Purification and characterization of endoglucanase S_s from *Clostridium thermocellum*

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The extracellular cellulolytic enzymes of the thermophilic anaerobe *Clostridium thermocellum* occur as a protein complex or aggregate known as the cellulosome. By using a combination of ion-exchange, adsorption and hydrophobic-interaction chromatography, it was possible to isolate from extracellular broth a specific endoglucanase of interest without the use of denaturants. The endoglucanase was identified as the cellulosomal subunit S_s by the use of specific antibodies. The enzyme has an *M_r* of 83000, an isoelectric point of 3.55, optimum pH of 6.6 and optimum temperature of 70 °C. It hydrolyses CM-cellulose and, at a higher rate, the cellodextrins, cellotetraose and cellopentaose, but does not hydrolyse a crystalline cellulose such as Avicel. Cellobiose and cellotriose are also immune to attack. It differs from endoglucanases previously isolated by others and a 76000-*M_r* endoglucanase recently isolated in this laboratory.

INTRODUCTION

Clostridium thermocellum, an anaerobic thermophile, secretes a highly potent cellulase (Johnson *et al.*, 1982). The system is composed of between 14 and 50 different polypeptides, which form a multimolecular complex (*M_r* 2.1×10^6) called the cellulosome (Lamed *et al.*, 1983; Coughlan *et al.*, 1985). Endoglucanase activity is constitutive and insensitive to O₂ (Garcia-Martinez *et al.*, 1980; Johnson *et al.*, 1985). On the other hand, hydrolysis of crystalline cellulose requires the activity of additional components, which are de-repressible, stimulated by Ca²⁺ and sensitive to oxidation, thiol-blocking agents and chelating reagents (Johnson *et al.*, 1982, 1985).

Isolation of the individual components of the complex in their native form is a major problem; the complex has been dissociated by treatment with denaturants such as SDS (Lamed *et al.*, 1983; Coughlan *et al.*, 1985; Mayer *et al.*, 1987; Wu *et al.*, 1988). Some endoglucanases have been purified from *C. thermocellum* culture supernatant fluids. One, termed EGA and of *M_r* 56000, was isolated from strain N.C.I.B. 10682 with the use of 6 M-urea (Petre *et al.*, 1981). Another, of *M_r* 83000–94000, was isolated from strain LQRI (Ng & Zeikus, 1981). No native exoglucanase has yet been purified.

One approach for analysing the components of the complex consists of cloning and expressing the genes coding for the various enzymes in a suitable host. The cloning of 20 or so individual genes encoding CM-cellulases, xylanases and β -glucosidases from *C. thermocellum* into *Escherichia coli* has facilitated isolation of genes and production of individual cellulase components (Schwarz *et al.*, 1985; Hazelwood *et al.*, 1988; Sakka *et al.*, 1989). A number of the recombinant cellulases, e.g. endoglucanases A, B, C and D, have been purified after expression in *E. coli* (Petre *et al.*, 1986; Beguin *et al.*, 1983; Joliff *et al.*, 1986; Schwarz *et al.*, 1986). Western-blot analysis showed that the *M_r* values of the *C. thermocellum* enzymes were higher than their counterparts synthesized in *E. coli*. The differences were relatively small for endoglucanases C (40000 versus 38000 respectively) and D (68000 versus 65000) (Petre *et al.*, 1986; Beguin & Gilkes, 1987). In the cases of endoglucanases A (56000

versus 40000) and B (66000 versus 55000), the differences were more substantial and may have been due to proteolysis in *E. coli* (Beguin & Gilkes, 1987; Beguin *et al.*, 1983).

Wu *et al.* (1988) showed that the combination of two cellulosomal proteins, namely S_L (*M_r* 210000–250000) and S_s (*M_r* 82000), could hydrolyse crystalline cellulose (Avicel) to cellobiose (Wu, 1987), albeit at a very low rate, probably because the proteins were isolated under denaturing conditions. S_L is thought to be the same protein as S₁ described by Lamed *et al.* (1983). We have recently prepared a simpler and more active cellulase preparation than the cellulosome of *C. thermocellum*. This 'sub-cellulosome' preparation is a macromolecular complex that is composed of six main protein subunits (*M_r* 210000–58000) as revealed by SDS/PAGE (Kobayashi *et al.*, 1990).

