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Cyclodextrin Glucanotransferase from *Bacillus stearothermophilus*: Purification by Affinity Chromatography and Its Properties

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*Bacillus stearothermophilus*가 생산하는 Cyclodextrin Glucanotransferase : Affinity Chromatography를 이용한 정제 및 성질

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The cyclodextrin glucanotransferase (CGTase) was purified from the culture broth of *Bacillus stearothermophilus* by ammonium sulfate precipitation and affinity chromatography. The specific activity of the CGTase increased by about 31-fold from 111.5 U/mg protein to 3445.0 U/mg protein. The SDS-PAGE indicated that the purified CGTase was homogeneous and the molecular weight of the purified CGTase was about 78,000. The optimum pH and temperature was 6.0 and 60°C, respectively. This enzyme was stable from pH 5.5-10.0. The enzyme retained its full activity at the incubation temperature up to 60°C and calcium ion increased the thermal stability. The isoelectric point was about 4.8.

Cyclodextrin exists mainly as three different forms; α-, β-, and γ-cyclodextrin, consisting, respectively of six, seven, and eight glucose monomers. These compounds have several unique properties as follows; a) as consequence of the cyclic arrangement, they have neither a reducing end nor non-reducing end group. Hence, they are not hydrolyzed by any aqueous alkali. b) they are resistant to acid hydrolysis. c) they are less susceptible to amylase action. d) due to the hydrophobic nature of the cavity, cyclodextrins are able to interact noncovalently to form stable inclusion complex (1, 2). Due to the cyclodextrin's properties above, cyclodextrin can be used to control flavor release, mask odors and tastes, stabilize emulsions, increase foaming power and control the color.

The cyclodextrin is formed from starch enzymatically by the cyclodextrin glucanotransferase (CGTase). In addition, the CGTase catalyzes the hydrolysis of cyclodextrin and transfer of the linear maltooligosaccharides to a cosubstrate and the disportion of small linear oligosaccharide (3-6). The CGTase has been found in many microorganisms. *Bacillus macerans* (7), *Bacillus megaterium* (8), *Bacillus circulans* ATCC 9995 (9), alkalophilic *Bacillus* sp. (10), and *Bacillus stearothermophilus* TC-60 (11) are reported to produce the CGTase. The CGTases from these microorganisms were purified and were examined the properties.

In this study, we purified the CGTase from *Bacillus stearothermophilus,* which was isolated from soil (12), by affinity chromatography and examined some properties of the CGTase.

Key words: *Bacillus stearothermophilus,* cyclodextrin glucanotransferase, affinity chromatography
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Materials and Methods

Materials

Epoxy-activated Sepharose 6B, and α, β, γ-cyclodextrin were purchased from Sigma. Defatted soybean meal was purchased from Dong Bang Yoo Ryang. Acrylamide and bis acrylamide were purchased from Bio Red. Ampholyte was purchased from Pharmacia. The other chemicals were purchased from Kan-

to Chemical Co.

Bacterial strain

Bacillus stearothermophilus was isolated from soil (12).

Immobilization of β-cyclodextrin

4g of epoxy-activated Sepharos 6B was reconstituted in distilled water for 1 hr. The gel was transferred to 0.1 M NaOH and excessive liquid was removed. The gel was reacted with a solution of 300 mg β-cyclodextrin in 12 m*l* 0.1 M NaOH at 45°C for 16 hrs. After incubation the gel was washed with distilled water for 1 hr and with 0.01 M sodium acetate buffer (pH 6.0) for 2 hrs. The gel was packed in 10×150 mm glass column and equilibrated with 0.01 M sodium acetate buffer (pH 6.0) (17).

Enzyme preparation

The stock culture of *Bacillus stearothermophilus* was cultivated at 55°C for 48 hrs in 500 m*l* Erlenmeyer flask (broth 100 m*l*). The medium used for the production of the CGTase was composed of 2% soluble starch, 0.5% defatted soybean meal, 0.1% $NaH_2PO_4 2H_2O$, and 0.015% $CaCl_2$ (12).

