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PURIFICATION AND FUNCTIONAL CHARACTERISTICS OF AN ENDOCELLULASE FROM *Chaetomium thermophile* VAR. COPROPHILE

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ABSTRACT

An endocellulase (1→4)-β-D-glucan 4-glucanohydrolase was isolated from the culture filtrates of *Chaetomium thermophile*. The enzyme was homogeneous by PAGE and SDS-PAGE. The molecular weight was 36 000 by SDS-PAGE and 38 000 by gel filtration. It was a glycoprotein. From the amino acid composition, it was found to be rich in glycine, threonine, and aspartic and glutamic acids, but contained only low proportions of histidine and sulfur-containing amino acids. It was optimally active at pH 6 and at 60°. The enzyme did not hydrolyze cellobiose and cellotriose, but hydrolyzed cello-tetraose, -pentaose, and -hexaose at comparable rates. It was specific for molecules containing β-(1→4) linkages. It showed high activity towards amorphous cellulose, and the reaction products contained cellobiose to cellopentaose, showing that it effects random cleavage of cellulose.

INTRODUCTION

The utility of cellulases for saccharification of cellulosic materials has assumed importance for the production of food and fuels. The hydrolysis of cellulose is effected by the cooperative action[1–3] of endocellulases [(1→4)-β-D-glucan 4-glucanhydrolase, E.C. 3.2.1.4], exocellulases [(1→4)-β-D-glucan cellobiohydrolase, E.C. 3.2.1.91] and β-D-glucosidases (E.C. 3.2.1.21). Because of their rapid growth and high rate of cellulose decomposition[4–6], thermophilic fungi are an attractive potential source of cellulases. Cellulases from the thermophilic fungi have been reported to be stable at high temperatures[6,7]. Considerable work has already been carried out on the isolation and characterization of endocellulases from mesophilic sources[8–10]. However, endocellulases from thermophilic fungi have not been investigated to the same extent. We now describe the purification of an endocellulase from the culture filtrates of the thermophilic fungus *Chaetomium thermophile* var. coprophile, and its characterization.

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MATERIALS AND METHODS

Chemicals and reagents. — DEAE-Sephadex A-50, Sephacryl S-200, polyacrylamide, SDS, lichenan, larchwood xylan, arabinogalactan, chitin, Sigmacell Type 20, *O*-(carboxymethyl)cellulose (CMC, medium viscosity), and baker's yeast glucan were obtained from Sigma. Ultrogel AcA-54 was from LKB, Sweden. Yeast extract and peptone were from Difco. Silica Gel G was from Merck. Phosphoric acid-swollen cellulose was prepared according to Wood[11], and cello-oligosaccharides were prepared according to the method of Miller *et al.*[12].

Organism. — *Chaetomium thermophile* var. coprophile IIS-110 was isolated from soil collected from the forests of Kerala, India, by enrichment culture on sugar-cane bagasse, and characterized[13].

Enzyme assays. — The substrate specificities of the principal enzymes of the cellulase system (exocellulase, endocellulase, and β-D-glucosidase) are known. Endocellulase hydrolyzes soluble CMC significantly, whereas exocellulase, when acting alone, can release a significant amount of reducing sugar from microcrystalline cellulose[14].

Endocellulase activity was determined by measuring the soluble sugars released during 30 min at 50° in a mixture containing 50mM sodium acetate buffer (pH 5.6), 5 mg of CMC, a suitable aliquot of the enzyme, and water to give a total volume of 2.0 mL. The reducing sugars were estimated according to the Somogyi–Nelson method[15]. Samples were centrifuged before reading the absorbance at 520 nm.

Exocellulase activity was measured in a reaction mixture containing 50mM sodium acetate buffer (pH 5.6), 50 mg of Sigmacell Type 20, a suitable aliquot of the enzyme, and water to give a total volume of 2.0 mL. Incubation was carried out in a shaking incubator for 30 min at 50°. The reaction was stopped by keeping the tube for 10 min in a boiling-water bath. The tubes were centrifuged at 2000*g* for 10 min, and the reducing sugars in the supernatant liquor were estimated colorimetrically as described by Somogyi[15].

One international unit (I.U.) of endocellulase or exocellulase activity is defined as the amount of the enzyme which produces one μmole of D-glucose equivalent per min at 50°.

