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Expression and Characterization of CMCax Having β-1,4-Endoglucanase Activity from *Acetobacter xylinum*

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The CMCax gene from *Acetobacter xylinum* ATCC 23769 was cloned and expressed in *E. coli*. With this gene, three gene products — mature CMCax, CMCax containing signal peptide(pre-CMCax), and a glutathione-S-transferase(GST)-CMCax fusion enzyme — were expressed. CMCax and pre-CMCax are aggregated to multimeric forms which showed high CMC hydrolysis activity, whereas GST-CMCax was less aggregated and showed lower activity, indicating that oligomerization of CMCax contributes to the cellulose hydrolysis activity to achieve greater efficiency. The enzyme was identified to be an β-1,4-endoglucanase, which catalyzes the cleavage of internal β-1,4-glycosidic bonds of cellulose. The reaction products, cellobiose and cellotriose, from cellopentaose as a substrate, were identified by HPLC. Substrate specificity of cellotetraose by this enzyme was poor, and the reaction products consisted of glucose, cellobiose, and cellotriose in a very low yield. These results suggested that cellopentaose might be the oligosaccharide substrate consisting of the lowest number of glucose. The optimum pH of CMCax and pre-CMCax was about 4.5, whereas that of GST-CMCax was rather broad at pH 4.5–8. The physiological significance of cellulose-hydrolyzing enzyme, CMCax, having such low β-1,4-endoglucanase activity and low optimum pH in cellulose-producing *A. xylinum* is not clearly known yet, but it seems to be closely related to the production of cellulose.

Keywords: *Acetobacter xylinum*, CMCax, β-1,4-endoglucanase.

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Introduction

Acetobacter xylinum has been known as a bacterium producing cellulose (Ross *et al.*, 1991). The biosynthesis of cellulose in the bacteria was considered a two-step process involving first polymerization and then crystallization of the individual glucan chains into native cellulose I. But cellulose produced by the crude extracts from the bacteria results in crystalline cellulose II, suggesting the requirement of a certain topological transformation (Bureau and Brown Jr., 1987). Recently, there was a report of an interesting result that *A. xylinum* has at least one gene encoding carboxymethylcellulose (CMC) hydrolyzing enzyme (CMCax) and this gene is localized to the upstream region of *bcs* operon (Standal *et al.*, 1994). *Bcs* operon encodes genes for cellulose synthase (Wong *et al.*, 1990). These results indicate that the expression of the CMC hydrolyzing enzyme may be essential for the biosynthesis of bacterial cellulose. However, little is known about the properties of the enzyme. In this paper we present the production of the enzyme by recombinant technology and its characterization.

Materials and Methods

Materials Restriction endonucleases were obtained from Boehringer Mannheim. These enzymes were used under the conditions recommended by the manufacturers. Pwo DNA polymerase was from Boehringer Mannheim, T4 DNA ligase from New England BioLabs Inc., Genomic tip-100 and Gel extraction kit from Qiagen Corp., Sequenase^{TM} kit from U.S. Biochemical Corp., pGEX 2T expression vector from Pharmacia Biotechnology Inc., and [γ-^{32}P] ATP from Amersham. Cellopentaose and CM-cellulose C-4888 were purchased from Sigma Chem. Co. Glutathione Sepharose 4B was from Pharmacia Biotechnology Inc. The μ-Bondapak^{TM} NH_2 column was from Waters.

Bacterial strains and growth conditions *A. xylinum* ATCC 23769 were grown aerobically in the medium at 30°C as

described by Hestrin and Schramm (1954). *E. coli* XL-1 blue MRF' {Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}*Z M15* Tn*10* (Tet^{r})]} was used as host strain for maintenance of the plasmid and for production of recombinant protein. The culture was grown aerobically in the Luria-Bertani medium (LB) (Sambrook *et al.*, 1989) at 37°C or 20°C.

