0273-2289/99/80/0121/$15.00

Molecular Cloning and Expression of an Endo-β-1,4-D-glucanase I (Avicelase I) Gene from *Bacillus cellulyticus* K-12 and Characterization of the Recombinant Enzyme

TAE-KYUN LEE AND CHEORL-HO KIM*

Department of Gynecology and Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, Sukjang-Dong #707, Kyungju-City, Kyungpook 780-714, Korea, E-mail: chkimbio@email.dongguk.ac.kr

Received July 20, 1998; Accepted November 4, 1998

Abstract

Bacillus cellulyticus K-12 Avicelase (Avicelase I; EC 3.2.1.4) gene (*ace* A) has been cloned in *Escherichia coli* by using the vector pT7T3U19 and *Hind*III–*Hind*III libraries of the chromosomal inserts. The libraries were screened for the expression of avicelase by monitoring the immunoreaction of the antiavicelase (immunoscreening). Positive clones (Ac-3, Ac-5, and Ac-7) contained the identical 3.5-kb *Hind*III fragment as determined by restriction mapping and Southern hybridization, and expressed avicelase efficiently and constitutively using its own promoter in the heterologous host. From the immunoblotting analysis, a polypeptide that showed a carboxymethylcellulase (CMCase) activity with an M_r of 64,000 was detected. The recombinant endo 1,4-β-$_D$-glucanase I was purified to homogeneity from an intracellular fraction of *E. coli* by DEAE-Toyopearl M650, Phenyl Toyoperal M650, and TSK gel HW50S chromatography. The enzyme had a monomeric structure, its relative molecular mass being 65 kDa by gel filtration and 64 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The p*I* was 5.3 and the optimal pH was 4.6, and the enzyme was stable at pH 4.0–10.5. The enzyme had a temperature optimum of 50°C and was stable at 55°C for 48 h, and retained approx 20% of its activity after 30 min at 70°C. It showed high activity toward carboxymethylcellulose (CMC) as well as *p*-nitrophenyl-β-D-cellobioside, 4-methylumbelliferyl cellobioside, Avicel, filter paper, and some cellooligosaccharides. K_m values for CMC and Avicel were 7.6 and 85.2 mg/mL, respectively, whereas V_{max} values were 201 and 9.2 μmol · min^{-1} · mg^{-1}, respectively. Cellotetraose (G4) was preferentially cleaved into cellobiose (G2) and cellopentaose (G5) was cleaved into G2 + cellotriose (G3),

*Author to whom all correspondence and reprint requests should be addressed.

whereas cellohexaose (G6) was cleaved into G4 + G2 and, to a lesser extent, into G3 + G3. G3 was not cleaved at all. G2 was the main product of Avicel hydrolysis. G2 inhibited whereas Mg^{++} stimulated the activity of CMCase and Avicelase. Hydrolysis of CMC took place with a rapid decrease in viscosity but a slow liberation of reducing sugars. Based on these results, it appeared that the cellulase should be regarded as endo type, although it hydrolyzed Avicel.

Index Entries: Gene cloning; expression; avicelase; endo-β-1,4-D-glucanase; cellobiohydrolase; xylanase; *Bacillus cellulyticus* K-12.

Introduction

The cleavage of crystalline cellulose is of great biotechnological and ecological potential, but is still quite poorly understood. It has been primarily studied using microcrystalline cellulose (Avicel), which has a chain length of 300–500 glucose units in comparison with 5000–15,000 in native cellulose *(1)*. Until now the cellulolytic system from the fungus *Trichoderma reesei* has been best characterized. Two endoglucanases and two cellobiohydrolases (CBHs) (EC 3.2.1.91) act synergistically to convert Avicel to cellobiose (G2) *(2,3)*. Only a few bacterial cellulases degrading the crystalline substrate have been studied genetically and biochemically in detail. For example, *Clostridium stercorarium* produces two avicelases that hydrolyze crystalline cellulose synergistically. Its Avicelase I has been identified as an endoglucanase *(4)*, whereas Avicelase II has been classified as an exoglucanase *(5)*. In *Streptomyces reticuli*, one unusual cellulase (Avicelase) has been identified *(1)*. The enzyme hydrolyzes crystalline and soluble cellulose as well as cellodextrins, predominantly to G2. In *Bacillus* species, Avicelases showing endo-β-1,4-D-glucanase (EGI) and CBH activities have been purified and characterized from *Bacillus circulans* F-2 *(6,7)*.

There are at least three different types of enzymes that are involved in the complete hydrolysis of native cellulose to glucose: 1,4-β-D-glucan CBHs, which specifically cleave cellobiosyl units from the nonreducing end of the cellulose chains; endo-1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.4) (endoglucanases), which cleave internal cellulosic linkages; and β-D-glucoside glucohydrolases (EC 3.2.1.21) or β-D-glucosidases (cellobiase), which specifically cleave glucosyl units from the nonreducing end of cellooligosaccharides. CBH has been widely described in the literature in terms of its activity on microcrystalline cellulose (Avicel) with G2 as the main product, and its inability to degrade the internal cellulose linkages of CMC. The term Avicelase is commonly regarded as synonymous with exoglucanase or CBH *(8)*. It is assumed that endoglucanases can only attack the amorphous regions of cellulose molecules. The degradation of the crystalline regions is thought to occur by CBH action catalyzing the release of G2 residues from the nonreducing ends of cellulose chains *(9)*. However, some inconsistencies with CBH behavior have been reported *(10,11)*, and the ability of some of the endoglucanases to also degrade xylan when others

only act on CMC or hydroxyethylcellulose indicates that there are two types of endoglucanases *(6,12)*.

Recently we have isolated *Bacillus cellulyticus* K-12, which produces powerful cellulases having β-1,4-glucanases for carboxymethyl cellulase (CMCase) (70–80 U/mL), avicelase (1.0–1.5 U/mL), filter paper hydrolase (0.5–1.0 U/mL), xylanase (3.5–4.0 U/mL), and β-glucosidase (0.3–0.5 U/mL) *(13)*. The homogeneous β-glucanase I preparation of *B. cellulyticus* K-12 acts very specifically and shows a specific substrate specificity toward β-1,4-glycosidic linkage of Avicel, a microcrystalline artificial cellulose substrate. The most interesting aspect of the enzyme was that it acted as not only endo β-glucanase, but also exo β-glucanase (Kim and Lee, unpublished data). It therefore appears that the cloning of a single homogeneous gene expressing the enzyme will be useful for elucidation of the enzyme-substrate relationship. Here we report the cloning of a cellulase (Avicelase) gene from *B. cellulyticus* K-12 in *Escherichia coli*, expressing Avicelase activity. This is the first report of a gene cloning of an endo–exo β-glucanase in *B. cellulyticus* K-12. Also, this article deals with the purification and characterization of the recombinant EGI (Avicelase I) and reports its mode of action on Avicel and cellodextrins.