Solid ammonium sulfate was added to the supernatant obtained by centrifugation of the culture to 20-80% saturation. Ammonium sulfate precipitate was dialyzed in deionized water overnight, at 4°C.

Affinity chromatography

The dialyzate from ammonium sulfate precipitation was loaded onto 10×150 mm column of β-cyclodextrin-bound-epoxy-activated Sepharose 6B whihc was pre-equilibrated with 0.01 M sodium acetate buffer (pH 6.0). The flow rate was 16 m*l*/hr and the fraction volume was 3 m*l* per cell.

The elution of the bound enzyme was carried out by 10 mg/m*l* solution of β-cyclodextrin in 0,.01 M sodium aceate buffer (pH 6.0). The fractions were assayed for the CGTase activity and protein content.

Assay of the enzyme activity and protein determination

Since the crude enzyme showed no apprecible amylolytic activity, dextrinizing activity was used as the enzyme activity.

The reaction mixture containing 0.5 m*l* of enzyme solution and 4.5 ml of 0.55% soluble starh in 0.1 M sodium acetate buffer (pH 5.5) was incubated at 55°C (13). After 10 min reaction, 0.5 m*l* was taken cut from the reaction mixture and added to 4 m*l* of 0.01 M iodine in 0.25 M potassium iodide. This mixture was diluted to 20 m*l* with the distilled water, and then the light transmission of the solution was measured at 660 nm in Beckman M-20 spectrophotometer. One unit of the enzyme was defined as the amount of the enzyme which gave a linear increase of 1% transmission per minute at 55°C.

The protein content was measured by the dye-binding method using bovine serum albumin as the standard protein (14).

SDS-Polyacrylamide gel electrophoresis and isoelectric focusing

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Choi and Dreyfuss (15) using myosin (MW 200,000), β-galactosidase (MW 116,250), phosphorylase B (MW 97,400), Bovine serum albumin (MW 66,200), and albumin (MW 45,000) as th standard proteins.

The isoelectric point of the enzyme was measured by O'Farrell's method (16).

Optimum pH and temperature

The optimum pH of the enzyme was determined with the reaction mixture following below. The mixtures consisted of 0.5 m*l* of the enzyme solution and 4.5 m*l* of 0.55% soluble starch in 0.1 M sodium acetate buffer (pH 3.5-6.0), 0.1 M tris-malate-NaOH buffer (pH 6.0-8.5) and 0.1 M glycine-NaOH (pH 8.5-10.5), respectively. Each reaction mixture was incubated at 55°C for 10 min.

The optimum temperature of the enzyme was determined with the mixture of 0.5 m*l* of the enzyme solution and 4.5 m*l* of 0.55% soluble starch in 0.1 M sodium acetate buffer (pH 6.0). The mixture was incubated from 40 to 80°C by 5°C interval for 10 min.

The pH and thermal stability

The pH stability was determined as follows; 0.5 m*l* of enzyme solution was incubated in 0.1 M sodium acetate buffer (pH 3.5-6.0), 0.1 M tris-malate-NaOH buffer (pH 6.0-8.5) and 0.1 M glycine-NaOH buffer (pH 8.5-10.5), respectively, for 30 min at 60°C. The remaining activity was assayed by the standard method.

The thermal stability was determined as follows; 0.5 m*l* of the enzyme solution was incubated from 50°C to 80°C by 5°C interval for 30 min in 0.1 M sodium acetate buffer (pH 6.0). The remaining activity was assayed by the standard method.

The effect of calcium ion on the thermal stability

The effect of calcium ion on the thermal stability was determined as follows; 0.5 m*l* of the enzyme solution was incubated from 50 to 95°C by 5°C interval for 30 min in 0.0043 M Ca^{2+} and 0.1 M acetate buffer (pH 6.0). The remaining activity was assayed by the standard method.