In the present paper we describe the purification of an endoglucanase from the culture supernatant fluid of *C. thermocellum* without the use of denaturants, and its characterization. In addition, we show that it is the endoglucanase referred to as S_s and is one of the components (J3) of the sub-cellulosome preparation.

EXPERIMENTAL

Enzyme source

A crude cellulase powder from *C. thermocellum* was furnished by Dr. N. B. Afeyan. It was obtained from the extracellular fluid of *C. thermocellum* S7-19 grown in a 200-litre fermenter on a complex Solka floc (SW 40) medium at 60 °C for 65 h. The powder had been prepared by sequential ultrafiltration and precipitation with 67% (v/v) acetone, followed by drying in a vacuum (Afeyan, 1987).

Protein purification

Unless otherwise stated, chromatography was carried out aerobically at 4 °C. Cation-exchange chromatography was done on a CM-Bio-Gel A column (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Column dimensions were 2.5 cm $\times$ 40 cm. The column was equilibrated with 10 mM-sodium succinate buffer, pH 4.5, containing 2.5 mM-CaCl₂ and 2.5 mM-2-mercaptoethanol

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(buffer I). Freeze-dried crude cellulase (660 mg) containing 329 mg of protein was dissolved in water and loaded on to two columns. The flow rate was 150 ml/h and 12 ml fractions were collected. The fractions were assayed for cellulase activity and protein content. The presence of endoglucanase S_8 was determined by cross-reaction with anti- S_8 antibodies with the use of a dot-blot format and subsequent analysis by SDS/PAGE. Elution of proteins was performed with buffer I, followed by buffer II (buffer I plus 0.5 M-KCl) and finally by buffer III (25 mM-Tris/HCl buffer, pH 7.0, containing 2.5 mM- CaCl_2 and 2.5 mM-2-mercaptoethanol). Fractions containing protein S_8 as determined by SDS/PAGE were concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation to 80% saturation at 4 °C.

Anion-exchange chromatography was done on a DEAE-Bio-Gel A column with a 40 ml bed volume. The column was equilibrated with 10 mM-Tris/HCl buffer, pH 7.1, containing 2.5 mM- CaCl_2 and 2.5 mM-2-mercaptoethanol. The concentrated fractions from the CM-Bio-Gel A column were dialysed against equilibration buffer, added to the column and eluted by a gradient of 0–250 mM-KCl in a total volume of 200 ml of equilibration buffer. The flow rate was 60 ml/h and 3 ml fractions were collected.

Fractions from DEAE-Bio-Gel A containing protein S_8 were concentrated by ultrafiltration (Omegacell Ultrafiltration Unit 10 K; Filtron Technology Corp., Clinton, MA, U.S.A.) and dialysed against equilibration buffer. Adsorption chromatography was performed on a hydroxyapatite column (Bio-Rad Laboratories) equilibrated with 1 mM-sodium phosphate buffer, pH 7.0. Column dimensions were 1 cm $\times$ 10 cm. Elution was carried out by a gradient of 1–200 mM-sodium phosphate buffer, pH 7.0. The flow rate was 35 ml/h and 2.5 ml fractions were collected.

Further purification was carried out with an f.p.l.c. system (Pharmacia, Uppsala, Sweden). Protein fractions were desalted on an Econo-Pak 10 DG desalting column (Bio-Rad Laboratories) and applied to the columns by means of a 10 ml superloop. Hydrophobic-interaction chromatography was performed with an alkyl-Superose (Pharmacia HR5/5) column equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 1.7 M- $(\text{NH}_4)_2\text{SO}_4$. Fractions were eluted with a 20 ml gradient of 1.7–0 M- $(\text{NH}_4)_2\text{SO}_4$. The flow rate was 0.3 ml/min and 1 ml fractions were collected.

Protein determination

Protein content was measured by the method of Bradford (1976), with BSA as standard. Protein was also detected by measuring absorbance at 280 nm.

