Characterization of the β-Cyclodextrin Glucanotransferase Gene of Bacillus firmus var. alkalophilus and Its Expression in E. coli

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Abstract The β-CGTase gene of alkalophilic *Bacillus firmus* var. *alkalophilus* was cloned into *E. coli* using pZErO™-2 as a vector. The cloned gene encoded a total of 710 amino acid residues consisting of 674 amino acids of the matured protein and 36 amino acids of the signal peptide, including 20 amino acids from the *lacZ* gene in the vector. Although the cloned β-CGTase gene did not contain the promoter and start codons, it was expressed by the *lac* promoter and *lacZ* start codon in the pZErO™-2 vector. A comparison was made with the amino acid sequence and ten other CGTases from *Bacillus* sp. Also, ten highly conserved regions, which are important amino acid residues in catalysis of CGTase, were identified. The *lac* promoter used for expression of the β-CGTase gene was induced constitutively in recombinant *E. coli* even without IPTG possibly because of a lack of the *lacI* gene in both host and vector, repressing the *lacZ* gene in the *lac* operon. Its expression was catabolically repressed by glucose, however, its repression was reduced by soluble starch, mainly because of the extremely high increase of the cAMP level. β-CGTase can be overproduced in the recombinant *E. coli* by maintaining intracellular cAMP levels mostly through the intermittent feeding of glucose during cultivation.

Key words: β-Cyclodextrin glucanotransferase, gene cloning, *Bacillus firmus* var. *alkalophilus*, nucleotide sequences, conserved regions of CGTase gene, expression of CGTase

Cyclodextrin glucanotransferase (CGTase, EC2.4.1.19) is an enzyme catalyzing the formation of cyclodextrins (CDs), that is derived from starch, and consists of α-, β-, and γ-CD having closed ring structures of six to eight glucose units linked with α-1,4-glucosidic bonds. It has unique abilities to solubilize hydrophobic materials and it can entrap the volatile compounds in their central cavity. For this reason, they have attracted a considerable amount

of commercial interest because they can be applied in several areas, such as foods, cosmetics, and pharmaceuticals.

CGTases are produced mainly from *Bacilli*, for example, *Bacillus macerans*, *B. circulans*, *B. stearothermophilus*, *B. ohbensis*, alkalophilic *Bacillus* sp., *Klebsiella pneumoniae* M5al, and *Thermoanaerobacter* sp. [15]. However, the alkalophilic *Bacillus* sp. have been receiving the major attention for industrial application because of their high activity and stability in a wide range of pH and temperature [9, 15]. In particular, alkalophilic *B. ohbensis*, *Bacillus* sp. KC201, and *Bacillus* sp. E1 have been regarded as the most promising strains for producing β-type CGTase, which creates mainly β-CD without accumulation of α-CD, which will facilitate the purification of β-CD from reaction mixture using the solubility difference between β-CD and γ-CD [14, 21, 31].

Fourteen CGTase genes derived from various strains have been cloned and their DNA sequences have been elucidated [1, 8, 9, 10, 13, 14, 28-31]. They consisted of about 700 amino acid residues with molecular weights of about 70-80 kDa. The cloned CGTases exhibit a relatively low overall similarity in their amino acid sequences with α-amylase. CGTases contain three distinct domains in the N-terminal that is similar to α-amylases (A, B, and C domains), however, they have an additional two domains (D and E) in the C-terminal [19]. The N-terminal region in CGTases contributes to the starch hydrolysis and the transglycosylation reactions combining both reducing and non-reducing ends of oligosaccharides [5]. Meanwhile, the C-terminal region of CGTases seems to be involved in cyclization activity and pH stability, but, unfortunately, their detail roles have not been fully defined at this time [9].

To elucidate the molecular structure for CGTase and its functional active sites, the site-specific chemical modification of enzyme, site-directed mutagenesis, and X-ray crystallography of CGTases have been recently attempted [17, 19, 20, 30]. Aspartic/glutamic acid residues, histidine residues, and aromatic amino acid residues such as tyrosine, phenylalanine, and tryptophan, played important

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roles in the catalysis of CGTase which included binding and hydrolysis of starch, and cyclization of CDs [19, 20, 30].

Previously, an alkalophilic Bacillus firmus var. alkalophilus excreting a novel β-CGTase, producing mainly β-CD and a slight amount of γ-CD without accumulation of α-CD, was screened [3] and its enzymatic characteristics were investigated using a purified β-CGTase [26]. Several mutant strains, including catabolite repression resistant mutant and constitutive mutant, were selected both for achieving overproduction of CGTase and for observing the regulation mechanism of CGTase [3, 12]. The key amino acid residues involved in the catalytic activities of β-CGTase were identified after the site-specific chemical modification took place [27].

