two proteins, whereas there was only one protein present in the 50–60%-satd.-$(NH_4)_2SO_4$ pellet (Fig. 3). Rechromatography of both the β-glucosidase and the cellulase fractions on Sephadex G-100 removed trace contaminants. Neither affinity chromatography on alkaline-swollen cellulose nor ion-exchange chromatography on DEAE-Sephadex A-50 was successful in fractionating the individual cellulases. However, the separation achieved by polyacrylamide-gel electrophoresis enabled pure enzymes to be obtained after slicing the gel.

Table 1. *$(NH_4)_2SO_4$ fractionation*

$(NH_4)_2SO_4$ saturation (%)	Total protein (mg)	Total activity (units)		
		10^{-3} × Filter-paper-degrading activity	10^{-3} × CM-cellulase activity	β-Glucosidase
10–20	36	0	2.75	66.6
20–30	51	5.50	5.75	266.6
30–40	86	15.75	200.00	300.0
40–50	120	37.80	136.00	31.6
50–60	76	10.00	2.75	8.2

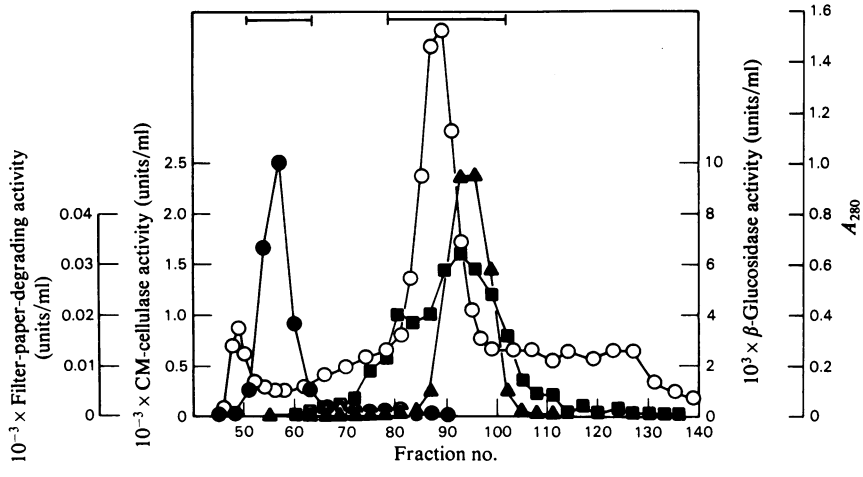

Fig. 1. *Elution profile for β-glucosidase and cellulases on a Sephadex G-100 column*
Details of the procedure are given in the text; 3.5 ml of the 10–40%-satd.-$(NH_4)_2SO_4$ pellet fraction was applied to the column. ○, A_{280}; ●, β-glucosidase activity; ■, filter-paper-degrading activity; ▲, CM-cellulase activity. The fractions under the bars were pooled.

A typical purification scheme for cellulase III is given in Table 2.

Enzyme purity

The homogeneity of the purified enzyme preparations was examined by analytical polyacrylamide-disc-gel electrophoresis in citrate/phosphate buffer, pH 5.5. The purified enzymes migrated as a single band, as shown in Fig. 4, indicating that the enzymes are electrophoretically homogenous. When unstained gels were scanned at 280 nm, only a single band was obtained for each of the purified enzymes.

For convenience, the cellulases are referred to below as cellulase I, cellulase II, and cellulase III, according to the positions they occupied in the gel in descending sequence.

Enzyme stability

The purified β-glucosidase and cellulases were stable for at least a year when stored as frozen solutions at −20°C. In addition, dilute solutions (5 μg/ml) could be kept at 4°C for several days without significant loss of activities.

Molecular-weight determination

The molecular weights of the purified enzymes were determined by gel filtration through a calibrated column of Bio-Gel P-60. The enzymes chromatographed as single peaks under these conditions. A linear relationship was obtained by plotting the elution volumes of the standard proteins against log(molecular weights) (Fig. 5), and the molecular weights of the enzymes determined from this curve were estimated to be 85000 for β-glucosidase, 78000 for cellulase I, 48000 for cellulase II and 34000 for cellulase III.

