Purification and characterization of a new endoglucanase from *Clostridium thermocellum*

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An endoglucanase (1,4- β -D-glucan glucanohydrolase, EC 3.2.1.4) from the thermophilic anaerobe *Clostridium thermocellum* was purified to apparent homogeneity without the use of denaturants. No carbohydrate is associated with the endoglucanase. A molecular mass of 76000 Da was determined by SDS/PAGE. The optimal pH is 7.0 and the enzyme is isoelectric at pH 5.05. The enzyme has a temperature optimum of 70 °C and retains approx. 50 % of its activity after 48 h at 60 °C. Hydrolysis of CM-cellulose takes place with a rapid decrease in viscosity but a slow liberation of reducing sugars, indicating an endoglucanase type of activity. The endoglucanase shows little ability to hydrolyse highly ordered cellulose. Cellobiose inhibits whereas Mg^{2+} and Ca^{2+} stimulate the activity. The enzyme is completely inactivated by 1 mM-Hg²⁺ and is inhibited by a thiol-blocking reagent.

INTRODUCTION

Clostridium thermocellum secretes a highly active cellulase system capable of completely solubilizing crystalline cellulose, and is therefore considered to have potential in the saccharification of lignocellulosic biomass. In general, enzymic hydrolysis of cellulose requires the co-operative action of different enzymes, including 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→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 of activity is β -glucosidase (β -glucoside glucanohydrolase, EC 3.2.1.21), which forms glucose by endwise cleavage of cello-oligosaccharides.

The cellulases of *C. thermocellum* are organized in a structure termed the cellulosome, with a molecular mass of about 2.1×10^6 Da (Lamed *et al.*, 1983), which exists on the extracellular surface of *C. thermocellum* vegetative cells (Lamed *et al.*, 1983). Cellulosomes are also found free in the extracellular medium or bound to cellulose. They can aggregate into macromolecular complexes termed polycellulosomes which range in size from 5×10^7 to 8×10^7 Da (Coughlan *et al.*, 1985; Mayer *et al.*, 1987). These polycellulosomal complexes exist on the surface of *C. thermocellum* cells as discrete structures (protuberances) (Lamed & Bayer, 1988). Cellulosomes of different strains are reported to contain from 14 to 50 proteins when analysed by SDS/PAGE, ranging in molecular mass from 20000 to 250000 Da (Lamed *et al.*, 1983; Mayer *et al.*, 1987). The major proportion of the cellulase activity of *C. thermocellum* resides within the cellulosomal complexes.

Isolation of individual components from undenatured cellulosomes has proven to be extremely difficult. Thus far, three endoglucanases have been purified from *C. thermocellum* culture supernatants. One, termed endoglucanase A, of molecular mass

56000 Da, was isolated from strain N.C.I.B. 10682 by using 6 M-urea (Petre *et al.*, 1981). Another, of molecular mass 83000–94000 Da, was isolated from strain LQRI without denaturants (Ng & Zeikus, 1981a,b). We have purified and characterized endoglucanase S_g, which has a molecular mass of 83000 Da (Fauth *et al.*, 1991) and is considered to be very important in the breakdown of crystalline cellulose (Wu *et al.*, 1988; Kobayashi *et al.*, 1990).

In the present paper we report the isolation without the use of denaturants of a new endoglucanase from the culture supernatant of strain *C. thermocellum* A.T.C.C. 27405 and its characterization.

MATERIALS AND METHODS

Culture and growth conditions

C. thermocellum strain A.T.C.C. 27405 was used throughout this study. The chemically defined medium, used for both seed development and fermentation, and the anaerobic cultivation techniques were those described by Johnson *et al.* (1981).