Results

Purification of the CGTase by ammonium sulfate precipitation and affinity chromatography

The two day-culture of *B. stearothermophilus* was centrifuged and the supernatant was recovered. Ammonium sulfate was added to the supernatant from 20 to 80%. The 65% of the activity in the crude enzyme was recovered by ammonium sulfate precipitation (Table 1).

The ammonium sulfate precipitate was dialyzed and the dialyzate was loaded onto β-cyclodextrin-bound-epoxy activated Sepharose 6B column. After being loaded, the column was washed with 0.1 M sodium acetate buffer (pH 6.0). The unwanted proteins being removed, the CGTase was eluted with 10 mg/m*l* β-cyclodextrin in 0.1 M sodium acetate buffer (pH 6.0) (Fig. 1). The individual fractions were determined for the enzyme activity and protein content. An almost symmetric peak in which protein content and enzyme activity overlapped was obtained. Fraction 31 to 34 were combined and dialyzed in distilled water. The result of the purification was summarized in Table 1. After the affinity chromatography, the enzyme was purified by 31-fold with 42% yield over the crude enzyme. The specific activity of the purified enzyme was 3445 (U/mg protein).

Some properties of the purified CGTase

The purified enzyme was assayed in various buffers (pH 3.5-10.5) under the standard condition. The enzyme was most active in pH 6.0 (Fig. 2).

The purified enzyme was dissolved in various buffers (pH 3.5-10.5) and incubated at 60°C for 30 min. After the enzyme was brought to pH 6.0, the

