Characterization of a Thermostable Cyclodextrin Glucanotransferase Isolated from *Bacillus stearothermophilus* ET1†

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A thermostable cyclodextrin glucanotransferase (CGTase) was isolated from a *Bacillus stearothermophilus* strain, ET1, which was screened from Korean soil. The corresponding CGTase gene cloned in *Escherichia coli* shared 84% and 88% identity with CGTase genes from other *B. stearothermophilus* strains at the nucleotide and amino acid sequence level, respectively. The enzyme was purified to apparent homogeneity by β-cyclodextrin (CD) affinity chromatography and high-performance liquid chromatography. The enzyme had an apparent molecular mass of 66,800 Da and a p*I* of 5.0. The optimum pH for the enzyme-catalyzed reaction was pH 6.0, and the optimum temperature was observed at 80 °C. Thermostability of the enzyme was enhanced by Ca^{2+}. A 13% (w/v) cornstarch solution was liquefied and converted to CDs solely using this enzyme. The cornstarch conversion rate was 44% and α-, β-, and γ-CDs were produced in the ratio of 4.2:5.9:1.

Keywords: *Cyclodextrin glucanotransferase; thermostability; cyclodextrin; Bacillus stearothermophilus*

INTRODUCTION

Cyclodextrins (CDs), industrially important compounds, are synthesized from starch by the action of cyclodextrin glucanotransferase [CGTase; 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)transferase, EC 2.4.1.19]. They have a closed ring structure of six, seven, or eight glucose units linked by α-1,4-glucosidic bonds, which are known as α-, β-, or γ-CDs, respectively (French and Rundle, 1942; Freudenberg and Cramer, 1948). Due to their structural characteristics, CDs can accommodate various organic molecules to form soluble or insoluble inclusion complexes. These increase the solubility of insoluble molecules, stabilize volatile flavors, remove noxious odors, and prevent oxidative degradation of molecules by heat or light (Fridrich et al., 1990; Bender, 1986). Therefore, production of CDs using CGTase has attracted increasing interest from the food and pharmaceutical industries.

The conventional procedure for the production of CDs includes liquefaction of starch using a thermostable α-amylase at 105 °C. After the reaction mixture is allowed to cool to ≈50 °C, it is treated with bacterial CGTases (Kitahata et al., 1974; Nakauma and Horikoshi, 1976; Bender, 1977; Kobayashi et al., 1978; Kitahata and Okada, 1982; Makela et al., 1988; Yagi et al., 1986), which have an optimum catalytic reaction temperature of 50−65 °C. However, this procedure is inefficient in many aspects: first, α-amylase should be inactivated before the addition of CGTase; second, microbial contamination is possible; third, it is time-consuming. Therefore, a CGTase with liquefying and cyclizing activities at high temperatures would be appropriate for efficient production of CDs. Norman and Jørgensen (1992) reported a thermostable CGTase isolated from a thermophilic anaerobe, *Thermoanaerobacter*. This thermostable CGTase with a temperature optimum of 90 °C produced CDs more efficiently than the CGTase isolated from *Bacillus macerans* with a temperature optimum of 55 °C (Yagi et al., 1986). However, the producing organism, *Thermoanaerobacter*, is not presently included in those organisms that are generally recognized as being safe for the production of food or pharmaceutical products of their applications. Inactivation of the enzyme upon the completion of the process would not be easy due to the thermostability of the enzyme.

In the present paper, we report the isolation of a thermostable CGTase and the corresponding gene from a *Bacillus stearothermophilus* strain, one of the bacterial species that are generally recognized as safe (GRAS). The physicochemical properties of the enzyme were characterized, and CDs were produced by treating starch solution with CGTase alone at 90 °C without the necessity of prior treatment with α-amylase to liquefy the starch.

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† Nucleotide sequence of the CGTase gene has been submitted to GenBank under Accession Number U83799.

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MATERIALS AND METHODS

Screening of Bacterial Strains. Bacterial strains isolated from Korean soil were tested for starch hydrolyzing activity on an agar plate containing 1% (w/v) soluble starch (Showa Chemical Co., Japan) and an iodine solution [0.2% (w/v) I_2 and 5.2% (w/v) KI in dH_2O]. The strains positive for starch hydrolyzing activity were screened for CD forming activity by the phenolphthalein test, which has been described previously (Park et al., 1989). One of the CGTase producing strains that was capable of growing at 55 °C was selected and used for this study.