Gel electrophoresis

SDS/PAGE was performed according to the procedure of Laemmli (1970). M_r values were calculated as described by Weber & Osborn (1969).

Isoelectric focusing

The protein was isoelectrically focused on Phast Gel IEF medium (Pharmacia), with the broad-range pI-calibration standards.

Cellulase assays

CM-cellulase activity was measured as described by Wu *et al.* (1988) except that 50 mM-sodium phosphate/citrate buffer, pH 6.0, was used. The substrate used was 0.61% (w/v) CM-cellulose 9M31, with a degree of substitution of 0.9 (Aqualon,

Wilmington, DE, U.S.A.). The reaction volume was 1.8 ml and the release of reducing sugar was determined by using the residual ferricyanide method of Park & Johnson (1949). Activity against Avicel (PH105; FMC Corp., Marcus Hook, PA, U.S.A.) and MN300 (Machery und Nagel, Darmstadt, Germany) was determined by adding 60 μ l of enzyme solution to 0.5 ml of a 1% (w/v) substrate suspension in 30 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 is defined as the amount of enzyme releasing 1 μ mol of glucose equivalent/min.

Optimum pH and temperature and thermal stability of the endoglucanase were determined by measuring the formation of reducing sugar from CM-cellulose by using the method described above; 50 mM-phosphate/citrate buffer, 50 mM-Tris/HCl buffer and 50 mM-sodium carbonate buffer were used for the pH ranges 2.6–7.6, 8.0–9.0 and 9.6–10.8 respectively. The optimum temperature and thermal stability experiments used 50 mM-phosphate/citrate buffer at pH 6.0.

Endoglucanase activity was also determined by a viscometric assay. 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. The incubations were stopped by boiling for 10 min to inactivate the enzyme. Viscosity was determined at room temperature with a 1 ml tuberculin syringe fitted with a blunt-ended 26-gauge needle.

Hydrolysis of cello-oligosaccharides

The specific activities of cello-oligosaccharide hydrolysis were determined by h.p.l.c. Endoglucanase (0.3 μ g/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. They were deionized by addition of Bio-Rad AG 501-X8 mixed-bed resin, and injected into a Bio-Rad HPX 87H column. The liquid phase was a dilute H_2SO_4 solution (0.54 ml of conc. H_2SO_4 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 by the use of authentic standards.

Preparation of anti- S_8 polyclonal antibodies and Western blotting of proteins

The S_8 protein of *C. thermocellum* was isolated by using the method of Wu *et al.* (1988). The active fractions from the Ultrogel AcA 34 column were subjected to SDS/PAGE under denaturing conditions. The gel was stained with the use of ice-cold 0.25 M-KCl plus 1 mM-dithiothreitol for 30 min, and destained in ice-cold 1 mM-dithiothreitol for 30 min. The S_8 band was identified and cut out of the gel. The protein was electro-eluted into 1 M-NaCl in a Pharmacia Extraphor unit. Before injection into a rabbit, the S_8 protein was heated at 100 °C for 15 min. The denatured S_8 protein was injected into a white rabbit with the use of the Ribl Adjuvant System (Ribl Immunochem Research, Hamilton, MT, U.S.A.). A booster injection was done 12 days after the first injection and the rabbit was bled 7 days after the booster injection. The antiserum was centrifuged for 15 min at 11000 g and 4 °C. The supernatant fluid was desalted over a 10 DG desalting column and purified by using a serum IgG purification column (Bio-Rad Laboratories).

Western blotting from SDS/PAGE gels on to nitrocellulose membranes was done by the method of Towbin *et al.* (1979). The

Table 1. Purification of endoglucanase S_8 from *C. thermocellum*

For experimental details see the text.

Stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Activity yield (%)	Purification (fold)
Crude cellulase	329	619	1.9	100	1.0
CM-Bio-Gel	26.4	130	4.8	21	2.5
DEAE-Bio-Gel	10.5	58.3	5.6	9.4	3.0
Hydroxyapatite	2.03	15.1	7.4	2.4	3.9
Alkyl-Superose	0.40	5.0	12.4	0.8	6.7

Fig. 1. CM-Bio-Gel A chromatography of crude cellulase

Peak I: elution with 640 ml of 10 mM-succinate buffer, pH 4.5, containing 2.5 mM- CaCl_2 and 2.5 mM-2-mercaptoethanol. Peak II: elution with 520 ml of the same buffer supplemented with 0.5 M-KCl. Peak III: elution with 20 mM-Tris/HCl buffer, pH 7.0, containing 2.5 mM- CaCl_2 and 2.5 mM-2-mercaptoethanol. ●, A_{280} ; □, CM-cellulase activity; ○, Avicelase activity.

membranes were blocked for 30 min at room temperature with 10 mM-Tris/HCl buffer, pH 8.0, containing 0.15 M-NaCl and 0.05% Tween 20 supplemented with 3% (w/v) non-fat dried milk. Antibody binding and development of bands with donkey anti-(rabbit Ig) serum conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, U.S.A.) were done according to the method of Rybicki & von Wechmar (1982), except that 10 mM-Tris/HCl buffer, pH 8.0, containing 0.15 M-NaCl and 0.05% Tween 20 was used throughout.

RESULTS

Purification

The procedure developed for the isolation of endoglucanase S_8 is shown in Table 1. Chromatography of the crude enzyme on a CM-Bio-Gel A cation-exchange column (Fig. 1) resolved a portion of the S_8 subunit (M_r 83 000) from the cellulase complex of *C. thermocellum* as detected by Western blotting. The fractions eluted with buffer I contained S_8 , whereas buffer II eluted no S_8 protein. Fractions eluted with buffer III contained S_8 , but they also contained subunit S_1 , which has the highest M_r (210 000–250 000) among the subunits of the cellulosome.

Fractions eluted with buffer I were concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (to 80% saturation), dialysed and applied to a DEAE-Bio-Gel A column. Gradient elution with 0–250 mM-KCl resulted in fractions that were free of a major contaminating

protein (M_r 100 000–150 000) present in the sample applied. Those fractions that were eluted at 100–175 mM-KCl contained S_8 as the major protein. Repeated anion-exchange chromatography with DEAE-Bio-Gel A separated the S_8 protein from almost all of the major contaminating protein.

By adsorption chromatography of the DEAE-Bio-Gel A eluate on hydroxyapatite with elution by 50 mM-phosphate buffer, fractions were obtained that contained S_8 protein and only a small amount of the major contaminating protein.

Use of hydrophobic-interaction chromatography with the f.p.l.c. system on an alkyl-Superose column resulted in proteins with M_r values lower than that of S_8 being eluted with high salt concentrations [1.1–0.7 M- $(\text{NH}_4)_2\text{SO}_4$], whereas most of the S_8 together with traces of the major contaminating protein was eluted with buffer containing no salt. Further elution with phosphate buffer yielded fractions with the S_8 protein as a single band (Fig. 2). Repetition of hydrophobic-interaction chromatography on fractions containing S_8 and the trace of the contaminating protein yielded additional pure S_8 . All fractions shown to contain S_8 by SDS/PAGE were enzymically active on CM-cellulose. Attempts to separate the 100 000- M_r protein- S_8 complex by a stronger hydrophobic matrix (phenyl-Superose) were not successful, since enzyme activity could not be recovered.

During the process of purification, it was noticed that the affinity and irreversible binding of protein S_8 to dialysis tubing increased with increased purity. Therefore desalting of such samples could only be achieved by desalting columns packed

Fig. 2. SDS/PAGE (10% gels) of the crude and purified S_8 endoglucanase (Coomassie Blue stain) and Western blots of the crude and purified protein using anti- S_8 antibodies

Lane A, crude cellulase (20 μ g); lane B, Western blot of crude cellulase (20 μ g); lane C, purified endoglucanase (0.5 μ g); lane D, Western blot of purified endoglucanase (0.5 μ g). Lanes A and B and lanes C and D were run at different times. Standard proteins: rabbit skeletal-muscle myosin (M_r 200 000); phosphorylase *b* (M_r 94 000); BSA (M_r 67 000); ovalbumin (M_r 43 000); carbonic anhydrase (M_r 30 000); trypsin inhibitor (M_r 20 000).