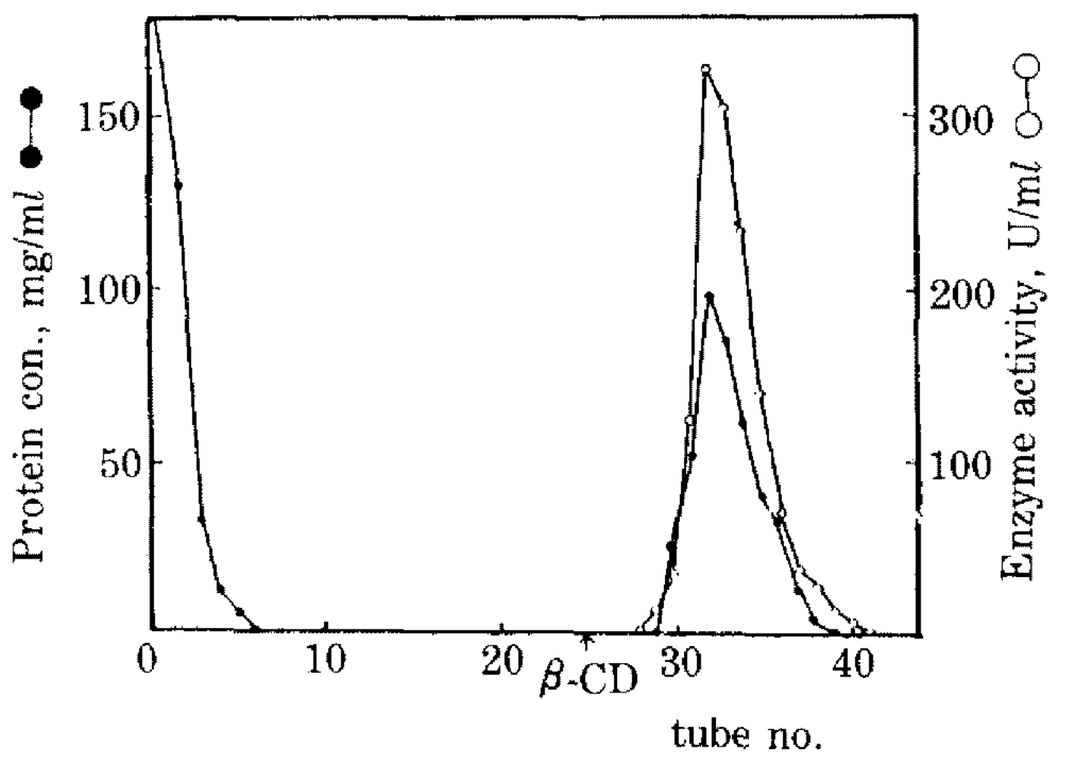

Fig. 1. Affinity chromatography of ammonium sulfate precipitates of the CGTase.

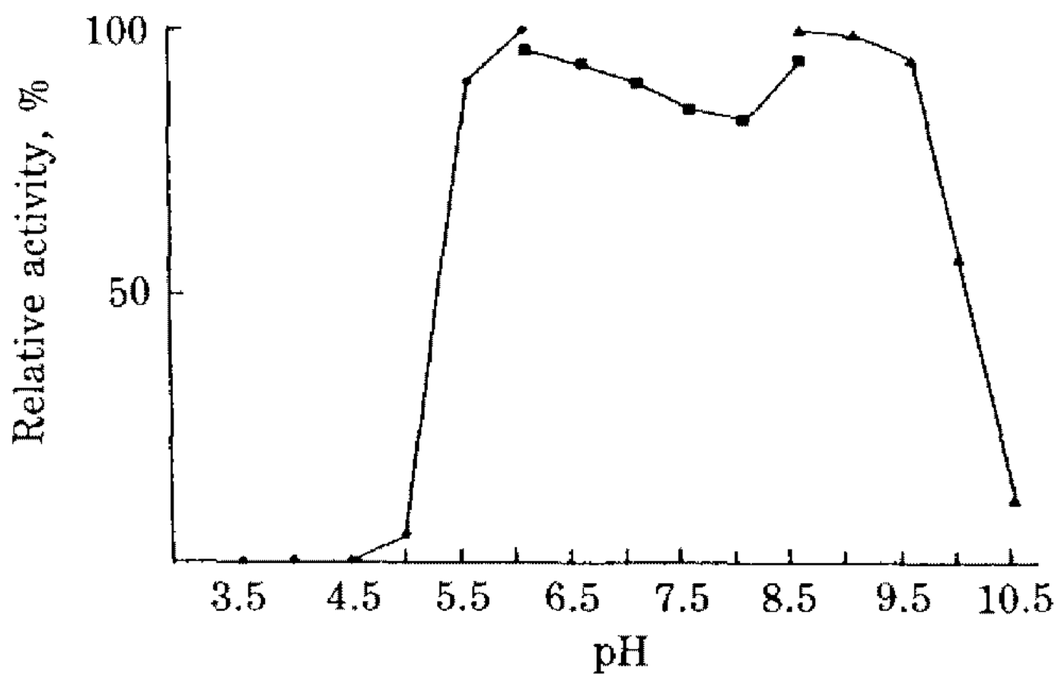

Fig. 2. Optimum pH of the CGTase.
●—● 0.1 M Acetate buffer, ■—■ 0.1 M Glycine-NaOH buffer, ▲—▲ 0.1 M Tris-malate-NaOH buffer

Table 1. Purification of the CGTase from *Bacillus stearothermophilus*

Step	Volume (m*l*)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Culture broth	900	80.1	8,298	111.5	100	1
$(NH_4)_2SO_4$Preci. 20-80%	87	36.2	5,394	149	65	1.34
Affinity chromatography	15.5	1.01	3,479.4	3,445	42	31

