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Purification and Properties of a Cellulase from *Aspergillus niger*

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A cellulolytic enzyme was isolated from a commercial cellulase preparation from *Aspergillus niger*. A yield of about 50mg of enzyme was obtained per 100g of commercial cellulase. The isolated enzyme was homogeneous in the ultracentrifuge at pH4.0 and 8.0, and in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis but showed one major and two minor bands in disc gel electrophoresis. No carbohydrate was associated with the protein. Amino acid analysis revealed that the enzyme was rich in acidic and aromatic amino acids. Data from the amino acid composition and dodecyl sulphate/polyacrylamide-gel electrophoresis indicated a molecular weight of 26000. The purified enzyme was active towards CM-cellulose, but no activity towards either cellobiose or *p*-nitrophenyl β-D-glucoside was detected under the assay conditions used. The pH optimum for the enzyme was pH 3.8–4.0, and it was stable at 25°C over the range pH 1–9; maximum activity (at pH4.0) was obtained at 45°C. The cellulase was more stable to heat treatment at pH8.0 than at 4.0. Kinetic studies gave p*K* values between 4.2 and 5.3 for groups involved in the enzyme–substrate complex.

It is well established that *Aspergillus niger* produces a number of cellulolytic enzymes. Commercial crude cellulase preparations, derived from culture filtrates of the fungus, have been fractionated by several workers (Whistler & Smart, 1953; Wolf *et al.*, 1959; Krishna Murti & Stone, 1961; Li & King, 1963; Pettersson, 1963; Clarke & Stone, 1965*a*; Ikeda *et al.*, 1967). Ikeda *et al.* (1973*a*,*b*) have reported the isolation of a homogeneous cellulolytic enzyme from *A. niger*, with an unusually low optimum pH (2.5). The other activities of *A. niger* have not, however, been fully characterized.

The aim of our study is to examine the mechanism of action of a cellulase, and the present paper describes the purification, physicochemical and enzymic properties of a 1,4-β-glucan glucanohydrolase (EC 3.2.1.4) from *A. niger*.

Experimental

Materials

Cellulase (type II, practical grade), *p*-nitrophenyl β-D-glucoside, 2-nitrophenylsulphenyl chloride and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. CM-cellulose 7LIXP was obtained from Hercules Inc., Wilmington, DE, U.S.A. *p*-Hydroxybenzoic acid hydrazide was from Fluka AG, Buchs, Switzerland. Glucose oxidase and cellobiose were from BDH Chemicals Ltd., Poole, Dorset, U.K. $(NH_4)_2SO_4$ (special enzyme grade) was obtained from Mann Research Laboratories, New York, NY, U.S.A. Cellulose powder (chromatography grade) was from J. T. Baker Chemical Co., Phillipsburgh, NJ, U.S.A. Bio-Gel P-60 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals used were of analytical-reagent grade.

Methods

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

Determination of reducing sugars. Reducing end groups were measured by a modification of the method of Lever (1973). *p*-Hydroxybenzoic acid hydrazide reagent was prepared immediately before use, by mixing 5ml of each of the stock solutions (1M-Na_2SO_3, 0.2M-$CaCl_2$, 0.5M-trisodium citrate and 5M-NaOH) and diluting to 90ml with water; 1.0g of *p*-hydroxybenzoic acid hydrazide was dissolved in the solution, which was then diluted to 100ml. *p*-Hydroxybenzoic acid hydrazide reagent (5ml) was added to 0.6ml of a solution of reducing sugar, mixed, and heated on a boiling-water bath for 10min. The samples were then cooled by immersing in cold water and the A_{240} was determined.

Assay of cellulase. Cellulase activity was measured

* Deceased.

by the appearance of reducing end groups in a solution of CM-cellulose. CM-cellulose was dissolved in 0.1 M-sodium acetate buffer, pH 4.0, to a final concentration of 10 mg/ml. Substrate solution (0.5 ml) and 0.1 ml of a suitable dilution of enzyme (diluted in the sodium acetate buffer) were mixed and incubated at 40°C for 30 min. The reducing sugars produced were then determined. An absolute definition of a unit of cellulase activity is difficult. This is because in the substrate the glucose molecules are substituted with carboxymethyl groups, and the products of the enzyme reaction are heterogeneous 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 number of 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 a product of the enzyme reaction. In the present work a unit of activity is defined as the amount of enzyme that produces an A_{420} of 0.10 under the conditions defined.