Crude enzyme preparation

Seed cultures were grown in Hungate tubes with 1 % (w/v) cellobiose as carbon source. Fermentations were carried out in 2.8-litre Fernbach flasks using 1 % (w/v) MN300 (Macherey and Nagel, Darmstadt, Germany) cellulose as the carbon source. Into each of two 1.6-litre batches of medium was inoculated 100 ml of exponentially growing seed culture. Before and during inoculation, the flasks were bubbled with CO₂. After 96 h of incubation at 60 °C, the whole broth (originally 3.4 l) was centrifuged at 16000 g for 20 min at 4 °C. The supernatant was decanted and re-centrifuged as above. It was then concentrated 10-fold by ultrafiltration (Omegacell Ultrafiltration Unit 10K, Pharmacia, Piscataway, NJ, U.S.A.). (NH₄)₂SO₄ (196 g) was added to the ultrafiltrate (350 ml) at 80 % saturation, and the precipitated proteins were collected by centrifugation (14000 g,

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45 min, 16 °C). The proteins were resuspended in a minimum volume of 20 mM-Tris/HCl, pH 7.1, and dialysed against 4 litres of the same buffer for 24 h with one change of buffer.

Enzyme purification

The crude enzyme [98.3 mg of protein in 20.7 ml of Tris/HCl buffer (pH 7.1)/10% (v/v) glycerol] was fractionated on a column (1.6 cm × 100 cm bed volume) of Sepharose 6B (Pharmacia) equilibrated with 20 mM-Tris/HCl buffer, pH 7.1, and eluted with 250 ml of the same buffer at a flow rate of 10 ml/h. Fractions (4 ml) were collected and assayed for protein and CM-cellulase activity and analysed by SDS/PAGE.

Peak II (80 ml) from the Sepharose 6B column (Fig. 1) was pooled and concentrated 4-fold by ultrafiltration, and dialysed against 2 litres of 20 mM-Tris/HCl, pH 7.1, for 24 h. Dialysis was always carried out at 4 °C. The protein was adsorbed on a Mono Q HR5/5 anion-exchange column (Pharmacia) using a 10 ml superloop. The column was washed with 4 ml of starting buffer (20 mM-Tris/HCl, pH 7.1) before being eluted with a 21 ml linear gradient (0–0.5 M) of KCl in the same buffer at a flow rate of 0.5 ml/min. The fractions were analysed by SDS/PAGE. Those containing endoglucanase activity, which were eluted in the initial column wash, were pooled.

(NH₄)₂SO₄ (to 1.7 M) was added to the pooled fractions and the material was adsorbed on an alkyl-Superose HP5/5 (Pharmacia) column. The protein was eluted with a 15 ml linear gradient (1.7–0 M) of (NH₄)₂SO₄ in 50 mM-potassium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. The endoglucanase fractions were eluted at the end of the gradient with 0 M-(NH₄)₂SO₄ in a volume of 10 ml.

Gel electrophoresis

Protein samples were analysed by electrophoresis in SDS/polyacrylamide slab gels according to Laemmli (1970). Molecular mass was calculated as described by Weber & Osborn (1969).

Protein determination

Protein concentration was measured by the Coomassie Blue-binding assay (Bradford, 1976), with BSA (fraction V) as standard.

Gel electrofocusing

Isoelectric focusing was performed on a Pharmacia Phastgel system using the Pharmacia broad-range calibration standards.

Cellulase assays

CM-cellulase was measured by using 0.61% (w/v) CM-cellulose (9M31, degree of substitution 0.9; Aqualon, Wilmington, DE, U.S.A.) as a substrate as described by Wu *et al.* (1988) except that 50 mM-phosphate/citrate buffer, pH 6.0, was used. Total volume was 1.8 ml and the release of reducing sugar was determined using the residual ferricyanide method of Park & Johnson (1949). The lower limit of detection was 4 µg of cellobiose or 2 µg of glucose. Reactions were run for 15 min, i.e. during the initial (linear) stage. Activity against Avicel (PH105; FMC Corp., Marcus Hook, PA, U.S.A.) and MN300 was determined by adding 60 µl of enzyme solution to 0.5 ml of a 1% substrate suspension in 50 mM-phosphate/citrate buffer, pH 6.0. The samples were incubated at 60 °C for periods between 1 and 48 h with occasional shaking. Reducing sugar was determined as mentioned above. One unit of endoglucanase activity is defined as the amount of enzyme catalysing the release of 1 µmol of glucose equivalent/min.