Amino acid analysis. — The purified protein was hydrolyzed in 6M HCl according to the method of Moore and Stein[16], and analyzed in an LKB 4400 automatic amino acid analyzer. Cysteine was determined following peroxyformic acid oxidation[17].

Gel electrophoresis. — Non-denaturing disc-gel electrophoresis was carried out at pH 4.5 on tube gels (7.5%, w/v) in Tris–glycine buffer, pH 8.3, according to Davis[18], and SDS-PAGE was done on 12.5% gels according to Laemmli[19]. The gels were stained with Coomassie Brilliant Blue for proteins, and for glycoproteins by the periodic acid–Schiff technique of Zacharius and Zell[20].

Thin-layer chromatography. — Sugars were chromatographed on plates of

Silica Gel G (0.25 mm) activated for 90 min at 110° just before use, in 3:3:1 (v/v) ethyl acetate–acetic acid–water. Sugars were made visible by spraying with 20% H_2SO_4 in ethanol followed by heating for 15–20 min at 110°.

Protein estimation. — Protein was estimated by the absorbance at 280 nm, or according to Lowry *et al.*[21], with bovine serum albumin as the reference standard.

Crude enzyme preparation. — *C. thermophile* was grown at 50° in a rotary shaker (250 r.p.m.) in Erlenmeyer flasks (500 mL) containing 150 mL of a medium that contained, per liter, $NH_4H_2PO_4$, 0.5 g; K_2HPO_4, 2 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; yeast extract, 1 g; peptone, 5 g; Sigmacell Type 20, 20 g; and Vogel trace elements, 0.01% (v/v). The pH of the medium was adjusted to 6.0, the solution was autoclaved for 20 min at 103.5 kPa, and inoculated with 60-h-old mycelial inoculum of the organism (5%, v/v). The mycelial inoculum was produced by adding spore suspension to 150 mL of the medium (D-glucose 2%, L-asparagine 0.4%, KH_2PO_4 0.1%, peptone 0.5%, and Vogel's trace element stock solution 0.01% v/v) contained in 500-mL Erlenmeyer flasks and allowed to germinate on a shaker (240 r.p.m.) at 50°. After 7 d of growth, the residual cellulose and mycelium in the culture medium were removed by centrifugation at 10 000 r.p.m. for 10 min. Solid $(NH_4)_2SO_4$ was added, with stirring, to 70% saturation and the precipitated proteins were collected by centrifugation, dissolved in water, and the solution desalted on a Sephadex G-25 column (100 × 2 cm) equilibrated with 0.1M acetate buffer, pH 6.0. The column was run at a flow rate of 15 mL/h, and 3-mL fractions were collected. The fractions containing proteins were pooled and lyophilized. From 7.8 L of the culture filtrate, 4.05 g of protein was obtained.

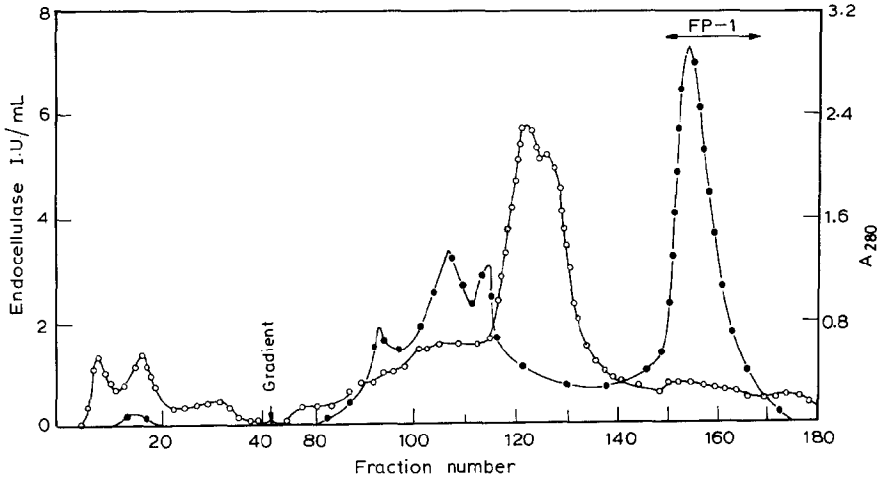


Fig. 1. Chromatography of crude proteins of *C. thermophile* on DEAE-Sephadex A-50. The experimental conditions are described in the text. Flow rate, 25 mL/h; fraction volume, 6 mL; (○), A_{280}; (●), endocellulase.