Cloning of CMCax gene Purification of total DNA from *A. xylinum* was performed by using Genomic tip-100. Oligonucleotide was synthesized and used for screening chromosomal DNA fragments of *A. xylinum*. Oligonucleotide was labeled with [γ-^{32}P] ATP by polynucleotide kinase catalysis. *A. xylinum* total DNA was digested by *Hin*dIII, and 9.9 kb fragments were eluted from 0.7% agarose gel by using a Qiagen gel extraction kit. Fragments were ligated with the dephosphorylated *Hin*dIII site of pBluescript II KS and then transformed into an *E. coli* XL-1 blue MRF' strain. Transformants were screened by the colony blot method using a ^{32}P-labeled oligonucleotide probe. The CMCax gene was confirmed by partial sequence with the dideoxynucleotide chain termination method using a Sequenase^{TM} kit.

CMCax enzyme assay CMC hydrolyzing activity of the enzyme was determined by measuring the generation of reducing sugar ends. Enzyme preparations were incubated at 37°C in 25 mM sodium phosphate (pH 5.5) containing 1% CMC. Aliquots of 0.25 ml were mixed with 0.75 ml of the dinitrosalicylic acid reagent described by Sumner (1924). After being heated at 100°C for 5 min, the tubes were cooled on ice, and the optical density at 550 nm of the solutions was finally determined. Glucose was used as a standard. CMC hydrolyzing activity was also determined on 1% agar plate containing 1% CMC. Among the enzyme solution, 10 μl was placed on the plate, incubated for 12 h at 37°C, stained with 1% Congo red solution, and destained with 1 M NaCl solution.

Activity staining of CMCax The enzyme was run on a 4–20% Tris-glycine gel (Novex, San Diego, CA, USA). The acrylamide gel was overlaid on agar plate containing 1% CMC and incubated for 1 h at 37°C. Then the agar plate was treated with 1% Congo red solution for 5 min and destained with 1 M NaCl solution (Morag *et al.*, 1990).

Polymerase chain reaction (PCR) amplification In order to prepare two forms of enzymes, mature CMCax and pre-CMCax (CMCax containing signal peptide), two forward primers, 5'-TCCACGGATCCGACCCCGCCC-3' (GAC is a start codon of mature CMCax gene), 5'-ACGGATCCATGTCGGTCAT-GGCGGC-3' (ATG is a start codon of pre-CMCax gene) and a reverse primer, 5'-AAGAATTCACTTTATAGTCTCCT-3' (TTA is a complementary sequence to a stop codon of the gene), were synthesized and used for PCR using pHB2 (a subclone containing 1.8 kb DNA segment encoding CMCax gene) as a template. Amplification was done by 25 cycles of PCR at standard reaction conditions: reaction volume, 50 μl; reaction composition, 50 ng of template, 10 pmol primer, and 0.25 units of DNA polymerase; cycle profile, 1 min at 95°C, 1 min at 45°C, 1 min at 72°C.

Construction of pGCX-1 and pGCX-2 The PCR products were purified and digested with *Bam*HI and *Eco*RI. These fragments were ligated with the over-expression vector pGEX-2T that had been digested with *Bam*HI and *Eco*RI. These recombinant DNA were named pGCX-1 for the production of mature CMCax and pGCX-2 for pre-CMCax, respectively (Fig. 1).

