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Purification and Properties of *Clostridium thermocellum* Endoglucanase 5 Produced in *Escherichia coli*

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ABSTRACT

Endoglucanase 5 (EG5) has been isolated from the strain of *E. coli* TG1 harboring recombinant plasmid pCU108, which contains the *cel5* gene of *C. thermocellum*. The enzyme has been produced with 98-fold purification and a final yield of 27% by using subsequent twofold high performance ion-exchange chromatography on Mono Q and high performance chromatofocusing on Mono P. The protein has a mol mass of 35 kDa and includes 3 multiple forms with pI 4.4–4.8 as evidenced by analytical gel isoelectrofocusing. EG5 cleaves CMC (K_m = 0.097 g/L, V_{max} = 8.2 mg/min·mg of protein), amorphous cellulose, xylan, lichenan as a substrate with an optimum temperature of 80°C and pH 6.0 and Avicel (K_m = 18.2 g/L, V_{max} = 0.035 mg/min·mg of protein) with an optimum temperature of 60°C and pH 6.0. Cellobiose in concentrations up to 200 μg/mL do not inhibit the hydrolysis of CMC by EG5, but 10–30 μg/mL of glucose significantly decrease the activity of this enzyme. The stimulating role of calcium chloride and concentration of protein in the system has been demonstrated for Avicel hydrolysis by EG5.

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Index Entries: *Clostridium thermocellum*; endoglucanase 5; purification; thermostability; inhibition; adsorption.

INTRODUCTION

Bacterial cellulases have become a subject of increased interest because to their high specific activity, high optimum temperatures, and weak inhibition by end products. The anaerobic thermophilic bacterium *Clostridium thermocellum* is one of the best studied microorganisms that produce thermostable cellulases. Since the cellulases of *C. thermocellum* are combined into a highly stable complex called cellulosome, isolation of the individual enzymes in the native form is complicated. Another way of obtaining the individual cellulases is to clone and express the gene in a different host. To date, the genes of at least 15 endoglucanases, 2 xylanases, 2 β-glucosidases, laminarinase, and lichenase have been cloned in *E. coli* (*1–4*).

Recently, selection and characterization of 8 endoglucanases, cellobiohydrolase, and exoglucanase of *C. thermocellum* F7 expressed in *E. coli* have been described (*5,6*) that differ from those studied previously by their mol mass and substrate specificities. We have shown that three of the enzymes—endoglucanases 5 (EG5) and 7 and cellobiohydrolase 3 (samples not purified)—act synergistically in cleaving Avicel (*7*).

The subject of the present work was the isolation of EG5 from the recombinant stain *E. coli*/pCU108 and the determination of some of its physicochemical characteristics.

MATERIALS AND METHODS

Bacterial Strain and Media

E. coli TG1 cells harboring pCU108 plasmid with *C. thermocellum* chromosomal DNA fragments were grown in L broth supplemented with 0.1 mg/mL ampicillin at 37°C.

Enzyme Assays

Endoglucanase activity was assayed at 60°C, pH 6.0 in 50 m*M* phosphate buffer with 1% carboxymethylcellulose (CMC) (Serva, medium viscosity), 0.5% lichenan, 1% xylan, 1% amorphous cellulose (all domestic supplies) as substrates. Reducing sugar groups were assayed with Somogyi and Nelson reagents and with dinitrosalicilic reagent (*8–10*). One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmole of glucosidic bonds/min. Viscosity reduction was detected with 0.5% CMC in the same buffer (*11*).

Cellobiosidase activity was assayed by using 1 m*M* *p*-nitrophenyl-β-D-cellobioside (pNPC) (Sigma) as a substrate. After incubation for 10–40 min the reaction was terminated by addition of 1*M* Na_2CO_3. Optical density was measured at 400 nm.

Hydrolysis of Avicel (Sigma) was carried out with shaking for 24–50 h at 60°C in 5 mL of the same buffer in the presence of 10 m*M* $CaCl_2$. Concentration of Avicel was 1–50 mg/mL, and of the enzyme 24 μg/mL.

Adsorption studies were carried out at 25°C under the same conditions as for hydrolysis of Avicel. Residual CMC-activity and protein concentration were determined in the supernatant after centrifugation.

Glucose concentration was measured with the aid of Sigma Diagnostics Kit (Cat. No. 510).

Thermostability of EG5 at 55–80°C was estimated in the same buffer without a substrate by measuring the residual CMC-activity.

Protein Determination

Protein was determined by the method of Bradford (*12*) using bovine serum albumin (BSA) as standard.