The molecular weights of the enzymes were also determined by SDS/polyacrylamide-gel electrophoresis by using the following proteins as standards (molecular weights in parentheses): bovine serum albumin (68000), ovalbumin (43000), chymotrypsinogen (25700), myoglobin (17500),

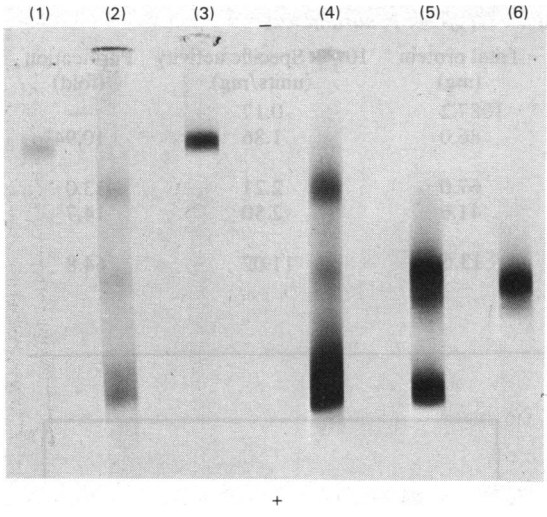

Fig. 2. *Polyacrylamide-gel electrophoresis*
Conditions for polyacrylamide-gel electrophoresis were as described in the Experimental section. The direction of migration was from top to bottom, and the electrode terminals were as shown. (1) Purified β-glucosidase from the 10–30%-satd.-$(NH_4)_2SO_4$ fraction; (2) 10–30%-satd.-$(NH_4)_2SO_4$ fraction after the β-glucosidase had been removed; (3) purified β-glucosidase from the 30–40%-satd.-$(NH_4)_2SO_4$ fraction; (4) 30–40%-satd.-$(NH_4)_2SO_4$ fraction after the β-glucosidase had been removed; (5) 40–50%-satd.-$(NH_4)_2SO_4$ fraction; and (6) 50–60%-satd.-$(NH_4)_2SO_4$ fraction.

lactoferrin (80000) and cytochrome *c* (12500). The relative mobility of β-glucosidase, cellulase II and cellulase III indicated mol.wts. of 89000, 51000 and 34000 respectively. Cellulase I showed several bands on SDS/polyacrylamide-gel electrophoresis, with mol.wts. ranging from 25000 to 80000.

Carbohydrate content

The carbohydrate contents of the purified enzyme preparations were determined by the method of Herbert *et al.* (1971) and are summarized in Table 3. Protein concentrations in the samples were determined by a modification of the Lowry method (Eggstein & Kreutz, 1967).

Kinetics

The K_m and catalytic-centre-activity values for β-glucosidase and the three cellulases were determined from saturation curves by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), and these data are summarized in Table 4.

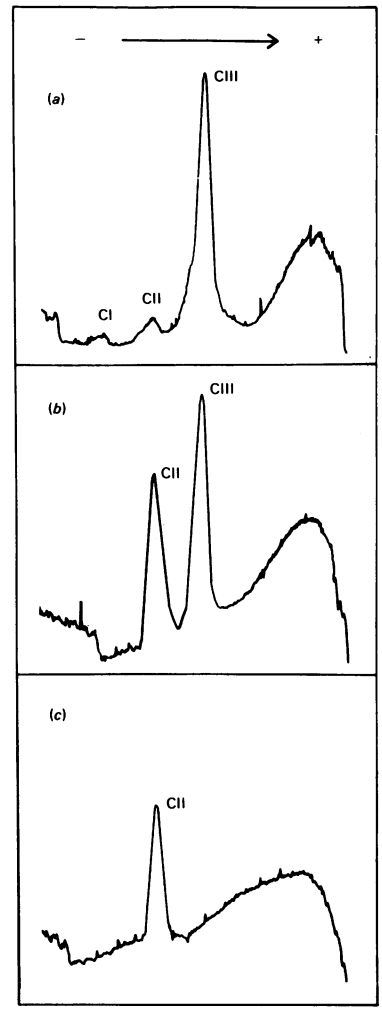

Fig. 3. *Densitometric tracing of polyacrylamide gels after electrophoresis*
Unstained gels were scanned under u.v. light on a Joyce–Loebel u.v. scanner. The direction of migration was left to right. Electrophoresis of pellets from (*a*) 10–40%-, (*b*) 40–50%-, (*c*) 50–60%-satd.-$(NH_4)_2SO_4$ fractions after chromatography on Sephadex G-100. Cellulase I (CI) was isolated from sliced gels after electrophoresis of the 10–40%-satd.-$(NH_4)_2SO_4$ fraction. Cellulase II (CII) was isolated from sliced gels after electrophoresis of the 40–50%- and the 50–60%-satd.-$(NH_4)_2SO_4$ fractions. Cellulase III (CIII) was isolated from sliced gels after electrophoresis of the 10–40%- and the 40–50%-satd.-$(NH_4)_2SO_4$ fractions.