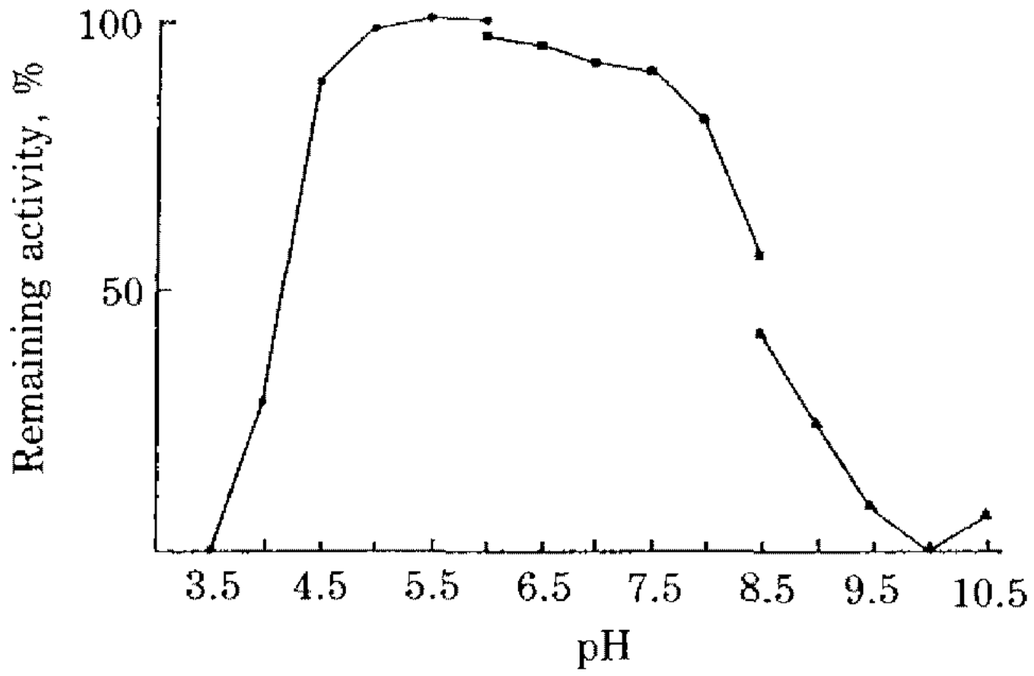

Fig. 3. pH stability of the CGTase.
●—● 0.1 M Acetate buffer, ■—■ 0.1 M Tris-malate-NaOH buffer, ▲—▲ 0.1 M Glycine-NaOH buffer