Assay of cellobiase. Cellobiase activity was determined by measuring the release of glucose from a solution of cellobiose. To 0.5 ml of cellobiose solution (10 mg/ml) in 0.1 M-sodium acetate buffer, pH 4.0, 0.1 ml of enzyme solution was added. The mixture was then incubated at 40°C for 30 min and the glucose released was measured by the glucose oxidase procedure of Lloyd & Whelan (1969).

Assay of β-glucosidase. The substrate for the determination of β-glucosidase activity was *p*-nitrophenyl β-D-glucoside. The assay mixture contained 2.0 ml of 1 mM-*p*-nitrophenyl β-D-glucoside in 0.1 M-sodium acetate buffer, pH 5.0, and 0.1 ml of enzyme solution. After incubation at 40°C for 30 min, 0.5 ml was removed and added to 10 ml of 0.1 M-Na_2CO_3. *p*-Nitrophenol released was measured by the A_{400}.

Determination of carbohydrate. Total carbohydrate was measured by the orcinol/H_2SO_4 method of Svennerholm (1956), by using glucose as standard.

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

Gel chromatography. A column (2.2 cm × 47 cm) of Bio-Gel P-60 was calibrated for molecular-weight determination by the method of Andrews (1964), by using 0.05 M-Tris/HCl/0.1 M-KCl buffer, pH 7.5.

Ultracentrifuge studies. Sedimentation-velocity analyses were performed in a Spinco model E analytical ultracentrifuge, equipped with schlieren optics, in a standard 12 mm single-sector cell and an analytical D rotor. Sedimentation coefficients were determined by the method of Schachman (1957).

Polyacrylamide-gel electrophoresis. The method of Hedrick & Smith (1968) was used for disc gel electrophoresis in 7.5% (w/v) polyacrylamide. Electrophoresis in 7.5% (w/v) polyacrylamide was carried out in continuous buffer systems (0.05 M-pyridine/acetic acid, pH 5.5, and 0.05 M-Tris/acetate, pH 8.0) by the method of Hjertén *et al.* (1965). SDS*/polyacrylamide-gel electrophoresis was performed by the technique of Weber & Osborn (1969). Gels were stained with Coomassie Brilliant Blue (Weber & Osborn, 1969). When required, unstained gels were scanned at 280 nm with a Joyce–Loebl u.v. scanner (type D8 MK2), frozen with solid CO_2 and sliced in 1 mm sections by using a Mickle gel slicer. Protein loads of up to 180–200 μg were used with all gel systems. The molecular weights of marker proteins used in the SDS/polyacrylamide-gel electrophoresis and gel-chromatography experiments were from tables compiled by Smith (1968).

Amino acid analysis. Three samples, each containing 0.5 mg of purified enzyme and a crystal of phenol, were hydrolysed under nitrogen in 6 M-HCl at 110°C for 24, 48 and 72 h respectively. The hydrolysates were analysed in a Jeol JLC-6AH amino acid analyser and the data computed in part by using a program developed for a Hewlett-Packard 9821A calculator. Average values were used for all amino acids except serine, threonine, phenylalanine and tyrosine, where zero-time values were calculated by extrapolation. Free thiol groups were determined spectrophotometrically by using 5,5′-dithiobis-(2-nitrobenzoic acid) in 8 M-urea or 2% (w/v) SDS (Habeeb, 1972). Cystine was measured as cysteine by using 5,5′-dithiobis-(2-nitrobenzoic acid) after the cellulase was reduced with $NaBH_4$ in the presence of 8 M-urea (Habeeb, 1972). Tryptophan was determined by the method of Edelhoch (1967) and by labelling with 2-nitrophenylsulphenyl chloride in 50% (v/v) acetic acid (Scoffone *et al.*, 1968).

Kinetic analyses. The kinetic parameters K_m and V were derived from data analysed by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), by using a program developed for the Hewlett–Packard calculator. Velocities were expressed as units/μg of protein and K_m values as mg of CM-cellulose/ml.

Purification of cellulase. All operations were carried out at 4°C. The crude cellulase powder (100 g) was suspended in 1 litre of 0.05 M-ammonium formate buffer, pH 4.0, stirred for 12 h and then insoluble material was removed by centrifugation (15 min at 20000*g*). The cellulase was then purified by the scheme given in Table 1. Column-chromatography eluates were concentrated by freeze-drying. After the $(NH_4)_2SO_4$ precipitation, the supernatant was desalted on a column (3 cm × 30 cm) of Sephadex G-25 equilibrated with the ammonium formate buffer. The eluate (approx. 600 ml) was then percolated through a column (5 cm × 20 cm) of DEAE-Sephadex A-25 equilibrated with the formate buffer

* Abbreviation: SDS, sodium dodecyl sulphate.