Culture Conditions for CGTase Production. *B. stearothermophilus* ET1 (deposited at Korean Collection for Type Cultures; KCTC 0114BP) was grown in Luria−Bertani broth [LB broth; 1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] containing 1% (w/v) soluble starch at 55 °C for 12 h. Twenty milliliters of the culture was transferred to 2 L of SH medium containing 4% (w/v) soluble starch, 0.35% $(NH_4)_2HPO_4$, 0.6% (w/v) yeast extract, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.2% (w/v) sodium citrate, and 0.008% (w/v) $CaCl_2 \cdot 2H_2O$ in a 5 L fermenter (Korean Fermenter Inc., Korea) and cultured at 50 °C for 24 h with agitation at 300 rpm. The supernatant was used for preparing the CGTase enzyme.

Purification of CGTase. Cells were removed from the culture by centrifugation (4700*g*) at 4 °C for 30 min, and the supernatant was used for fractionation of extracellular CGTase by 70% (w/v) $(NH_4)_2SO_4$ at 4 °C overnight. The precipitate was resuspended in 20 mM Tris-HCl buffer (pH 7.0) and dialyzed against 50 mM Tris-HCl (pH 8.0) and the crude enzyme solution then applied to a β-CD affinity column (donated by Miwon Inc., Korea). The β-CD affinity column was prepared as follows: 4 g of epoxy-activated Sepharose 6B (Sigma Co., St. Louis, MO) was reconstituted in distilled water for 1 h. The matrix was transferred to 0.1 M NaOH, and then excessive liquid was removed. The matrix was reacted with a solution containing 300 mg of β-CD in 12 mL of 0.1 M NaOH at 45 °C for 16 h, followed by washing with distilled water for 1 h and subsequently with 20 mM Tris-HCl buffer (pH 7.0) for 2 h. The affinity column was prepared by packing the matrix in a 10 × 150 mm column and equilibrated with the Tris-HCl buffer. The crude enzyme solution was loaded onto the column and washed with 20 mM Tris-HCl buffer (pH 7.0) for 30 min. Contaminating proteins adsorbed to the column were removed by washing with 20 mM Tris-HCl buffer (pH 7.0) containing 0.5 M NaCl. CGTase bound to the column was eluted with 20 mM Tris-HCl buffer (pH 7.0) containing 1% (w/v) β-CD at a flow rate of 2 mL/min. The eluted enzyme was further purified by ion exchange high-performance liquid chromatography (HPLC; Waters 600E, Milford, MA) using a Protein-Pak DEAE-8HR column (Waters) and a UV−vis detector (SLC-200, Samsung Electornics Devices, Korea). Active fractions for CGTase were collected after elution using a NaCl gradient from 0 to 0.5 M.

Assay of CGTase Activity. CGTase activity was assayed according to the phenolphthalein test described by Kaneko et al. (1987). Enzyme solution (2 units/20 μL) was added to 1 mL of gelatinized soluble starch solution [4% (w/v) in 50 mM maleate−NaOH buffer (pH 6.0)] and incubated at 60 °C for 10 min. The reaction was stopped by adding 3.5 mL of 30 mM NaOH and then 0.5 mL of phenolphthalein solution [0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3] was added to the reaction mixture and left to stand at room temperature for 15 min. The concentration of β-CD was determined by the change in absorbance at 550 nm to a standard β-CD curve. One unit of enzyme activity was defined as the amount of enzyme producing 1 mg of β-CD/min.

Assay of Reducing Sugar. The amount of reducing sugar released by the enzyme was assayed according to the method of Miller (1959) with some modification. Three milliliters of 3,4-dinitrosalicylic acid solution (DNS; 3,4-dinitrosalicylic acid, 10.6 g; NaOH, 19.8 g; potassium sodium tartrate, 306 g; phenol, 7.6 mL; sodium metabisulfate, 8.3 g; and distilled water, 1416 mL) were added to 1 mL of reaction mixture containing CGTase and substrate. The mixture was boiled for 5 min and cooled immediately by placing the test tube under cold running water. Absorbance of the mixture was measured (575 nm) and the amount of reducing sugar was determined using a DNS standard curve.

Temperature and pH Optima. Optimal temperature for the enzyme activity was determined by incubating CGTase for 10 min with 4% (w/v) starch in 50 mM maleate−NaOH buffer (pH 6.0) at various temperatures. Thermal stability of the enzyme was studied by incubating it for 10, 20, 30, and 60 min with 4% (w/v) starch in 50 mM maleate−NaOH buffer (pH 6.0) at temperatures between 60 and 90 °C. Optimal pH of the enzyme was determined by incubating the reaction in citrate (pH 5.0), phosphate or maleate−NaOH (pH 6.0), Tris-HCl (pH 7.0−8.5), and glycine−NaOH (pH 10.5) buffers at 80 °C for 10 min.