Temperature and pH optimum

The effect of both temperature and pH on enzyme activity was studied with the substrate most active for each enzyme: β-glucosidase on *p*-nitrophenyl β-

Table 2. *Purification of a cellulase (cellulase III) from T. aurantiacus*

Fraction	Volume (ml)	10^{-3} x Total activity (units)	Total protein (mg)	10^{-3} x Specific activity (units/mg)	Purification (fold)
Crude Extract	1510.0	185	1087.2	0.17	—
30–40%-satd.-$(NH_4)_2SO_4$ ppt.	20.0	160	86.0	1.86	10.94
Bio-Gel P-2 eluate	57.3	148.5	67.0	2.21	13.0
Sephadex G-100 eluate second chromatography	130.0	104	41.6	2.50	14.7
Disc-Gel-Electrophoresis eluate	20.0	150	13.6	11.02	64.8

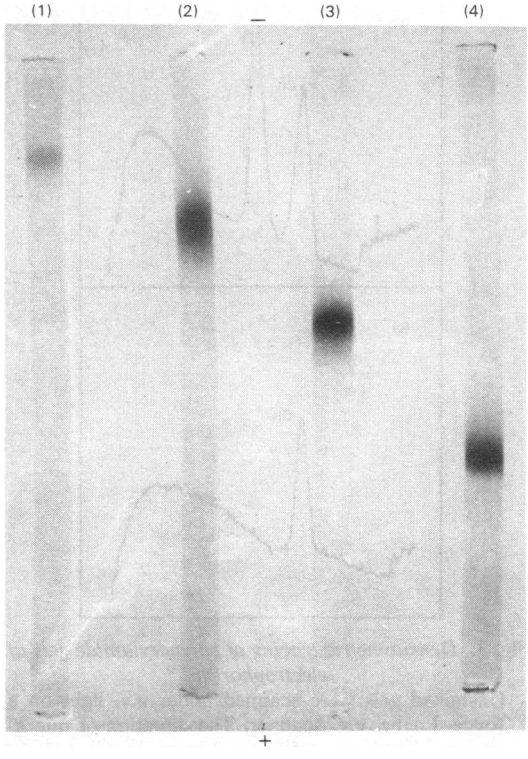

Fig. 4. *Polyacrylamide-gel electrophoresis of the purified enzymes*
Purified enzyme solutions were subjected to electrophoresis in 7.5% (w/v) polyacrylamide gel for 23h at 7.0mA/tube (0.7cm x 12.0cm). Protein loads were (1) 21.0; (2) 48.0; (3) 25.0; (4) 20.4 μg respectively. Migration was from top to bottom. Electrode terminals were as shown. (1) β-Glucosidase; (2) cellulase I; (3) cellulase II; (4) cellulase III.

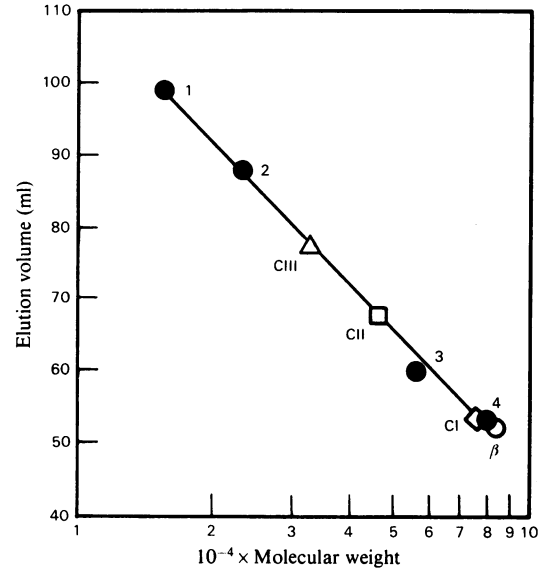

Fig. 5. *Estimation of the moelcular weights of the β-glucosidases and cellulases from T. aurantiacus on Bio-Gel P-60*
Standard proteins were: (1) α-amylase; (2) α-chymotrypsin; (3) bovine serum albumin; and (4) lactoferrin. The molecular weights of marker proteins (except lactoferrin) were taken from the results of Smith (1968). ○, β-Glucosidase; ◇, cellulase I; □, cellulase II; △, cellulase III.