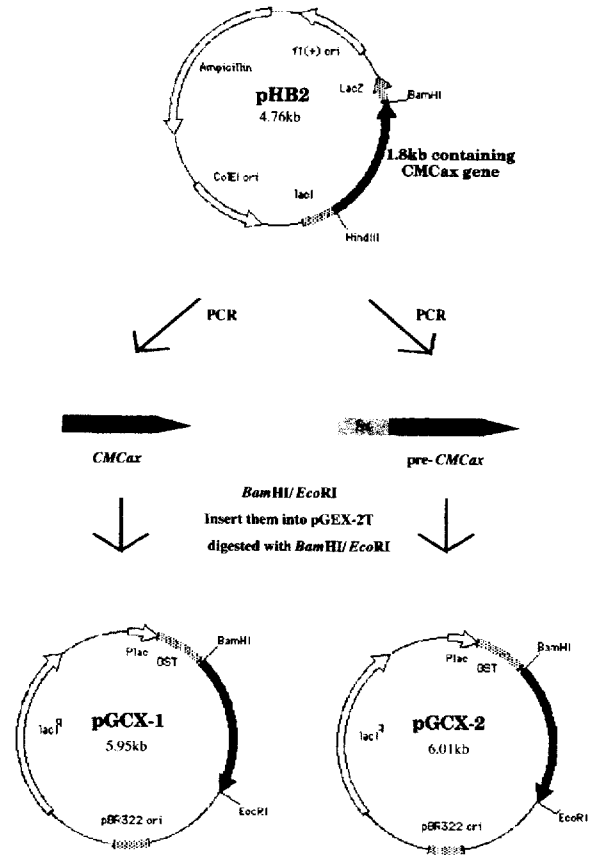

Fig. 1. Construction of pGCX-1 and pGCX-2. Two different PCR products, *CMCax* and pre-*CMCax*, were prepared with pHB2 as a template. *CMCax* is the coding region for the mature CMCax, whereas pre-*CMCax* is the region for the proform of CMCax including signal sequence (Sx). The PCR fragments were cut with *Bam*HI and *Eco*RI, and then inserted them into pGEX-2T vector digested with *Bam*HI and *Eco*RI to yield pGCX-1 and pGCX-2

Over-expression of mature CMCax-gene and pre-CMCax gene The transformed *E. coli* XL-1 blue MRF' with pGCX-1 or pGCX-2 were cultured in a 3 ml LB medium containing ampicillin (50 μg/ml) for 12 h, respectively and then transferred into 200 ml of the same medium. The culture was incubated at 37°C for 2.5 h and isopropylthiogalactoside (IPTG) (1 mM final conc.) was added, followed by incubating the culture at 20°C for 24 h.

Purification of overexpressed recombinant proteins Cells were harvested at 4,000 × *g*, resuspended in phosphate-buffered saline (PBS), and disrupted by sonication. Cellular debris was removed by centrifugation (20,000 × *g*, at 4°C) and the supernatant (30 mg protein) was used for purification of the protein. Glutathione-Sepharose 4B beads (3 ml) were added to

the supernatant, and after an incubation for 12 h at 4°C with gentle shaking, the beads were washed with 10 volumes of PBS. Then, five units of thrombin (units/ml) were added to this slurry to separate the GST (glutathione-S-transferase) part of the fusion protein. After the incubation for 12 h at 25°C with gentle shaking, this slurry was packed, and recombinant protein was washed out with five bed-volumes of PBS.

Product analysis by HPLC Synthetic substrates, cellotetraose and cellopentaose, were used to determined substrate specificity (Anderson, 1986). The reaction mixture, 20 μl, containing 80 μg of the substrates, 8.4×10^{-4} U CMCax in 0.1 M sodium phosphate buffer, pH 5.5, was incubated for 1 h at 37°C. The reaction mixture was mixed with 5 μl of 10% trichloroacetic acid and 25 μl of 1% (w/v) dansyl hydrazine in 78% acetonitrile solution, and was heated at 80°C for 10 min (Takemoto and Ikenaka, 1985). Samples were analyzed with a μ-Bondapak^{TM} NH_2 column (3.9 × 30 cm) (Verhaar and Kuster, 1981). The Dansyl-hydrazones of oligosaccharides were eluted by 78% acetonitrile in ddH_2O at the flow rate (1.5 ml/min) and monitored by a fluorescence detector.