In this study, a β-CGTase gene from B. firmus var. alkalophilus was cloned into E. coli using pZErO™-2 as a vector to determine its nucleotide sequences. Several highly conserved regions, which are known to have possible significance in catalysis, have been identified through comparison of the homology with other cloned CGTases. The extracellular, periplasmic, and intracelluar fractions were analyzed to trace down the excretion processing of CGTase in E. coli. The expression characteristics of the β-CGTase gene in E. coli were studied from the point of view of the induction and catabolite repression by cAMP and glucose, mostly for getting a total understanding for the reaction mechanism and active site of β-CGTase, but also for overproduction of β-CGTase of B. firmus var. alkalophilus.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Culture Media

Alkalophilic B. firmus var. alkalophilus was cultured at 37°C for 2 days in a Horikoshi's alkaline medium II [3, 9]. E. coli TOP10 [F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ 80lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL, endA1, nupG] was used as a cloning host, and plasmid pZErO™-2 (Invitrogen Co. CA, U.S.A.) was used as a cloning vector. Host E. coli strain for transformation was cultivated in LB medium and kanamycin (25 μg/ml) was added as a selection marker for transformants harboring recombinant plasmid.

Preparation and Manipulation of DNA

The total genomic DNA of B. firmus var. alkalophilus was isolated according to the method of Saito and Miura [23], and the plasmid DNA was isolated from the E. coli using the method developed by Birnboim and Doly [2]. General recombinant DNA manipulation was carried out according to the protocol suggested by Sambrook et al. [24].

Preparation of CGTase Gene Probe

DNA probe for screening the CGTase gene was prepared using a polymerase chain reaction (PCR) according to the procedure developed by Griffin and Griffin [6]. To amplify the DNA probe, upstream and downstream primers were designed based on the conserved regions of the CGTases from various Bacilli.

Cloning Procedures for the β-CGTase Gene

Genomic DNA of B. firmus var. alkalophilus was partially digested with Hind III, and DNA fragments potentially containing the β-CGTase gene were identified by Southern hybridization using a DNA probe prepared by PCR [24]. The genomic DNA fragments showing a positive signal of CGTase gene were eluted, and ligated with pZErO™-2 plasmid DNA which were cleaved with Hind III. The ligation mixture was then transformed into E. coli TOP10 according to the protocol of Sambrook et al. [24]. The transformants were spread onto LB agar plates containing 25 μg/ml of kanamycin, and after an overnight growth period at 37°C, the survived colonies were hybridized with a DNA probe, and then the positive colonies were selected as the clones potentially containing the β-CGTase gene. After cultivation in LB containing 1.0% soluble starch, CGTase activities of these positive clones were measured [11], and then the clones exhibiting CGTase activity were finally selected as the recombinant harboring β-CGTase gene.

DNA Sequence Analysis

The DNA sequence was determined by using the dideoxy-chain termination method of Sanger et al. [25] that utilizes the Sequenase version of 2.0 DNA sequencing kit (Amersham Co., England). PCR sequencing was also completed by using the TOPD™ DNA sequencing kit (Bioneer Co., Korea).

Measurement of CGTase Activity

CGTase activity was determined according to the color reduction method that measures the phenolphthalein entrapped by β-CD [11]. One unit of enzyme was defined as the amounts of enzyme that can form 1 mg of β-CD from a soluble starch per min.

Measurement of Cyclic AMP Level and Glucose Concentration

Intracellular level of cyclic AMP was measured using a cAMP enzyme-linked immunoassay kit (EIA kit, Amersham Inc., U.K.) after harvesting about 10^7 of cells according to the procedure described by Notley-McRobb et al. [18]. Glucose concentration in the culture medium was measured by the peroxidase/glucose oxidase method (PGO method, Sigma Co., U.S.A.).

Immunoblot Analysis

Polyclonal antibodies were raised against the purified β-CGTase of B. firmus var. alkalophilus [26] according to the

method described by Harlow and Lane [7]. Protein samples were separated by SDS-PAGE, blotted onto the nitrocellulose paper, and then processed according to the method of Sambrook *et al.* [24]. A goat anti-rabbit second antibody labeled with alkaline phosphatase was employed and nitro-blue-tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as detection reagents (Sigma Co., St. Louis, U.S.A.).

Gel Electrophoresis and Zymography

SDS-PAGE was performed as described by Laemmli [17]. For zymography, the gel was incubated at 50°C for 30 min in a solution containing 1.0% of the soluble starch in a 50 mM Tris-maleic acid-NaOH buffer (pH 6.0) after SDS-PAGE, and then it was stained with iodine solution (0.02% I_2 and 0.2% KI) for identifying the starch-degrading activity.

Nucleotide Sequence Accession Number

The nucleotide sequence data reported in this paper will appear in the KRIBB (Korea Research Institute of Bioscience and Biotechnology) Genome Center with the accession number of GP02245.