D-glucoside, cellulase I on CM-cellulose and yeast glucan, cellulase II on filter paper and cellulase III on CM-cellulose.

As shown in Fig. 6, optimum temperatures for the β-glucosidase and cellulolytic activities fell within the range 60–75°C. The highest optimum temperature was observed with cellulase I acting on CM-cellulose (75°C), whereas hydrolysis of yeast glucan by the same enzyme was most efficient at 65°C. It should be noted that the incubation time for the enzyme assay was 24h with yeast glucan compared with 0.5h on CM-cellulose. The optimum temperature for cellulase II acting on filter paper decreased from 68 to 60°C when the incubation period of the assay

was increased from 2 to 24h. Activity on *p*-nitrophenyl β-D-glucoside was optimal at 70°C. Cellulase III showed a temperature optimum at 65°C with CM-cellulose as substrate, which is slightly lower than that recorded with the crude enzyme preparation (C. C. Tong, M. G. Shepherd & A. L. J. Cole, unpublished work). It is noteworthy

Table 3. *Carbohydrate content of the β-glucosidase and the cellulases of T. aurantiacus*

The protein and carbohydrate contents were determined on 0.5ml samples of each of the enzymes as described in the Experimental section. The values shown are averages from duplicate samples.

Enzyme	Protein (μg)	Carbohydrate (μg, as glucose)	Carbohydrate (%, w/w)
β-Glucosidase	28	14	33.0
Cellulase I	12	0.7	5.5
Cellulase II	109	3.0	2.6
Cellulase III	136	2.5	1.8

Table 4. *K_m and catalytic-centre activity values for β-glucosidase and cellulases I, II and III from T. aurantiacus*

K_m and catalytic-centre activities were determined by the procedures outlined in the Experimental section. The K_m values for the cellulases are expressed as mg of substrate/ml; for β-glucosidase the units of K_m are μmol of *p*-nitrophenol/ml. The catalytic-centre activity for the cellulases have been determined after converting the ΔA_{560} to μg of reducing sugar and the units are mol of glucose equivalent produced/s per mol of cellulase. For the β-glucosidase the units are mol of *p*-nitrophenol/s per mol of β-glucosidase.

	K_m	Catalytic-centre activity
β-Glucosidase (on *p*-nitrophenyl D-glucoside)	0.52	6.5×10^4
Cellulase I (on CM-cellulose)	3.9	6.3
Cellulase I (on yeast glucan)	1.2	1.1
Cellulase II (on filter paper)	34.4	0.34
Cellulase III (on CM-cellulose)	1.9	33

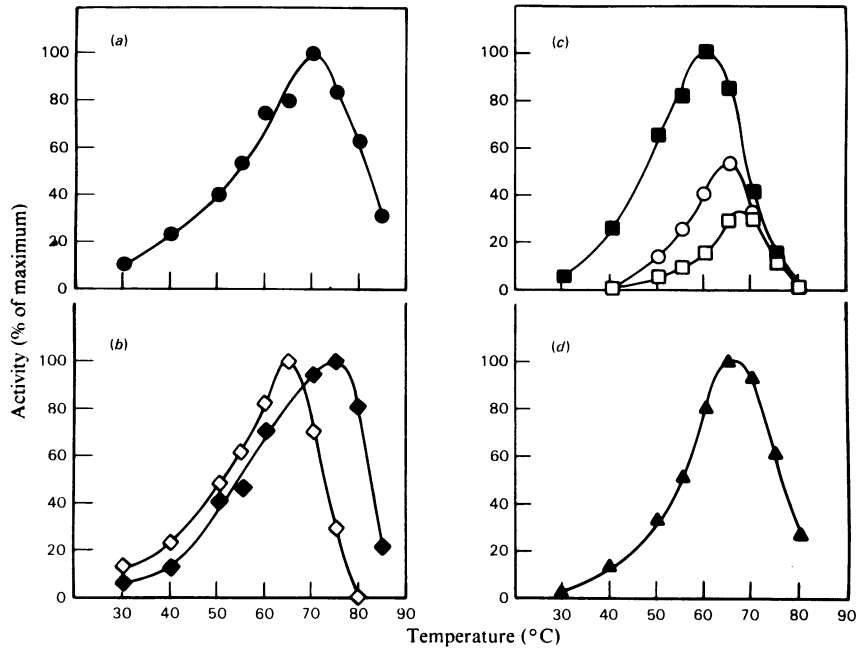

Fig. 6. *Effect of temperature on the activities of β-glucosidase (a) and cellulases I (b), II (c) and III (d) of T. aurantiacus*