Endoglucanase activity was also determined by a viscometric assay (Fauth *et al.*, 1991). Reaction mixtures containing 0.5% (w/v) CM-cellulose in 50 mM-phosphate/citrate buffer, pH 6.0,

were incubated for up to 60 min at 60 °C. Incubations were stopped by boiling for 10 min to inactivate the enzyme. The viscosity was determined at room temperature with a 1 ml Tuberculin syringe fitted with a 26-gauge blunt needle.

The pH and temperature optima of the endoglucanase were determined by measuring the formation of reducing sugars from CM-cellulose. Endoglucanase activity was assayed at 60 °C in 50 mM-phosphate/citrate buffer, 50 mM-Tris/HCl buffer and in 50 mM-sodium carbonate buffer for the pH ranges 3.0–7.6, 8.0–9.0 and 10.0–10.8 respectively. The temperature optimum and thermal stability were determined in 50 mM-phosphate/citrate buffer, pH 6.0. The pH values of buffers were measured at 25 °C.

Enzymic hydrolysis of cellodextrins

Specific activity of cellodextrin hydrolysis was determined by h.p.l.c. Endoglucanase (0.3 µg/ml) was incubated with 20 mM-cellobiose, 25 mM-cellobiose, 50 mM-cellobiose or 50 mM-cellobiose in 50 mM-phosphate/citrate buffer, pH 6.0, at 60 °C. Reaction mixtures were shaken continuously. Samples were withdrawn for analysis, inactivated by heating at 100 °C for 5 min and centrifuged at 10000 g for 5 min. Samples were deionized by addition of Bio-Rad AG501-X8 mixed-bed resin. Portions were injected on to a Bio-Rad HPX874 column. The liquid phase was dilute H₂SO₄ (0.54 ml conc. H₂SO₄ in 2 litres of water), with a flow rate of 0.5 ml/min; detection was by differential refractometry. The rates of hydrolysis were calculated at points in time when formation of reaction products was linear with time and the system was saturated with substrate. Identity of products and their concentrations were determined using authentic standards.

N-Terminal sequencing

N-Terminal protein sequencing was performed using an Applied Biosystems (Warrington, Cheshire, U.K.) 470A Gas Phase protein sequencer equipped with a 120A on-line phenylthiohydantoin analyser. Protein bands cut from Immobilon blots (Immobilon P; Millipore Corp., Bedford, MA, U.S.A.) were placed directly on to the sequencer reaction cartridge and held in place with trifluoroacetic acid-etched glass-fibre filter discs (Hunkapillar *et al.*, 1983).

RESULTS

Purification and size

Results on purification are shown in Table 1. Protein peak II from the Sepharose 6B column (Fig. 1) contained only about 40% of the total starting protein. However, analysis by SDS/PAGE revealed that it contained only small amounts of the glycosylated S_L (= S₁) (210 000 to 250 000 Da) protein (Wu *et al.*, 1988). In contrast, peak I contained a large amount of S_L protein. As expected, the presence of S_L protein agreed with the finding of Avicelase activity in peak I and the absence of S_L protein correlated with the absence of Avicelase activity in peak II. Since we found that the presence of this protein complicates the purification of individual endoglucanases, we chose peak II for further purification. The pooled peak II fractions were adsorbed on a Mono Q (anion-exchange) column. The endoglucanase was not tightly bound and could be eluted by washing the column with starting buffer. The final column was alkyl-Superose, a hydrophobic interaction column. The protein was eluted at the end of the (NH₄)₂SO₄ gradient, indicating that it is reasonably hydrophobic. The contaminating proteins were less hydrophobic and were eluted from the column much earlier than the endoglucanase. The enzyme preparation migrated as a single band on SDS/PAGE, indicating an homogeneous protein with a mol-

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

Stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Activity yield (%)
Crude supernatant	98.3	172.0	1.75	1.0	100
Sepharose 6B					
Peak I	26.4	73.1	2.77	1.6	42
Peak II	37.5	76.4	2.32	1.4	44
Mono Q	8.9	58.8	6.61	3.8	34
Alkyl Superose	1.1	11.3	10.25	5.9	6.6

Fig. 1. Sepharose 6B chromatography of the *C. thermocellum* crude enzyme preparation

●, A_{280} ; ▲, CM-cellulase activity; ×, Avicelase activity.