Materials and Methods

Materials

Restriction enzymes and modification enzymes were purchased from BM Korea, and were used as recommended by the suppliers. Molecular marker kits for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nitrocellulose membrane filter for immunodetection and Western blotting analysis were obtained from Bio-Rad (Tokyo, Japan). Nylon membrane filter (Hybond-C) for DNA hybridization was purchased from Amersham Japan (Tokyo, Japan). DEAE Toyopearl M650, TSK Phenyl M650, and TSK Gel HW 50S were obtained from Tohso (Tokyo, Japan). Avicel SF and cellulose powder, carboxymethyl cellulose ([CMC], with a degree of substitution [DS] = 0.65 and average molecular weight = 110,000), were supplied by Asahi (Tokyo, Japan). *p*-Nitrophenyl-β-D-cellobioside (pNPC), *p*-nitrophenyl-β-xylopyranoside (pNPX), *p*-nitrophenyl-β-arabinofuranoside, arabinose, *p*-nitrophenyl-β-D-glucopyranoside (pNPG), 4-methylumbelliferyl cellobroside [MeUmb$(Glc)_2$], and G2 were purchased from Sigma (St. Louis, MO). Cellooligosaccharides and xylodextrins were purchased from Seikagaku Kougyo K. K. (Tokyo, Japan). Filter paper (1 × 0.6 cm) was from Whatman (Maidstone, England). Other chemicals used were of the purest grade commercially available.

Bacterial Strains and Plasmids

B. cellulyticus K-12 was used on the source of the gene that codes for the avicelase. The following bacterial strains and plasmids were used: strains *E. coli* MV1184, JM109; and plasmids pT7T3U19 and pBR322.

Media and Culture Conditions

B. cellulyticus K-12 was maintained in induction medium containing 10 g of a filter paper strip (13×1 cm^2) and 2 g of avicel, 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, 1.0 g of bactopeptone, 1 g of yeast extract, and 1 mL of trace element in 1 L of water *(13)*. *E. coli* MV1184, JM109 was maintained in Luria broth (LB) (1% peptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2), whereas JM109, MV1184/plasmid was maintained in LB containing 40 µg/mL of ampicillin. Cultures were preserved in 25% glycerol at –70°C.

DNA Isolation

Chromosomal DNA was extracted from *B. cellulyticus* K-12 according to the method of Saito and Miura *(14)*. Large-scale preparation of plasmid DNA was carried out by the method of Birnboim and Doly *(15)*. Restriction endonuclease (BRL, Gaithersburg, MD) was used under the assay conditions described by the manufacturers. Agarose gel electrophoresis of DNA fragments was carried out in Tris-acetate buffer, pH 7.8, containing EDTA as described elsewhere *(9)*. DNA from the gel was transferred onto Hybond-C membrane (Amersham) and used for hybridization. The DNA probe was nick translated with [α-^{32}P]dCTP as described by Maniatis et al. *(16)*.

Gene Library Construction and Cloning of Avicelase Gene

Chromosomal DNA from *B. cellulyticus* K-12 was partially digested with *Hind*III. After removal of proteins, the resulting fragments were ligated to *Hind*III-digested pT7T3U19 DNA using T4 DNA ligase *(17)*. *E. coli* MV1184 was transformed using these recombinant plasmids according to the method described by Mandel and Higa *(18)*. The transformants were transferred to a nitrocellulose membrane plate for avicelase genes by immunoreacting with antiavicelase antiserum *(19)*, affinity-purified goat antimouse IgG-alkaline phosphatase conjugate, and substrate of alkaline phosphatase (Vector, Burlingame, CA). Original colonies showing immunoreactivity on the nitrocellulose membrane were selected and screened for their enzyme activity.

Enzyme Assays and Preparation of Polyclonal Antibody

Avicelase activity was determined by incubating the appropriately diluted enzyme with 2% Avicel SF (Asahi) with shaking for 30 min at 50°C. After incubation, the remaining sugars released were estimated as G2 equivalents by the modified dinitrosalicylic acid method described previously *(20)*. One International unit was defined as 1 µmol of product formed per minute under the given temperature and pH conditions. For preparation of the substrate used for avicelase, 2% Avicel suspension in 50 m*M* phosphate buffer, pH 6.5, was left standing at room temperature for 20 min and then centrifuged. After the supernatant was discarded, the pellet was used as substrate.

CMCase or xylanase activity was determined as described previously *(6)* using the dinitrosalicylic acid (DNS) method *(21)*. One unit of each activity corresponds to the release of 1 µmol equivalents of reducing groups per minute. For detection of β-glucosidase, β-xylosidase, and pNPC-hydrolase activities, the substrates pNPG, pNPX, and pNPC, respectively, were used according to the method as described in refs. *6* and *7*. Assays containing 1 mL of enzyme suitably diluted in water, 1.5 mL of 100 m*M* sodium acetate buffer (pH 4.5), and 0.5 mL of 5 m*M* *p*-nitrophenyl (pNP)-derivative in water were incubated at 50°C for 15 min. *p*-Nitrophenol produced by the addition of 2.0 mL of 1 *M* $NaCO_3$ was measured spectrophotometrically at 410 nm. One unit of enzyme activity is defined as the amount of enzyme that liberated 1 µmol of pNP in 1 min at 40°C. For hydrolysis of G2, the glucose released was determined by the glucose-oxidase method (Glucose-oxidase detection kit AR-II, Wako, Tokyo, Japan).

Filter paper–hydrolyzing activity (F.P.A.) *(22)* was assayed as described previously *(7)*. To 10 mg (1 × 6 cm^2 strip) of Whatman no. 1 filter paper were added 0.5 mL of 0.1 *M* sodium acetate buffer (pH 4.5) and 0.3 mL of enzyme solution. The mixture was incubated at 40°C for 1 h, and the reducing sugar liberated was then measured by the DNS method. One unit of activity corresponds to the release of 1 µmol of G2 equivalent/min under the above conditions.

The procedure for measuring the activities of other glycan-hydrolyzing enzymes was essentially the same as described for CMCase. Endoglucanase activity was also determined by a viscometric assay. Reaction mixtures containing 0.5% (w/v) CMC in 50 m*M* sodium acetate buffer (pH 4.5) were incubated for up to 60 min at 50°C. The viscosity was determined at room temperature with a 1-mL Tuberculin syringe fitted with a 26-gage blunt needle by using a digital viscosimeter (Ubbelohde microviscosimeter, Kaburagi Scientific, Tokyo, Japan). A solution (1 mL) containing CMC (2.0% [w/v] in 0.1 *M* acetate buffer, pH 4.5) was added to the sample container and heated to maintain a temperature of 40°C. The reaction was initiated by addition of different amounts of purified enzyme. Reduction of viscosity was monitored by continuous readout of viscosity vs liberation of reducing sugars by recording devices connected to the viscosimeter. The reciprocal of the specific viscosity (fluidity) $1/\eta$ was calculated by the formula

$$1/\eta = t_0/(t - t_0)$$

in which t_0 is the flow time of the buffer and t is the flow time of reaction mixture *(4)*.