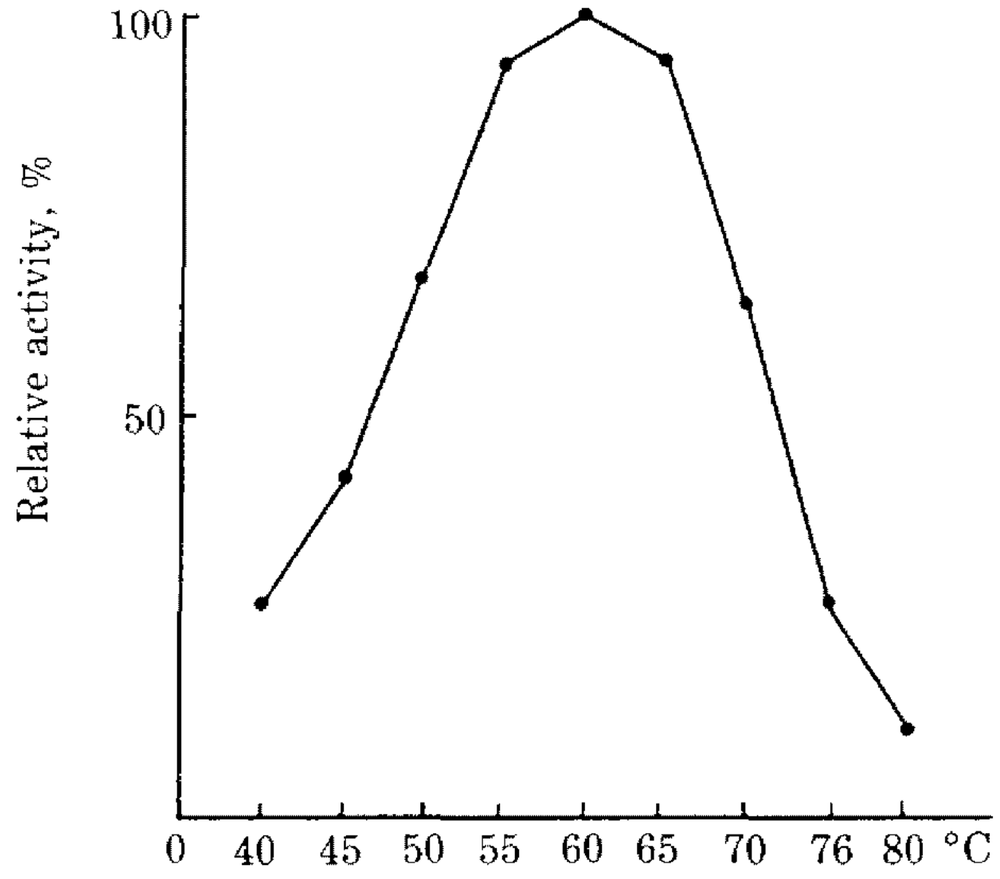

Fig. 4. Optimum temperature of the CGTase.

remaining activity of the enzyme was determined by the standard assay condition. This enzyme was stable from pH 5.5 to 9.5 (Fig. 3).

The purified enzyme activity was measured at various temperatures by the standard assay method. The optimum temperature for the enzyme was about 60°C (Fig. 4).

The enzyme was dissolved in 0.1 M sodium acetate buffer (pH 6.0) and incubated for 30 min at various temperatures. The remaining activity was measured by the standard method. The enzyme was stable at temperatures below 55°C as shown in Fig. 5. The reaction was also done in the presence of 0.0043 M Ca^{2+} (in the form of $CaCl_2$). The CGTase was stable at temperatures below 65°C (Fig. 5). The thermal stability of the enzyme was increased by calcium ion.

