Purification and characterization of an endoglucanase (1,4- β -D-glucan glucanohydrolase) from *Clostridium thermocellum*

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An endoglucanase (1,4- β -D-glucan glucanohydrolase, EC 3.2.1.4) was purified from *Clostridium thermocellum* by procedures that included centrifugation, ultrafiltration, selective precipitation, ion-exchange Sephadex chromatography and preparative gel electrophoresis. The 22-fold-purified enzyme behaved as a homogeneous protein under non-denaturing conditions. The enzyme represented a significant component (>25%) of total extracellular endoglucanase activity, but was purified in low yield by the procedures employed. The native molecular weight of the endoglucanase was determined by ultracentrifugational analysis, amino acid composition and polyacrylamide-gel electrophoresis, and varied between 83 000 and 94 000. The enzyme contained 11.2% carbohydrate and was isoelectric at pH 6.72. The pH and temperature optima of the endoglucanase were 5.2 and 62°C respectively. The enzyme lacked cysteine and was low in sulphur-containing amino acids. The purified endoglucanase displayed: high activity towards carboxymethylcellulose, celloheptaose, cellohexaose and cellopentaose; low activity towards Avicel microcrystalline cellulose and cellotetraose; no detectable activity towards cellotriose or cellobiose; increased activity towards cello-oligosaccharides with increasing degree of polymerization. The internal glycosidic bonds of cello-oligosaccharides were cleaved by the enzyme in preference to external linkages. The apparent Michaelis constant ($[S]_{0.5V}$) and $V_{\max}$ for cellopentaose and cellohexaose hydrolysis were 2.30 mM and 39.3 μ mol/min per mg of protein, and 0.56 mM and 58.7 μ mol/min per mg of protein, respectively.

Cellulase is the key enzymic activity produced by cellulolytic micro-organisms for the biodegradation of cellulose (Siu, 1951). The utility of cellulase for industrial saccharification of cellulosic biomass has been recently reviewed (Linko, 1977; Meyers, 1978; Ryu & Mandels, 1980; Emert *et al.*, 1980).

Cellulase is a complex comprising at least two major components with cellulose-hydrolysing activity, each of which displays a certain degree of multiplicity (Halliwell, 1979). These include both endoglucanases (1,4- β -D-glucan glucanohydrolase, EC 3.2.1.4), which form free reducing chain-ends and short-chain oligosaccharides by random cleavage of the β -(1 $\rightarrow$ 4)-bonds of cellulose, and exoglucanases (1,4- β -D-glucan cellobiohydrolase, EC 3.2.1.91), which form mainly cellobiose by endwise hydrolysis of the free reducing end of

cellulose or cello-oligosaccharides. Another major component activity associated with microbial cellulase is β -glucosidase (β -glucoside glucohydrolase, EC 3.2.1.21), which forms glucose by endwise cleavage of cello-oligosaccharides (Emert *et al.*, 1974). The different enzymic components of cellulase act synergistically and completely solubilize crystalline cellulose to glucose and/or cellobiose (Eriksson, 1975; Berghem *et al.*, 1976; Wood & McCrae, 1978). Despite the fact that many cellulolytic bacteria produce very active cellulases, most of what is known about the mechanism of cellulase activity is derived from previous studies on purified fungal cellulase and especially that of *Trichoderma* (Halliwell, 1979).

Thermophilic cellulolytic bacteria have been recognized for their prolific growth rate on cellulose and the ability of anaerobic species to ferment cellulose directly to ethanol (Zeikus, 1979, 1980; Zeikus *et al.*, 1981). Several strains of thermophilic clostridia have been characterized that produce an

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active extracellular cellulase (Enebo, 1954; Lee & Blackburn, 1975; Ng *et al.*, 1977; Ait *et al.*, 1979; Shimyo *et al.*, 1979; Garcia-Martinez *et al.*, 1980). Recently, the extracellular cellulase activities of *Clostridium thermocellum* strain LQRI were compared with those of the *Trichoderma reesei* strain QM9414, a hyper-cellulase-producing mutant (Ng & Zeikus, 1981). Most notably, *C. thermocellum* cellulase had a higher ratio of endoglucanase to exoglucanase activity, a different mode of action towards cello-oligosaccharide hydrolysis, an equivalent rate of activity for celohexaose conversion into cellotriose, and the enzyme was not significantly inhibited by glucose or cellobiose.