(flow rate approx. 60ml/h) and concentrated. A gelatinous precipitate was removed by centrifuging and the supernatant was layered on to a column (5cm×100cm) of Sephadex G-75 and eluted with the formate buffer at a rate of 50ml/h. Fractions 90–130 (12.5ml) associated with the cellulolytic activity were pooled and concentrated.

The freeze-dried residue was dissolved in 10ml of 0.05M-ammonium acetate buffer, pH5.5, and applied to an alkali-swollen cellulose column (2.5cm×90cm). The column was eluted with acetate buffer at a rate of 20ml/h. Fractions containing more than 50% of the activity found in the peak fraction were pooled. This solution was freeze-dried and dissolved in 10ml of 5mM-sodium phosphate buffer, pH6.5, and equilibrated with that buffer by passage through a column (2.2cm×22cm) of Sephadex G-25. This eluate was then applied to a column (5cm×15cm) of hydroxyapatite–Sephadex G-25. The ratio of hydroxyapatite/Sephadex G-25, added to improve the flow rate, was 1:2 (v/v) of swollen components. The column was equilibrated with 5mM-sodium phosphate buffer and eluted stepwise with 5, 10, 50, 100 and 500mM-sodium phosphate buffers, pH6.5, at the rate of 20ml/h. All cellulolytic activity appeared in the 5mM-buffer eluate.

Carbohydrate associated with the enzyme was removed by a Bio-Gel P-60 column. The enzyme was concentrated and dissolved in the acetate buffer, then applied (in two separate 3.5ml portions) to a column (2.2cm×47cm) of Bio-Gel P-60 and eluted with the acetate buffer at a rate of 20ml/h. Complete separation of protein from carbohydrate was achieved. The cellulase fractions were pooled (total vol. 60ml) and freeze-dried. The purified cellulase was dissolved in 6ml of 0.1M-sodium acetate buffer, pH4.0.

Results

Purification of cellulase

Typical results of a purification scheme are given in Table 1. Enzyme fractions were concentrated by freeze-drying; in this procedure the salt concentration did not increase because of the volatile nature of the buffers. When desalting was necessary, for example after the $(NH_4)_2SO_4$ precipitation, dialysis in cellulose acetate tubing was avoided by the use of gel filtration. As well as removing a large quantity of protein, the DEAE-Sephadex column also removed most of the dark-brown pigment found in the crude extract. The Sephadex G-75 column separated most of the protein from the main cellulolytic peak. The elution profile of the affinity chromatography showed that although the cellulase was retarded there was incomplete separation from the contaminating protein. Some 60% of the remaining protein and the last traces of pigment were removed by adsorption on hydroxyapatite. No loss of total activity was encountered on these columns; the decrease in total units recovered is due entirely to the selection of the fractions to be pooled. Efficient removal of the carbohydrate associated with the enzyme preparation, which probably resulted from the digestion of the alkali-swollen cellulose, was achieved by gel filtration on Bio-Gel P-60.

Enzyme stability

The purified cellulase was stable indefinitely when stored as a frozen solution at −15°C. In addition, dilute solutions (<10μg/ml) could be kept at 4°C for several days without significant loss of activity.

Gel-electrophoresis analyses

Analysis of the purified enzyme by gel electrophoresis at pH5.5 revealed one major and two minor protein bands. Similar results were obtained at pH8 (continuous gels) and with disc gel electrophoresis. Unstained gels of the latter type were sectioned and all three bands showed cellulolytic activity. Sufficient material for kinetic analysis was obtained by running several disc gels and pooling the eluted protein from these gels. Pooling was based on the profile of enzyme activity in each gel. Each enzyme band exhibited the same pH optimum and the same K_m value towards CM-cellulose when assayed at pH4.0, as described below.

Table 1. *Purification of a cellulase from A. niger*

Fraction	Volume (ml)	10^{-5}×Total activity (units)	Total protein (mg)	10^{-3}×Specific activity (units/mg)	Purification (fold)
Crude extract	1010	283	15900	1.8	—
80%-satd.-$(NH_4)_2SO_4$ ppt.	355	256	7800	3.3	1.8
Desalted resuspended $(NH_4)_2SO_4$ ppt.	600	192	5700	3.4	1.9
DEAE-Sephadex eluate	50	80	2250	3.6	2.0
Sephadex G-75 eluate	14	50	415	12.0	6.7
Affinity-column eluate	15	33	138	23.9	13.3
Hydroxyapatite eluate	7.7	24	55.4	43.3	23.9
Bio-Gel P-60 eluate	5.8	23.5	52.0	45.2	25.1

SDS/polyacrylamide-gel electrophoresis of the purified enzyme (25 μg) revealed one protein band with a molecular weight of 26000 ± 500 calculated from a standard curve.