RESULTS AND DISCUSSION

DNA Probe and Cloning of the β-CGTase Gene from *B. firmus* var. *alkalophilus*

Nucleotide sequences of primers used as a DNA probe for cloning the β-CGTase gene (A) and the DNA band of the

(A) Primer sequences

Primer 1 (N-terminal)
5'-GGCTATACCTCCTACCATGG
ATATTGG-3'
Primer 2 (C-terminal)
5'-GTTTTGGGGATCAACTTCTC
CTGATCC-3'

(B) PCR product
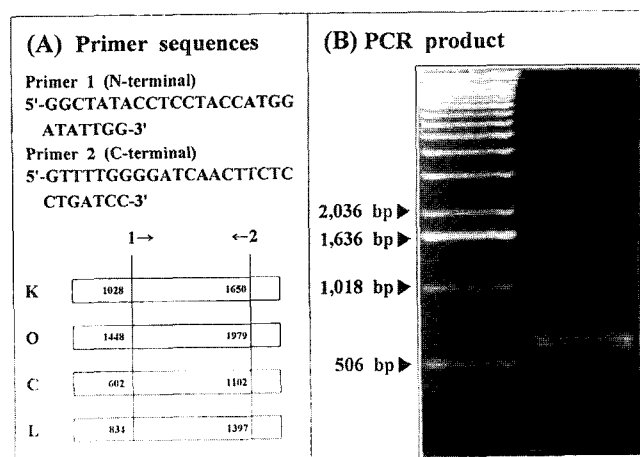
Fig. 1. Amplification of the β-CGTase gene fragment by PCR. DNA fragment for the β-CGTase gene probe was amplified from genomic DNA of *B. firmus* var. *alkalophilus* used as a template DNA by PCR according to the procedure of Griffin and Griffin [6]. The number of (A) is the DNA sequence number of each CGTase gene listed as follows: K, *Bacillus* sp. KC201 CGTase; O, *Bacillus obensis* CGTase; C, *Bacillus circulans* 251 CGTase; and L, *Bacillus licheniformis* CGTase.

nucleotide sequence after polymerase chain reaction (B) are illustrated in Fig. 1. The nucleotide sequences were constructed from several conserved regions of the amino acid sequences of β-CGTases of other *Bacilli* [8, 15, 18, 28]. About 0.6 kb region was amplified using the genomic DNA of *B. firmus* var. *alkalophilus* as a template by PCR. The PCR product exhibited a high homology with other CGTases, especially, the high homology of 98% with the CGTase gene from *Bacillus* sp. KC201 [15]. In
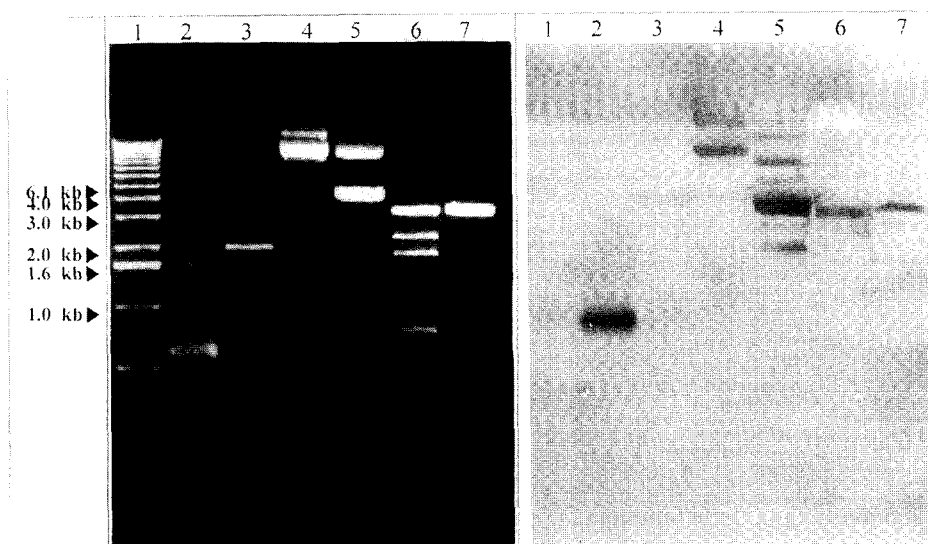
Fig. 2. Southern blot analysis of pZeCD4 and pZeCD53 harboring the β-CGTase gene from *B. firmus* var. *alkalophilus*. pZeCD4 and pZeCd53 were digested with *Hin*d III, separated by 0.8% agarose gel electrophoresis, and transferred onto nylon membrane. The blot was hybridized with the β-CGTase gene probe labeled with DIG-11-dUTP. Lane 1, 1 kb ladder; Lane 2, β-CGTase gene probe; Lane 3, pZErO™-2 vector; Lane 4, pZeCD4; Lane 5, pZeCD53; Lane 6, pZeCD4 digested by *Hin*d III; Lane 7, pZeCD53 digested by *Hin*d III.

addition, it was used as the DNA probes for further β-CGTase gene cloning.