Fig. 3. Effect of temperature on the activity of endoglucanase S_8 towards CM-cellulose

The reaction mixture contained 1.6 μ g of purified enzyme. Formation of reducing sugars was measured.

with a polyacrylamide matrix; dialysis resulted in loss of almost all enzyme activity.

Properties of the enzyme

The enzyme has a temperature optimum of 70 °C (Fig. 3) and retains 50% of maximum activity through a temperature range of 40–90 °C. The pH optimum is pH 7.0 (Fig. 4). Activity fell dramatically below pH 5.0 and above pH 8.0. The enzyme is quite stable at 60 °C, retaining 33% of activity after 50 h (Fig. 5). CaCl_2 (5 mM) moderately stabilized the enzyme.

Slab-gel electrophoresis of the purified endoglucanase in the presence of SDS yielded a single band of protein corresponding

Fig. 4. Effect of pH on the activity of endoglucanase S_8 towards CM-cellulose

Buffers: $\square$, phosphate/citrate; $\times$, Tris/HCl; $\blacksquare$, sodium carbonate. Reaction mixture contained 1.6 μ g of enzyme. Formation of reducing sugars was measured.

Fig. 5. Stability of S_8 endoglucanase activity towards CM-cellulose at 60 °C

At the times indicated, 1.8 ml portions were taken (containing 1.6 μ g of protein) for determination of CM-cellulase activity. $\blacksquare$, Enzyme; $\times$, enzyme plus 5 mM- CaCl_2 . Formation of reducing sugars was measured.

to an M_r of 83 000 (Fig. 2, lane A). Western blotting with anti- S_8 antibody confirmed that the S_8 endoglucanase had been purified to homogeneity. The isoelectric point of the enzyme was determined by electrofocusing to be 3.55. The endoglucanase did not stain pink with Schiff reagent after resolution by SDS/PAGE, indicating a lack of carbohydrate.

Effects of various compounds on the endoglucanase activity

Effects of metal ions and chemical agents on enzyme activity are shown in Table 2. Ca^{2+} (5 mM) was found to stimulate activity, whereas Mg^{2+} at the same concentration inhibited activity by approx. 50%. NaCl at 100 mM also greatly inhibited activity. Hg^{2+} inhibition may be due to complex-formation with, or catalysis of oxidation of, specific residues by thiol groups, or

Table 2. Effects of various chemicals on the activity of endoglucanase S₈

The reaction mixtures contained 1.6 µg of enzyme. The chemicals were added before initiation of the reaction by the addition of the enzyme.

Chemical	Concentration (mM)	Relative activity (%)
NaCl	5	65
	50	43
	100	13
CaCl ₂	5	120
MgCl ₂	5	55
	10	12
HgCl ₂	1	13
SDS	1	0
<i>p</i> -Chloromercuribenzoate	0.05	79
	0.2	66

Table 3. Inhibition of CM-cellulose hydrolysis by glucose and cellobiose

CM-cellulose hydrolysis was assayed by the viscometric method. Reaction mixtures contained 0.2 µg of enzyme in a total volume of 1 ml.

Compound	Concentration (mM)	Relative activity (%)
Glucose	100	100
	200	100
	300	91
Cellobiose	1.25	100
	2.5	100
	5.0	97
	25	86
	100	68

simply the result of non-specific salt formation. Inhibition by the thiol-specific inhibitor *p*-chloromercuribenzoate was comparatively potent. These data indicate the presence of essential thiol groups in the enzyme.

The effects of glucose and cellobiose on endoglucanase activity, as determined by the viscometric method, as shown in Table 3. Little to no inhibition of enzyme was observed with glucose. Cellobiose inhibition was greater but still relatively mild.