Purification of Recombinant EG5

EG5 was isolated by following the scheme below.

1. *Disintegration of biomass*. Biomass was resuspended in 50 m*M* phosphate buffer, pH 6.3, supplemented with 2 m*M* phenylmethylsulphanylfluoride (PMSF), then disrupted by sonication using UZDI-1*M* disintegrator (Russia), heated at 60°C for 30 min, and centrifuged at 9500 rpm for 1 h.
2. *Precipitation with ammonium sulfate*. Ammonium sulfate was added to the supernatant (30% saturation) with stirring in an iced-water bath. The precipitate was removed by centrifugation (9500 rpm, 1 h), and then ammonium sulfate was added again (up to 60% saturation). The suspension was stored for 12–20 h at 4°C and then centrifugated, the precipitate was resuspended in a small volume of 60% ammonium sulfate.
3. *FPLC ion-exchange chromatography*. The desalted preparation was loaded on a HR10/10 column (FPLC, Pharmacia) packed with Mono Q equilibrated with 25 m*M* histidine buffer, pH 6.9. The column was eluted by an 0–0.3*M* NaCl gradient. Active fractions were collected and concentrated by ultrafiltration in an Amicon cell (PM 10 membrane).
4. *FPLC ion-exchange rechromatography*. The desalted protein solution was loaded on the same column for rechromatography. The active fraction was eluted by 0.18*M* NaCl.
5. *FPLC chromatofocusing*. Active fractions were collected and loaded on a HR5/20 column (Pharmacia, Sweden) packed with Mono P equilibrated with the same buffer, pH 6.3. Elution was carried out by 10% Polybuffer P-74, pH 4.2.

Table 1
Purification of Recombinant Endoglucanase 5 Produced by *E. coli* TG1 (pCU108)

Stage of purification	Total protein, mg	Total activity,* U	Specific activity,* U/mg	Yield, %	Purification, fold
Crude extract	4360	1308	0.3	100	1.0
Heat treatment/$(NH_4)_2SO_4$ precipitation	1243	932	0.75	71	2.5
Mono Q	124	681	5.5	52	18.3
Mono Q (rechromatography)	21.2	484	22.8	37	76.7
Mono P	12.0	351	29.3	27	98

*Activity was measured using 1% CMC as a substrate.

Gel Electrophoresis of Proteins

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini-Protean II Multi-Casting Chamber (Bio-Rad, USA) according to the instruction manual (*13*).

Isoelectric Focusing

Isoelectric focusing was performed using IEF-cell (Bio-Rad) in polyacrylamide gel plates with ampholine gradient (pH 3.5–6.0) according to the instruction manual (*14*).

RESULTS AND DISCUSSION

We tested a number of procedures for purification of EG5. The results of the most successful isolation are presented in Table 1.

It should be noted that heat treatment and precipitation with ammonium sulfate afforded a removal of significant amount of *E. coli* proteins without substantial loss of activity of EG5. Since ion-exchange chromatography on DEAE-sepharose and gel-filtration on Toyopearl and Superose 12 (Pharmacia, Sweden) were ineffective, we used high-performance ion-exchange chromatography on Mono Q immediately after precipitation with ammonium sulfate. The protein with CMC-ase activity was eluted in a two-head peak (Fig. 1a and b). Chromatofocusing on Mono P gives an elution profile of EG5 with a single almost symmetrical peak (Fig. 1c). Finally, a 98-fold purified protein with the yield of 27% was obtained.

According to SDS-PAGE data, the mol mass of EG5 is about 35 kDa, along with the main band there is also a minor band, which is probably produced by proteolysis of EG5 (Fig. 2). It is interesting to note that according to the data of gel-filtration on Superose 12 the mol mass of the purified protein is about 160 kDa. This disagreement may at attributed to the subunit structure of this protein.