Fig. 2. SDS/PAGE of the crude and purified endoglucanase

A, Crude cellulase (20 µg of protein); B, Purified endoglucanase (0.5 µg); C, molecular mass standards.

ecular mass of 76000 Da (Fig. 2). The purified endoglucanase was stable for at least 6 months when frozen at -20°C in solution.

Influence of temperature and pH on activity

The effect of temperature on the initial velocity of CM-cellulose hydrolysis was studied using an incubation period of 15 min. Enzyme activity gradually increased up to 70°C , and then decreased. However, even at 80°C , 90% of the activity remained. Above 80°C the activity rapidly declined. As shown in

Fig. 3. Effect of pH on the activity of the endoglucanase on CM-cellulose

The reaction mixture contained 50 mM-buffer. Buffers: ×, pH 3.0–7.6, citrate/phosphate; □, pH 8.0–9.0, Tris/HCl; △, pH 10.0–10.8, sodium carbonate. Reducing sugars were estimated by the ferricyanide method; 1.2 µg of purified enzyme was used.

Fig. 4. Thermal stability of endoglucanase activity at 60°C

Incubation was in citrate/phosphate buffer (pH 6.0). At the times indicated, a sample was removed (equivalent to 1.2 µg of protein) for determination of CM-cellulase activity by reducing sugar liberation. ■, Enzyme; ×, enzyme plus 5 mM- MgCl_2 .

Fig. 3, maximum activity was observed at pH 7.0. The activity fell to low values below pH 6.0 and above pH 8.0.

Temperature stability

Enzyme solutions were incubated in citrate/phosphate buffer, pH 6.0, at 60°C for various lengths of time up to 48 h. A parallel set of reactions contained 5 mM- MgCl_2 in addition. It can be seen from Fig. 4 that the enzyme is reasonably stable at 60°C , 47% of its activity remaining after 48 h. MgCl_2 slightly enhanced the stability of the enzyme.

Isoelectric pH and lack of carbohydrate

The isoelectric point of the pure enzyme, determined by electrofocusing, was estimated to be pH 5.05 (results not shown). The endoglucanase, when resolved by SDS/PAGE, did not stain with the Schiff stain, indicating that the enzyme is not glycosylated (results not shown).

Effect of various compounds

The effects of metal ions and chemical agents on enzyme activity were determined. Both Ca^{2+} and Mg^{2+} stimulated activity. In the case of MgCl_2 , there was a 100% increase in the rate of the hydrolysis of CM-cellulose at 5 mM. At the same concentration, CaCl_2 stimulated by 50%. Enzyme activity was not affected by up to 100 mM- NaCl , but was totally inhibited by 1 mM- HgCl_2 and SDS. Inhibition by the thiol-specific inhibitor *p*-chloro-mercuribenzoate was comparatively strong (40% at 0.2 M), and,

as there was no preincubation of the enzyme and inhibitor, inactivation must occur rapidly, suggesting the presence of essential thiol groups in the enzyme. There was no inhibition by 200 mM-glucose. In contrast, there was moderate inhibition by cellobiose (50 % inhibition at 100 mM-cellobiose).