Enzyme Purification

Unless stated otherwise, all purification steps were carried out at 4°C. The crude cell extracts from 2 L of cultured cells were used for initial purification. The harvested cells were sonicated and resuspended in 200 mL of 20 m*M* Tris-HCl buffer (pH 8.0) and dialyzed against 2 L of the same buffer

Table 1
Avicelase Activities Expressed
in *E. coli* Carrying pAV-3
and Its Derivative Plasmids

Plasmid	Activity (U/mL culture)[a]	
	+IPTG	–IPTG
pAV-3	6.80	1.70
pAV-5	8.30	1.80
pAV-7	2.40	0.70
pAV-9	ND	ND
pAVR-3	1.70	1.60
pAVR-11	1.70	1.90
pAB-3	0.56	0.57
pABR-3	0.65	0.53

[a]ND, not detected.

for 24 h with one change of buffer. The crude enzyme was subjected to 30–60% ammonium sulfate fractionation, and the precipitates were resuspended in a minimum volume of 20 m*M* Tris-HCl buffer (pH 8.0) and dialyzed against 2 L of the same buffer for 24 h with one change of buffer. The crude enzyme (110 mL; 340 mg of protein) was applied to a DEAE Toyopearl M650 column (1.6×64 cm^2) previously equilibrated with 20 m*M* Tris-HCl buffer at pH 8.0. Elution was effected with 400 mL of linear gradient (0.0–0.5 *M* NaCl) in equilibration buffer at a flow rate of 36 mL/h. Fractions (5 mL) were collected, assayed for protein and enzyme activities, and analyzed by SDS-PAGE.

One peak of enzyme activity was resolved by DEAE Toyopearl M650 ion chromatography, and active fractions (120 mL) from the column were pooled, concentrated 10-fold by ultrafiltration with a Diafulo YM30 membrane (Amicon, Beverly, MA) and dialyzed against 3 L of 20 m*M* Tris-HCl (pH 8.0) for 24 h. Dialysis was always carried out at 4°C. Dialyzed enzyme was concentrated by ultrafiltration, dissolved in a small volume of 1 *M* ammonium sulfate in 0.1 *M* sodium phosphate buffer (pH 7.5), and applied to a hydrophobic interaction chromatography column of TSK Gel Phenyl 5PW previously equilibrated with 1 *M* ammonium sulfate in 0.1 *M* sodium phosphate buffer (pH 7.5). The enzyme was eluted with a decreasing $(NH_4)_2SO_4$ gradient in 0.1 *M* phosphate buffer (pH 7.5). Protein and avicelase activities were eluted as a single peak at 0.40 *M* ammonium sulfate. The enzyme obtained as described above was successfully rechromatographed on a high-performance liquid chromatography (HPLC) TSK Gel Toyopearl HW-65S column (1.6×90 cm^2, Tohso, Tokyo, Japan) equilibrated with 50 m*M* sodium chloride. Protein and enzyme activities were eluted as a single peak. The active fractions of Avicelase were pooled, concentrated, dialyzed against 50 m*M* acetate buffer (pH 4.5), and subjected to the following experiments as the purified enzyme. Table 1 summarizes the purification of the enzyme.

SDS-PAGE and Western Blotting

SDS-PAGE of the cell extracts was carried out on 10% slab gels *(23)*. The separated proteins were then transblotted onto Hybond sheets and processed according to Towbin et al. *(24)* using antiavicelase antibodies raised in mice. The polyclonal antiavicelase antibodies used were raised against avicelase that was purified from *B. cellulyticus* K-12. For immunization, the purified avicelase (100 μg) was periodically boosted three times in four mice (Balb/c). After the fourth boosting, the antibodies were titrated by double diffusion assay. Details of enzyme purification and production of polyclonal antibody will be reported elsewhere.

Kinetic Determinations

Initial rates of Avicel, CMC, or xylan hydrolysis were determined at various substrate concentrations (0.1–20.0 mg/mL). The reaction mixtures contained 5 μg of purified enzyme. The kinetic constants K_m and V_{max} were estimated by the method of Lineweaver and Burk *(25)*.

Analytical Methods

Protein content was determined by the method of Lowry et al. *(26)* with bovine serum albumin (BSA) as standard. Protein in the column eluants was routinely followed by the absorbance at 280 nm. The amount of total sugars was determined by the phenol–sulfuric acid method *(27)* with G2 as standard. The following M_r standards were used to calibrate the column: blue dextran, 2000 kDa; thyroglobulin, 660 kDa; apoferritin, 443 kDa; γ-globulin, 160 kDa; ovalbumin, 48 kDa; and RNase A, 18.4 kDa (Sigma). SDS-PAGE was performed in 7.5 and 15% (w/v) gels with SDS by the method of Laemmli *(23)*. SDS-PAGE with 15% homogeneous PhastGel was also performed with a Phast-System (Pharmacia, Uppsala, Sweden). The sample buffer was 0.01 *M* Tris-HCl (pH 8.0) containing 2.5% SDS and, in some cases, 5% (v/v) β-mercaptoethanol. PhastGels were silver stained *(28)*. CMCase or xylanase activities in mildly denaturing gels and isoelectric focusing point were detected as described previously *(6,7)*. For determination of molecular weight, the standards (Bio-Rad, Richmond, CA) used were myosin, β-galactosidase, phosphorylase B, BSA, and ovalbumin. Time course hydrolysis of CMC, G2, xylan, Avicel, filter paper, and cotton was carried out according to our previous method *(6)*. For separation of reaction mixture on thin-layer chromatography (TLC), samples were spotted on precoated silica gel plates (Merck, Darmstardt, Germany) and developed 3 × 3 h at room temperature with chloroform:acetic acid:H_2O (6:7:1, v/v/v). Detection of sugars was carried out as described previously *(29)*.

Effect of pH and Temperature on Enzyme Activities and Stabilities

Enzyme activities were measured at pHs from 4.0 to 10.0 under standard assay conditions with Avicel SF and CMC as substrates. Buffers used were 100 m*M* citrate (pH 2.5–3.0), 100 m*M* sodium acetate (pH 3.0–5.5),

100 m*M* MES-NaOH (pH 5.5–7.0), 100 m*M* potassium phosphate (pH 6.0–8.0), and 100 m*M* glycine-NaOH (pH 8.0–11.0). Enzyme activities were also assayed at temperatures from 30 to 70°C at pH 4.5. Assays of enzyme stabilities were done as described previously *(30,31)*.

Enzymic Hydrolysis of Cellodextrins and Avicel

Specific activity of cellodextrin hydrolysis was determined by TLC. The reaction mixture consisting of 20 µL of 1% cellooligomer and 20 µL of purified enzyme (26 µg) in 0.05 *M* acetate buffer (pH 4.5) was incubated at 40°C. The reaction mixture of each sample after 2 h of incubation was spotted on a thin layer of silica (Merck). The reaction mixture consisting of 0.2 mL of 0.1% Avicel suspension in 0.05 *M* acetate buffer (pH 4.5) and 0.1 mL of enzyme solution (80 µg of purified enzyme) was incubated at 40°C. Reaction mixtures were shaken continuously. The reaction mixture was sampled at time intervals and analyzed by TLC.