Gel chromatography

The molecular weight of the cellulase from the calibrated P-60 column was 26000 ± 2000, by using ribonuclease (12600), α-chymotrypsin (21600), α-amylase (45000) and bovine serum albumin (68400) as standards (Smith, 1968).

Ultracentrifugal analysis

A single symmetrical peak of material was observed when the cellulase was sedimented in the ultracentrifuge (rotor speed 56000 rev./min; temperature 20°C). The $s_{20,w}$ of the cellulase (10 mg/ml) was 2.4S in 0.05 M-sodium acetate buffer, pH 5.0, and 2.6S (9 mg/ml) in 0.1 M-Tris/HCl buffer, pH 8.0.

Amino acid analysis

The results of the amino acid analysis are given in Table 2. The enzyme contains a high proportion of acidic and aromatic amino acids, but is low in sulphur-containing and basic amino acids. The total number of residues was calculated as 241 ± 1, which indicated a molecular weight of about 26000.

Table 2. *Amino acid composition of a cellulase from A. niger*
Total number of residues = 241; molecular weight from closest integer = 25900.

Amino acid	Content (residues/molecule) Determined	Closest integer
Lysine	8.3	8
Histidine	3.6	4
Arginine	2.0	2
Aspartic acid	28.0	28
Threonine	23.8	24
Serine	26.2	26
Glutamic acid	23.8	24
Proline	9.7	10
Glycine	27.9	28
Alanine	20.3	20
Half-cystine	0*, 0.8†	0–1
Valine	15.9	16
Methionine	2.7	3
Isoleucine	9.0	9
Leucine	6.6	7
Tyrosine	15.2	15
Phenylalanine	10.8	11
Tryptophan	6.2‡, 6.3§	6

* Free thiol group in native or denatured cellulase.
† Free thiol group in reduced cellulase.
‡ Determined in guanidine hydrochloride.
§ Determined with 2-nitrophenylsulphenyl chloride.

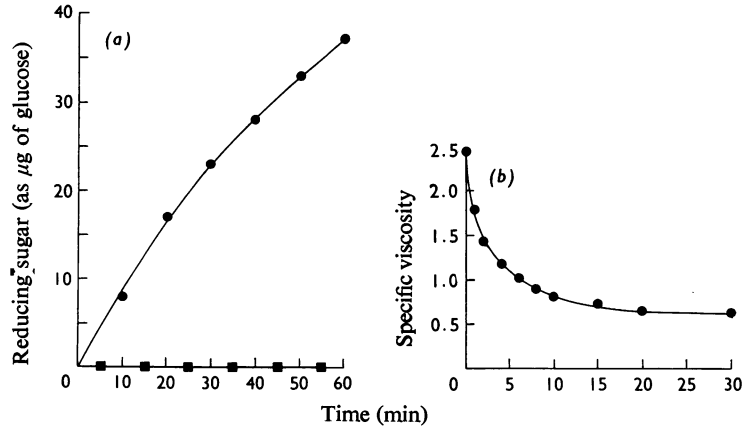


Fig. 1. *Hydrolysis of CM-cellulose*
(*a*) Production of reducing sugars during the hydrolysis of CM-cellulose. Each assay contained 0.1 ml of enzyme solution (0.25 μg) and 0.5 ml of CM-cellulose (10 mg/ml) in 0.1 M-sodium acetate buffer, pH 4.0 at 40°C. Tubes were removed from the incubation bath at the times indicated, kept on ice and reducing sugars (●) and glucose (■) determined as described under 'Methods'. (*b*) Effect of cellulase on the viscosity of CM-cellulose solution. Solutions of CM-cellulose (20 mg/ml, in 0.1 M-sodium acetate buffer, pH 4.0, 30 ml) and cellulase (25 μg/ml, in the same buffer, 5 ml) were mixed and 5 ml samples were removed at the times indicated for the determinations with an Ostwald viscometer. The specific viscosity at zero time was determined by substituting buffer for the enzyme solution. The experiment was carried out in a constant-temperature room at 40°C.