RESULTS

Purification of endocellulase. — The crude protein preparation (300 mg) was initially fractionated on a column (60 × 2 cm) of DEAE-Sephadex A-50 equilibrated with Tris–HCl buffer (pH 7.4; 50mM). After washing the column with 250 mL of the buffer, a linear gradient of sodium chloride (30–300mM) in the same buffer was applied. It may be seen from Fig. 1 that the crude protein contained four endocellulases. Fractions (148–168) having major amounts of the enzyme activity were pooled, lyophilized, and desalted by gel filtration as before. The procedure was repeated three times and the pooled endocellulase-proteins were rechromatographed on a DEAE-Sephadex A-50 column (60 × 1.6 cm) equilibrated with 50mM Tris-HCl buffer (pH 7.4). The proteins were eluted by applying a downward pH gradient of 7.4 to 6 with a simultaneous NaCl gradient of 100 to 300mM; the total volume of the gradient was 400 mL. It may be seen from Fig. 2 that the endocellulase was resolved into two peaks. Fractions 99–114, containing most of the endocellulase activity, were pooled, concentrated by lyophilization, desalted on Sephadex G-25, and lyophilized to dryness. This preparation was chromatographed on a column (110 × 2 cm) of Ultrogel AcA-54 equilibrated with ammonium acetate–acetic acid buffer (50mM; pH 5.5). Fractions 62–73 (see Fig. 3), containing the endocellulase, were pooled and concentrated by lyophilization. The concentrate was then chromatographed on a column (90 × 1.25 cm) of Sephacryl S-200 equilibrated with 50mM ammonium acetate–acetic acid buffer (pH 6.5). This resulted in the separation of small amounts of contaminating proteins, and endocellulase was eluted as a symmetrical peak. Fractions 51–63, containing the enzyme activity, were pooled, concentrated by lyophilization, and desalted on Sephadex

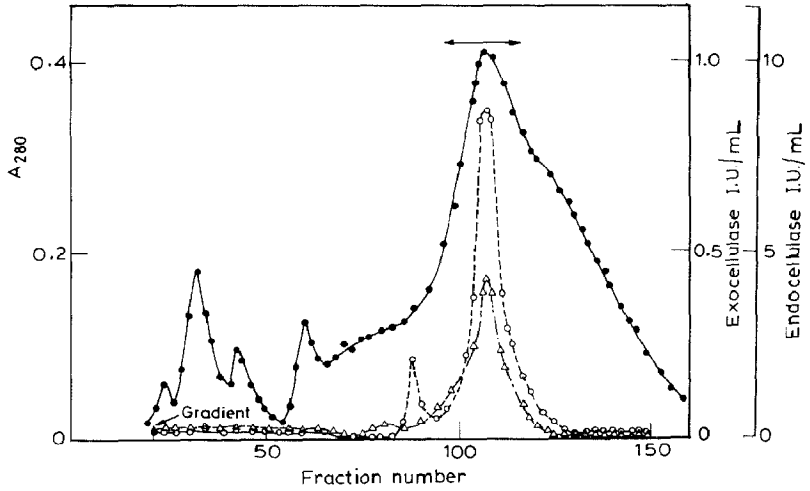


Fig. 2. Chromatography of endocellulase on DEAE-Sephadex A-50. The experimental conditions are described in the text. Flow rate, 15 mL/h; fraction volume, 3 mL; (○), A_{280}; (●), endocellulase; (△), exocellulase.