Results and Discussion

Construction of plasmid pHB2 According to the nucleotide sequence of the region upstream of *bcs* operon from *A. xylinum* reported previously (Standal *et al.*, 1994), oligonucleotide, 5′-ATGTGACCGCAGATGGTCG-3′ was synthesized and used for screening chromosomal DNA fragments of *A. xylinum* ATCC 23769. A 9.9 kb DNA fragment was successfully isolated from *Hin*dIII-digested chromosomal DNA fragments. This fragment containing the region upstream and segment of *bcs* genes in *A. xylinum* was inserted into pBluescript II KS (pKSC2). Again the insert DNA was digested by *Hin*dIII/*Bam*HI and subcloned into pBluescript II KS. Among them, a subclone, pHB2, containing 1.8 kb nucleotide insert was found to have the gene encoding CMCax. The CMCax gene in the subclone was confirmed by DNA sequencing. CMC-hydrolyzing activity was detected from the extracts of *E. coli* XL-1 blue MRF′ containing pHB2 without any induction system. This result indicates that the self-promoter of CMCax on *Acetobacter* DNA is properly operated by an *E. coli* transcription system.

Preparation of GST-CMCax pGCX-1 was introduced into *E. coli* XL-1 blue MRF′. GST-CMCax was translated well by IPTG as an inducer in the transformed *E. coli*. GST-CMCax (specific activity 2.6×10^{-2} U/mg) has CMC hydrolyzing activity even though it is low. This result indicates that the modification of N-terminal amino acid deeply influences on its activity.

Preparation of CMCax and pre-CMCax Intact CMCax and pre-CMCax containing signal peptide were prepared by thrombin cleavage of GST-CMCax and GST-pre-CMCax fusion protein. Pre-CMCax (37,800 Da), CMCax (35,600 Da) and GST-CMCax (61,600 Da) were run on a native PAGE. This PAGE gel was used for zymogram to visualize CMC hydrolyzing activity. As shown in Fig. 2, pre-CMCax and CMCax were aggregated more than GST-CMCax. Furthermore, CMC hydrolyzing activities of aggregated enzymes were higher than that of less aggregated GST-CMCax. This result indicates that aggregation of CMCax contributes to the CMC-hydrolyzing activity to achieve greater efficiency, and masking of N-terminal such as GST-CMCax hinders the oligomerization and thus manifest lower enzyme activity. Pre-CMCax, which had signal peptide, was aggregated like CMCax, indicating that the signal peptide consisted of 20 amino acids could not hinder oligomerization. Specific CMC hydrolyzing activities of CMCax and pre-CMCax were 8.8×10^{-2} U/mg and 9.1×10^{-2} U/mg, respectively. They are higher than those of GST-CMCax but it is much lower than 6.9×10^{-1} U/mg and 1.3 U/mg of EG II and EG III from *P. verruculosum*, respectively (Kim *et al.*, 1992).

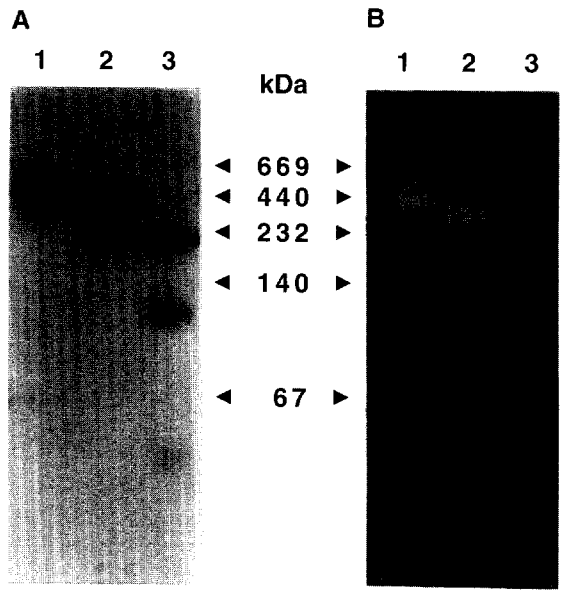

Fig. 2. PAGE for pre-CMCax, CMCax, and GST-CMCax and activity staining. Pre-CMCax (lane 1), mature CMCax (lane 2), and GST-CMCax (lane 3) were run on 4–20% acrylamide-Tris-glycine gradient gel (panel A) and the gel was overlaid on 1% agarose gel (panel B) containing 1% CMC for activity staining.