Standard assays systems were used, except that the incubation temperatures were varied. The amounts of enzyme used in each assay were: 0.14μg for β-glucosidase; 2.4 and 0.6μg for cellulase I with CM-cellulase and yeast glucan as substrates respectively; 6.25μg for cellulase II and 0.16μg for cellulase III. Further details are given in the text. Points represent averages of two determinations. (*b*): ◇, yeast glucan as substrate: ◆, CM-cellulase as substrate. (*c*): □, 2h incubation; ○, 4h incubation; ■, 24h incubation.

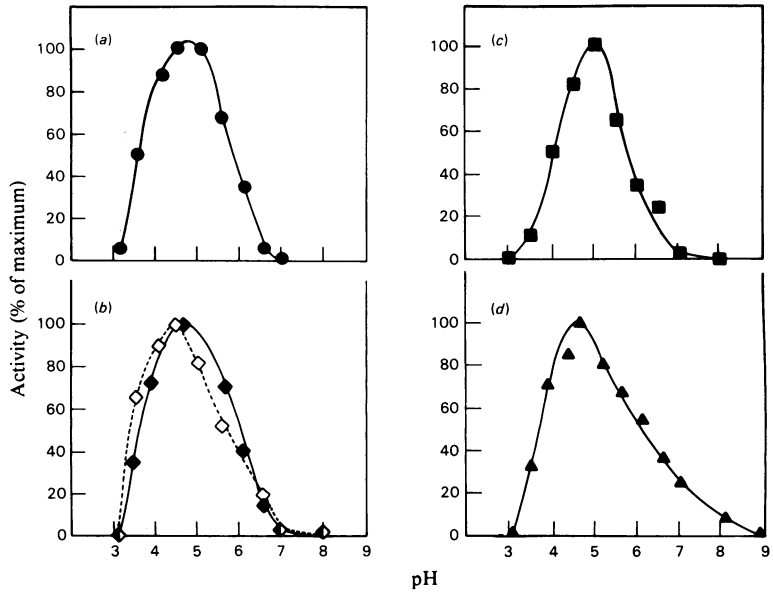

Fig. 7. *Effect of pH on the activities of β-glucosidase (a) and cellulases I (b), II (c) and III (d) from T. aurantiacus* The enzyme activities were measured in the standard assay systems, except that the buffer and pH were varied. Buffers used were citrate/phosphate, pH 3–7, and Tris/HCl, pH 8 and 9. The amount of enzyme used in each assay was as stated for Fig. 6. Points represent the average of results from two determinations. With cellulase I, both yeast glucan (◊) and CM-cellulose (♦) were used as substrates.