Results and Discussion

Cloning of Avicelase Gene

Of 25,000 recombinants derived from a library, 3 were found to produce avicelase. These recombinant plasmids with endoglucanase gene were analyzed by *Hind*III digestion and classified into one category according to distinct DNA. The digestion pattern of *Hind*III on the insert DNA fragment was common. Restriction site analysis revealed that the 3.5-kb fragment is the same, respectively, indicating that 3.5-kb DNA fragment is responsible for Avicelase expression in *E. coli*.

A physical map of the 3.5-kb insert DNA showed that the insert has two different restriction sites for *Eco*RI, *Dra*I, and *Pvu*II, and one site for *Sma*I and *Bam*HI. No sites for the enzymes *Pst*I, *Sst*I, and *Xho*I could be seen within this *Hind*IV site (Fig. 1).

Expression of Avicelase Gene

To localize the structural gene in a 3.5-kb insert fragment of pAV-3 (6.3 kb), *Eco*RI- or *Sma*I-digested DNA fragments were religated and avicelase-positive clones were selectively screened by immunoreacting with antiavicelase antiserum as described in Materials and Methods. Plasmids of pAV-5 (5.9 kb), pAV-7 (5.1 kb), and pAV-9 (4.5 kb) were newly created as shown in Fig. 2. *E. coli* carrying plasmids of pAV-3 and pAV-5 showed their high enzyme activities (1.7 U/mL for pAV-3 and 1.8 U/mL for pAV-5) whereas *E. coli* (pAV-7) produced only 0.7 U/mL of avicelase. However, pAV-9 did not result in any avicelase activity. These results clearly indicate that plasmid pAV-7 carried the DNA fragment responsible for the avicelase-encoding structural gene without its own promoter and that a 0.8-kb DNA fragment of *Eco*RI–*Eco*RI site might be its own 5'-promoter region for the gene transcription. Since the pT7T3U19 as a plasmid vector has T7 and T3

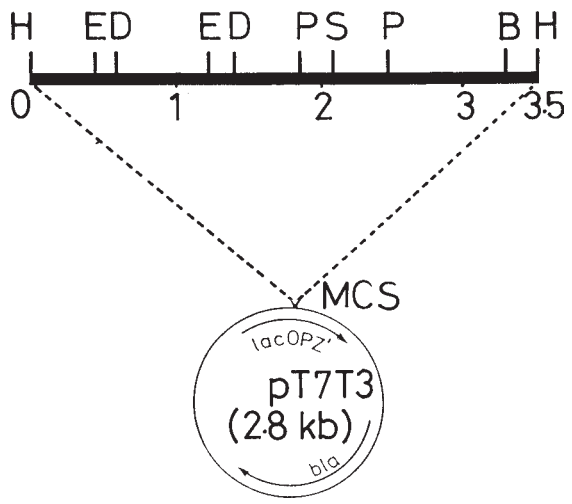

Fig. 1. Physical map of pAV-3 constructed with pT7T3 U19 and the *Hind*III fragment containing the *B. cellulyticus* K-12 avicelase gene. MCS, multiple cloning site.

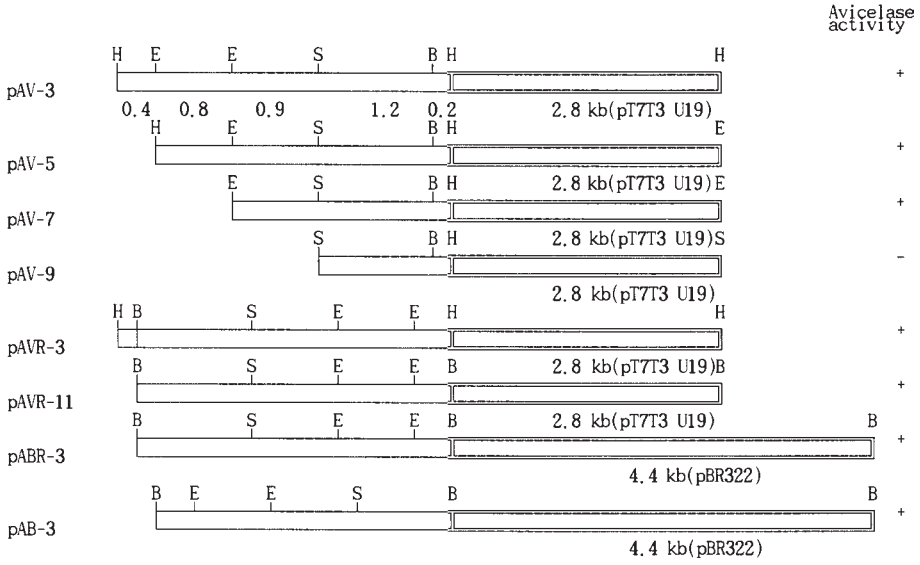

Fig. 2. Restriction enzyme maps of the fragments cloned in recombinant plasmid pAV-3 and their derivative fragments. Solid bars represent *B. cellulyticus* K-12 DNAs. Plasmid pABR-3 was constructed by subcloning of the 3.3-kb *Bam*HI fragment to pBR322. There is no *Pst*I site in these inserts. H, *Hind*III; E, *Eco*RI; B, *Bam*HI; S, *Sma*I; +, avicelase activity was detected on Congo red plates; –, avicelase activity was not detected on Congo red plates.

transcriptional promoter, there was not any significant difference between the enzyme activities produced by the two plasmids of pAV-3 and pAV-5. *E. coli* JM109 carrying the derivative plasmid pAV-7 expressed 2.4-fold

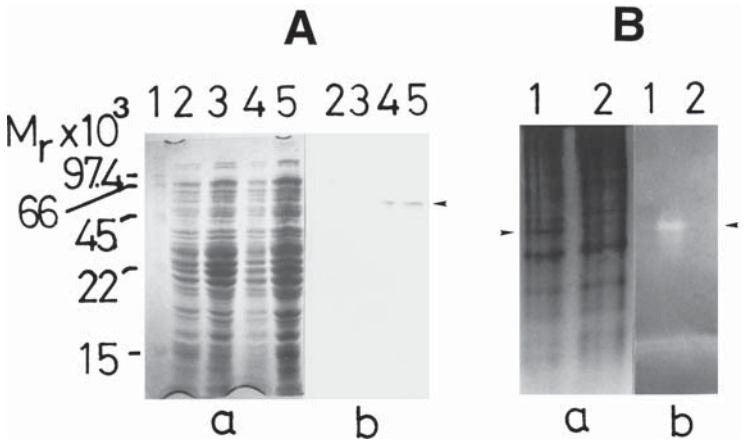

Fig. 3. SDS-Western blotting analysis of the cloned avicelase samples expressed in *E. coli* JM109 (pAV-3). **(A)** SDS-PAGE. The cell extracts were separated by 12.5% SDS-PAGE, transferred, and treated with anti-avicelase. Reaction was carried out by the method described in Materials and Methods. (a) Cell extracts of *E. coli* JM109 with pT7T3 U19 or pAV-3 were stained with Coomassie Blue R250. Lane 1, molecular sizes of standard marker (in kilodaltons) are indicated at the left; lane 2, *E. coli* JM109 (pT7T3 U19) as a negative control (6 µg); lane 3, *E. coli* JM109 (pT7T3 U19) as a negative control (12 µg); lane 4, *E. coli* JM109 (pAV-3) as a positive control (6 µg); lane 5, *E. coli* JM109 (pAV-3) as a positive control (12 µg). **(B)** Zymography of SDS-PAGE. (b) Immunoreacted samples corresponding to lanes of a.