Products analysis In order to find the mode of substrate binding, cellotetraose and cellopentaose were used as a substrate separately. The reaction products were properly measured by the detection of fluorescence of sugar dansyl hydrazone. As shown in Fig. 3A, cellotetraose was hydrolyzed into glucose, cellobiose, and cellotriose in a very low yield by CMCax and GST-CMCax respectively. However, cellopetaose was hydrolyzed completely into cellobiose and cellotriose by the enzymes (Fig. 3B). This

result indicated clearly that cellopentaose is the minimum size of oligosaccharide substrate for this enzyme, that there are at least five substrate binding sites for the glycosyl units on the active site, and that this enzyme is a typical β-1,4-endoglucanase. The catalytic domain of cellobiohydrolase CBHII of *T. reesei* was the first cellulase structure to be solved, and consequently most of the structure-function studies so far have been carried out with this protein. For this enzyme, the active site tunnel is about 20 Å long, containing at least four binding sites for the glycosyl units (Rouvinen *et al.*, 1990). However, for cellobiohydrolase CBHI of *T. reesei* seven putative subsites for substrate binding were recently proposed (Divne *et al.*, 1994). In addition to these results, it is of interest to know that GST-CMCax showed almost the same catalytic activity on soluble cellopentaose as a substrate even though it showed much lower hydrolytic activity of CMC.

pH dependence of CMCax, pre-CMCax, and GST-CMCax Native cellulose is most efficiently degraded by filamentous fungi such as *T. reesei* which produce a set of cellulases active in the acidic pH range. Another efficient cellulolytic fungus, *Humicola insolens*, produces a very similar set of enzymes with broad pH profiles from 5 to 10 (Teeri and Koivula, 1995). *Acetobacter* secretes acid to medium naturally, and the pH of the culture medium decreases gradually to 3.3. Therefore it was of interest to determine the optimum pH of the enzymes. As shown in Fig. 4, CMCax and pre-CMCax showed its optimum pH at about 4.5, whereas GST-CMCax showed broad pH profiles from 4.5 to 8.0, respectively. This suggests that CMCax is designed properly for acidic environment which is natural for *Acetobacter*, and that the N-terminal blocking of the enzyme by GST disturb the acidic optimum pH of CMCax.

In conclusion, this paper presents clearly that cellulose-producing *A. xylinum* also produces the cellulose-hydrolyzing enzyme, CMCax. Oligomerization of this enzyme contributes to the cellulose hydrolysis activity to achieve greater efficiency. This enzyme is a typical β-1,4-endoglucanase which has five glycosyl binding sites. Its optimum pH was about 4.5 which is the natural environmental pH for *Acetobacter*.