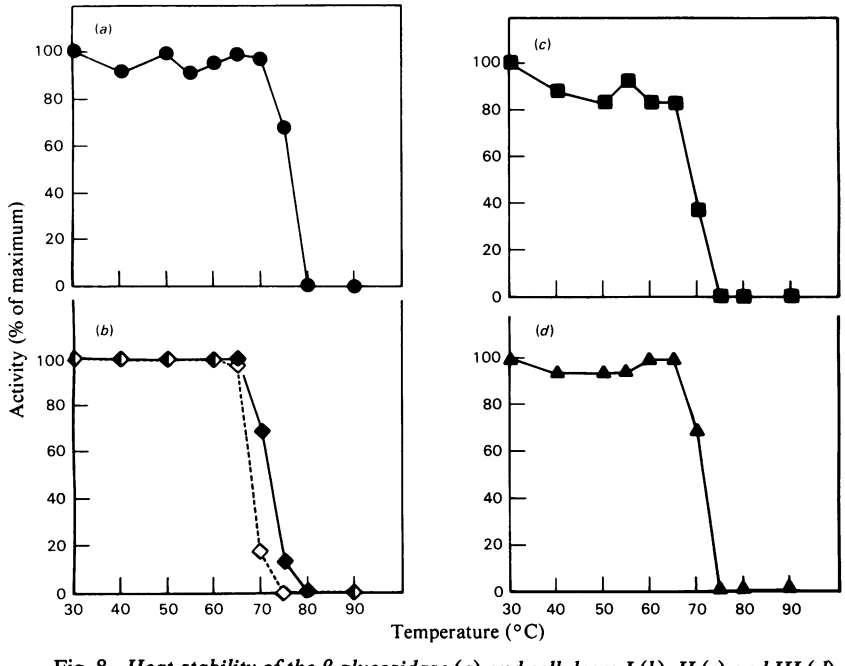

Fig. 8. *Heat-stability of the β-glucosidase (a) and cellulases I (b), II (c) and III (d)* Enzyme solutions of the same concentration as those used in the temperature- and pH-optimum experiments were used in these studies. After incubation for 1 h at the temperatures indicated, the activity of each enzyme was then determined at the optimum temperature and pH of each purified enzyme. Points were the average of two determinations. With cellulase I, yeast glucan (◊) and CM-cellulase (♦) were used as substrates.

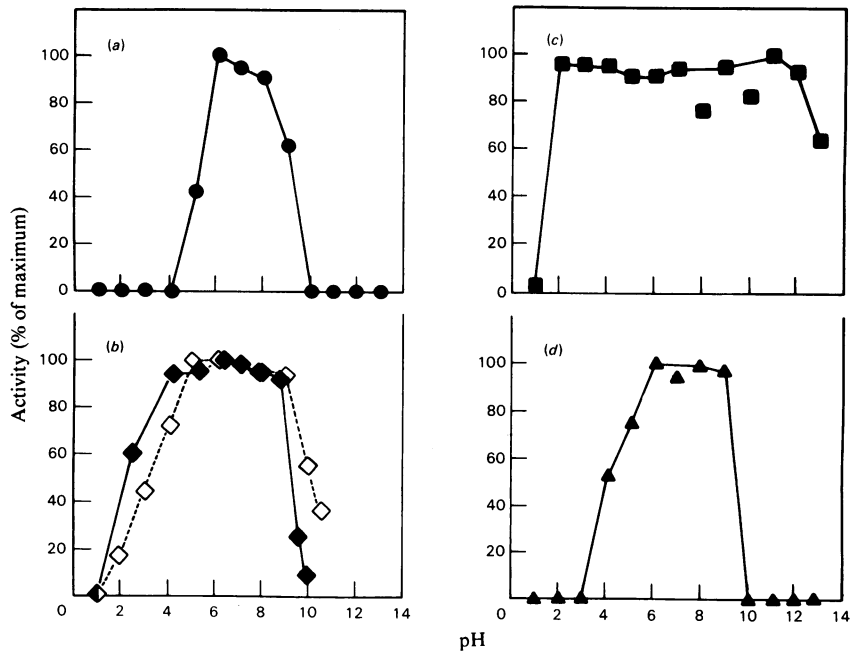

Fig. 9. *pH-stability of the β-glucosidase (a) and cellulases I (b), II (c) and III (d)*

Purified enzyme solutions were mixed with the appropriate buffers to give the required final pH and incubated at 25°C for 24h. Substrates prepared in double-strength assay buffers were then added and the activities determined at the optimum temperature and pH of the purified enzymes. The amount of enzyme used in each assay was as stated for Fig. 6. The pH of each assay was checked after adding the substrate. Buffers used were: 0.4M-KCl/HCl, pH 1.0 and 2.0; 0.2M-citric acid/0.1M-dibasic sodium phosphate, pH 3.0, 4.0, 5.0, 6.0 and 7.0; 0.05M-$Na_2B_4O_7$,$10H_2O$/0.2M-NaOH, pH 10.0 and 11.0; 0.4M-KCl/NaOH, pH 12.0 and 13.0. Points represent the average of two determinations. With cellulase I, both yeast glucan, (◇) and CM-cellulase (◆) were used as substrates.

that, at the temperature normally used for determining enzyme activity (30°C), these enzymes, from a thermophile, exhibited less than 10% of the optimal activity.

The optimum pH for each of the enzyme was determined over the range pH 3–9 (Fig. 7). The temperatures of incubation for β-glucosidase, cellulase II and cellulase III were 70, 60 and 65°C respectively. The activity of cellulase I on CM-cellulose was determined at 75°C, whereas on yeast glucan a temperature of 65°C was used. The hydrolysis catalysed by each of the purified enzymes was confined to acid pH, with optima between pH 4.5 and 5.0.

Temperature- and pH-stability

Fig. 8 shows that the three cellulases were completely stable at temperature up to 65°C, but above this temperature they were very quickly denatured. At 75°C, less than 10% of any of the cellulolytic activity remained. β-Glucosidase was more thermostable than the cellulases. The enzyme was totally stable at 70°C and still retained 70% of its activity after 1h incubation at 75°C.