Through Southern blot analysis, the *Hin*d III was identified as the most suitable restriction enzyme for partial digestion of genomic DNA of *B. firmus* var. *alkalophilus*. About 3.6 and 4.3 kb of the *Hin*d III-digested genomic DNA were eluted with consideration for not only the sizes of CGTase genes (usually 2.0-2.5 kb) but also the cloning efficiency. Then, they were ligated to pZErO™-2 that was cleaved by *Hin*d III. The ligation mixture was transformed into *E. coli* TOP10, and then about 100 colonies were selected as the potential clones containing the β-CGTase gene from around 10,000 colonies. Two clones, pZeCD4 and pZeCD53, with insert sizes of about 6.6 and 3.0 kb, respectively, were finally selected.

Figure 2 illustrates the results of the Southern blot analysis in regards to the above two recombinant plasmids, and the strong positive signals for the β-CGTase gene were detected within the same band of about 3.4 kb in *Hin*d III-digested pZeCD4 and pZeCD53, indicating identical β-CGTase genes.

Figure 3 illustrates SDS-PAGE (A), zymography (B), and immunoblot analysis (C) of the CGTase produced by the parent strain *B. firmus* var. *alkalophilus* and two recombinant *E. coli* strains harboring pZeCD4 and pZeCD53, respectively. All CGTases exhibited strong starch hydrolyzing activities. Furthermore, nearly identical molecular weights

were found as can be compared in Figs. 3A and 3C, and this indicates that the β-CGTase of the parent strain was successfully expressed in *E. coli*. In addition, a slightly larger band than purified β-CGTase was also found in the case of recombinant *E. coli* strains harboring pZeCD4 and pZeCD53, and this band seemed to be premature enzyme, which is not spliced the signal peptides. The β-CGTase gene in pZeCD53 was selected for nucleotide sequence analysis upon considering its insert size (about 3.4 kb), which was identified as a sufficient size for the full β-CGTase gene.

Nucleotide and Amino Acid Sequences of the Cloned β-CGTase Gene

Figure 4 shows the nucleotide sequence of the insert DNA (about 3.4 kb) of pZeCD53. Neither translation initiation codon nor promoter sequences was observed within a cloned DNA fragment initially, therefore further sequence analysis, including nucleotide sequences of the vector, was carried out. It was discovered that the cloned β-CGTase gene was expressed by a *lac* promoter and an initiation codon (ATG) of *lacZ* of pZErO™-2 vector. The open reading frame (ORF) of the cloned β-CGTase gene was composed of 710 amino acid residues, including 674 amino acids of the matured β-CGTase gene (≒ 75,539 dalton) and 36 amino acids of signal peptide containing 16 amino acids from the cloned gene, and 20 amino acids of the *lacZ* gene from the pZErO™-2 vector.
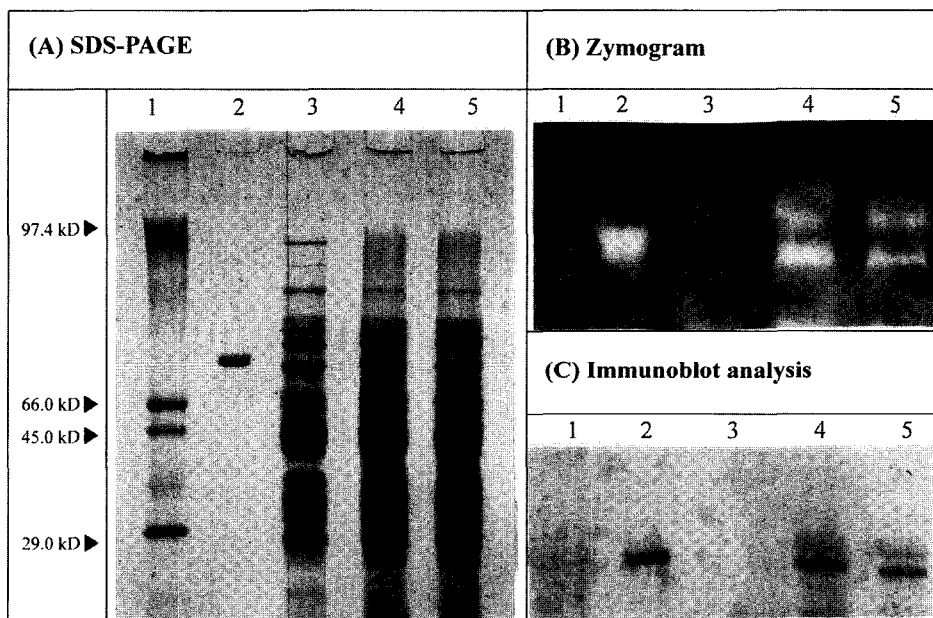
Fig. 3. SDS-PAGE, zymogram, and immunoblot analysis of the β-CGTases expressed by the parent strain, recombinant *E. coli* harboring pZeCD4 and pZeCD53.