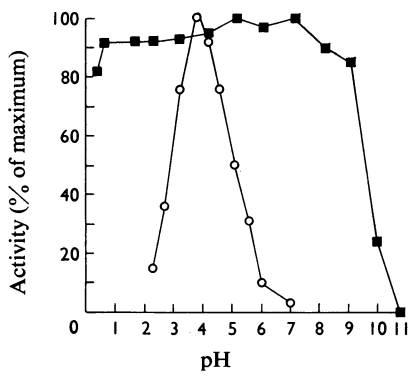


Fig. 2. *Effect of the pH on the activity and stability of cellulase*

The effect of pH on the enzymic hydrolysis of CM-cellulose (○) was measured in the assay system consisting of 0.25ml of CM-cellulose (20mg/ml) in water, 0.25ml of an appropriate buffer and 0.1ml of cellulase (0.25μg). Buffers used were: 0.2M-glycine/HCl (pH1.5, 2.0, 2.5); 0.2M-citric acid/NaOH (pH3.0, 3.5); 0.2M-acetic acid/NaOH (pH4.0, 4.5, 5.0, 5.5); 0.2M-succinic acid/NaOH (pH5.0, 5.5, 6.0); 0.2M-imidazole/HCl (pH4.0). Points were the average of three determinations. In determining the stability of the enzyme (■), cellulase was incubated at 25°C for 24h in a mixture containing 0.5ml of buffer, 0.4ml of water and 0.1ml of enzyme solution (250μg/ml). After incubation, 0.1ml samples were transferred to 1.0ml of 0.25M-sodium acetate buffer, pH4.0. Duplicate assays were carried out on 0.1ml portions. Buffers used were: 0.2M-glycine/HCl (pH1.5, 2.0); 0.2M-citric acid/NaOH (pH3.0, 4.0, 5.0, 6.0); 0.2M-imidazole/HCl (pH7.0); 0.2M-Tris/HCl (pH8.0, 9.0); 0.2M-glycine/NaOH (pH10); 0.2M-$NaHCO_3$/NaOH (pH11.0). For pH values below 1 dilute HCl solutions (approx. 0.5M) were used. The pH of each incubation was measured after mixing the component solutions.

Nature of the enzyme action

The purified cellulase was tested for the presence of both cellobiase and β-glucosidase as described under 'Methods'; neither of these activities, however, could be detected. The progress of the hydrolysis of CM-cellulose was followed by monitoring reducing end-group production (Fig. 1*a*). No glucose was detected, which suggested an endo- rather than exo-cellulolytic mode of action. Further, hydrolysis of CM-cellulose was accompanied by a rapid decrease in the viscosity of the solution (Fig. 1*b*).

Effect of pH

The effect of the H^+ concentration on the activity and stability of the cellulase is shown in Fig. 2. Hydrolysis of CM-cellulose was confined to acid media, being maximal at pH3.8–4.0. In contrast with the sharp pH optimum seen in the activity profile, the cellulase was stable in the absence of substrate, over a wide range of pH values.

Effect of temperature

A temperature of 40°C was used in the standard assay; however, the temperature of maximum activity under these conditions proved to be 45°C (Fig. 3*a*). At pH4.0, close to the optimum for activity, the cellulase was extremely sensitive to temperatures above 60°C (Fig. 3*b*). In contrast, at pH8.0 enzyme activity decreased gradually at temperatures above 40°C, but was not completely lost until 100°C.

Results of a subsequent experiment, designed to yield data on the effect of pH on heat-denaturation (at two fixed temperatures) of the purified cellulase, are shown in Fig. 3(*c*). The cellulase was remarkably unstable at 60°C in the pH range for greatest activity. At 50°C, however, the cellulase was relatively stable over the pH range used.

Kinetics

Typical saturation curves of the purified enzyme acting on CM-cellulose at different pH values indicated that substrate saturation of the enzyme was not achieved above pH3.5. Table 3 summarizes K_m and V values from several different experiments. The highest V was obtained at pH4.5–5.0, whereas the pH optimum under the standard assay conditions

Table 3. *Variation in kinetic parameters of cellulase with pH*

V and K_m values were determined at different pH values. Each assay contained 0.5ml of buffered substrate solution and 0.1ml of enzyme solution (0.1μg of protein). Stock solutions (40mg/ml) of CM-cellulose were prepared in 0.1M buffers adjusted if necessary with either 6M-HCl or 5M-NaOH, then diluted with the appropriate buffer to 30, 25, 20, 15, 10, 7.5, 5.0 or 2.5mg of CM-cellulose/ml. Buffers used were 0.1M-glycine/HCl, pH2.5; 0.1M-citric acid/NaOH, pH3.0, pH3.5; 0.1M-acetic acid/NaOH, pH4.0, pH4.5, pH5.0, pH5.5; 0.1M-succinic acid/NaOH, pH6.0. Values given are the ranges obtained from the number of determinations shown in parentheses.