lower avicelase activity than the original plasmid pAV-3 (Table 1). This possibly suggests that the deletion of the 1.2-kb fragment caused less-efficient read-through transcription. The addition of isopropyl-β-D-thiogalactopyranoside (IPTG), an inducer of the *lac* promoter, into the cell extract stimulated expression of the avicelase gene in pAV-7, pAV-5, and pAV-3, indicating that the functional orientations of the avicelase and *lac* promoter of the vector are the same in this plasmid and that the *lac* promoter also participates in expressing the gene (Table 1). On the other hand, a 3.5-kb insert DNA of plasmid pAV-3 was subcloned into pT7T3U19 in reverse orientation and the *Bam*HI-digested DNA fragment was religated. The newly designated plasmids, pAVR-3 and pAVR-11, conferred the same avicelase activities as those produced by pAV-3 to *E. coli*, indicating that transcription of the gene arose in part from a *B. cellulyticus* K-12 promoter. For further characterization of the gene and of the promoter localization for avicelase, a *Bam*HI-digested 3.3-kb DNA fragment of pAV-3 was subcloned to promoter-negative pBR322. Two derivative plasmids, pABR-3 and pAB-3, in which the pAV-3 insert was in direct and reverse orientation, revealed that a promoter was present in this fragment.

The culture supernatant of *B. cellulyticus* K-12 contained multiple forms of cellulase, which probably act synergistically, whereas the *E. coli* (pAV-3) made only one of these active forms. This could be one of the possible reasons for relatively low avicelase activity in the pAV-3 cell

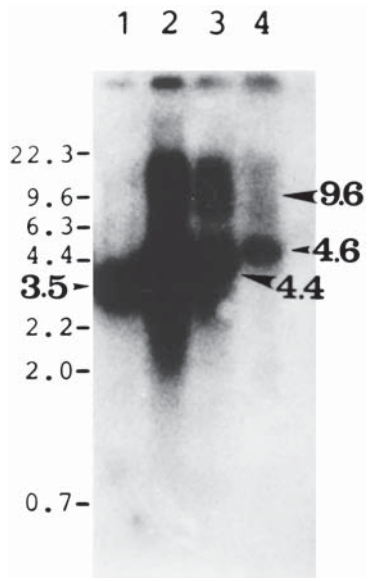

Fig. 4. Southern hybridization analysis using the cloned plasmids as probes against avicelase-digested *B. cellulyticus* K-12 genomic DNA. Lane 1, *Hind*III-digested DNA of *B. cellulyticus* K-12 (5 µg); lane 2, *Eco*RI-digested DNA of *B. cellulyticus* K-12 (10 µg); lane 3, *Eco*RI-digested DNA of *B. cellulyticus* K-12 (5 µg); lane 4, *Pst*I-digested DNA of *B. cellulyticus* K-12 (5 µg). The numbers on the left indicate kilobase scale; the numbers on the right indicate the hybridized bands.

extract. It is well known that multiple cellulases are produced by some microorganisms *(32–34)*. These are mainly owing to either posttranslational modification such as proteolysis/glycosylation or multiple genes for these enzymes *(29,35)*. In the case of *B. cellulyticus* K-12, in the present study, even though multiple activity bands corresponding to slow- and fast-migrating proteins were observed on zymogram staining after PAGE, the pAV-3-coded avicelase was a fast-migrating protein (Fig. 3). The size of the insert also indicated the presence of only one gene in the clone.

Southern Hybridization

The cloned avicelase gene was used as a probe to carry out Southern hybridization analysis of genomic DNA from *B. cellulyticus* K-12. The genomic DNA was digested with *Hind*III, *Eco*RI, or *Pst*I and hybridized with a ^{32}P-labeled 3.5-kb insert fragment of pAV-3. The pAV-3 insert probe was found to hybridize with a single 3.5-kb *Hind*III fragment, with two *Bam*HI fragments of 4.4 and 9.6 kb, and a single 4.6-kb *Pst*I fragment (Fig. 4). DNA sequencing of the 3.5-kb fragment is now under way in our laboratory to compare amino acid sequences among endoglucanase and avicelase genes in *B. cellulyticus* K-12 as well as to determine the precise homologous region to the gene to other *Hind*III fragments.

Crossreactivity of the Cloned Avicelase with Antiavicelase Produced by B. cellulyticus *K-12*

To determine the chemical relation between the cloned avicelase component and the avicelase produced by *B. cellulyticus* K-12, the cell extracts of *E. coli* were subjected to immunodiffusion. *E. coli* JM109 with and without pAV-3 was cultured on LB medium. Each cell extract of *E. coli* transformed was tested for reactivity against antiavicelase antiserum. Avicelases of the cell extracts of all the strains except *E. coli* JM109 without plasmids crossreacted with the antiserum, and the precipitin lines fused completely with each other (data not shown). Additionally, cell extracts produced by *E. coli* JM109 (pAV-3) or without plasmid were subjected to SDS-PAGE and blotted to nitrocellulose. The crossreactivity toward antiserum was examined by Western methods (Fig. 3). Results shown in Fig. 3 indicate that *E. coli* producing avicelase has a common antigenity to *B. cellulyticus* K-12 avicelase. Furthermore, from the result of SDS-PAGE, the molecular weight of the cloned avicelase was calculated to be 64,000. This value is similar to that of the avicelase (M_r approx 64,000) of *B. cellulyticus* K-12.

Purification and Biochemical Characteristics of the Cloned Enzyme

Cell extracts of recombinant *E. coli* were concentrated by ammonium sulfate fractionation, and EGI was purified by DEAE ion-exchange chromatography on a DEAE Toyopearl M650 column, HIC on a TSK Gel Phenyl 5PW column, and gel filtration chromatography on a TSK-HW 50 S column, as summarized in Table 2. It can be seen that the ratio of CMCase:avicelase activity remained roughly constant in the course of the purification procedure. Avicelase and CMCase activity eluted as a single peak with an apparent molecular mass of 64 kDa. Analysis by SDS-PAGE, which was used as a criterion for purity, revealed a single protein band with a molecular mass of about 64 kDa (Fig. 5). The identity of this protein band with the purified endoglucanase was confirmed by *in situ* activity staining (Fig. 5).