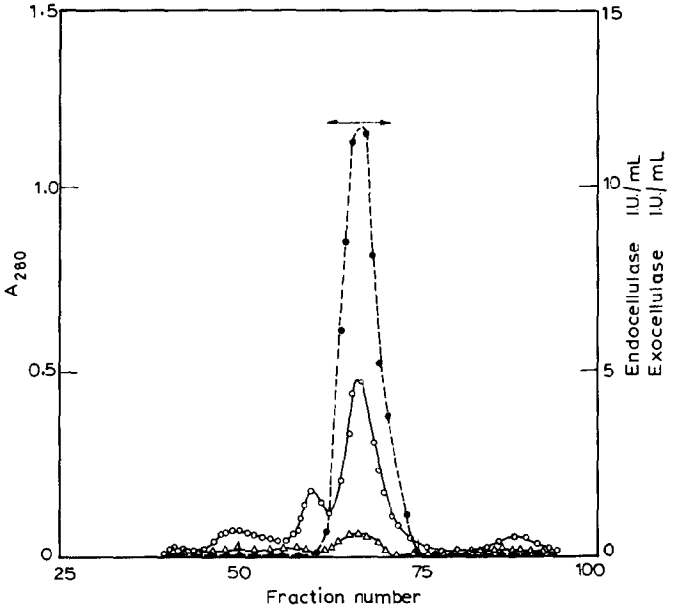


Fig. 3. Chromatography of endocellulase on Ultrogel AcA-54. Experimental conditions are described in the text. Flow rate, 12 mL/h; fraction volume, 3 mL; (○), A_{280}; (●), endocellulase; (△), exocellulase.

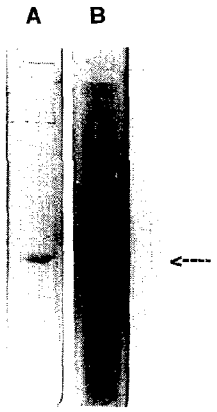
TABLE I

PURIFICATION OF ENDOCELLULASE FROM CULTURE FILTRATE OF *C. themrophile*

Purification step	*Total activity (μmol/min)*	*Total absorbance (A) at 280 nm*	*Specific activity μmol/min/A*	*Yield (%)*
Crude culture-filtrate	5334	5736	0.93	100
Ammonium sulfate precipitation (0–70%)	4833	2856	1.69	91
Sephadex G-25 chromatography	3,915	1,836	2.13	73.4
DEAE-Sephadex A-50 chromatography	400	74	5.14	7.5
DEAE-Sephadex A-50 chromatography	205	22	9.3	3.8
Ultrogel AcA-54 chromatography	186	8.5	22.0	3.5
Sephacryl S-200 chromatography	138	5.9	23.4	2.6

G-25. This final preparation was used for further studies. It may be seen from Table I that this procedure led to a 25-fold purification of the enzyme, with 2.6% recovery.

The purified endocellulase moved as a homogeneous protein on PAGE (see



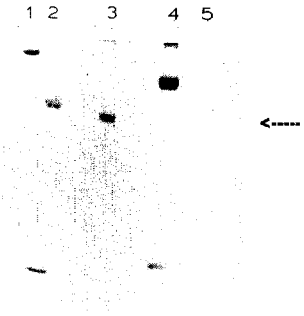


Fig. 4. Polyacrylamide gel electrophoresis of endocellulase. Electrophoresis was carried out as described in Methods. A, Coomassie Brilliant Blue staining; B, glycoprotein staining of proteins.

Fig. 5. SDS-PAGE of endocellulase. Electrophoresis was done as described in Methods. Lane 1, phosphorylase B (96K); Lane 2, ovalbumin (45K); Lane 3, endocellulase (36K); Lane 4, BSA (67K); and Lane 5, lysozyme (14.3K).

TABLE II

AMINO ACID COMPOSITION OF ENDOCELLULASE ISOLATED FROM *C. thermophile*

Amino acid residue	*Mole percent*
Aspartic acid[a]	8.3
Threonine	16.0
Serine	8.3
Glutamic acid[a]	8.8
Proline	3.8
Glycine	14.8
Alanine	8.0
Cysteine[b]	1.8
Valine	6.7
Methionine	0.9
Isoleucine	3.8
Leucine	5.6
Tyrosine	3.2
Phenylalanine	1.6
Histidine	1.8
Lysine	2.5
Arginine	1.95
Tryptophan	N.D.[c]

[a]Includes L-asparagine and L-glutamine. [b]Cysteine determined as cysteic acid by peroxyformic acid oxidation. [c]N.D., not determined.