Activity on different cellulosic substrates

The relationship of substrate to specific activity of the purified endoglucanase is shown in Table 4. With regard to long-chain polymers, the enzyme displayed its highest activity towards CM-cellulose, and significantly lower activity towards MN300 cellulose; no activity was detected with Avicel. There was no activity towards either cellobiose or cellotriose, but cellotetraose was hydrolysed to cellobiose with a specific activity of 13.4 units/mg, and cellopentaose to cellobiose and cellotriose with a specific activity of 18.5 units/mg. The *K_m* for cellopentaose was determined to be 1.8 mM by a Lineweaver-Burk plot (results not shown), suggesting that the enzyme has a reasonable affinity towards this substrate. The enzyme released a relatively small amount of reducing sugar for a large decrease in viscosity, e.g. 0.5% release of reducing sugar at 50% decrease in viscosity, which is typical of an endoglucanase (Fig. 6).

Endoglucanase S₈ alone is not able to hydrolyse Avicel, but S_L

Table 4. Activity of endoglucanase S₈ towards various substrates

The amount of enzyme used for CM-cellulose and the cello-oligosaccharides was 0.3 µg; for MN300 and Avicel it was 10 µg. Total volume was 1 ml for the cello-oligosaccharides, 5 ml for MN300 and Avicel and 1.8 ml for CM-cellulose. Hydrolysis of cello-oligosaccharides was followed by h.p.l.c. Hydrolysis of CM-cellulose and MN300 was determined by the increase in reducing sugar. Abbreviation: N.D., not detected.

Substrate	Specific activity (units/mg)
Cellobiose	N.D.
Cellotriose	N.D.
Cellotetraose	13.4
Cellopentaose	18.5
CM-cellulose	12.5
MN300	0.27
Avicel	N.D.

Fig. 6. Hydrolysis of CM-cellulose as measured by viscometry and release of reducing sugar

Each reaction mixture consisted of 5 mg of CM-cellulose in 1 ml of 50 mM-citrate/phosphate buffer and 0.3 µg of enzyme. At the specified times, the reaction was stopped by boiling for 10 min. The percentage reducing sugar release was calculated on the basis of complete hydrolysis releasing 3.25 mg of glucose. x, Change in viscosity; ■, reducing end groups released.

Table 5. Effect of combining S₈ with S_L on hydrolysis of Avicel

The reaction was carried out at 60 °C for 5 days (volume 5 ml).

Enzyme	Concentration (µg)	Cellobiose released (µg)
S ₈	10	0
S _L	20	100
S ₈ + S _L	10 + 20	175

subunit (Romaniec *et al.*, 1991) slowly hydrolyses Avicel; in the experiment of Table 5, it released 100 μ g of cellobiose over a period of 5 days. When S_s was added to S_L , there was a 75% increase in the amount of cellobiose released.

DISCUSSION

By using a combination of ion-exchange and hydrophobic-interaction columns, it was possible to purify a specific endoglucanase, termed S_s by Wu *et al.* (1988), without the use of denaturants. The purified enzyme was homogeneous as judged by SDS/PAGE and isoelectric focusing. The enzyme has an apparent M_r of 83000, which is close to the value of 82000 previously published (Wu *et al.*, 1988). The enzyme is clearly different in size from the 56000- M_r protein purified by Petre *et al.* (1981), but its size is similar to the 83000–94000- M_r endoglucanase found by Ng & Zeikus (1981). However, the S_s endoglucanase contains no carbohydrate, unlike the latter enzyme. The S_s endoglucanase has an M_r very similar to that of another endoglucanase recently purified by us, i.e. 76000 (M. P. Romaniec, U. Fauth, N. Huskisson, P. Barker & A. L. Demain, unpublished work). However, anti- S_s antibodies do not cross-react with the 76000- M_r protein, showing that they are distinct proteins.