The purpose of the present study was to purify and characterize the general kinetic and mechanistic features of a major endoglucanase component from the extracellular cellulase complex of *C. thermocellum* strain LQRI.

Materials and methods

Organism

C. thermocellum strain LQRI was isolated and cultured as described previously (Lamed & Zeikus, 1980; Ng & Zeikus, 1981).

Crude enzyme preparation

C. thermocellum strain LQRI was grown at 60°C in 14-litre fermenters (New Brunswick, Edison, NJ, U.S.A.) with 12 litres of medium that contained, per litre of distilled water: K_2HPO_4 , 0.5 g; $KH_2PO_4 \cdot 3H_2O$, 1.0 g; urea, 2.0 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $FeSO_4 \cdot 5H_2O$, 0.5 mg; Mops (4-morpholinepropanesulphonic acid), 10 g; cysteine hydrochloride hydrate, 1 g; freeze-dried yeast-extract diffusate, 2 g; cellobiose (Sigma Chemical Co., St. Louis, MO, U.S.A.), 5 g. Freeze-dried yeast extract was pretreated to remove proteins by dialysis of a 40% yeast-extract suspension in a hollow-fibre cartridge (Amicon Corp., Lexington, MA, U.S.A.) against double-distilled water. The diffusate was collected and freeze-dried at -80°C. The culture was incubated (approx. 24 h) without stirring until the late exponential phase, when all the cellobiose was consumed. The culture broth was centrifuged at 10000 g for 30 min. The supernatant was concentrated 10-fold by ultrafiltration with a PM10 membrane (Amicon Corp.). $(NH_4)_2SO_4$ was added to the ultrafiltrate at 80%-saturation final concentration and the precipitated proteins were collected by centrifugation at 9000 g for 30 min. The proteins were resuspended in a minimal volume of 20 mM-ammonium acetate buffer, pH 5.0, and dialysed in a 2-litre ultrafiltration cell with a PM10 membrane until the conductivity matched that of the buffer.

Enzyme purification

The crude enzyme was initially fractionated on a 4.5 cm × 50 cm (bed volume) column of DEAE-Sephadex A-50 (Pharmacia, Piscataway, NJ, U.S.A.). The column was eluted in steps with 50 mM-, 100 mM-, 600 mM- and 800 mM-ammonium acetate buffer, pH 5.0. A constant elution rate of 24 ml/h was obtained by using a peristaltic pump, and 10 ml fractions were collected. Subsequent chromatography with DEAE-Sephadex A-50 was performed on a 2.6 cm × 30 cm (bed volume) column with a constant elution rate of 23 ml/h, and 2.5 ml fractions were collected. The column was eluted with a stepwise increase of NaCl (50 mM, 100 mM and 150 mM) in 50 mM-Tris/HCl buffer, pH 7.2. Sulphopropyl-Sephadex C-50 fractionation employed a 2.6 cm × 30 cm (bed volume) column that was eluted with a continuous gradient of NaCl (0–300 mM) in 20 mM-sodium citrate buffer, pH 3.5. A constant flow rate of 15 ml/h was used, and 3.0 ml fractions were collected.

Preparative gel electrophoresis was performed with the Buchler Poly Prep 200 apparatus (Fort Lee, NJ, U.S.A.) designed as described by Jovin *et al.* (1964). A 4 cm 8% separating gel with a 0.5 cm 4% stacking gel was used. The upper reservoir buffer consisted of 0.8 M-Tris/sulphate, pH 8.0. The ionic strength of the lower-reservoir buffer was twice that of the upper-reservoir buffer. Both reservoirs were continuously fed with buffer at a rate of 1.0 ml/min. The proteins were eluted by sweeping the elution chamber with 0.36 M-Tris/HCl buffer, pH 7.6, at a rate of 1.0 ml/min. Protein content was monitored by a Uvicord II recording spectrophotometer (LKB Instruments, Bromma, Sweden) at 280 nm. Fractions (2.5 ml) were collected and assayed for endoglucanase activity.