pH	V (units/μg of protein)	K_m (mg of CM-cellulose/ml)
2.5	10.8–11.2 (3)	1.3–1.4 (3)
3.0	25.7–31.5 (4)	4.0–6.3 (4)
3.5	46.2–60.9 (4)	5.3–9.2 (4)
4.0	85–106 (5)	14.5–17.3 (5)
4.5	108–140 (4)	30–39 (4)
5.0	127–207 (4)	52–80 (4)
5.5	36.5–47.5 (4)	40–55 (5)
6.0	11.0–16.4 (4)	21–30 (4)

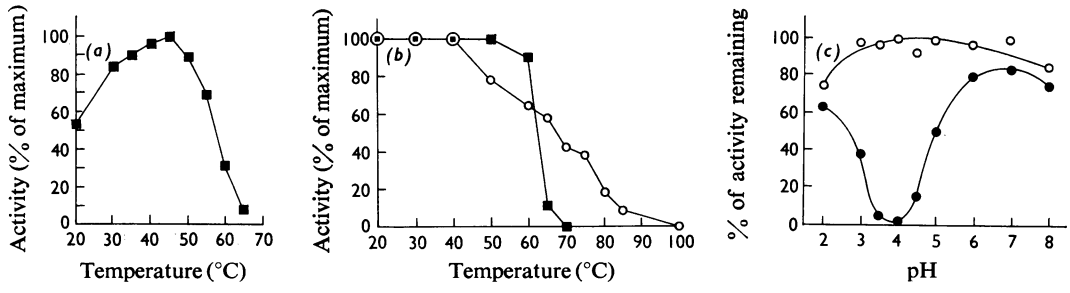


Fig. 3. *Effect of heat on cellulase*

(a) Variation of cellulase activity with temperature. Enzyme (0.2 μg) was assayed in the standard system, except that the temperatures were varied as shown. Points are the average of duplicate determinations. (*b*) Stability of cellulase at different temperatures and two pH values. Cellulase solutions (1.0 ml, containing 24 μg) in 0.1 M-acetic acid/NaOH, pH 4.0 buffer (■), or 0.1 M-phosphoric acid/NaOH, pH 8.0 buffer (○), was incubated for 1 h at the temperatures indicated, diluted with 9.0 ml of cold 0.1 M-acetate buffer, pH 4.0, and then 0.1 ml samples were assayed by the standard procedure. Points are average of duplicate determinations. (*c*) Effect of pH on the stability of cellulase at 65°C and 50°C. Buffered solutions of cellulase (1 ml containing 20 μg) were incubated for 30 min, at 65° (●) or 50°C (○), cooled and diluted with 9.0 ml of 0.1 M-sodium acetate buffer, pH 4.0. Duplicate assays were carried out with 0.1 ml samples; 100% activity remaining represents the sample treated at 20°C and pH 4.0. Buffers used were: 0.1 M-glycine/HCl, pH 2.0; 0.1 M-formic acid/NaOH, pH 3.0, 3.5; 0.1 M-acetic acid/NaOH, pH 4.0, 4.5, 5.0; 0.05 M-Mes (4-morpholineethanesulphonic acid)/NaOH, pH 6.0; 0.05 M-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulphonic acid})/NaOH, pH 7.0; 0.05 M-Tris/HCl, pH 8.0.

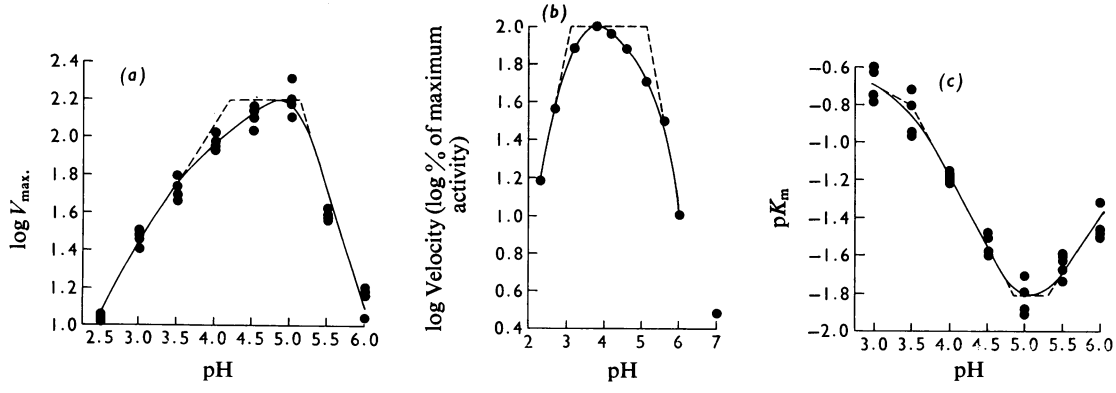


Fig. 4. *Effect of pH on the kinetic parameters of cellulase*

(*a*) Variation of log V with pH. log V values were derived from the data in Table 3. The points represent different experimental determinations. (*b*) Variation of log v with pH. The log of relative v values shown in Fig. 2 are plotted versus pH. (*c*) Variation of pK_m with pH. pK_m ($-\log K_m$) values obtained from experiments as described in Table 3 are plotted versus pH. Each point represents a separate experimental determination. In (*a*), (*b*) and (*c*) the dashed lines are extrapolations of the linear portions of the curves, used in the estimation of pK values.