Activity on different cellulosic substrates

The activity of the endoglucanase was assayed on different substrates (Table 2). The enzyme displayed a high activity towards CM-cellulose, and had significantly lower activity on a cellulose which partially contains crystalline regions, i.e. MN300; no activity was detected on microcrystalline Avicel. Both cello-tetraose and cello-pentaose were hydrolysed. The specific activity on cello-pentaose was higher than that on CM-cellulose. The K_m for cello-pentaose was found to be 1.2 mM by a Lineweaver-Burk plot (results not shown), indicating a reasonable affinity of the enzyme towards cello-pentaose. Cellobiose was the only product of cellotetraose hydrolysis in short time incubations (results not shown). This indicates that the second glycosidic bond (counting from the non-reducing end) is selectively cleaved by the endo-glucanase. Likewise, cello-pentaose yielded only cellobiose and cellotriose, indicating that the second and/or the third bonds are preferentially cleaved over the first and fourth. However, on prolonged incubations (> 12 h at 60 °C), glucose was also detected. Small amounts of glucose were detected in the hydrolysis of cellotriose to cellobiose, of cellotetraose to cellobiose and cello-pentaose to cellotriose and cellobiose. It should be emphasized that glucose was always a minor product.

Table 2. Activity of the endoglucanase on various substrates

Substrate	Specific activity (units/mg)
Cellobiose	N.D.*
Cellotriose	N.D.*
Cellotetraose	17.8
Cello-pentaose	33.1
CM-cellulose	18.5†
Cellulose MN300	0.13†
Avicel	N.D.*†

* N.D., not detected.

† Assayed by release of reducing sugars; others by h.p.l.c.

Fig. 5. Hydrolysis of CM-cellulose as measured by viscosity and release of reducing sugar

Each reaction mixture consisted of 50 mg of CM-cellulose in 1 ml of 50 mM-citrate/phosphate buffer, pH 6.0, and 0.5 µg of purified enzyme. At the specified times, the reaction was stopped by boiling for 10 min. The percentage reducing sugar was calculated assuming that complete hydrolysis would release 3.25 mg of glucose. x, Change in viscosity; ■, reducing groups released.

The purified endoglucanase enhanced the activity of protein S_L (= S_1) on Avicel. Purified *C. thermocellum* S_L protein (isolated by electroelution from an SDS/polyacrylamide gel; Romaniec *et al.*, 1991) released 100 µg of cellobiose from Avicel (5 days, 60 °C, 5 ml volume, 20 µg of S_L protein). When 10 µg of the purified endoglucanase was added, cellobiose release increased to 155 µg; with 40 µg of purified endoglucanase, 310 µg of cellobiose was released.

The viscometric assay showed that the purified enzyme rapidly decreases the viscosity of a CM-cellulose solution. As shown in Fig. 5, a large decrease in viscosity was accompanied by only a small increase in the number of reducing end groups. This is typical of an endoglucanase type of activity.

N-Terminal sequence

The N-terminal sequence of the enzyme was found to be: Ser-Pro-Thr-Phe-Ala-Glu-Thr-Ala-.

DISCUSSION

Cellulases in general are very difficult to purify, but those of *C. thermocellum* are extremely so. This is due to the fact that the cellulolytic enzymes of *C. thermocellum* exist in the form of large stable protein complexes termed cellulosomes (Lamed *et al.*, 1983; Lamed & Bayer, 1988), which can only be dissociated by SDS.

We have shown that it is possible to utilize an initial fractionation of crude *C. thermocellum* A.T.C.C. 27405 supernatant over a Sepharose 6B column. The crude material is distributed into two major fractions (peaks I and II). One of these (peak I) contains a large amount of a 210 000–250 000 Da glycoprotein termed S_L (Wu *et al.*, 1988), whereas the other (peak II) contains a relatively low amount. It was possible to purify the endoglucanase from peak II.

The purified enzyme was homogeneous, as judged by SDS/PAGE and isoelectric focusing. The apparent molecular mass obtained under dissociating conditions is 76 000 Da. This value is different from the 56 000 Da reported for the *C. thermocellum* N.C.I.B. 10682 endoglucanase purified by Petre *et al.* (1981), the 83 000–94 000 Da *C. thermocellum* LQR1 endoglucanase isolated by Ng & Zeikus (1981a,b), and the 99 000 Da endoglucanase isolated from *Clostridium stercorarium* by Creuzet & Frixon (1983).