Production of CD. Cornstarch (13%, w/v) was liquefied by CGTase (4.27 units/g of substrate) in 50 mM maleate−NaOH buffer (pH 6.0) at 90 °C for 30 min to have dextrose equivalent (DE) 10. The DE value represents the percentage of reducing sugar (g) over total soluble starch (g) in the solution. The amount of reducing sugar was determined according to the DNS method. The reaction mixture was then cooled to 60 °C, and cyclization was allowed to occur at the temperature.

Analysis of End Products. End products synthesized by the action of CGTase from soluble starch (Showa Chemical Co.) were analyzed by HPLC and thin-layer chromatography (TLC). The HPLC system (SLC-100, Samsung Electron Inc., Korea) was equipped with a Lichrosorb NH_2 column (10 μm, 4 × 250 mm; Merck, Darmstadt, Germany) and a Waters differential refractometer (Waters 400R). The samples were eluted at a flow rate of 1.0 mL/min using a mixture of acetonitrile and water (65:35, v/v). TLC analysis of CDs was carried out on silica gel 60 plates (Merck Co.) with a mobile phase of 1-butanol, ethanol, water, and ammonia (5:3:4:2). HPLC and TLC were carried out as described previously (Kim et al., 1992).

Protein Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using 10% (w/v) polyacrylamide gels as described by Laemmli (1970), and the gel was visualized by staining with Coomassie brilliant blue. Isoelectric focusing (IEF) was done using PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden) and PhastGel (IEF 3−9) at 15 °C with 2.5 mA current as described previously (Kim et al., 1992).

Differential Scanning Calorimetry (DSC). Thermal denaturation of CGTase was analyzed using DSC 120 (Seiko Co., Japan). DSC analysis was performed in the range of 30−120 °C by raising the temperature at a rate of 1 °C min. CGTase was concentrated by ultrafiltration and resuspended in 50 mM Maleate−NaOH buffer (pH 6.0) at a concentration of 10% (w/v). A sample aluminum pan was filled with 20 μL of the enzyme solution and a reference pan with the same volume of buffer solution.

Cloning and Nucleotide Sequence Analysis of the CGTase Gene. A 3.2 kb *Bam*HI−*Xba*I fragment containing the CGTase gene of *B. stearothermophilus* ET1 was cloned on pUC119. The clone, designated pVBX32, was isolated by probing a subgenomic DNA library with a 0.78 kb PCR product that was generated using two primers (5′-TATCATGGTTATTGGGC-3′ and 5′-TACGCCACGGGATGTCAA-3′) with sequences derived from two previously reported CGTase gene sequences (Fujiwara et al., 1992). Nucleotide sequence analysis was carried out by Sanger's chain termination method (Sanger et al., 1977) using an autosequencer (ALF express, Pharmacia). Nucleotide sequence of the CGTase gene was deposited in the Genbank with the Accession Number U83799.

RESULTS

Identification of the CGTase Producing Strain. The CGTase producing bacterial strain isolated from

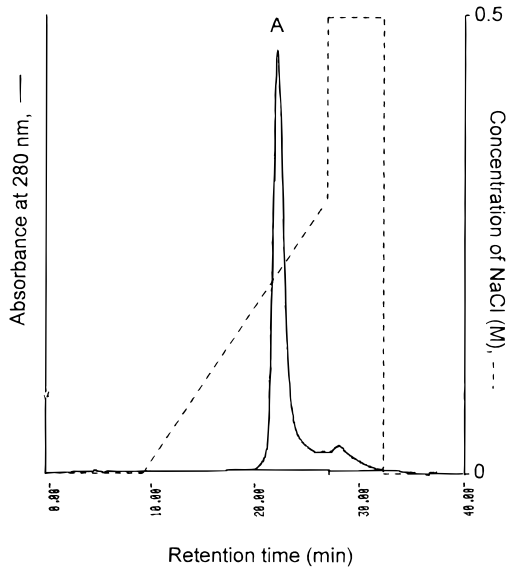

Figure 1. High-performance liquid chromatography of CGTase. Proteins eluted from the β-CD affinity column were loaded onto a Protein-Pak DEAE-8HR column and eluted by 20 mM Tris-HCl buffer with NaCl gradient from 0 to 0.5 M. The fraction collected from peak A retained the CGTase activity.

Korean soil was identified as *Bacillus stearothermophilus* on the basis of the results of various physiological tests and the data obtained using an automatic microbial identification system (Vitek System, Biomerieux Vitek Inc.) and according to the criteria of *Bergey's Manual of Systematic Bacteriology* (Claus and Berkeley, 1986). The organism was a facultative anaerobic Gram-positive Bacillus (1.05 × 2.6 μm) with motility. The isolate formed spores and grew at 65 °C. Also, the organism was negative for the Voges−Proskauer test and produced acid from D-glucose, D-arabinose, and D-xylose but not from D-mannitol. From these, the bacterial strain was designated *B. stearothermophilus* ET1.