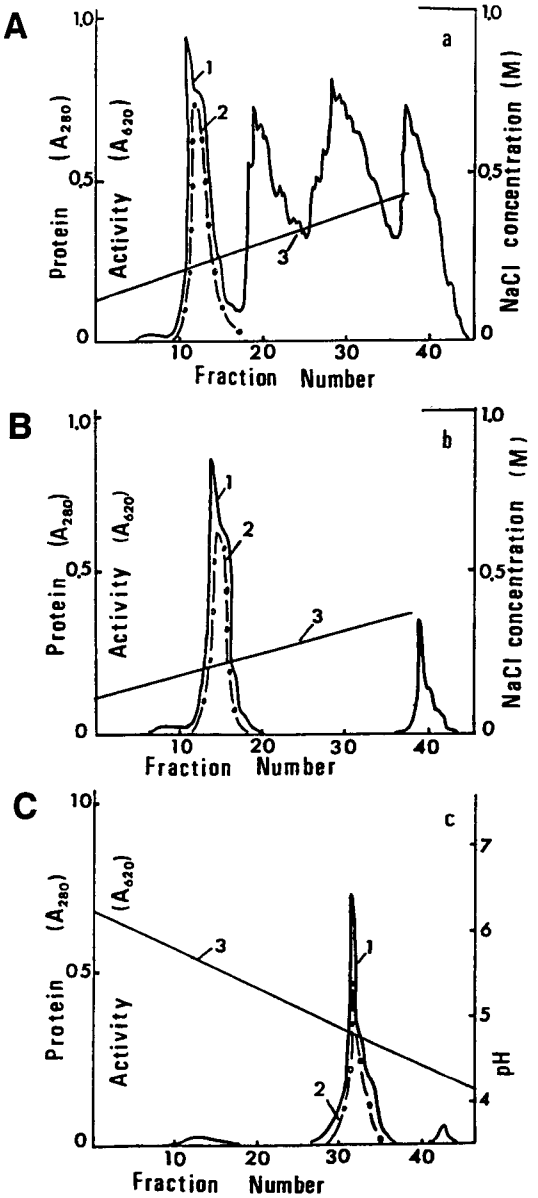

Fig. 1. a. Elution profile of FPLC chromatography on Mono Q column after precipitation with ammonium sulfate (experimental conditions are described in Materials and Methods): 1. absorption at 280 nm; 2. CMC-ase activity; 3. gradient of NaCl. b. Elution profile of rechromatography on Mono Q column (for explanations, *see* a. above. c. Elution profile of FPLC chromatofocusing on Mono P column (conditions are described in Materials and Methods): 1. absorption at 280 nm; 2. CMC-ase activity; 3. pH gradient.

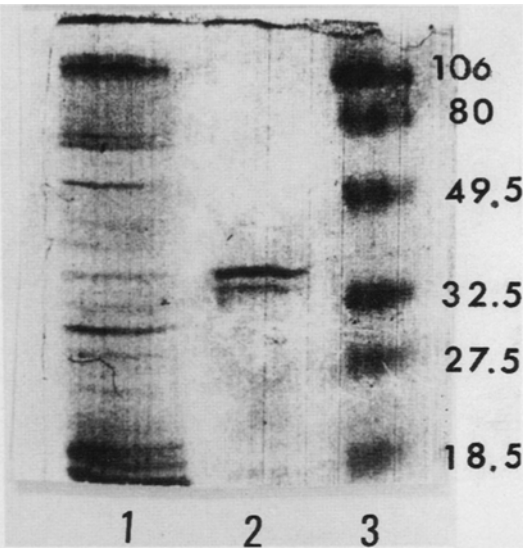

Fig. 2. SDS-PAGE of EG5: 1. nonpurified preparation; 2. after chromatofocusing; 3. mol mass markers (106 kDa, 80 kDa, 49.5 kDa, 32.5 kDa, 27.5 kDa, 18.5 kDa).

Table 2
Substrate Specificity of EG 5

Activity, U/mg (60°C)							
CMC, 1%, reduc. sug.	CMC, 0.5%, reduc. sug.	CMC, 0.5%, viscosity reduction	lichenan, 0.5%	xylan, 1%	amorphous cellulose, 1%	Avicel, 1%	pNPC 0.5 mg/mL
29	22.4	26.5	42	11.2	5.4	0.04	0.0

Isoelectrofocusing pattern of the purified EG5 shows three bands with the values of pI of 4.4, 4.6, and 4.8. Similar results has recently been obtained for recombinant cellulase (*15*), and a detailed study of this fact is yet to be performed.

Endoglucanase from the same strain with similar mol mass and similar pI values has previously been described (*5,15,16*). However, according to blot-hybridization data these proteins are the products of different genes and they display significantly different properties. For example, the thermostability of EG5 is higher than that of endoglucanase (*5,15,16*) (temperature optima of CMC-ase activity are 80 and 60°C, respectively). The proteins also have different optimal pH values and substrate specificities.

The data on substrate specificity of EG5 are presented in Table 2. The enzyme exhibits significant activity against CMC and lichenan; it also cleaves xylan, amorphous cellulose, and Avicel. It does not use pNPC as a substrate. The fact that the ratio of CMC-ase activity obtained with Somogyi and Nelson reagents to that estimated from viscosity reduction data is close to unity that characterizes EG5 as a typical endoglucanase.