Fig. 4) and SDS-PAGE (see Fig. 5). It was a glycoprotein (see Fig. 4). The molecular weight of the endocellulase was calculated to be 38 000 by gel filtration on Sephacryl S-200, and 36 000 by SDS-PAGE.

Amino acid composition. — Amino acid composition of the endocellulase

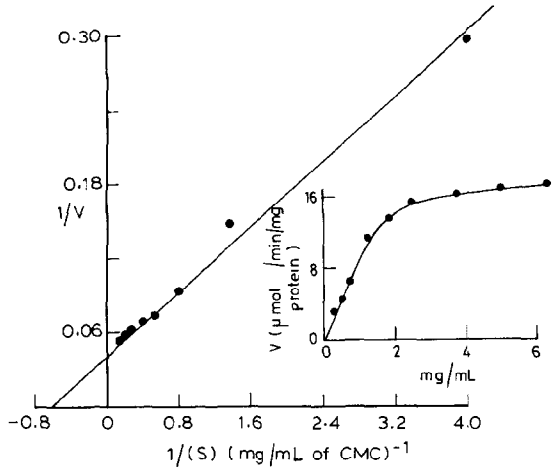
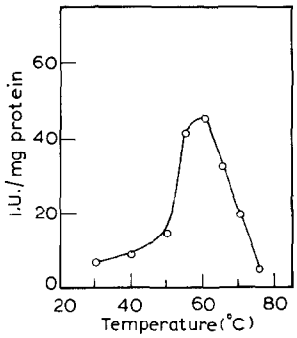


Fig. 6. Lineweaver–Burk plot of endocellulase action on *O*-(carboxymethyl)cellulose.



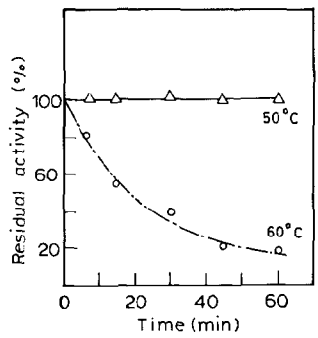


Fig. 7. Effect of temperature on endocellulase activity. Experimental conditions are as described in Methods, except that 0.25 μg of enzyme was used per assay, and the tubes were incubated for 30 min at the temperatures indicated.

Fig. 8. Thermostability of endocellulase. Endocellulase (10 μg) was taken in 10 mL of 50mM sodium acetate buffer, pH 5.6, and incubated in closed tubes at 50 or 60°. Aliquots were withdrawn at regular intervals, and the enzyme activity at 50° was determined.

(see Table II) showed that the protein is rich in threonine, glycine, aspartyl and glutamyl residues, serine, and alanine, which together account for 65% of the total amino acids, and poor in the sulfur-containing amino acids, which is very much different from the amino acid composition of the five isozymes of endocellulase isolated from *Sporotrichum pulverulentum*[22].

Properties of the enzyme. — Endocellulase showed optimal activity between pH 5.8 to 6.2. It showed no activity at pH 3.8 but surprisingly showed 64% of the maximal activity at pH 8.8. The enzyme activity increased with protein concentration up to 0.5 mg/reaction, and the formation of product was linear, up to 30 min

TABLE III

ACTION OF ENDOCELLULASE ON DIFFERENT POLYSACCHARIDES

Substrate[a]	*Concentration of substrate*		*Linkage type(s)*	*Activity*
	mg/mL	*μmol/mL*		*μmoles/min/mg of protein*
O-(Carboxymethyl)cellulose	5		β-(1→4)	14.5
Phosphoric acid-swollen cellulose	5		β-(1→4)	6.5
Lichenan	5		β-(1→4); β-(1→3)	19.6
Baker's yeast D-glucan	2		β-(1→3)	N.D.[b]
Dewaxed cotton	25		β-(1→4)	0.05
Sigmacell Type 20	25		β-(1→4)	1.4
Xylan	2		β-(1→4)	N.D.
Chitin	5		β-(1→4)	N.D.
Starch	5		α-(1→4); α-(1→6)	N.D.
Cyclomaltoheptaose	5		α-(1→4)	N.D.
Arabinogalactan	5		β-(1→3); β-(1→6)	N.D.
Cellobiose		2	β-(1→4)	N.D.
Cellotriose		2	β-(1→4)	N.D.
Cellotetraose		2	β-(1→4)	5.76
Cellopentaose		2	β-(1→4)	6.25
Cellohexaose		2	β-(1→4)	6.45

[a]The enzyme (0.3 μg) was incubated with each of the substrates in a total volume of 1 mL for 30 min at pH 5.6 and 50°. The reaction was terminated by keeping the tubes in a boiling-water bath for 10 min, and the amounts of reducing sugars liberated were estimated according to Somogyi[15]. [b]N.D., not detected.