Production of CGTase in *B. stearothermophilus* ET1. The cells began to produce CGTase after 5 h of growth when they were cultured in a complex medium containing 4% (w/v) soluble starch. The enzyme production increased as the cells grew exponentially and then reached the highest level as the cells entered stationary phase. However, the strain ET1 produced only a relatively small amount of CGTase (10 mg/10 L of culture). All of the enzyme produced was extracellular as has been found with other CGTase producing bacteria (Akimaru et al., 1991; Jamuna et al., 1993; Kitamoto et al., 1992; Makela et al., 1988; Sin et al., 1993).

Properties of CGTase. CGTase was purified from the culture medium to apparent homogeneity using a β-CD affinity column and a Protein-Pak DEAE-8HR column (Figure 1). The purification procedure is summarized in Table 1. The enzyme was purified 90.8-fold with a yield of 31.6%. The specific activity of the purified enzyme was 443.8 units/mg. The apparent molecular mass of the purified CGTase was determined as 66 800 Da by SDS−PAGE (Figure 2, lane 1) and the p*I* as 5.0. Optimum temperature for the enzyme activity was tested by incubating CGTase for 10 min with 4% (w/v) starch in 50 mM maleate−NaOH buffer (pH 6.0) at each temperature setting. The enzyme showed the highest activity at 80 °C (Figure 3). The enzyme activity was not enhanced by $CaCl_2$ at the temperatures tested. The optimum pH of CGTase was tested in the range of pH 5.0−10.5 and determined as pH 6.0 (data not shown). It was stable only in a narrow range (pH 6.0−8.0).

Thermostability of CGTase. More than 90% of the enzyme activity remained after 60 min of incubation at 60 °C, and almost 50% remained after 10 min of incubation at 80 °C (Figure 4). The enzyme was stabilized by Ca^{2+}, similar to other amylolytic enzymes (Vihinen and Mantsala, 1989). Over 90% of enzyme activity remained after 60 min of incubation at 70 °C in the presence of 15 mM $CaCl_2$ (Figure 4). Thermal inactivation of the enzyme followed first-order kinetics in the range of temperature between 70 and 90 °C. The thermodynamic data are summarized in Table 2. In the presence of Ca^{2+} ion, enthalpy and entropy changes for enzyme inactivation, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, respectively, increased while Gibb's free energy, $\Delta G^{\ddagger}$, was not changed. Thermostability of the enzyme was also confirmed by DSC analysis (data not shown). Denaturation of the enzyme commenced at 76.6 °C (T_0), and the rate of denaturation occurred maximally at 79.1 °C (T_p). The conclusion temperature (T_c) of phase transition was 81.7 °C . These data correlated well with the results described above.

Production of CD from Soluble Starch by CGTase. The CD mixture produced by CGTase was analyzed by HPLC (Figure 4) and TLC. In an hour of reaction, 44% (w/w) of starch was converted to CDs. α-(Figure 5, peak 1), β- (Figure 5, peak 2), and γ-CDs (Figure 5, peak 3) were produced in the ratio of 4.2:5.9:1. Reaction temperatures for cyclization between 60 and 90 °C did not affect the yield of CD production. Conversion of starch to CD by the enzyme was tested using 5, 9, 13, 17, 20, and 23% (w/v) starch slurries. The CD conversion rate decreased as the concentration of starch increased, even though the net amount of CD obtained was highest when 13 or 17% (w/v) starch slurry was used. Considering the conversion rate and the amount of CD produced, the most appropriate substrate concentration for the production of CD using the enzyme was 9−13% (w/v).

The effect of DE value on CD yield was also studied. Cornstarch (13%, w/v) was liquefied by CGTase as described above. During the liquefaction process, aliquots were removed at several time points to determine DE values. Yields of CD were measured after 1 h of incubation with the enzyme at 60 °C. Conversion to CD was also affected by degree of starch hydrolysis; oligosaccharides with DE value about 10 was most effective for the cyclization reaction in the range of DE 1−22. The conversion rate dropped sharply when DE of oligosaccharides was controlled to 13 and continued to decrease as DE increased over 20. The conversion was faster when there were more reducing ends per mole of CGTase as starch was liquefied to shorter length. However, when liquefaction was allowed to proceed further to produce oligosaccharides with <6 glucose units, it resulted in poor conversion to CDs.