The same amounts of protein samples were separated on 10% SDS-PAGE and stained with Coomassie brilliant blue in (A), and the starch degrading activity was detected *in situ* with I₂ solution in (B). A gel run in parallel in (A) was transferred onto nitrocellulose membrane and probed with anti-β-CGTase antibody in (C). Lane 1, molecular weight marker; Lane 2, purified CGTase from *B. firmus* var. *alkalophilus* [26]; Lane 3, culture broth of *E. coli* TOP10; Lane 4, culture broth of *E. coli* harboring pZeCD4; Lane 5, culture broth of *E. coli* harboring pZeCD53.
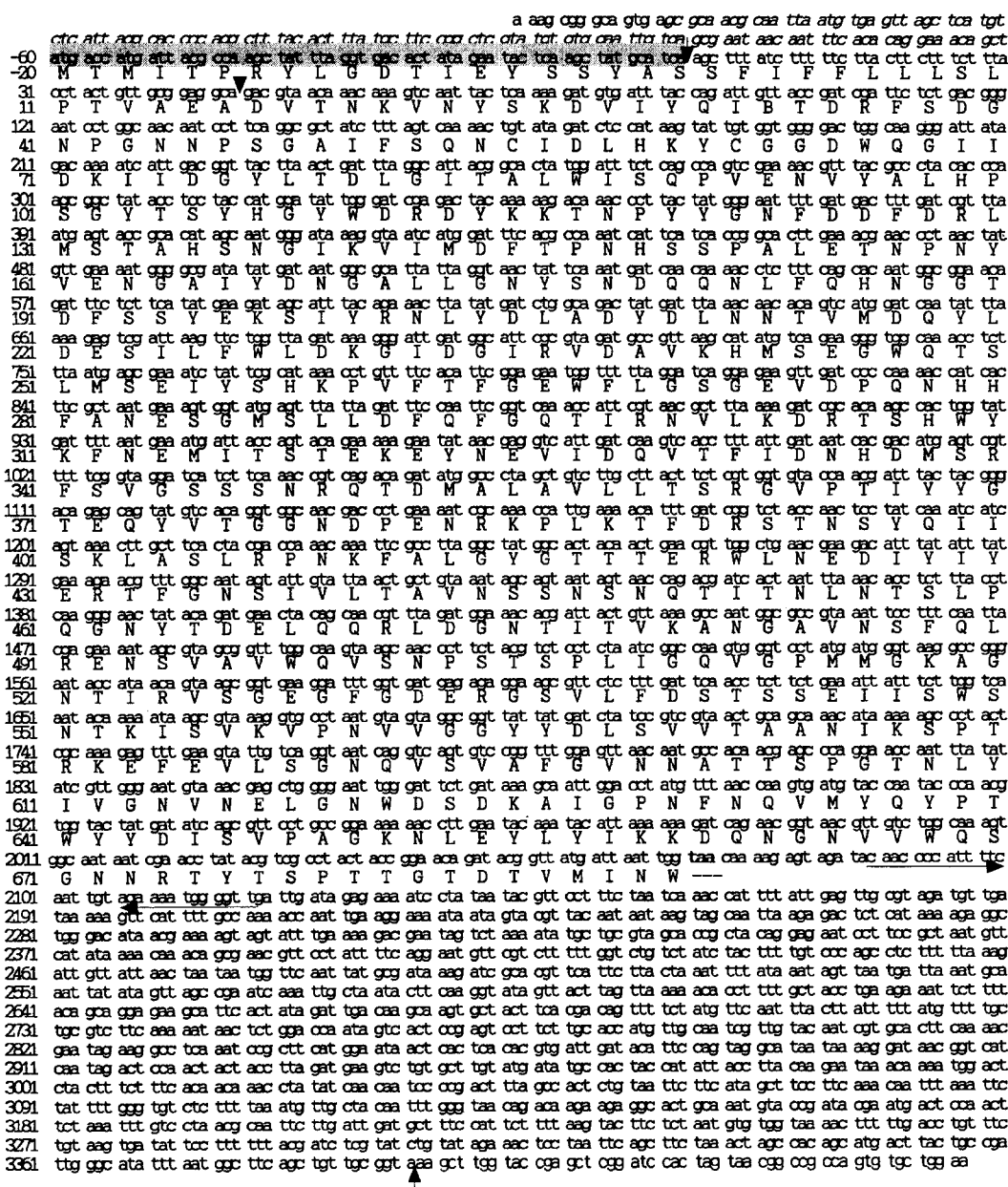
Fig. 4. Nucleotide and deduced amino acid sequence of the clone pZeCD53 encoding the β-CGTase gene of *Bacillus firmus* var. *alkalophilus*.
Numbering of nucleotide and amino acid sequences start from the *Hin*d III-digested site of the N-terminal of the β-CGTase gene, and the vertical arrows indicate the *Hin*d III-digested site. The italicized sequence is the lac promoter, and the shaded sequence is derived from the *lacZ* gene in pZErO™-2 vector. The processing site for the signal peptide sequence (arrow head) was assigned by comparing with other CGTases from *Bacilli*. The palindromic sequence at downstream is shown by horizontal arrows under the sequence.