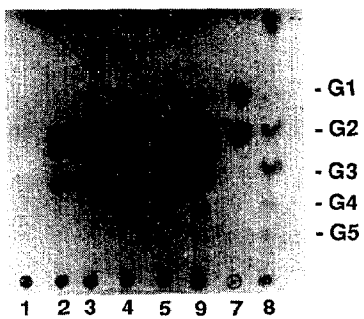


Fig. 9. T.l.c. of products of hydrolysis of cellulose by endocellulase. Reaction mixture containing phosphoric acid-swollen cellulose (30 mg) and endocellulase (20 μg) was incubated at 50°. Aliquots were withdrawn at regular intervals, and the reactions stopped by keeping the tubes for 10 min in a boiling-water bath. They were centrifuged, the clear, supernatant liquor was evaporated, and the residue dried. The residue was dissolved in a small amount of water, and the solution used for t.l.c. as described in Methods. Lane 1: unhydrolyzed sample; Lanes 2, 3, 4, 5, and 9 represent samples withdrawn at 5, 15, 30, 60, and 120 min of incubation, respectively. Lanes 7 and 8; standard oligosaccharides.

of incubation. The Lineweaver–Burk plot of endocellulase gave a K_m of 1.67 mg of CMC/mL and a V_{max} of 25 mmol of reducing equivalents liberated/min/mg of protein (Fig. 6). With the assay protocol used, the enzyme showed maximal activity at 60° (see Fig. 7). The enzyme was unstable at this temperature in the absence of substrate (see Fig. 8), since it steadily lost activity over a period of 60 min with $t_{1/2}$ of 18 min. This indicates that *O*-CMC partially protects the enzyme from heat inactivation.

Hydrolysis of different polysaccharides and oligosaccharides by endocellulase. — Endocellulase showed maximal activity on lichenan, followed by CMC and phosphoric acid-swollen cellulose. It had limited ability to degrade such crystalline cellulosic materials as Sigmacell Type 20 or dewaxed cotton, and did not hydrolyze any of the other polysaccharides, showing that it is specific for β-(1→4)-D-glycosidic linkages. It also did not hydrolyze cellobiose and cellotriose, but hydrolyzed cellotetraose, -pentaose, and -hexaose at comparable rates, indicating that a minimum of four D-glucose residues linked in β-(1→4) fashion is required for enzyme action (see Table III).

To be able to understand the mode of action of endocellulase, it was incubated with phosphoric acid-swollen cellulose, and analysis of the products formed was made. It may be seen from Fig. 9 that cellobiose and cellotriose are the major products of hydrolysis, even at 5 min of incubation, with traces of cellotetraose and cellopentaose. It is possible that cello-tetraose and -pentaose and other higher oligosaccharides are the immediate products of hydrolysis of cellulose by endocellulase, because the rate of hydrolysis of phosphoric acid-swollen cellulose by endocellulase is comparable to that on the higher oligosaccharides (G_4–G_6), so that these oligosaccharides are immediately hydrolyzed to cellobiose and cellotriose, which would accumulate, as endocellulase does not hydrolyze cellobiose and cellotriose.

DISCUSSION

Extracellular cellulolytic components from *C. thermophile* var. coprophile reported here exist in multiple forms. It is shown that the organism secretes at least four endocellulases into the culture medium when grown on cellulose. This is in contrast to the report on *C. thermophile* var. dissitum[23], which secreted only one endocellulase into the medium, but is similar to *Trichoderma viride*[24] and *Trichoderma koningii*[25], which produced six endocellulases, and to *Penicillium funiculosum*[26], which secreted three or more of the endocellulases. Among various endocellulase components produced by *C. thermophile* var. coprophile, the major endocellulase component was purified to homogeneity as judged by PAGE, SDS–PAGE, and gel filtration. It consists of a single polypeptide chain with a molecular weight of 36 000, which is similar to the endocellulases isolated from *Fusarium solani*[8], *Sporotrichum pulverulentum*[9] and *Thermoascus aurantiacus*[27].