CGTase Gene of *B. stearothermophilus* ET1. The structural gene for CGTase was cloned in *Escherichia coli*. The structural gene had capacity to encode a protein of 711 amino acid residues with a molecular mass of 79 300 Da. The nucleotide sequence of the gene and the deduced protein sequence for the corresponding gene product shared 84 and 88% identity, respectively,

Table 1. Purification of CGTase from the Supernatant of *B. stearothermophilus* ET1 Culture

	vol (mL)	act. (units)	protein (mg)	sp act. (units/mg)	purifn (fold)	yield (%)
culture broth	10,000	639	920	4.89	1	100
70% $(NH_4)_2SO_4$ precipitate	380	504	608	5.84	1.2	78.9
β-CD affinity column	7.5	345	7.03	346	70.8	54.0
DEAE-8HR column	13.0	201	3.20	443.8	90.8	31.6

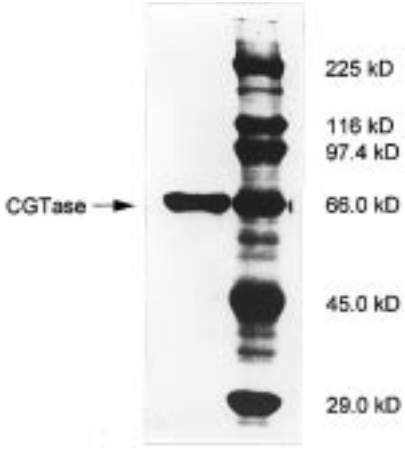

Figure 2. SDS−PAGE analysis of purified CGTase. Purity of CGTase harvested from peak A of Figure 1 was tested by electrophoresis using a 10% SDS−polyacrylamide gel. Lane 1 was loaded with purified CGTase; lane 2 indicates size markers. The size markers used are myosin from rabbit muscle (205 kDa), β-galactosidase of *E. coli* (116 kDa) phosphorylase *b* from rabbit muscle (97.4 kDa), bovine albumin (66 kDa), yolk albumin (45 kDa), and carbonic anhydrase from bovine erythrocytes (29 kDa).

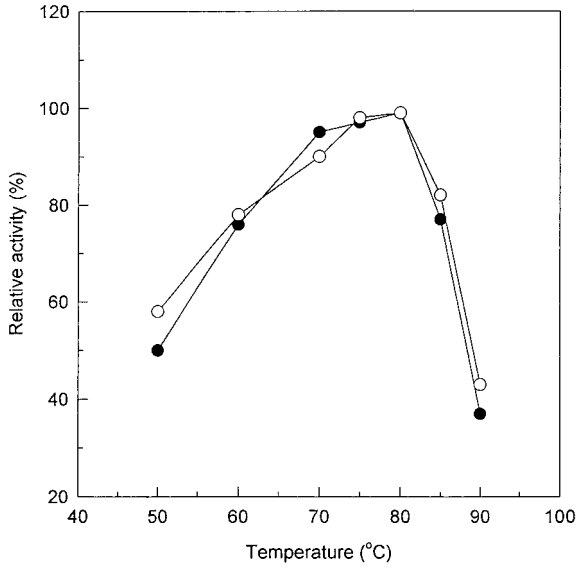

Figure 3. Effect of temperature on CGTase activity. Temperature optimum of the CGTase enzyme was determined using 4% (w/v) soluble starch in 50 mM maleate−NaOH buffer (pH 6.0) with 15 mM (○) or without $CaCl_2$ (●).

with the sequences of *B. stearothermophilis* NO2 strain that was reported by Fujiwara et al. (1992; Figure 6) However, the upstream and downstream DNA sequences of the two structural genes showed lower homology: upstream, 78%; downstream, 36%.

Expression of the CGTase gene was poor and the product unstable in *E. coli*, even though the gene was preceded by a putative ribosome binding site, GGAGGA, seven nucleotides upstream and a good consensus sequence (TTGACA for −35 and TATTAT for −10 with

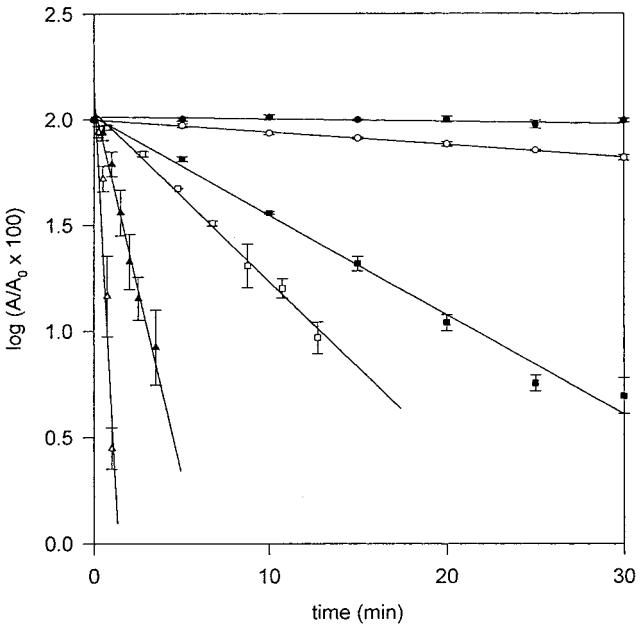

Figure 4. Thermostability of CGTase at various temperatures. Thermostability of the enzyme was tested at 70 (○), 80 (□), and 90 (△) °C. Solid symbols represent thermostability of the enzyme in the presence of 15 mM $CaCl_2$ at corresponding temperatures. Residual enzyme activity at each time point was represented as *A* and initial enzyme activity at time zero as A_0. The bars represent standard deviations.