The effects of pH on the stability of β-glucosidase and cellulase enzymes are shown in Fig. 9. In general, the pH–stability curves of the enzymes are much broader than the pH–activity curves (Fig. 7). Cellulase II was found to be stable under both acidic (pH 2) and alkaline (pH 12) conditions. Although all of the enzymes were stable at pH 8, the activity at this pH is negligible. All of the enzymes were stable in the range pH 6–8. However, at the pH of optimal activity (pH 4.5–5.0), both β-glucosidase and cellulase III lost 50% of the activity after a 24h incubation.

Discussion

The cellulolytic activity of fungi and bacteria has been shown to be a mixture of hydrolytic enzymes (Mandels & Reese, 1964; Li *et al.*, 1965; Halliwell, 1965; Selby & Maitland, 1967; Pettersson *et al.*, 1963; Wood & Phillips, 1969). Generally, however, these enzymes have resisted purification, and there is a paucity of information available on all of the purified cellulases from any particular organism. The

studies on *Trichoderma* species have provided most of the information in this area. The mode of action of the cellulase complex can best be understood by a study of the purified individual enzymes. We report here the purification of the cellulases from the thermophile *T. aurantiacus*.

Extensive purification was achieved by stepwise $(NH_4)_2SO_4$ precipitation. Although ion-exchange and affinity chromatography were not successful in achieving final purification, preparative gel electrophoresis produced electrophoretically homogenous enzymes. This purification scheme produced a β-glucosidase and three cellulase components, designated cellulase I, II and III.

Table 2 shows that cellulase III has been purified 65-fold with respect to the crude extract. This value is undoubtedly an underestimate of the effectiveness of the purification. The assay does not distinguish between cellulolytic activities, and hence the usefulness of a particular purification step in isolating a single activity may not be immediately apparent. It could well be that the purification achieved was in reality several-hundredfold. Evidence for the homogeneity of the three cellulases comes from gel electrophoresis, which indicated a single protein species. On SDS/polyacrylamide-gel electrophoresis, cellulase I showed a number of bands. Further, the cellulases migrated as a single symmetrical peak on the gel-filtration column used for molecular-weight estimation.

The molecular weight of cellulase is low, ranging from 5600 (Selby & Maitland, 1965) to 76000 (Li *et al.*, 1965). The molecular weights of the purified enzymes from *T. aurantiacus* obtained by gel filtration were calculated to be 85000 for β-glucosidase, 78000 for cellulase I, 48000 for cellulase II and 33000 for cellulase III. All three cellulases were retarded on Sephadex G-100 and eluted as a single broad peak. The anomalous behaviour of proteins on Sephadex columns have been observed previously (Whitaker, 1963; Andrews, 1964). Chromatography on polyacrylamide (Bio-Gel) was therefore used to eliminate these effects. The molecular weights obtained from SDS/polyacrylamide-gel electrophoresis were 89000 for β-glucosidase, 51000 for cellulase II and 34500 for cellulase III. Cellulase I was shown as a number of bands with mol.wts. ranging from 25500 to 80000. These results therefore indicate that the enzymes β-glucosidase, cellulase II and III are single polypeptide chains. The cellulases all have carbohydrate bonded to them, but the nature of the association of the protein and the carbohydrate has not been determined. The carbohydrate may be covalently linked to the protein moiety in some cases (Okada *et al.*, 1966; Eriksson & Pettersson, 1971), although present as dissociable complexes in others (Wood & Phillips, 1969; Eriksson & Pettersson, 1968). It is not known whether the carbohydrate plays a role in the catalytic reaction or merely a residue indicating that the enzyme has been associated with the cell wall. Jermyn (1955) showed that a β-glucosidase preparation from *Stachybotrys atra* contained a carbohydrate component that was essential for the stability of the enzyme but not for its activity. Complexes between enzyme and polysaccharide have been shown to have caused the apparent heterogeneity observed in some cellulase systems (Jermyn, 1955; Eriksson & Pettersson, 1968); the presence of carbohydrate may also have been the reason for the heterogeneity of the cellulases isolated from *Trichoderma koningii* (Wood & McCrae, 1972), *Trichoderma reesei* (Berghem & Pettersson, 1973) and *Fusarium solani* (Wood, 1969).