(Fig. 2) was pH 3.8. By using the analysis of Dixon & Webb (1964), the log V versus pH plot (Fig. 4*a*) gave pK_{ES} values of 4.2 and 5.1 for groups in the enzyme–substrate complex. The plot of log v versus pH (Fig. 4*b*) revealed pK values of 3.1 and 5.1, which approximate to the pK values of groups on either the free enzyme or free substrate. Further indication of pK_{ES} values was obtained from the pK_m versus pH graph (Fig. 4*c*), which gave values of 4.8 and 5.3. The break at pH 3.6 may be due to the ionization of the substrate (King & Smibert, 1963). Slopes of ± 1 were obtained only from the log v versus pH plot; the slopes obtained from the log V versus pH and pK_m versus pH plots were +0.6, −1.2 and +0.6, −0.7 respectively.

Discussion

Determination of cellulase activity with *p*-hydroxybenzoic acid hydrazide was preferred to other methods for two reasons. One was the greater sensi-

tivity of the method compared with the commonly used dinitrosalicyclic acid procedure (Sumner & Sisler, 1944; Miller *et al.*, 1960); the latter has a lower limit of sensitivity of 100 μg of glucose equivalent, compared with 1–2 μg for the *p*-hydroxybenzoic acid hydrazide. Further, destruction of reducing sugars is not encountered with *p*-hydroxybenzoic acid hydrazide, but is apparent with the dinitrosalicylic acid method (Miller *et al.*, 1960). Second, although the copper-reagent method of Nelson (1944) and Somogyi (1952) has sensitivity comparable with that of *p*-hydroxybenzoic acid hydrazide, the former suffers from the disadvantage that the assay involves three successive manipulations. When large numbers of assays are to be performed, for example in the elucidation of a purification scheme, it is desirable to have a rapid assay system involving a minimum number of steps; this criterion is met by the *p*-hydroxybenzoic acid hydrazide method.

Fractionation of the crude cellulase preparation into as many different cellulolytic components as possible was not the objective of this work. The purification scheme was designed for the isolation of one homogeneous cellulase from several elaborated by *A. niger*. Ikeda *et al.* (1967) obtained four cellulases from *A. niger*, but the purity of these fractions was not described. Subsequently a modified procedure was designed for the isolation of a specific homogeneous cellulase (Ikeda *et al.*, 1973*a*). Clarke & Stone (1965*a*) had reported the purification of a β-(1 $\rightarrow$ 4)-glucan hydrolase from a crude *A. niger* extract, and subsequently described some enzymic properties of that cellulase (Clarke & Stone, 1965*b*). The physicochemical and enzymic properties reported in the present paper indicate that this enzyme is different from previously described *A. niger* cellulases.

Table 1 shows that the cellulase has been purified 25-fold compared with 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 in the present work is, in reality, several hundredfold.

Evidence for the homogeneity of the cellulase comes from the SDS/polyacrylamide-gel electrophoresis experiments and the sedimentation studies: both of these techniques indicated a single protein species. Further, the cellulase migrated as a single symmetrical peak on the gel-filtration columns used for molecular-weight estimation.

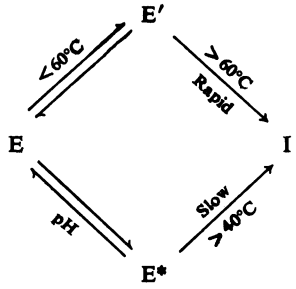
Generally the mol.wt. of cellulases is low, ranging from 5600 (Selby & Maitland, 1965) to 76000 (Li *et al.*, 1965). The mol.wt. of 26000 obtained from the SDS/polyacrylamide-gel electrophoresis, amino acid composition and gel-filtration data is compatible with the determined sedimentation coefficient of 2.5 $\pm$ 0.1 S. These experiments, together with the chemical analyses, indicate that the cellulase is a single polypeptide chain devoid of disulphide linkages and carbohydrate. A cellulase from *A. niger*, with a mol.wt. and sedimentation coefficient of 46000 and 3.27 S respectively, containing both glucosamine and arabinose has been reported (Ikeda *et al.*, 1973*a*,*b*).