17 nucleotides between) for a putative promoter. Expression and stability of CGTase were improved when the gene was subcloned on a *Bacillus* vector, pUB140, and expressed in *B. subtilis*. *B. subtilis* cells harboring the CGTase clone produced 10 times as much of the enzyme as *B. stearothermophilus* ET1. The CGTase produced from the clone exhibited the same properties as the enzyme purified from the mother strain.

DISCUSSION

B. stearothermophilus ET1 produced at least two extracellular enzymes and an intracellular amylolytic enzyme, a classical amylase, a CGTase, and a maltogenic amylase (unpublished data). Synthesis of CGTase was initiated at the onset of exponential growth in the presence of starch and remained constant for the rest of growth. This correlated well with the data reported by Jamuna et al. (1993) on the pattern of CGTase production by *Bacillus cereus*. In *B. cereus*, CGTase was produced during the early exponential growth and reached maximum during the midsporulating stage (16 h). However, only a small amount of purified CGTase (3.2 mg from 10 L of culture) could be obtained from the culture of *B. stearothermophilus* ET1, indicating very weak expression of the gene encoding the enzyme in the cells. This suggests that isolation and manipulation of the corresponding gene are desirable for industrial application of the enzyme.

The CGTase of *B. stearothermophilus* ET1 was purified more efficiently by using β-CD affinity chromatog-

Table 2. Kinetic Analysis of CGTase Inactivation

	without Ca^{2+}			with Ca^{2+}		
	70 °C	80 °C	90 °C	70 °C	80 °C	90 °C
rate constant (k; s^{-1})	$(9.90 \pm 0.20) \times 10^{-5}$	$(1.34 \pm 0.10) \times 10^{-3}$	$(2.90 \pm 0.17) \times 10^{-2}$	$(1.88 \pm 0.15) \times 10^{-5}$	$(7.79 \pm 0.16) \times 10^{-4}$	$(2.44 \pm 0.24) \times 10^{-3}$
$\Delta H^{\ddagger}$ (kJ/mol)		284.6 ± 3.9			250.0 ± 9.3	
$\Delta S^{\ddagger}$ (J/mol·K)		540.6 ± 11.5			429.7 ± 26.9	
$\Delta G^{\ddagger}$ (kJ/mol)		93.8 ± 4.4			98.3 ± 3.9	

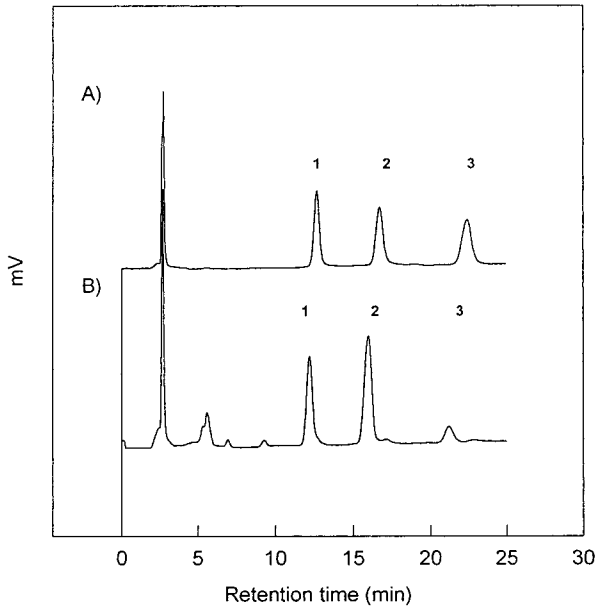

Figure 5. HPLC chromatogram of CD mixture produced by the action of CGTase. The CD mixture produced by CGTase was analyzed using a Lichrosorb NH_2 column. Panel A represents a chromatogram of standard: peak 1, α-CD; peak 2, β-CD; peak 3, γ-CD. Panel B represents a chromatogram of the CD mixture produced.

raphy followed by HPLC than any other method tested in the laboratory. Ion exchange chromatography was not efficient in removing other amylolytic enzymes and the yield was very poor. The enzyme was not eluted from the affinity column by a buffer containing high concentration of salt (0.5 M), but by a buffer with higher concentration of β-CD. Ahn et al. (1990) reported that CGTase has at least two binding sites for β-CD and elution with higher concentration of β-CD is a result of mass action effects.