Kinetics for Cellulose Hydrolysis and Properties of Recombinant Enzyme

Maximum enzyme activity toward Avicel and CMC was observed between pH 4.5 and 6.0. The optimum pH for activity was pH 4.5, with decreasing activity at pH values higher than 4.7 (data not shown). Stability of the enzyme at various pHs was examined for 24 h at 30°C. The purified enzyme activity was found to be stable at pHs between 4.0 and 10.5 (data not shown). The optimum temperature of the enzyme based on the initial velocity of Avicel and CMC-hydrolysis was 50°C. The thermal stability of the enzyme was also measured; enzyme activity was stable up to 55°C, with 78% of its activity remaining after 72 h, and the enzyme was inactive at 70°C. No differences in thermal inactivation were observed between avicelase and CMCase activities of the enzyme (data not shown).

Table 2
Purification Procedure of the Cloned Enzyme

Purification step	Volume (mL)	Protein (mg)	Total activity (U)		Specific activity (U/mg)		Yield (%)		Avicelase/CMCase (ratio)
			CMCase	Avicelase ($\times 10^{-2}$)	CMCase	Avicelase ($\times 10^{-2}$)	CMCase	Avicelase	
Culture broth	4000	1320	1440	158.4	1.1	0.1	100.0	100.0	0.0011
$(NH_4)_2SO_4$ (30–60%)	350	930	1280	130.2	1.4	0.1	88.9	82.2	0.0010
DEAE-Toyopearl M650	54	670	1120	126.4	1.7	0.2	77.8	79.8	0.0011
TSK Phenyl M650	16	8.4	424	42.8	50.1	5.1	29.4	27.0	0.0010
TSK HW 50S	8	5.8	411	38.2	70.9	6.9	28.5	24.1	0.0009

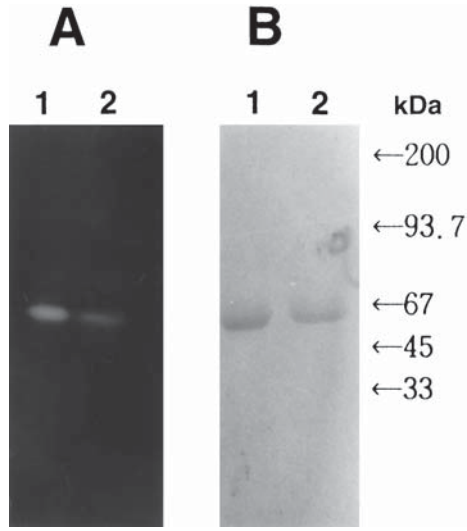

Fig. 5. **(A)** SDS-PAGE and **(B)** activity staining of the purified enzyme. The proteins were separated by 10% SDS-PAGE and CMCase activity was stained. Molecular markers are indicated on the right. Lane 1, purified enzyme (7 μg); lane 2, purified enzyme (4 μg).

K_m values for the enzyme action on CMC and Avicel were 7.6 and 85.2 mg/mL, respectively. V_{max} values for the action on CMC and Avicel were 201 and 9.2 μmol · min^{-1} · mg^{-1}, respectively. This result shows that endoglucanase I was considerably more active toward CMC than Avicel. The pI of the pure enzyme, determined by electrofocusing, was estimated to be pH 5.3 (data not shown). When the effects of metal ions and chemical reagents in enzyme activity were determined, both Mg^{++} and Ca^{++} stimulated activity (Table 3). $MgCl_2$ slightly enhanced the stability of the enzyme, and at 2 m*M* $MgCl_2$ there was a 100% increase in the rate of the hydrolysis of CMC. $CaCl_2$ stimulated the CMCase activity by 40%. Enzyme activity was totally inhibited by 1 m*M* $HgCl_2$, $CuCl_2$, and 10 m*M* SDS (Table 3). Inhibition by thio-specific inhibitor *p*-chloromercuribenzoic acid (PMCB) was strong (60% at 0.5 *M*), and even without preincubation of the enzyme and inhibitor, the activity decreased rapidly, suggesting the presence of essential thiol groups in the active site of the enzyme (data not shown).

Action Mode of Recombinant Enzyme on Cellodextrins

A series of cellooligosaccharides was incubated with purified enzyme, and hydrolysis products were analyzed by TLC (Fig. 6). EGI acted very rapidly on cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6), but not on G2 and cellotriose (G3). The hydrolyzate of G4 was only G2; however, G6 was mainly hydrolyzed to G2 and a trace of G3. This may indicate that the second glycosidic bond (counting from the nonreducing end) can be cleaved by EGI. Likewise, G5 yielded only G2 and G3, indicating that the second and/or third bonds are preferentially cleaved over the

Table 3
Comparison of Enzyme Effectors on Purified Enzyme Activity

Reagent	Concentration (m*M*)	Relative activity (%)
None		100
$MgCl_2$	1	146
	2	201
$CaCl_2$	1	140
	2	154
KCl	1	102
NaCl	1	107
$CoCl_2$	1	82
$NiCl_2$	1	97
$NiSO_4$	1	86
$ZnCl_2$	1	118
$CuCl_2$	1	6
$CuSO_4$	1	2
$FeSO_4$	1	64
$FeCl_2$	1	76
$MnCl_2$	1	118
$HgCl_2$	1	1
EDTA	1	61
SDS	10	2

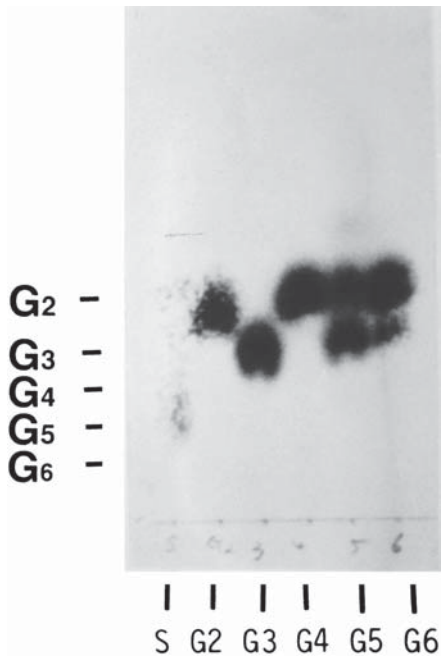

Fig. 6. Digestion of cellooligosaccharides by the purified enzyme. The reaction mixture was incubated for 30 min at 30°C and was analyzed on a thin-layer silica gel (Merck) as described in Materials and Methods. S, standard markers.