The homogeneity of the purified endoglucanase was analysed by polyacrylamide-slab-gel (4–8%) electrophoresis in accordance with the procedures of Maurer (1971). The proteins were located by staining with Coomassie Brilliant Blue R-250 (Chrambach *et al.*, 1967), and glycoproteins were detected with the periodic acid/Schiff reagent (Fazekas *et al.*, 1963). Sodium dodecyl sulphate (1%) /polyacrylamide-gel electrophoresis was performed in accordance with the method of Weber & Osborn (1964). Molecular-weight standards (Pharmacia) consisted of phosphorylase (mol.wt. 94 000), albumin (mol.wt. 67 000), ovalbumin (mol.wt. 43 000), carbonic anhydrase (mol.wt. 30 000), soya-bean trypsin inhibitor (mol.wt. 20 000) and β -lactalbumin (mol.wt. 14 400).

Isoelectric focusing was performed with polyacrylamide gels in accordance with the procedures described by Pharmacia (Anonymous, 1978). A Corning 130 pH-meter, equipped with a micro pH probe, was used for pH determination. Gels were

scanned by a Zenith Soft Laser Scanning Densitometer (LKB Instruments).

General procedures

Endoglucanase, exoglucanase and cellulose-solubilizing activities were determined on Avicel, CM-cellulose and Remazol Brilliant Blue-dyed Avicel at 60°C in 0.2M-sodium acetate buffer, pH 5.2, as described previously (Ng & Zeikus, 1981). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co.) as standard. NaCl concentration was calculated from a calibration curve of conductivity versus standard NaCl concentration with a Radiometer conductivity meter P10 (London Corp., Cleveland, OH, U.S.A.). Total carbohydrates present in reaction mixtures or the purified enzyme were determined after hydrolysis by the phenol/H₂SO₄ method (Dubois *et al.*, 1956).

The molar absorption coefficient of the purified endoglucanase was determined with a Gilford 240 spectrophotometer at 280nm in 50mM-sodium acetate buffer, pH 5.0, with a 1cm-light-path quartz cuvette (Hellma, Jamaica, NY, U.S.A.). The protein concentration was determined from analysis of the amino acid content.

The pH and temperature optima of the endoglucanase were determined by measuring the formation of reducing sugars from CM-cellulose (Miller *et al.*, 1960). Endoglucanase activity was assayed at 60°C in 50mM-sodium citrate buffer and in 50mM-Mops/NaOH buffer for the pH ranges 3.0–6.0 and 6.0–9.0 respectively. The temperature optimum of the endoglucanase was determined by measuring activity in 50mM-sodium citrate buffer, pH 5.0.

Enzymic hydrolysis of cellooligosaccharides

The specific rates of cello-oligosaccharide hydrolysis by endoglucanase were determined by high-pressure liquid chromatography as described previously (Ng & Zeikus, 1981). The apparent kinetic constants $[S]_{0.5V}$ and V_{max} for cellopentaose and cellohexasaccharide hydrolysis were obtained by determining the rate of cellobiose and cellotriose production from these substrates. Cello-oligosaccharide substrates were prepared and quantified by the high-pressure liquid-chromatographic procedures reported previously (Ng & Zeikus, 1981).

Ultracentrifugation analysis

Sedimentation-equilibrium analysis was performed on a Beckman Spinco model E ultracentrifuge by Dr. J. Blair of the Enzyme Institute, University of Wisconsin, Madison. The endoglucanase sample (0.5mg/ml) was dialysed against 50mM-sodium citrate buffer, pH 5.0, and was centrifuged at 23 159 rev./min (approx. 39 000g) for 19 h at 27°C. Rayleigh interference optics were employed to deter-

mine fringe displacement. The sedimentation-equilibrium coefficient was calculated by the meniscus-concentration-depletion techniques of Yphantis (1964). The partial specific volume was calculated from the amino acid and carbohydrate content of the enzyme by the method of Cohn & Edsall (1943).