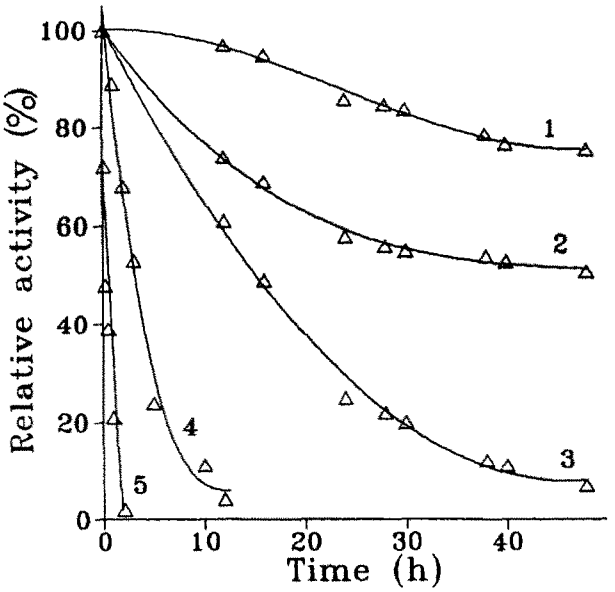

Fig. 3. Thermostability profiles of EG5 (°C): 1. 55; 2. 60; 3. 65; 4. 70; 5. 80 (conditions are described in Materials and Methods).

The values of K_m and V_{max} of 0.097 g/L and 8.2 mg/min·mg of protein, respectively, were obtained with CMC as a substrate. The corresponding values for Avicel are 18.2 g/L and 0.035 mg/min·mg of protein.

Optimal activity of EG5 with CMC, amorphous cellulose, lichenan, and Avicel as the substrates was found at pH 6.0–6.5.

As mentioned above, 80°C is the optimum temperature for EG5 to cleave CMC, amorphous cellulose, and lichenan. However, the optimum temperature decreases to 60°C when assayed in the course of a prolonged (24–50 h) incubation with Avicel as a substrate, probably because of enzyme inactivation.

It was interesting to study the thermostability of EG5. The results of the experiments without a substrate are presented in Fig. 3. The half-inactivation times are: 8 min at 80°C; 3 h at 70°C; 24 h at 60°C; and more than 60 h at 55°C. It is likely that the half-inactivation times increase in the presence of a substrate because of the formation of an enzyme–substrate complex. It should be noted that the thermostable endoglucanase 1 of the same origin has been described (*17*), but EG5 studied by us is the only enzyme from *C. thermocellum* that has such a high optimum temperature.

Since both glucose and cellobiose are among the end products of CMC hydrolysis, we studied the inhibition of EG5 by them. The enzyme activity was measured by using the viscosity reduction method with glucose and cellobiose added to the initial solution of CMC. We found that cellobiose in concentrations up to 200 μg/mL does not inhibit hydrolysis, but 10–30 μg/mL of glucose significantly decreases the activity of EG5 (Fig. 4).

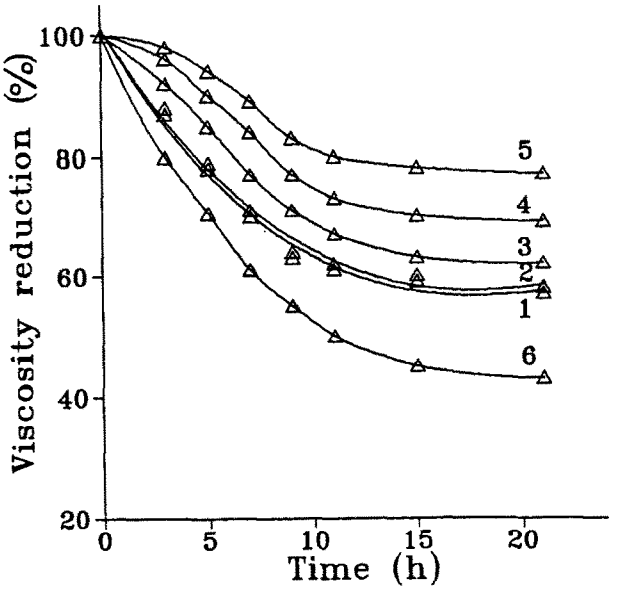

Fig. 4. Reduction of viscosity of CMC produced by EG5: 1. without additives; 2. with cellobiose, 200 μg/mL; 3, 4, 5. with glucose, 10, 20, 30 μg/mL respectively; 6. with 10 m*M* $CaCl_2$.