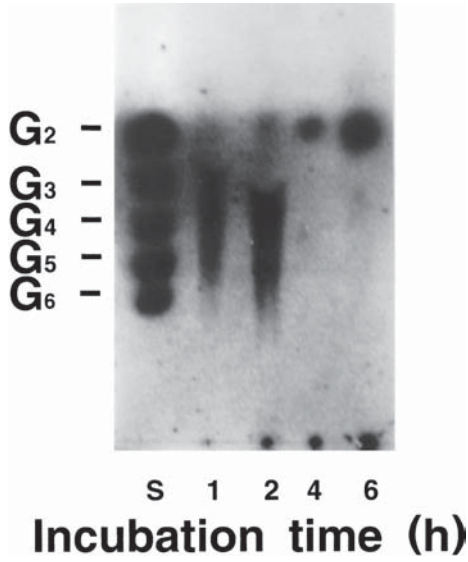

Fig. 7. Hydrolysis of Avicel SF by the purified enzyme. The reaction mixture was incubated as described in Materials and Methods. At various time intervals, the reaction mixture was sampled and analyzed by TLC. S, standard markers.

first and fourth. Furthermore, glucose was not detected in the hydrolysis of G6 to G2, of G4 to G2, and of G5 to G3 and G2. With pNPC, glycosidic bond cleavage was detected at a low rate. These results suggest that the purified endoglucanase I seems to be a unique cellulolytic enzyme. It can be seen that G4 was rapidly cleaved into G2 + G2, and that G5 was cleaved into G2 + G3. On the other hand, G6 was cleaved into G2, and, to a lesser extent, into G3 + G3 (Fig. 6).

Enzymatic Digestion and Action Mode of Recombinant Enzyme on Various Cellulosic Substrates

To elucidate the substrate specificity of the enzyme, various cellulosic substrates such as CMC, Avicel, and some oligosaccharides were separately incubated with the purified enzyme containing 0.05 *M* sodium acetate buffer (pH 4.5). Avicel was incubated with EGI to investigate the hydrolysis of highly ordered substrate. Figure 7 shows the time course of product formation from Avicel. Qualitative analysis of the products of the reactions by TLC showed that G2 was the principal product, and this was confirmed by quantitative analysis by using the phenol/H_2SO_4 method. This result is similar to the results obtained with the CBHs of both *Aspergillus aculeatus (36)* and *Trichoderma koningii (37)*.

Table 4 lists the activities of purified enzyme on several model substrates and cellooligosaccharides. EGI was active on CMC, filter paper, Avicel, MN300, xylan, and insoluble cellooligosaccharides. A remarkable

Table 4
Enzymatic Activity of the Cloned Enzyme
on Various Cellulosic Substrates[a]

Substrates	Enzyme activity (U/mg protein)[b]
Hydroxyethylcellulose	65.0
CMC (DS = 0.65, DP[c] = 500)	77.2
Filter paper (Whatman no. 1)	2.7
Avicel SF	7.6
MN300	7.8
Xylan	1.2
Insoluble cellooligosaccharide (DP = 20)	10.6
Cellohexaose (DP = 6)	16.2
Cellopentaose (DP = 5)	41.7
Cellotetraose (DP = 4)	52.4
Cellotriose (DP = 3)	<0.001 (ND)
Cellobiose (DP = 2)	<0.001 (ND)
Salicin	<0.001 (ND)
pNPC	0.16
$MeUmb(Glc)_2$	0.12
pNPG	ND
pNPX	ND

[a]Activity on polymeric substrates is expressed on the basis of the amount of G2 (or xylobiose for xylan) produced. For cellotetraose, cellopentaose, and cellohexaose, equimolar amounts of the cellooligosaccharides were incubated at 40°C with 0.5 mL of 0.1 *M* sodium acetate buffer, pH 4.5, enzyme solution (40 µg of protein) and water to give a total volume of 1 mL. After 30 min a sample was assayed for reducing sugar by the same method described *(8)*. Using cellobiose and cellotriose as substrates, the amount of glucose formed was determined by the glucose oxidase method *(42)*. The degree of substitution is expressed as DS as described in ref. *45*.

[b]ND, not detected.

[c]DP, degree of polymerization.

property of the enzyme was the prominently high activity toward G5 and G4; those activities were more than 2.1 and 2.4 times those of CMCase, respectively. The most preferred substrates for EGI were therefore G5 and G4. In all of the reaction mixtures with G2, glucose was never detected even after 24 h (Table 4, Fig. 5). On the other hand, the random hydrolysis of substrates by this cellulase was also shown by the relationship between the degree in viscosity and the increase in the reducing sugars from CMC. The cellulase has a straight-line slope (specific fluidity, $1/\eta_{sp}$/reducing sugar) with a roughly 45° angle (data not shown). This suggests that the enzyme should be an endo β-1,4-glucanase although it appeared to be a type of CBH in view of its substrate specificity. If CMC is hydrolyzed by an enzyme in an exo-fashion, the slope against abscissa would be a smaller angle, since the drop in viscosity of CMC would be small compared with the simultaneous production of reducing sugar.

Conclusion

Recombinant EGI of *B. cellulyticus* was purified to homogeneity as judged by SDS-PAGE and gel filtration of HPLC. Extensive purification of EGI confirmed that both avicelase and CMCase activity reside in the same monomeric protein of about 64 kDa (Fig. 3). EGI had molecular mass of 65 kDa when determined by gel filtration of TSK Gel G3000 xL.

Apparently, the chemical modification of sugar hydroxyl groups severely interfered with enzyme action. The enzyme had a strong capacity for solubilizing CMC and a relatively strong activity for degrading microcrystalline cellulose such as Avicel and MN300; in each case, the principal product of the hydrolysis was G2. These results suggest that the enzyme is similar to that of CBH (Fig. 6). On the other hand, the properties of the purified enzyme were tested by analyzing the hydrolyzates of various cellulosic components. The dominant product from G4, G5, or G6 and insoluble celluloses such as Avicel and MN300 was G2 (Fig. 5). These results appear to be the same as those obtained for the CBHs of *T. viride (38)* and *Chaetomium thermophile (39)*. Possibly, this suggests that enzyme splits off G2 units from the nonreducing end of cellooligomers. However, in view of the present study, it was concluded that the CBH from the fungus *Trichoderma* reported by Wood and McCrae *(37)*, Berghem and Pettersson *(38)*, and Ogawa et al. *(40)* may be similar to the cellulase obtained in the present work. Recently, it has been suggested that a cellulase derived from a mesophilic fungus, strain Y-94, and readily hydrolyzed Avicel, should be classified as a specific endo-cellulase rather than an exo-cellulase *(41)*. The results suggest that a cellulase cloned here from *B. cellulyticus*, which appears so far to be a CBH according to the nomenclature used, should belong to both endo β-1,4-glucanase and avicelase. According to the nomenclatural definition, CBH must produce only G2 in the hydrolyzate of cellulose and G4.

The ability of *B. cellulyticus* EGI to degrade microcrystalline cellulose is interesting. Recently, several endoglucanases as single enzyme forms able to act efficiently on crystalline cellulose have been reported from *Clostridium cellulolyticum (42)*, *Thermomonospora fusca* E4 *(43,44)*, *Cellulomonas fimi* CenC *(45)*, and *C. stercoraium (4)*. By contrast, activity toward Avicel has previously been reported for the endoglucanase I from *T. reesei (46)*, endoglucanase from *C. stercorarium (47)*, and endoglucanase from *Clostridium thermocellum (48)*. However, these findings have been questioned and attributed to the impurity of the enzyme. Similar objections can be raised against the avicelase associated with the *Bacillus circulans* endoglucanase preparations utilized in a previous study *(30)*.