The maximal temperature for the hydrolysis of CMC by the endocellulase

from *C. thermophile* is 60°, which is higher than that reported for the thermophilic fungus *Humicola insolens*[28] and some of the mesophilic sources[29–31], but is similar to those reported for other thermophiles such as *Clostridium thermocellum*[32] and *C. thermophile* var. dissitum[23], and lower than those for the endocellulases from *Talaromyces emersonii*[33] and *Thermoascus aurantiacus*[27]. It was found to be stable at 50°, but unstable at its maximal temperature for activity (*i.e.*, 60°), and it lost 50% of its activity in 18 min in the absence of substrate.

The endocellulase reported here is similar to those isolated from other sources in its ability to degrade such soluble forms of polysaccharides as CMC and lichenan more readily than the crystalline celluloses, Sigmacell and cotton[10,23,34–36]. Activity towards lichenan, a mixed D-glucan containing β-(1→3) and β-(1→4) linkages[37], is higher compared to CMC, which is similar to the results reported by Hurst *et al.*[38] and Gilkes *et al.*[39]. The presence of carboxymethyl substitution in the case of CMC may be responsible for lower rates of hydrolysis[40].

Because the enzyme was unable to degrade baker's yeast glucan [(1→3)-β-D-glucan], cyclomaltoheptaose or starch [(1→4)-α-D-glucans], it appears that the enzyme is specific for (1→4)-β-D-glucans. The enzyme did not hydrolyze larchwood xylan, indicating that it requires the hydroxymethyl group on C-5 for activity. In this respect, it differs from the endocellulases from *T. viride*[41] and *H. insolens*[42], both of which were shown to hydrolyze xylan.

An analysis of the products of hydrolysis of phosphoric acid-swollen cellulose showed that the enzyme cleaves the cellulose molecule randomly to produce oligosaccharides ranging from cellobiose to cellopentaose, indicating that it is an endocellulase. It appears that the minimum chain-length required for hydrolysis by *C. thermophile* endocellulase is cellotetraose. This is similar to the endocellulase from *C. thermocellum*[32] and a marine shipworm bacterium[43]. Although the enzymes from *Aspergillus niger*[37], *Myrothecium verrucaria*[44], and *T. viride*[45] hydrolyze cello-oligosaccharides with a degree of polymerization of four and above, they differ from the endocellulase described here. Thus, the enzyme from *T. viride*[44] was maximally active on cellohexaose, whereas the enzyme from the two other organisms showed optimal activity against cellopentaose.

The endocellulase purified in the present study represents only a portion of the total endocellulase activity of the *C. thermophile*. The purification, characterization and subsequent comparison of other endocellulase components may provide instructive insight on the diversity and mechanism of cellulose degradation by *C. thermophile* var. coprophile.

REFERENCES

1 T. M. Wood, *Biotechnol. Bioeng. Symp.*, 5 (1975) 111–136.
2 C. S. Gong, M. R. Ladisch, and G. T. Tsao, *Adv. Chem. Ser.*, 181 (1979) 261–287.
3 E. T. Reese, *Rec. Adv. Phytochem.*, 11 (1977) 311–367.
4 S. L. Rosenberg, *Can. J. Microbiol.*, 21 (1975) 1535–1540.
5 B. Hagerdal, J. D. Ferchak, and E. K. Pye, *Biotechnol. Bioeng.* 22 (1980) 1515–1526.
6 A. Margaritis and R. Merchant, *Biotechnol. Bioeng. Symp.*, 13 (1983) 299–314.