It has previously been shown (*18,19*) that addition of dithiothreitol (DTT) and calcium ions increases the yield of reducing sugars during hydrolysis of Avicel by cellulosome and by some individual cellulases. In the present work we showed that $CaCl_2$ stimulates the reduction of viscosity of CMC (10% increase of activity) but DTT (1–10 m*M*) does not have any effect on hydrolysis (Fig. 4). In hydrolysis of Avicel a 1.3-fold increase of the yield of end products was observed when 10 m*M* $CaCl_2$ was added.

The kinetics of adsorption of EG5 on Avicel (Fig. 5) shows that after equilibrium is reached in this system (after about 40 h), approx 15% of the enzyme stays in the solution.

It is known that the rate of enzymatic hydrolysis of cellulose depends on a protein concentration in the system. We investigated the influence of two nonenzymatic proteins, BSA and human albumin (added in concentrations of 0.1–5.0 mg/mL) on hydrolysis of Avicel. A stimulating effect of both proteins on the cleavage process is well pronounced (Fig. 6). This fact may be explained by the stabilizing effect of increased protein concentration.

In conclusion a new endoglucanase from *C. thermocellum* different from those previously described was purified and some of its properties were studied. Since contribution of EG5 to the synergism on hydrolysis of Avicel mentioned above is significant, a more detailed investigation of this phenomenon is necessary, and will be put forward in our future studies.

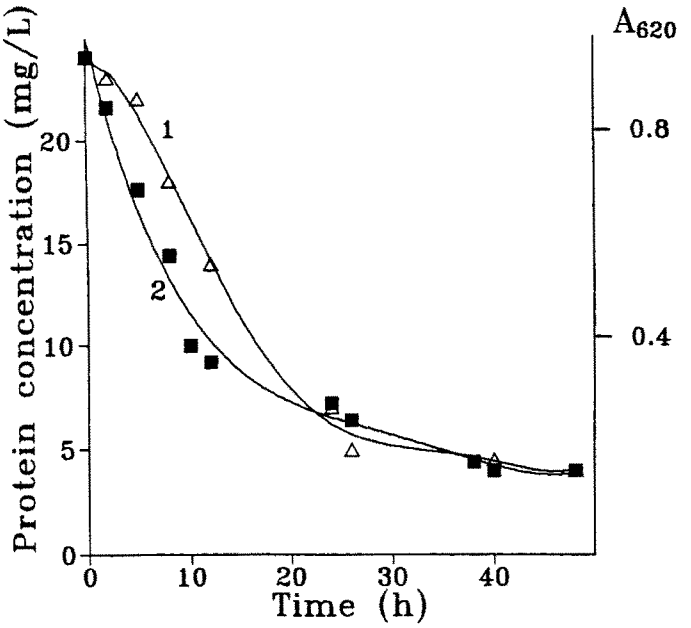

Fig. 5. Decrease of protein concentration (1) and residual CMC-ase activity (2) in solution during adsorption of EG5 on Avicel (30 mg/mL).

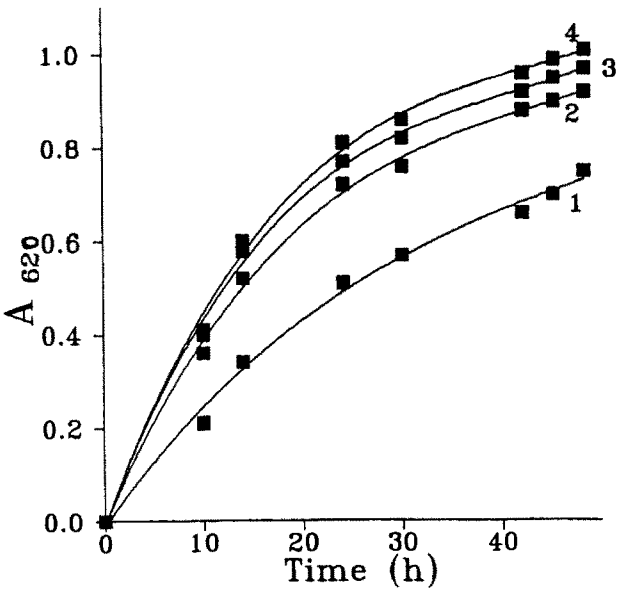

Fig. 6. Accumulation of reducing sugars in the presence of different concentrations of BSA: 1. without BSA; 2. 0.1 mg/mL; 3. 1 mg/mL; 4. 2 or 5 mg/mL of BSA.

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