In the fungus *T. reesei*, two types of endoglucanases were produced: the specific endoglucanases that act on the terminal glucosidic bonds of cellulase chains only, and the nonspecific endoglucanases that are also capable of hydrolyzing xylans and the heterosidic bonds of $MeUmb(Glc)_2$ and pNPC. It has also been shown that high specific activities on CMC are

not characteristic of all endoglucanases and that the enzyme referred to in the literature as CBH in fact behaves like a specific endoglucanase *(31)*.

Analysis of the reaction products of the cloned enzyme revealed the formation of G2 as a primary and final product. The absence of cellodextrins larger than G3 is surprising. It is possible that such intermediates could not be detected because of their rapid conversion to G2. Alternatively, the action of EGI on crystalline cellulose may occur in a nonrandom fashion, resulting in the progressive release of G2.

Acknowledgment

This work was supported in part by research grants from the Dongguk University Research Foundation.

References

1. Schlochtermeier, A., Walter, S., Schroder, J., Moorman, M., and Schrempf, H. (1992), *Mol. Microbiol.* **6,** 3611–3621.
2. Montenecourt, B. S. (1983), *Trends Biotechnol.* **1,** 156–161.
3. Tomme, P., Van Tilbeurgh, H., Perterson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. (1988), *Eur. J. Biochem.* **170,** 575–581.
4. Bronnenneier, K. and Staudenbauer, W. L. (1990), *Enzyme Microb. Technol.* **12,** 431–436.
5. Bronnenmeier, K., Rucknagel, K. P., and Staudenbauer, W. L. (1991), *Eur. J. Biochem.* **200,** 379–385.
6. Kim, C. H. and Kim, D. S. (1995), *Enzyme Microb. Technol.* **17,** 248–254.
7. Kim, C. H. (1995), *Appl. Environ. Microbiol.* **61,** 959–965.
8. Wood, T. M. and Bhat, K. M. (1988), *Methods Enzymol.* **160,** 87–112.
9. Enari, T. M. and Niku-Paavula. M. L. (1987), *CRC Crit. Rev. Biotechnol.* **5,** 67–87.
10. Fagerstam, L. G. and Pettersson, L. G. (1980), *FEBS Lett.* **119,** 97–100.
11. Shoemaker, S. P., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., and Annis, M. (1983), *Bio/Technology* **1,** 691–696.
12. Gilbert, H. J. and Hazlewood, G. P. (1993), *J. Gen. Microbiol.* **139,** 187–194.
13. Kim, D. S. and Kim, C. H. (1992), *J. Microbiol. Biotechnol. (Seoul)* **2,** 7–13.
14. Saito, H. and Miura, K. (1963), *Biochim. Biophys. Acta* **72,** 619–629.
15. Birnboim, H. C. and Doly, J. (1979), *Nucleic Acids Res.* **7,** 1513–1523.
16. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982), *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring, NY, pp. 68, 150–161.
17. Sugino, A., Goodman, H. M., Heynekar, H. L., Shine, J., Boyer, H. W., and Cozzarelli, N. R. (1982), *J. Biol. Chem.* **252,** 3987–3994.
18. Mandel, M. and Higa, A. (1970), *J. Mol. Biol.* **53,** 159–162.
19. Yoshigi, N., Taniguchi, H., and Sasaki, T. (1990), *J. Biochem.* **108,** 388–392.
20. Bucht, B. and Eriksson, K. E. (1968), *Arch. Biochem. Biophys.* **124,** 135–141.
21. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
22. Kyriacou, A., Mackenzie, C. R., and Neufeld, R. J. (1987), *Enzyme Microb. Technol.* **9,** 3322–3325.
23. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
24. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
25. Lineweaver, H. and Burk, D. (1934), *J. Am. Chem. Soc.* **56,** 658–666.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
27. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Analytical Chem.* **28,** 350–356.
28. Heukeshoven, J. and Dernick, R. (1985), *Electrophoresis* **6,** 103–112.

29. Kim, C. H., Kwon, S. T., Taniguchi, H., and Lee, D. S. (1992), *Biochim. Biophys. Acta* **1122,** 243–250.
30. Kim, C. H. and Kim, D. S. (1993), *Appl. Biochem. Biotechnol.* **42,** 83–94.
31. Kim, C. H. and Ko, Y. H. (1993), *Biochim. Biophys. Acta* **1202,** 200–206.
32. Estaban, R., Villaneuva, J. R., and Villa, T. G. (1982), *Can. J. Microbiol.* **28,** 733–739.
33. Marui, M., Nakanishi, K., and Yasui, T. (1985), *Agric. Biol. Chem.* **49,** 3399–3407.
34. Wong, K. K. Y., Tan, L. U. L., Saddler, J. N., and Yagushi, M. (1988), *Microbiol. Rev.* **52,** 305–317.
35. Gilbert, H. J., Sullivan, D. A., Jenkis, G., Kellet, L. E., Minton, N. P., and Hall, J. (1988), *J. Gen. Microbiol.* **134,** 3239–3247.
36. Murao, S., Sakamoto, R., and Arai, M. (1985), *Agric. Biol. Chem.* **49,** 3511–3517.
37. Wood, T. M. and McCrae, S. I. (1972), *Biochem. J.* **128,** 1183–1192.
38. Berghem, L. E. and Pettersson, L. G. (1973), *Eur. J. Biochem.* **37,** 21–30.
39. Ganju, R. K., Murthy, S. K., and Vithayathil, P. J. (1989), *Biochim. Biophys. Acta* **993,** 266–274.
40. Ogawa, K., Toyama, D., and Fujii, N. (1991), *J. Gen. Appl. Microbiol.* **37,** 249–259.
41. Yamanobe, T., Mitsuishi, Y., and Yagisawa, M. (1988), *Agric. Biol. Chem.* **52,** 2493–2524.
42. Gal, L., Gaudin, C., Belaich, A., Pages, S. C., Tardif, C., and Belaich, J. P. (1997), *J. Bacteriol.* **179,** 6595–6601
43. Sakon, J., Irwin, D., Wilson, D. B., and Karplus, P. A. (1997), *Nat. Struct. Biol.* **4,** 810–817.
44. Irwin, D., Shin, D. H., Zhang, S., Barr, B. K., Sakon, J., Karplus, P. A., and Wison, D. B. (1998), *J. Bacteriol.* **180,** 1709–1714.
45. Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y., and Steitz, T. A. (1996), *EMBO J.* **15,** 5739–5751.
46. Kubata, B. K., Suzuki, T., Horitsu, H., Kawai, K., and Takamizawa, K. (1994), *Appl. Environ. Microbiol.* **60,** 531–535.
47. Bronnenmeier, K. and Staudenbauer, W. L. (1988), *Appl. Microbiol. Biotechnol.* **27,** 432–436.
48. Wu, J. H. D., Orme-Johnson, W. H., and Demain, A. L. (1988), *Biochemistry* **27,** 1703–1709.