Amino acid analysis

The amino acid composition of the endoglucanase was analysed at the Amino Acid Analysis Laboratory, Department of Cellular and Molecular Biology, University of Wisconsin, Madison. Samples (1.5mg each) were hydrolysed in sealed and evacuated ampules with 6M-HCl at 110°C for 24 and 72h. Values for serine and threonine were obtained by linear extrapolation to zero time of hydrolysis. Tryptophan content was determined by the spectrophotometric method of Bencze & Schmid (1957).

Results

Purification

Initial separation of crude enzyme by anion-exchange chromatography resolved the protein into four protein components (Fig. 1). The protein eluted in starting buffer (component I, fractions 27–83) and at 600mM buffer (component III, fractions 195–230) contained more than 80% of the total endoglucanase activity. Component I had the highest specific and total endoglucanase activity and was further purified. Chromatography of pooled fractions 27–83 on a DEAE-Sephadex A-50 column yielded four major protein peaks (Fig. 2). The protein eluted by 50mM-NaCl (peak IIa, fractions 80–112) contained approx. 60% of the endoglucanase activity present in component I. The remaining activity resided mainly in proteins eluted at starting buffer (peak Ia, fractions 16–32); peak IIIa (fractions 155–172) and peak IVa (fractions 216–224) contained little endoglucanase activity. Cation-exchange chromatography was employed to purify peak IIa protein further. As shown in Fig. 3, three protein components were obtained by elution with a linear gradient of NaCl. The major endoglucanase activity coincided with the smallest protein peak (peak IIb, fractions 116–127), and this was eluted at approx. 200mM-NaCl. The amount of protein recovered from this peak was so low that further purification on conventional gel-permeation chromatography was not feasible. Further purification of the endoglucanase was achieved by preparative gel electrophoresis (Fig. 4). However, 47% of the endoglucanase activity (63% of the protein) was lost during this purification step. The purified endoglucanase was recovered from fractions 384–389.

Fig. 1. Separation of crude extracellular cellulase from *C. thermocellum* on a DEAE-Sephadex A-50 column (4.5 cm $\times$ 50 cm)

The sample contained 3.34 g of protein (400 ml); the elution buffer was 50 mM-, 100 mM-, 600 mM- and 800 mM-ammonium acetate, pH 5.0, added as indicated by arrows; the fraction volume was 10 ml. O, Endoglucanase activity; Δ , protein.

Fig. 2. Separation of component I on a DEAE-Sephadex A-50 column (2.6 cm $\times$ 30 cm)

The sample contained 0.31 g of protein (10 ml); the elution buffer was 0 mM-, 50 mM-, 100 mM- and 150 mM-NaCl in 50 mM-Tris/HCl, pH 7.2, added as indicated by arrows; the fraction volume was 2.5 ml. O, Endoglucanase activity; Δ , protein.

A quantitative representation of the scheme used for purification of endoglucanase is summarized in Table 1. A 22-fold increase in endoglucanase specific activity was obtained, but only 2.5% of the original

total activity and 0.12% of the starting protein was recovered. The greatest loss of endoglucanase activity occurred during initial DEAE-Sephadex A-50 chromatography.

Fig. 3. Separation of peak IIa material on a sulphopropyl-Sephadex C-50 column (2.6 cm $\times$ 30 cm). The sample contained 0.12 g of protein (10 ml); the elution buffer was a continuous NaCl gradient in 50 mM-sodium citrate buffer, pH 3.5; the fraction volume was 3.0 ml. O, Endoglucanase activity; Δ , protein; $\bullet$, concn. of NaCl.

Fig. 4. Separation of peak IIIb material by 8% preparative polyacrylamide-gel electrophoresis. The sample contained 43 mg of protein (2 ml); the elution buffer was 0.36 M-Tris/HCl, pH 7.6; the fraction volume was 2.5 ml; the gel length was 4 cm. O, Endoglucanase activity; Δ , protein.

As shown in Fig. 5, the purified endoglucanase displayed a single protein component, in contrast with the numerous protein bands present in cellulase on 4–8% polyacrylamide gels. Soft-laser scanning of the gel confirmed one band in the purified protein. Analysis of the purified endoglucanase by iso-electric focusing also demonstrated a single band,

indicating the absence of isoenzymes. Analysis of purified endoglucanase by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed enigmatic results. Namely, the enzyme displayed one band (apparent mol.wt. 90 000) on gels containing 0.1% sodium dodecyl sulphate, but two additional minor bands (apparent mol.wt. 60 000–80 000) were

Table 1. *Purification of endoglucanase from C. thermocellum*

For full experimental details see the text. Units represent μmol of reducing sugars formed from CM-cellulose per min at 60°C .