The rapid decrease in the specific viscosity of CM-cellulose solutions accompanied by a small increase in reducing sugars (Fig. 1) is compatible with a random hydrolysis of the substrate. In this respect the cellulase resembles components 3, 4, 5 and 6 from *A. niger* of King & Smibert (1963) and the *A. niger* cellulase of Clarke & Stone (1965*b*). Although Ikeda *et al.* (1967) observed similar results with their 'glycol cellulose' as substrate, they concluded that *A. niger* cellulases were exoglucanases because release of reducing sugars from filter paper did not result in visual paper breakdown. The validity of their conclusion is questionable.

The pH–activity profile (Fig. 2) showed a sharp optimum at pH 3.8–4.0. Clarke & Stone (1965*b*) published a profile with a rather broad optimum in the range pH 4–6 obtained with an *A. niger* cellulase and CM-cellulose. The cellulase from *A. niger* reported by Ikeda *et al.* (1973*a*,*b*) is characterized by a pH optimum for activity towards their 'glycol cellulose' at pH 2.5.

Although the cellulase retained 90% of its activity after heating at 60°C and pH 4 for 1 h (Fig. 3*b*), the enzymic effectiveness was much decreased at that temperature (Fig. 3*a*). This observation implies that the decrease in activity from 45° to 65°C cannot be fully explained by enzyme instability at these temperatures. Hence, the temperature optimum determined in this experiment cannot be classically described as the balance between the effect of temperature on catalysis and the effect of temperature on the rate of enzyme denaturation. The presence of substrate did not affect the heat stability of the enzyme. Temperatures greater than 60°C (at pH 4) resulted in irreversible inactivation. In contrast, gradual loss of stability with increasing temperature at pH 8 suggested that the H^+ concentration had a role in the temperature stability of the cellulase. This was substantiated by the results in Fig. 3(*c*): at 65°C the stability of the enzyme was markedly pH-dependent, being least stable at pH values where activity (at 40°C) is maximal. At 50°C, however, enzymic activity was retained throughout the greater part of the pH range used. These results can be explained by postulating modified forms of the enzyme as shown in Scheme 1.

E, as the low-temperature form of the enzyme at about pH 4, is reversibly converted into an inactive or partially active form (E′) at temperatures up to 60°C. At temperatures greater than 60°C (pH 4) this state is



Scheme 1. *Possible modified forms and related pathways of the cellulase*
For details see the text.

rapidly and irreversibly denatured (I). E* is a species of enzyme that predominates at pH values greater than 6, where catalysis is poor, and is characterized by a relatively slow rate of heat inactivation. A corresponding state may exist at low pH values. In view of the remarkable similarity of the curve obtained at 65°C (Fig. 3*c*) and the inverse of the pH-activity profile (Fig. 2), it is tempting to speculate that the ionization of those groups on the enzyme responsible for the catalysis is also involved in the temperature stability of the enzyme.

Graphical analyses gave four pK values for groups involved in the enzyme–substrate complex. It is not possible to say with certainty that these values are indicative of two, three or four unique amino acid residues. Comparisons of Fig. 4(*b*) with Figs. 4(*a*) and 4(*c*) show that the interaction of the enzyme and substrate results in significant perturbation of the pK of one of the groups in the enzyme (3.1 increases to either 4.2 or 4.8). The observation that the slopes from the plots log V against pH and pK_m against pH are not ± 1 implies that a simple model of the pH-dependence of cellulase action is insufficient to describe the cellulase–CM-cellulose system. Wakim *et al.* (1969) come to a similar conclusion when they reported gross divergence from unit slopes in the log V versus pH plot obtained with pig pancreatic α-amylase acting on starch. Hydrolysis of the nitrophenyl glycoside of *N*-acetylglucosaminyl-β-(1,4)-glucose by lysozyme showed complex pH-dependence of the kinetic constants in the acid region; a slope of 0.6 was obtained from the log V/K_m versus pH plot by Rand-Meir *et al.* (1969) and they suggested that this effect might have been due to the interaction of two ionizable groups in lysozyme.

The pK values obtained indicate that carboxyl groups may be involved in cellulase catalysis. A wealth of evidence exists for the involvement of carboxyl groups in the catalytic mechanism of lysozyme (for reviews see Osserman *et al.*, 1974). In view of the functional similarity between lysozyme and cellulase (both cleave β-1,4-glucosidic bonds) it is tempting to speculate that a similar situation occurs in cellulase, with a carboxyl group acting as a general acid, protonating the leaving groups.

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