The inhibitory effect of the thiol-blocking reagent suggests the existence of essential thiol groups in the endoglucanase. This is similar to that reported for the endoglucanase isolated by Creuzet & Frixon (1983). The endoglucanase isolated by Petre *et al.* (1981) was unaffected by thiol-blocking reagents. Ng & Zeikus (1981b) found that the activity of their crude enzyme was inhibited by Ag^+ and Mg^{2+} but it was not clearly established whether this inhibition was due to inactivation of essential thiol groups on the purified enzyme itself.

Unlike the 56 000 Da endoglucanase from *C. thermocellum*, and the 99 000 Da enzyme from *C. stercorarium*, our new endoglucanase is stimulated by both Mg^{2+} and Ca^{2+} ions. Johnson *et al.* (1982) reported that Ca^{2+} ions stimulate hydrolysis of crystalline cellulose by the *C. thermocellum* cell-free supernatant.

The rapid decrease in the specific viscosity of the CM-cellulose solution accompanied by a small increase in reducing sugar is compatible with a random hydrolysis of substrate. It was therefore concluded that the purified enzyme was an endo-β-1,4-glucanase. The release of reducing sugar by the endoglucanase is similar to that reported by Petre *et al.* (1981) for the 56 000 Da protein. The purified enzyme could only weakly hydrolyse MN300, and Avicel not at all. It exhibited activity against cellotetraose and cello-pentaose, which is the same as with the

endoglucanases previously isolated from *C. thermocellum* (Ng & Zeikus, 1981a; Petre *et al.*, 1981).

The endoglucanase is not inhibited by 100 mM-glucose, and retains 48 % of its activity in the presence of 100 mM-cellobiose, the end product of cellulose hydrolysis. The endoglucanase purified by Petre *et al.* (1981) is not inhibited by glucose, but suffers only 25 % inhibition by 100 mM-cellobiose. These two enzymes are in contrast with that reported by Creuzet & Frixon (1983) isolated from *C. stercoarium*, which retains only 6 % of its activity in the presence of 25 mM-cellobiose, although not inhibited by 100 mM-glucose. The endoglucanase has a specific activity of 10.3 units/mg of protein when measured on CM-cellulose. This compares with 12.4 units/mg for another endoglucanase (S_8) purified from strain S7-19, a mutant of A.T.C.C. 27405 (Fauth *et al.*, 1991), and 65 units/mg for the cellulase purified by Ng & Zeikus (1981a) from a supernatant of strain LQRI. However, the specific activity is considerably lower than that reported for two recombinant *C. thermocellum* N.C.I.B. 10682 endoglucanases: endoglucanase A has a specific activity of 580 units/mg (Schwarz *et al.*, 1986) and endoglucanase D, 428 units/mg (Joliff *et al.*, 1986). On the other hand, recombinant endoglucanase B was reported to have a specific activity of 60 units/mg (Beguín *et al.*, 1983) and recombinant endoglucanase C, 27 units/mg (Petre *et al.*, 1986). The specific activity reported for purified enzymes from other micro-organisms is in general lower than 100 units/mg. The endoglucanase purified from *C. stercoarium* has a value of 45 units/mg (Creuzet & Frixon, 1983), and a cellulase from an alkalophilic *Bacillus* sp., 59 units/mg (Fukumori *et al.*, 1985). The new enzyme also differs from a *C. thermocellum* endoglucanase isolated by Hazlewood *et al.* (1990). It is smaller than this 86000 Da endoglucanase E and has a different N-terminal amino acid sequence.

The enzyme purified in the present study represents only a portion of total endoglucanase activity in the *C. thermocellum* cellulase complex. The cloning of 22 CM-cellulases from *C. thermocellum* (Schwarz *et al.*, 1985; Hazlewood *et al.*, 1988; Sakka *et al.*, 1989) is impressive, but alone is incapable of explaining either the need for this number of enzymes or their modes of action. The purification and characterization of the native enzymes and subsequent comparison with the recombinant proteins and identification of their genes will provide a much needed insight into the mechanism of the *C. thermocellum* cellulase system.

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