Procedure	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification
Cell-free supernatant	12000	6720	20300	3.0	—	—
Ultrafiltration	1000	4750	17800	3.7	87.6	1.2
$(\text{NH}_4)_2\text{SO}_4$ precipitation	400	3370	13800	4.2	68.3	1.4
DEAE-Sephadex A-50 chromatography (peak I)	400	624	6400	10.3	31.6	3.4
DEAE-Sephadex A-50 chromatography (peak II)	60	119	2750	23.0	13.6	7.6
Sulphopropyl-Sephadex C-50 chromatography (peak I)	25	21	975	45.9	4.8	15.2
Preparative gel electrophoresis	10	7.9	514	65.1	2.5	21.5

Fig. 5. Polyacrylamide-gel (8.4%) electrophoresis of the crude and the purified endoglucanase (A) Crude cellulase ($200\mu\text{g}$); (B), (C) and (D) purified endoglucanase (40 , 10 and $5\mu\text{g}$ respectively). Arrow indicates top of gels.

observed when the enzyme was pretreated with 1% sodium dodecyl sulphate at 90°C for 5 min before electrophoresis (results not shown).

Physical properties

Native enzyme homogeneity was examined by

analytical ultracentrifugational analysis. A plot of $\log J$ versus γ^2 for the endoglucanase was linear. Linear-regression analysis of the data points gave a correlation coefficient of 0.99 and indicated only one protein component, with an apparent molecular weight of $83000 (\pm 4000)$. The apparent molecular weight obtained for endoglucanase by amino acid composition analysis (88000 ± 2000) was in agreement with the value obtained by sedimentation-equilibrium analysis. The higher apparent molecular-weight value (94000) obtained by polyacrylamide-gel electrophoresis may be a result of the common lower electrophoretic mobility of glycoproteins (Hashimoto *et al.*, 1978) compared with the protein standards. The endoglucanase contained approx. 11% carbohydrate. The purified endoglucanase displayed a molar absorption coefficient at 280nm of $53750\text{M}^{-1}\cdot\text{cm}^{-1}$ and an isoelectric point of 6.72 ± 0.02 . The pH and temperature optima for the enzyme were 5.2 and 62°C respectively (see Fig. 6). The amino acid composition of purified endoglucanase is given in Table 2. Notably, the enzyme contained methionine (2.5%) but lacked cysteine.

Catalytic properties

The relationship of substrate chemistry to specific activity of the purified endoglucanase is shown in Table 3. The enzyme displayed highest activity towards CM-cellulose and significantly lower activity toward Avicel or dyed Avicel. Notably, *C. thermocellum* endoglucanase was 33 times more active towards dyed Avicel than towards Avicel. The activity towards cello-oligosaccharides increased with higher degree of polymerization of substrates. The endoglucanase was not active on cellobiose or cellotriose, but activity increased from cellotetraose to celloheptaose.

The rate of specific glycosidic bond hydrolysis by the purified enzyme was examined by determining the rate of formation of specific products from different cello-oligosaccharides (see Table 4). Cellobiose was the only detected product of cello-tetraose hydrolysis, and this indicated that the

Fig. 6. Effects of pH and temperature on purified endoglucanase activity

(a) Endoglucanase activity was measured in citrate buffer (□) and Mops buffer (△); 100% activity corresponds to 5 units of endoglucanase activity on CM-cellulose. (b) Endoglucanase activity was measured at 20–75°C; 100% activity corresponds to 14.3 units of activity.

Table 2. Amino acid composition of *C. thermocellum* endoglucanase

The values are based on a molecular weight of 88 000, of which 11% was carbohydrate and 89% protein. Abbreviation: N.D., not detected.