The effect of the thiol-blocking reagent suggests the existence of essential thio groups in the endoglucanase, unlike that of the 56000- M_r endoglucanase (Petre *et al.*, 1981). Ng & Zeikus (1981) found that the activity of crude extracellular cellulase of strain LQRI was inhibited by Ag^+ and Hg^{2+} , but it was not clearly established whether this inhibition was due to inactivation of essential thiol groups. Unfortunately, they never tested their purified endoglucanase with these metal ions or thiol-blocking reagents. A 99000- M_r endoglucanase isolated from another thermophilic *Clostridium* (Creuzet & Frixon, 1983) was also found to be sensitive to thiol-blocking reagents. Interestingly, the S_s endoglucanase is stimulated by Ca^{2+} . Johnson *et al.* (1982) reported the stimulatory effect of Ca^{2+} on the hydrolysis of crystalline cellulose by crude cellulase in a *C. thermocellum* filtrate. In addition, Wu *et al.* (1988) reported that Ca^{2+} stimulated the hydrolysis of Avicel by the combination of endoglucanases S_L and S_s . We also found the S_s endoglucanase to be very sensitive to the presence of Mg^{2+} , and to be inhibited by 100 mM-NaCl.

The enzyme was very resistant to glucose and was only mildly inhibited by cellobiose, the end product of *C. thermocellum* cellulose hydrolysis. These results are very similar to those reported by Petre *et al.* (1981) for the 56000- M_r endoglucanase.

Besides CM-cellulose, the isolated enzyme could hydrolyse cellulose MN300, albeit at a much lower rate, the end product being cellobiose. No hydrolysis of Avicel could be detected. The S_s endoglucanase hydrolysed neither cellobiose nor cellotriose, but did hydrolyse cellotetraose to cellobiose, and cellopentaose to cellobiose and cellotriose, both with slightly higher specific activities than it showed towards CM-cellulose. This substrate pattern is different from those of the other two endoglucanases purified from *C. thermocellum* (Petre *et al.*, 1981; Ng & Zeikus, 1981).

The rapid decrease in the specific viscosity of a CM-cellulose solution accompanied by a very small increase in reducing sugars is compatible with a random endoglucanase-type hydrolysis of the substrate.

The S_s enzyme is most active at pH 6.6 and 70 °C when assayed against CM-cellulose. The pH optimum is similar to that of the sub-cellulosome preparation isolated in this laboratory (Kobayashi *et al.*, 1990), which has a broad pH optimum of

5.5–7.0. The latter also has a temperature optimum of 70 °C. The S_s endoglucanase is thought to be the J3 constituent of the sub-cellulosome preparation. When endoglucanase S_s was combined with endoglucanase S_L , there was a 75% increase in the amount of cellobiose released from Avicel, as compared with S_L alone. This result is similar to the result previously published by Wu *et al.* (1988) except that we now find the S_L enzyme to have some activity on its own. The discrepancy is probably due to different degrees of denaturation during isolation of S_L .

The S_s endoglucanase was isolated from fraction I of the CM-Bio-Gel fractionation, although analysis by SDS/PAGE revealed that most of the S_s enzyme was eluted from the column in fraction III. Fraction III contains all the S_L glycoprotein eluted from the column and is used as the starting material for the preparation of the sub-cellulosome fraction (Kobayashi *et al.*, 1990).

The relationship between S_s and the S components of the cellulosome as defined by Lamed *et al.* (1983) is unclear. At one time we considered S_s to be equivalent to S_8 (Wu & Demain, 1988). However, Morag *et al.* (1991) has found S_8 to have an M_r of 75000 and is therefore smaller than S_s . Also S_8 attacks Avicel to a greater extent than CM-cellulose and is a cellobiohydrolase rather than an endoglucanase. Component S_7 (Morag *et al.*, 1990) has an M_r of 84000 (which is only slightly higher than that of S_s) but acts as an exoglucanase rather than an endoglucanase. The endoglucanase EGE studied by Hazelwood *et al.* (1990) probably has too high an M_r (86000) to be equivalent to endoglucanase S_s .

This work was funded by National Science Foundation Grant EET-8711725 and U.S. Army Research Office Contract DAAL03-88-K-0064. Support for T.K. was supplied by the Kao Corporation. M.P.M.R. was a Merck Postdoctoral Fellow and U.F. was a scholar of the Deutsche Forschungsgemeinschaft. We sincerely appreciate the gift of crude cellulase powder and encouragement from Noubar B. Afeyan. We thank Claus von der Osten and Anthony J. Sinskey for use of their facilities and R. Longin for celloextrins.

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Received 27 December 1990/3 April 1991; accepted 12 April 1991