The molecular weight of the cloned β-CGTase was found to be consistent with the value estimated from the SDS-PAGE of purified β-CGTase of *B. firmus* var. *alkalophilus* as reported previously [26]. The homology comparison of the N-terminal sequences was somewhat puzzling because the cloned β-CGTase gene contained 20 amino acids derived from the *lacZ* gene of the vector. However, the region of the remnant amino acids (1 to 16)

was identical with the signal peptide sequences of *Bacillus* sp. KC201 [14] and *Bacillus* sp. E1 CGTases [31]. Meanwhile, 56% identified with that of *B. ohbensis* CGTase [28]. A palindromic sequence that could form a stable stem-and-loop structure (nucleotides 2,088 through 2,120) followed by an AT-rich sequence is a characteristic of the ρ-independent transcriptional terminators of *E. coli*. [22].

Table 1. Relative homology (percent of identity) between the deduced amino acid sequences of various CGTase genes from *Bacilli.*

Bacillus Strain	Percent of identity (%)											
	BCGFA	BKC201	BCGE1	BCGO	BCG251	BCG38	BCG17	BMCG	BSCG	BLCG	BCG1011	BCG290
BCGFA	100	97.0	91.5	79.3	55.9	55.9	54.8	54.1	59.7	55.3	54.7	53.1
BKC201		100	95.4	79.7	56.8	56.3	55.5	54.5	61.3	55.4	55.3	55.5
BCGE1			100	78.1	56.3	55.5	54.6	54.1	60.0	55.0	54.9	52.7
BCGO				100	58.4	57.2	56.7	56.8	63.2	57.0	57.0	55.5
BCG251					100	86.4	89.8	67.3	65.6	90.7	70.2	50.3
BCG38						100	83.4	66.2	62.5	69.3	97.2	50.1
BCG17							100	66.6	64.0	71.6	84.2	50.8
BMCG								100	59.3	62.4	63.9	50.8
BSCG									100	60.0	60.5	56.5
BLCG										100	70.3	50.0
BCG1011											100	50.9
BCG290												100

BCGFA, CGTase from *Bacillus firmus* var. *alkalophilus* [this study]; BKC201, CGTase from *Bacillus* sp. KC201 [14]; BCGE1; CGTase from *Bacillus* sp. E1 [31]; BCGO, CGTase from *Bacillus ohbensis* [28]; BCG251, CGTase from *Bacillus circulans* 251 [17]; BCG38, CGTase from *Bacillus* sp. 38-2 [10]; BCG17, CGTase from *Bacillus* sp. 17-1 [9]; BMCG, CGTase from *Bacillus macerans* [29]; BSCG, CGTase from *Bacillus stearothermophilus* [5]; BLCG, CGTase from *Bacillus licheniformis* [8]; BCG1011, CGTase from *Bacillus* sp. #1011 [20]; BCG290, CGTase from *Bacillus* sp. 290 [8].

Comparison of CGTase of *B. firmus* var. *alkalophilus* with Other CGTases

Amino acid homology. The amino acid homology of above cloned β-CGTase was compared with eleven other CGTases from *Bacilli* in order to classify the types of CGTases based on amino acid sequences as shown in Table 1. The cloned CGTase showed a high similarity of 97% with CGTase from *Bacillus* sp. KC201 [14], 91.5% with alkalophilic *Bacillus* sp. E1 [31], and 79.3% similarity with *B. ohbensis* [28], having common characteristics of producing β-type CGTase.