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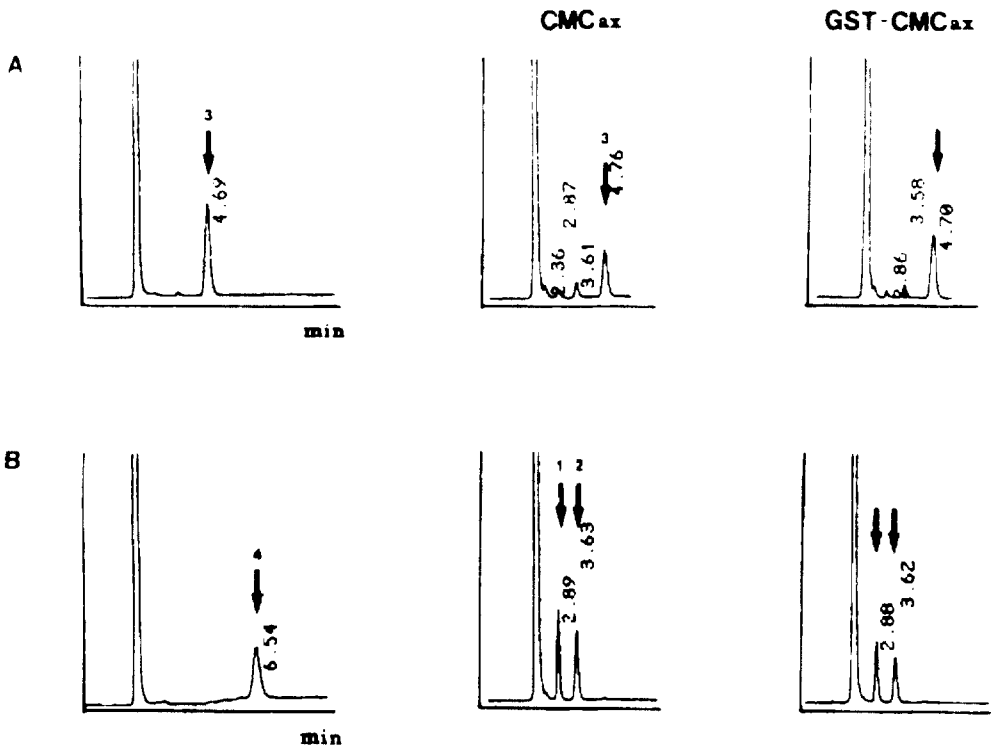

Fig. 3. HPLC chromatogram of degradation products by CMCax and GST-CMCax. HPLC analysis of products digested by CMCax and GST-CMCax from cellotetraose (A) and cellopentaose (B) as enzyme substrates. The numbers 1 to 4 represent the dansyl hydrazones of: 1, cellobiose; 2, cellotriose; 3, cellotetraose; and 4, cellopentaose.

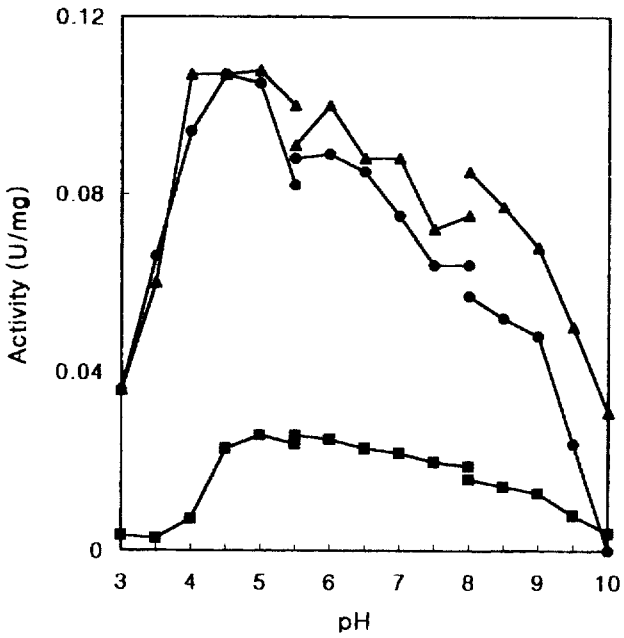

Fig. 4. pH dependence on pre-CMCax, CMCax, and GST-CMCax. The activity of pre-CMCax (–▲–), CMCax (–●–), and GST-CMCax (–■–) was determined at various pH. Citrate-NaOH buffer (from pH 3.0 to 5.5), sodium phosphate buffer (from pH 5.5 to 8.0), and Tris-HCl buffer (from pH 8.0 to 10.0), were used, respectively. The concentration of the buffers was 100 mM.

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