Amino acid	Composition	
	(mol/mol of protein)	(mol/100 mol of residues)
Aspartic acid	80	11.0
Threonine	59	8.1
Serine	41	5.7
Glutamic acid	42	5.8
Proline	37	5.1
Glycine	60	8.2
Alanine	63	8.7
Valine	57	7.9
Half-cysteine (or cysteic acid)	N.D.	N.D.
Methionine	18	2.5
Isoleucine	44	6.1
Leucine	52	7.2
Tyrosine	33	4.6
Tryptophan	6	0.8
Phenylalanine	29	4.0
Lysine	67	9.3
Histidine	12	1.7
Arginine	24	3.3
Total	724	100

Table 3. Substrate–activity relationships for purified endoglucanase from *C. thermocellum*

Products measured with respective substrates were: glucose (cellobiose); cellobiose (cellotriose, cello-tetraose and cellopentaose); cellotriose (cello-hexaose); cellotetraose (celloheptaose); reducing sugars (CM-cellulose and Avicel); dyed oligo-saccharides (dyed Avicel). For experimental details see the text. Abbreviation: N.D., not detected.

Substrate	Specific activity (μmol/min per mg of protein at 60°C)
Cellobiose	N.D.
Cellotriose	N.D.
Cellotetraose	0.63
Cellopentaose	11.21
Cellohexaose	19.06
Celloheptaose	25.13
CM-cellulose	65.05
Dyed Avicel	5.33
Avicel	0.17

second glycosidic bond (counted from the non-reducing end) was selectively cleaved by the endoglucanase. Likewise, hydrolysis of cellopentaose yielded mainly cellobiose and cellotriose, indicating that the second and/or the third bonds were preferentially cleaved over the first and fourth. In a similar fashion, preferential cleavage of the third

bond of cellohexaose and the fourth and fifth bonds of celloheptaose by the endoglucanase produced mainly cellotriose and cellotriose plus cellotetraose. Kinetic constants for cellopentaose and cellohexaose hydrolysis were obtained from Lineweaver–Burk plots, and the results are shown in Table 5. Linearity of the reciprocal plots was observed for both substrates whereas plots of V versus $[S]$ represented a right-angle hyperbola where $[S]_{0.1V}/[S]_{0.9V}$ values were 0.79 and 0.80 for cellopentaose and cellohexaose hydrolysis respectively. These results indicated that the hydrolysis of cellopentaose and cellohexaose by the endoglucanase followed Michaelis–Menten kinetics. The endoglucanase

Table 4. Relationship between oligosaccharide hydrolysed and the rate of formation of specific products by endoglucanase purified from *C. thermocellum*

For experimental details see the text. Abbreviations: N.D., not detected; —, not determined.

Rate of product formation ($\mu\text{mol}/\text{min}$ per mg of protein at 60°C)

Substrate	Glucose	Cellobiose	Cellotriose	Cellotetraose	Cellopentaose
Cellotetraose	N.D.	0.63	N.D.	—	—
Cellopentaose	0.11	11.21	10.57	0.37	—
Cellohexaose	N.D.	2.65	19.06	2.33	—
Celloheptaose	N.D.	5.06	25.13	22.20	4.27

Table 5. Kinetic properties of endoglucanase purified from *C. thermocellum*

Substrate	$[S]_{0.5V}$ (mM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg of protein at 60°C)
Cellopentaose	2.30 ± 0.27	39.3 ± 4.4
Cellohexaose	0.56 ± 0.05	58.7 ± 6.7

showed a higher affinity towards cellohexaose than towards cellopentaose. The enzyme turnover rate as represented by V_{max} was higher for cellohexaose than cellopentaose. The low activity of the endoglucanase towards cellotetraose rendered the determination of the kinetic properties impracticable, whereas accurate determination of the kinetic properties for celloheptaose was not possible because the apparent K_m value was well below the detectability limit of the differential refractometer.