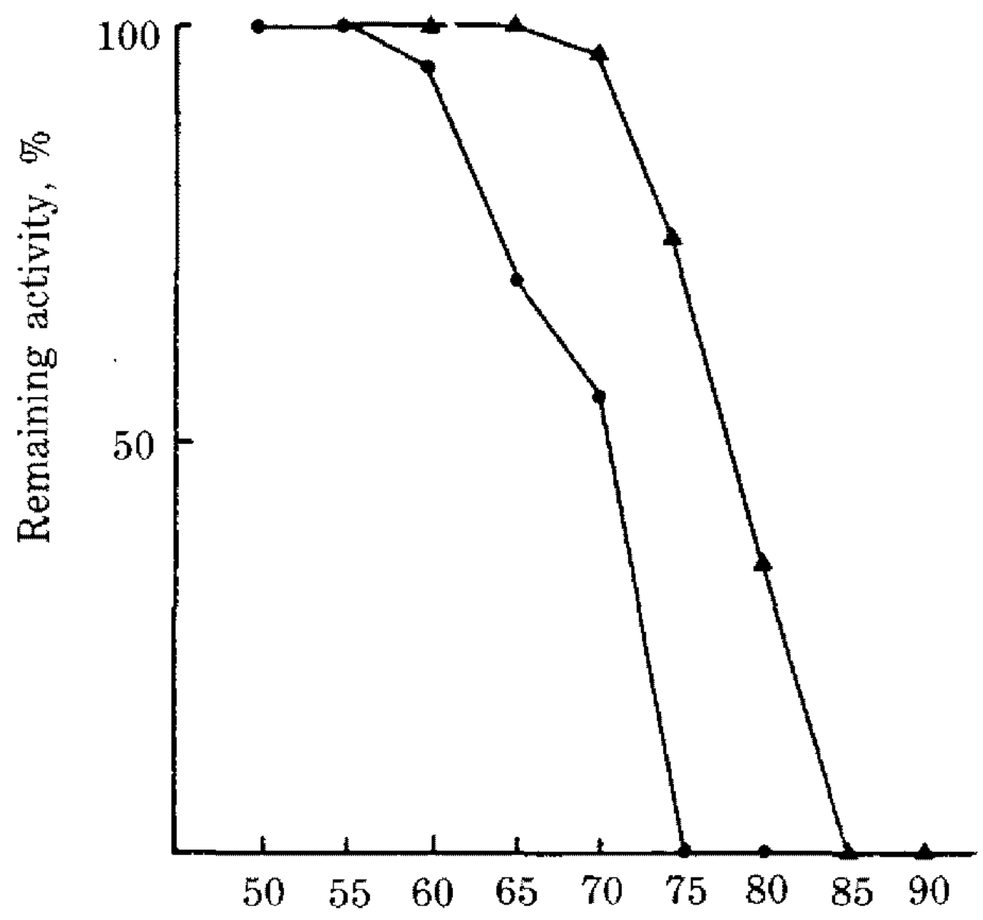

Fig. 5. Thermal stability of the CGTase with and without calcium ion.
●—● No Ca^{2+}, ▲—▲ 4.3 mM Ca^{2+}

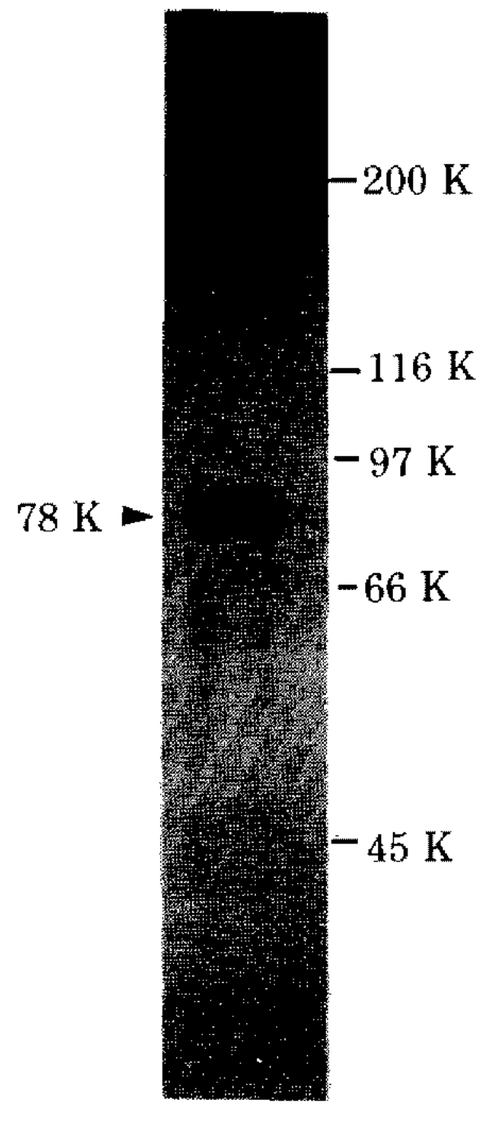

Fig. 6. SDS-PAGE of the purified CGTase.

SDS-Polyacrylamide gel electrophoresis and isoelectric focusing

To examine the molecular weight of the purified enzyme, the SDS-polyacrylamide gel electrophoresis was performed. The 10 ug of purified enzyme was applied to 7.5% gel. The molecular weight of this enzyme was 78,000 when calibrated with standard protein (Fig. 6). The figure shows single band, which suggests

the homogeneity of the purified enzymes.

To examine the isoelectric point of the purified enzyme, we used the ampholyte in range of pH 3.5-10.0. The isoelectric point of this enzyme was about 4.8.