Purity of the enzyme was confirmed by SDS−PAGE followed by Coomassie blue staining (Figure 1). The gel was also visualized by silver staining to confirm the purity of the preparation. A single protein band was observed where proteins with molecular mass of 66 800 Da would appear, indicating the enzyme preparation was not contaminated by an α-amylase. Molecular mass of CGTases isolated from various bacteria vary greatly from 64 000 to 200 000 Da (Hofman et al., 1989; Kitahata et al., 1974; Kitahata and Okada, 1982; Kobayashi et al., 1978; Makela et al., 1988; Nakauma and Horikoshi, 1976; Tomita et al., 1993; Yagi et al., 1986; Marechal et al., 1996). They are also distinctive from each other not only in p*I*, optimum pH, and temperature but also in the major products they generate. Among these properties, optimum reaction temperature and major products are the most important properties that should be considered for commercial application. Most of the CGTases isolated to date are active around 55 °C and below (Hofman et al., 1989; Lee and Kim 1991; Sabioni and Park, 1992; Yagi et al., 1986). Only a few of them have optimum temperature above 70 °C. A CGTase of *Thermoanaerobacter* has a temperature optimum of 90−95 °C at pH 6.0, which is the highest of all CGTases known so far (Norman and Jørgensen, 1992). The enzyme was capable of liquefying 35% (w/v) starch slurry at pH 4.5. Starnes et al. (1991) and Wind et al. (1995) also reported the isolation of thermostable CGTases from *Thermoanaerobacterium thermosulfurigenes*. Optimum temperatures and pH values were slightly different among these CGTases.

The CGTase of *B. stearothermophilus* ET1 showed the highest activity at 80 °C and could liquefy starch slurry without the addition of α-amylase. Several research groups have reported CGTases of *B. steartothermophilus* species. CGTases reported by Fujiwara et al. (1992) and Ahn et al. (1990) had optimum temperatures of 65 and 60 °C, respectively. The stabilizing effect of Ca^{2+} on CGTase shown in Table 2 and Figure 4 indicated that Ca^{2+} molecules increased the heat stability of CGTase. Akimaru et al. (1991) observed that saturation of CGTase with Ca^{2+} resulted in an increase of heat stability and the optimum temperature of the enzyme shifted from 60 to 75 °C. The onset temperature of CGTase denaturation was determined by the DSC method. A single transition peak was observed with an onset temperature of 76.6 °C. Maximum temperature (T_p; 79.1 °C) in a DSC thermogram does not mean the inactivation rate is the highest but the reaction proceeds at the maximum in the DSC pan as a reaction vessel. The DSC data were in good agreement with those of the thermal inactivation curve stating a significant inactivation at 80 °C.

Nucleotide sequence analysis of the CGTase clone indicated that the two *B. stearothermophilus* strains, ET1 and NO2 (Fujiwara et al., 1992), shared extensive homology at both DNA sequence and predicted amino acid sequence levels. The NO2 strain reported by Fujiwara et al. (1992) contains at least three structural CGTase genes with different nucleotide sequences encoding the same amino acid sequences. The amino acid sequence of CGTases in the NO2 strain was 99% identical with that of *B. stearothermiphilus* TC-91 strain (Sakai et al., 1987). Therefore, the ET1 strain was likely to have undergone more rearrangement in the CGTase gene than the other two strains in the course of evolution. Four domains known to be conserved among amylolytic enzymes had identical sequences in these strains. The difference in thermostability and CD production profile between the CGTases of these strains might be due to the small differences in the amino acid sequence.

The CGTase clone was expressed very poorly in *E. coli* despite the presence of good signals for translation and transcription. It could be due to high A+T content of the gene: 68% in the upstream region and 62% in the structural gene. Codon usage bias, which could be another reason for poor expression of the gene in *E. coli*,