In general, cellulases have high temperature optima when compared with other enzyme systems. The cellulases and β-glucosidase of the thermophile, *T. aurantiacus* have an optimum temperature of about 70°C. When cellulase II was assayed on filter paper over a period of 24h, the optimum temperature decreased to 60°C. This was probably due to inactivation of the enzyme at elevated temperatures over the longer incubation period. The temperature optima determined in these experiments can be classically described as the balance between the effect of temperature on the rate of reaction and its effect on the rate of enzyme destruction, as stated by Dixon & Webb (1964). The high thermostability of the enzyme appears to be a characteristic of most fungal cellulases (Mandels & Reese, 1965). This is an important property of cellulases, since the hydrolysis of cellulose will proceed faster at higher temperatures. The cellulases and the β-glucosidases described here were completely stable at 65°C for at least 1h, and there was little difference observed between the thermal-stability curves of the four enzymes.

The pH optima of fungal cellulases are generally between pH4.0 and 6.0 (Mandels & Reese, 1965), and the optimal pH observed for the β-glucosidase and cellulases of *T. aurantiacus* seems consistent with this. The enzymes all showed a sharp optimum on the pH–activity profile between pH4.5 and 5.0. The pH-stability of the cellulase enzymes differed: cellulase II exhibited a remarkably broad range of stability from pH2 to 12 and cellulase III a narrower range of pH6–9. β-Glucosidase was recorded as having a pH-stability range of pH6–8.

The kinetic parameters K_m and $V_{max.}$ of the purified enzymes were determined. K_m is normally determined for enzymic reactions acting on simple, well-defined soluble substrates in which the product formed is known. In the case of highly ordered forms of cellulose such as filter paper, not only is the substrate insoluble and poorly defined, but the products of the enzyme reaction are

heterogeneous. Moreover, the rate of hydrolysis on different parts of the substrate may vary. In the present work, the K_m values determined for the enzymes hydrolysing filter paper, CM-cellulose and yeast glucan serve to denote the amount of substrate required to achieve half the maximal initial reaction velocity. There is a paucity of information available on K_m values of cellulose hydrolysis by cellulases. Values of 0.5 and 1.6 mg/ml have been published in studies on CM-cellulose hydrolysis by cellulases of *Myrothecium verrucaria* (Halliwell, 1961) and *T. reesei* (Reese & Mandels, 1963) respectively, but the effect of substrate concentration on reaction rate is complicated by the adsorption of the enzyme on to the substrate (whether it be soluble or insoluble cellulose), in such a way that it is rendered inactive when the ratio of enzyme to substrate is relatively low (Reese & Mandels, 1963). Hurst *et al.* (1977) analysed the pH-dependence of K_m and $V_{max.}$ of a cellulase from *Aspergillus niger* on CM-cellulose. Four pK values between 4.2 and 5.3 were obtained for groups involved in the enzyme–substrate complex, indicating the probable importance of carboxy groups in catalysis. In the present work, a K_m value of 1.9 mg/ml was obtained with cellulase III component acting on CM-cellulose (degree of substitution = 0.75; degree of polymerization = 3200). It has been shown that the K_m for CM-cellulose increases with an increase in degree of substitution (Eriksson & Hollmark, 1969). This probably explained the high K_m value of 19.0 mg/ml calculated by Stutzenberger (1971) for the endo-cellulase hydrolysing a CM-cellulose with a degree of substitution of approx. 1.2. Hydrolysis of filter paper by cellulase II was much slower. The K_m for this reaction was recorded as 34.4 mg/ml, and a catalytic-centre activity of 0.34 mol of glucose equivalents/s per mol of enzyme was calculated (Table 4). A K_m value of 0.52 μmol/ml was obtained with β-glucosidase acting on *p*-nitrophenyl β-D-glucoside and the catalytic-centre activity for this reaction was calculated to be 6.5×10^4 mol of *p*-nitrophenol/s per mol of enzyme, and this compares with a catalytic-centre activity of 6×10^4 for the β-glucosidase from *T. reesei* also acting on *p*-nitrophenyl β-D-glucoside (Berghem & Pettersson, 1974). For the cellulases, the catalytic-centre activity was calculated as shown in Table 4. From the relative activity of cellulases I and III acting on CM-cellulose, it was found that the catalytic-centre activity of cellulase III (33) is five times greater than that of cellulase I (6.3) for this substrate. The catalytic-centre activities for cellulases I, II and III acting on filter paper are 0.12, 0.34 and 0.18 respectively. For cellulase III there is a 180-fold difference between the catalytic-centre activities on CM-cellulose and filter paper.

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