Discussion

The CGTases from *B. macerans* (3), *B. megaterium* (4), *B. circulance* ATCC 9995 (5). alkalophilic *Bacillus* sp. (6), *B. stearothermophilus* TC-60 (7) were purified and characterized. All CGTases from the *Bacillus* species were purified by starch adsorption method, ammonium sulfate precipitation, ion exchange and gel filtration chromatography. We purified the CGTase from *B. stearothermophilus* (12) by using the β-cyclodextrin-bound-epoxy activated Sepharose 6B. This method could purify the CGTase in one step after ammonium sulfate precipitation without other purification procedures.

The affinity chromatography using α-cyclodextrin-bound-epoxy activated Sepharose 6B was used in purifying α-amylase (14). In this experiment, α-amylase was eluted with α-cyclodextrin-containing buffer. In our preliminary study, α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin were bound to epoxy activated Sepharose 6B, respectively. The binding capacity of the CGTase was better to β-cyclodextrin than to any other cyclodextrins. The interaction between CGTase and β-cyclodextrin which is the substrate of the CGTase was so specific that we could obtain the highly-purified CGTase.

The mechanism of the interaction between the β-cyclodextrin and CGTase is not clear. When high concentration of salt was used to elute the bound-CGTase, the CGTase was not eluted. It indicates that the interaction is not a result of charge effects. The enzyme, however, was eluted with the more concentrated β-cyclodextrin solution. It suggests that the CGTase has at least two binding sites for β-cyclodextrin and it is a result of mass action effects.

The estimated molecular weight of the purified enzyme was about 78,000 by SDS polyacrylamide gel electrophoresis. The molecular weight of the CGTase from *B. stearothermophilus* TC-60 was 70,000 (11). The gene coding the CGTase from *B. stearothermophilus* was cloned and determined its nucleotide sequence. The molecular weight of the CGTase from the predicted amino acid sequence was 75,459 (18). The CGTase of *B. macerans* consists of two subunits, molecular weight of each being 74,000 (7). The molecular weight of the CGTase from other *Bacillus* sp. was determined. In *B. circulans,* it was about 103,000 (9), and in alkalophilic *Bacillus* sp., it was 88,000 (10). It was known that the CGTase from *Bacillus* sp. except that of *B. macerans* was not composed of multi subunits.

S. Kitahata and S. Okada also purified the CGTase from *B. stearothermophilus* TC-60 (7). They used the starch-absorption method, the DEAE-Sephadex and the Biogel P-150 method. The properties of the CGTase between two differently-purified enzyme were similar. The optimum pH was 6.0. The difference of the optimum temeperature between two was 10°C. The pH and thermal stability of ours were better. The isoelectric point between two-differently purified enzyme was slightly different; 4.5 and 4.8 respectively. Whether two diffrently-purified enzymes mentioned above are the same is not clear. More reseaches are needed.

The addition of calcium ion to enzyme solution increased the thermal stability. The CGTase from an alkalophilic *Bacillus* sp. also showed the increased the thermal stability when 0.01 M $CaCl_2$ was added to (6). The above results suggested that calcium ion in creased the thermal stability of the CGTase.

요 약

Bacillus stearothermophilus 가 생산하는 cyclodextrin glucanotransferase (CGTase)를 ammonium sulfate 법과 affinity chromatography 법에 의해 정제하였다. 이 CGTase의 비활성은 111.5 U/mg protein에서 33445.0 U/mg protein으로 증가하였다. 이 효소의 분자량을 측정하기 위해 SDS-PAGE를 실시한 결과 분자량은 약 78,000이었다. 이 효소의 최적 pH와 온도는 각각 6.0과 60℃이었다. 이 효소는 pH 5.5와 10.0에서 안정하였다. 이 효소에 calcium ion을 첨가하였더니 열안정성이 증가하였다. 이 효소의 등전점은 약 4.8이었다.

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