Kitamoto *et al.* [14] classified the β-type CGTases from alkalophilic *Bacilli* into two groups. Group I was represented by alkalophilic *Bacillus* sp. 38-2 β-CGTase producing β-CD with small amounts of α- and γ-CD, and group II was represented by *Bacillus sp.* KC201 producing negligible amount of α-CD. Accordingly, cloned β-CGTase can be classified as group II type β-CGTase that produces negligible amounts of α-CD. Meanwhile, the cloned β-CGTase exhibited a low similarity of 54.1–59.7% compared to other CGTases from neutrophilic *Bacilli*, such as *B. macerans*, *B. stearothermophilus*, and *B. licheniformis* [8, 29]. **Conserved regions.** Four conserved regions which were involved in an active and/or substrate-binding site was suggested by Kitamoto *et al.* [15] and Nakamura *et al.* [20] after comparing the alignment of amino acid sequence between CGTases and amylase. However, it may be premature at this time to get a full understanding of the active site and the various modes of reactions of CGTase, particularly cyclization and coupling reactions, since the above comparison was based on and made by simple comparisons of CGTase and amylases. Therefore, in order to identify additional conserved regions of CGTase in *B.*

firmus var. *alkalophilus*, which were not found in amylases, a further homology analysis besides the above conserved regions was performed.

Figure 5 illustrates amino acid sequences of ten highly conserved regions of *B. firmus* var. *alkalophilus* and those of CGTases from various *Bacilli*. Regions II-VIII in *B. firmus* var. *alkalophilus* and other CGTases can be recognized as the active and/or substrate-binding sites of enzymes, which are also found in many amylases as α-amylase, glucoamylase, maltotetraose-forming amylase, and raw-starch digesting amylase [15]. Three histidines, two aspartic acids, and one glutamic acid, along with four aromatic amino acid residues most commonly seen in all conserved regions of II-VIII have been located at or near active sites of CGTases. They are involved in substrate binding or various catalytic functions, such as cyclization and starch hydrolysis [19, 20, 30]. On the other hand, regions I, IX, and X, located in the N- and C-terminus of CGTase, were not conserved in α-amylase and other amylases [15, 30]. The region I is known to participate in calcium-binding, and regions IX and X are also known to involve the raw starch-binding, where, region X is especially known as a maltose-binding site I rough X-ray crystallographic studies of CGTase [17, 30]. Cyclization reaction of CGTase is unique among starch hydrolyzing enzymes, and the deletion of the N-terminal or C-terminal regions in CGTase affected both the cyclization activity and the enzymatic properties, such as pH or thermal stability [4, 9, 28]. These facts definitely indicated some involvement of these conserved regions in the transglycosylation reaction of the CGTase.

The aspartic/glutamic acid, histidine, and tryptophan residues have been identified as the critical amino acid residues in our previous work [27], in which we

Fig. 5. Comparison of the deduced amino acid sequences of the CGTase gene from *Bacillus firmus* var. *alkalophilus* with other CGTases.

The ten highly conserved regions in different CGTases from *Bacilli* are described. The conserved three His residues, four aromatic residues, and three carboxylic amino acid residues are in bold. BMCG, *B. macerans* CGTase [29]; BSCG, *B. stearothermophilus* CGTase [5]; BLCG, *B. licheniformis* CGTase [8]; BCG38, *Bacillus* sp. 38-2 CGTase [10]; BCG17, CGTase from *Bacillus* sp. 17-1 [9]; BCG251, *B. circulans* 251 [17]; BCG1011, *Bacillus* sp. 1011 [20]; BCGFA, *B. firmus* var. *alkalophilus* CGTase [this study]; BCGE1, *Bacillus* sp. E1 CGTase [31]; BKC201, CGTase from *Bacillus* sp. KC201 [14]; BCGO, *B. ohbensis* CGTase [28]; BCG290, *Bacillus* sp. 290 [8].

Table 2. Localization of CGTase activity in different fractions of recombinant *E. coli* harboring pZeCD53.

	Cell fraction		
	Extracellular	Periplasmic	Intracellular
CGTase activity (units/ml)*	0.415	0.119	0.085
Fraction of total activity (%)	67.0	19.2	13.8

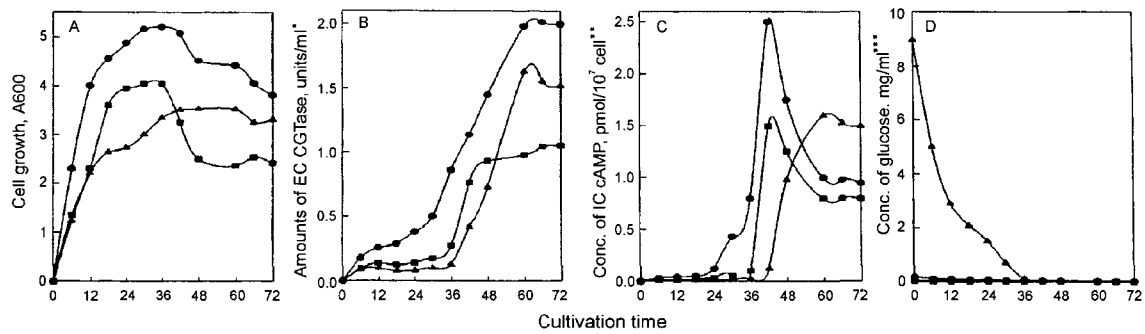*CGTase activity on the basis of culture broth.