Discussion

Cellulases in general are very difficult enzymes to purify. *C. thermocellum* extracellular cellulase, as well as those reported for other organisms (Halliwell, 1979), displays a strong affinity towards cellulose. Thus after adsorption on cellulose enzyme activity is not eluted by distilled water, 0.1% Triton X-100 or 1 M-NaOH (results not shown). The scheme used in the present work to purify cellulase incorporated procedures to avoid the loss of cellulase activity caused by strong binding to native cello-oligosaccharides. Thus *C. thermocellum* was grown on cellobiose, and the endoglucanase was purified by techniques that did not include DEAE-cellulose chromatography or dialysis with cellulose membranes. *C. thermocellum* strain LQRI produces the same amount of carboxymethylcellulase activity when grown on cellulose or cellobiose (Lamed & Zeikus, 1980). In addition, Sephadex and Bio-Gel chromatography were not employed because low mobility of cellulase on these gel-permeation

matrices resulted in very poor resolution of the protein. Similar problems were reported during purification of cellulase from thermophilic clostridia (Lee & Blackburn, 1975; Ait *et al.*, 1979).

The data clearly indicate that the procedures employed enabled purification of the endoglucanase to ultracentrifugational homogeneity. The results of isoelectric focusing and polyacrylamide-gel electrophoretic analysis also confirmed that the purified endoglucanase behaved as a single protein under non-denaturing conditions. The structural basis that accounts for two additional components after treatment of native enzyme with sodium dodecyl sulphate is not known. Classical subunits cannot be suggested at present because cysteine was absent from the protein (and hence thiol-bond interactions), and the molecular weights of the denatured protein components were too close to that of native enzyme. Perhaps sodium dodecyl sulphate treatment influenced the carbohydrate and/or carbohydrate-protein interactions of the glycoprotein; however, this speculation requires further characterization.

In comparison with other glycoprotein endoglucanases purified from other micro-organisms (Goksoyr *et al.*, 1975; Enari & Markkanen, 1977), the *C. thermocellum* enzyme had a higher molecular weight and a more basic isoelectric point. The temperature optimum of the enzyme (62°C) is higher than that reported for other purified endoglucanases and correlates well with the optimum temperature for growth of *C. thermocellum* (Ng *et al.*, 1977). As evidenced during enzyme purification, multiple forms of endoglucanase were fractionated from crude extracellular cellulase of *C. thermocellum*. This feature is similar to that reported for *Trichoderma reesei* (Shoemaker & Brown, 1978b; Berghem *et al.*, 1976; Okada, 1975, 1976), *Trichoderma koningii* (Wood & McCrae, 1978), *Sporotrichum pulverulentum* (Eriksson & Pettersson, 1975), *Fusarium solani* (Wood, 1971), *Iprex lacteus* (Kanda *et al.*, 1976), *Cellvibrio gilvus* (Storvick & King, 1960) and *Pseudomonas fluorescens* var. *cellulosa* (Yoshikawa *et al.*, 1974). The endoglucanase of *C. thermocellum* shares

similar amino acid composition and common catalytic features with the enzyme purified from *Ps. fluorescens* (Suzuki *et al.*, 1969; Yamane *et al.*, 1970).

The mode of action of purified endoglucanase from *C. thermocellum* is also similar to that reported by *Cellvibrio gilvus* (Cole & King, 1964), where enzyme-substrate complex formation favours cello-triosyl units over cellobiosyl units, and that from glucosyl units is rare. The preference for cleavage of internal glycosidic bonds in cello-oligosaccharides appears as a common property for endoglucanases from *C. thermocellum* as well as those of *T. reesei* (Pettersson, 1975; Shoemaker & Brown, 1978a) and *Sporotrichum pulverulentum* (Eriksson & Pettersson, 1975). It is apparent that the endoglucanase properties alone cannot account for the different end products observed (Ng & Zeikus, 1981) from long-term hydrolysis of cello-oligosaccharides by crude cellulases of *C. thermocellum* (i.e. cello-triose and cellobiose) and *T. reesei* (i.e. cellobiose and glucose). This difference is probably a reflection of significant differences in exoglucanase and β -glucosidase activities in the two organisms.

The endoglucanase purified in the present study represents only a portion of total activity in the *C. thermocellum* cellulase complex. The ability to actively hydrolyse cellotetraose and cello-triose (Ng & Zeikus, 1981) could reside in other endoglucanase components. The purification, characterization and subsequent comparison of other endoglucanase components in the cellulase complex of *C. thermocellum* may provide instructive insights on the diversity and mechanism of cellulose degradation by bacterial cellulase.

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