7 S. HAYASHIDA AND H. YOSHIOKA, *Agric. Biol. Chem.*, 44 (1980) 481–487.
8 T. M. WOOD, *Biochem. J.*, 115 (1969) 457–464.
9 K. E. ERIKSSON AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1975) 213–218.
10 L. E. R. BERGHEM, L. G. PETTERSSON, AND U. B. AXIO-FREDRICKSSON, *Eur. J. Biochem.*, 61 (1976) 621–630.
11 T. M. WOOD, *Biochem. J.*, 121 (1971) 353–362.
12 G. L. MILLER, J. DEAN, AND R. BLUM, *Arch. Biochem. Biophys.*, 91 (1960) 21–26.
13 R. K. GANJU, Ph.D. Thesis, Indian Institute of Science, Bangalore, India (1986).
14 T. M. WOOD AND K. M. BHAT, *Methods Enzymol.*, 160 (1988) 87–111.
15 M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19–23.
16 S. MOORE AND W. H. STEIN, *Methods Enzymol.*, 6 (1953) 819–831.
17 C. H. W. HIRS, *J. Biol. Chem.*, 219 (1956) 611–621.
18 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404–411.
19 U. K. LAEMMLI, *Nature (London)*, 227 (1970) 680–685.
20 R. M. ZACHARIUS AND T. E. ZELL, *Anal. Biochem.*, 30 (1969) 148–152.
21 H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
22 K. E. ERIKSSON AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1975) 193–206.
23 J. ERIKSSON AND J. GOKSOYR, *Eur. J. Biochem.*, 77 (1977) 445–450.
24 G. BELDMAN, F. M. SEARLE-VAN LEUWEN, R. M. ROMBOUTS, AND G. J. VORAGEN, *Eur. J. Biochem.*, 146 (1985) 301–308.
25 T. M. WOOD AND S. I. MCCRAE, *Adv. Chem. Ser.*, 181 (1979) 181–209.
26 T. M. WOOD, S. I. MCCRAE, AND C. C. MACFARLANE, *Biochem. J.*, 189 (1980) 51–65.
27 C. C. TONG, A. L. COLE, AND M. G. SHEPHERD, *Biochem. J.*, 191 (1980) 83–94.
28 S. HAYASHIDA AND H. YOSIOKA, *Agric. Biol. Chem.*, 44 (1980) 1721–1728.
29 H. BJÖRNDAL AND K. E. ERIKSSON, *Arch. Biochem. Biophys.*, 124 (1968) 149–153.
30 W. A. LINDNER, C. DENNISON, AND R. K. BERRY, *Biochim. Biophys. Acta*, 746 (1983) 160–167.
31 G. OKADA, *J. Biochem. (Tokyo)*, 80 (1976) 913–922.
32 J. K. NG AND J. G. ZEIKUS, *Biochem. J.*, 199 (1981) 341–350.
33 M. P. COUGHLAN AND A. P. MOLONEY, *Methods Enzymol.*, 160 (1988) 363–368.
34 G. PETTERSSON, *Arch. Biochem. Biophys.*, 123 (1968) 307–311.
35 J. KANDA, K. WAKABAYASHI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 79 (1976) 977–988.
36 C. S. GONG AND G. T. TSAO, *Annu. Rep. Ferm. Proc.*, 3 (1979) 111–140.
37 S. PEAT, W. J. WHELAN, AND J. G. ROBERTS, *J. Chem. Soc.*, (1957) 3916–3924.
38 P. L. HURST, P. A. SULLIVAN, AND M. G. SHEPHERD, *Biochem. J.*, 169 (1978) 387–395.
39 N. R. GILKES, M. L. LANGSFORD, D. G. KILBURN, R. C. MILLER, JR., AND R. A. J. WARREN, *J. Biol. Chem.*, 259 (1984) 10 455–10 459.
40 K. NISIZAWA, *J. Ferm. Technol.*, 51 (1973) 267–304
41 S. TODA, H. SUZUKI, AND K. NISIZAWA, *J. Ferm. Technol.*, 49 (1971) 499–521.
42 U. SUBRAHMANYESWAR RAO AND S. K. MURTHY, *Indian J. Biochem. Biophys.*, 25 (1988) 687–694.
43 R. V. GREENE, H. L. GRIFFIN, AND S. N. FREER, *Arch. Biochem. Biophys.*, 267 (1988) 334–341.
44 E. G. HANSTEIN AND D. R. WHITAKER, *Can. J. Biochem. Physiol.*, 41 (1963) 707–718.
45 L. H. LI, R. M. FLORA, AND K. W. KING, *Arch. Biochem. Biophys.*, 111 (1965) 439–447.