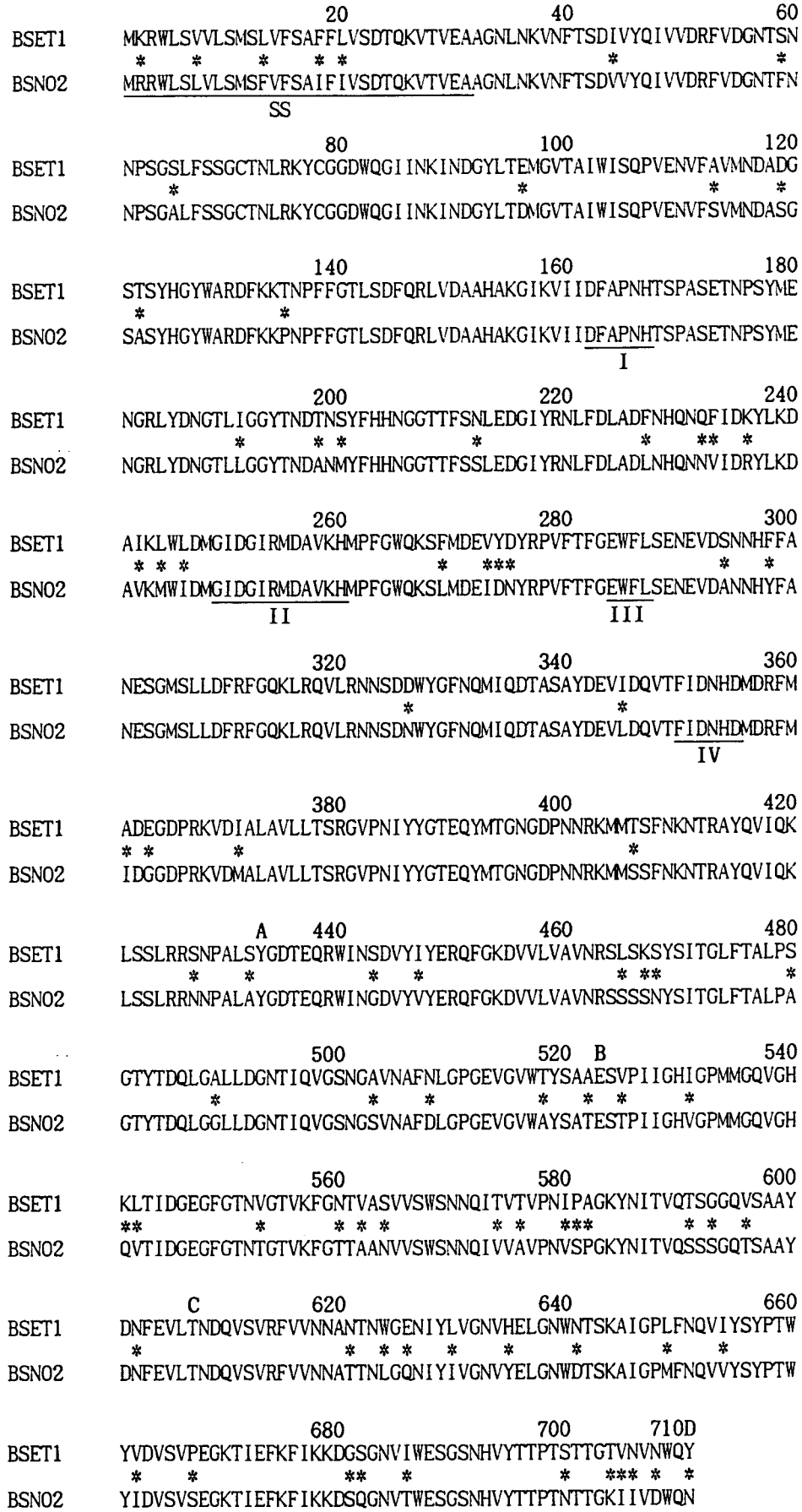

Figure 6. Comparison of the predicted amino acid sequences of CGTases from *B. stearothermophilus* strains ET1 and NO2. The amino acid residues were aligned without a gap, and those not identical are marked by asterisks. The signal sequence and four conserved domains are underlined. BSET1 represents the amino acid sequence of *B. stearothermophilus* ET1 and BSNO2 that of *B. stearothermophilus* NO2.

was observed for several amino acids including tyrosine, glutamine, lysine, aspartic acid, glutamic acid, threonine, and phenylalanine.

The CGTase enzyme isolated from *B. stearothermophilus* ET1 would be useful in commercial production of CDs in that it is thermostable and effective in starch liquefaction. Utilization of this enzyme for CD production would be superior to the CGTase from *Thermoanaerobacter* in a couple of aspects. First, it is originated from a bacterial strain that is affirmed as generally recognized as safe by the U.S. Food and Drug Administration (FDA; Docket 83G-0227). The gene encoding CGTase of *Thermoanaerobacter* was cloned, and the enzyme was produced from a heterogeneous bacterial host strain carrying the clone due to difficulties in cultivating the anaerobic mother strain. However, *Thermoanaerobacter* is not recognized as a safe strain by FDA to our knowledge. Second, the CGTase of *B. stearothermophilus* ET1 seemed to be more favorable for the process control, since it is less thermostable than the CGTase of *Thermoanaerobacter*. The former would be inactivated more readily by heat upon the completion of the CD manufacture process. Site-directed mutagenesis of the gene and fusion of the structural gene to a stronger promoter would improve the enzyme properties and production of the enzyme, respectively.

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