cultured in LB medium containing 1.0% soluble starch for 2 days; subsequently, the localized CGTase activity in each cellular fraction was carefully measured. As shown in Table 2, the majority of the enzyme activity was observed in a culture broth, and a small amount of enzyme was localized in both the periplasmic and intracellular fractions. The β-CGTase produced in recombinant *E. coli* appeared to be mobilized from the intracellular fraction to the periplasm, and then the most of the enzyme excreted into the extracellular medium as a parent strain *B. firmus* var. *alkalophilus*.

Most of the CGTases from other strains expressed in *E. coli* remained mostly in the periplasm that was caused by another spatial barrier, an outer-membrane [14, 15, 28, 29], with an exception of *K. pneumoniae* and *Bacillus* sp. E1 CGTases [1, 31]. The efficient excretion of β-CGTase encoded in plasmid pZeCD53 at the recombinant *E. coli* has drawn great interest and, thus, further research to clarify the excretion mechanism is needed.

Regulation of Expression of β-CGTase in *E. coli*

The regulation mechanism of β-CGTase production in *E. coli* harboring plasmid pZeCD53 was investigated after cultivating in three different media: LB medium (LB), LB medium containing 1.0% glucose (LBG), and LB medium containing 1.0% soluble starch (LBS), at 37°C for 2 days. Figure 6 illustrates the cell concentration and CGTase activity during the cultivation process of transformant *E. coli*. β-CGTase was mostly produced only after the stationary phase of the cell growth, and it was also constitutively expressed in all three different kinds of medium, even in LB and LBG not containing soluble starch, or other inducers such as IPTG or lactose. This suggests that the β-CGTase gene, controlled by *E. coli lac* promoter in pZErO vector, can be constitutively expressed even without any inducers, and considering that neither the host cell nor vector contains a *lac I* gene which represses the *lac* promoter.

The highest excretion, which is about 2.0 units/ml, comparable to the amount of excretion by a parent strain *B. firmus* var. *alkalophilus* [4], occurred in the LBS medium. What this indicates is that the intracellular cAMP concentration activating the *lac* promoter related to the

investigated the role of amino acid residues in the catalytic act of β-CGTase of *B. firmus* var. *alkalophilus* through site-specific chemical modification. In particular, the tryptophan residues are involved in a substrate-binding activity rather than as a catalytic site of enzyme, whereas the histidine residues played an important role in catalysis for cyclization and starch hydrolysis reaction. In addition, the tyrosine and phenylalanine are both involved in determining the profiles of α, β, γ-CD and cyclization reaction of CGTases. The roles of these conserved regions will be identified after point-mutation on β-CGTases by a site-directed mutagenesis, and the subsequent characteristic of β-CGTases will be further studied.

Expression and Localization of β-CGTase in *E. coli*

To localize the expression of β-CGTase in *E. coli*, the transformant *E. coli* harboring plasmid pZeCD53 was

Fig. 6. Cell growth (A), β-CGTase production (B), concentration of the intracellular cAMP (C), and extracellular glucose concentration (D) by transformed *E. coli* harboring pZeCD53 in different media.

E. coli harboring pZeCD53 cultivated in LBS was innoculated 5.0% (v/v) into LB, LBG, and LBS, respectively, and cultivated at 37°C for 3 days. LB (■), LB containing 1% glucose (LBG, ▲), and LB containing 1% soluble starch (LBS, ●). *Amounts of extracellular CGTase, units/ml; **Concentration of intracellular cAMP, pmol/10⁷ cell; ***Concentration of glucose, mg/ml.

expression of β-CGTase was elevated successfully by soluble starch. Meanwhile, the least amounts of β-CGTase was excreted in the LBG medium prior to the late stationary phase where glucose depletion started, and this suggests that the *lac* promoter controlling the β-CGTase gene was catabolite-repressed by glucose.

Recently, Notley-McRobb *et al.* [18] have reported that the growth of *E. coli* in a minimal medium containing a micromolar level of glucose was more effectively stimulated, when compared to both excess glucose and without any carbon sources that is higher in intracellular cAMP levels. An effective production of β-CGTase in *E. coli* harboring plasmid pZeCD53 can also be explained by the above observation. The overproduction of β-CGTase using recombinant *E. coli* seems to be achieved by controlling the intracellular cAMP level strictly through the selection of a suitable cultivation method, such as a fed-batch wise addition of glucose.

Acknowledgments

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