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Purification and Characterization of Cyclodextrin Glycosyltransferase from Alkalophilic *Bacillus* sp.

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호알카리성 *Bacillus* sp.의 cyclodextrin glycosyltransferase의 정제와 특성

정용준 · 공인수 · 강윤숙 · 유주현*

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Alkalophilic *Bacillus* sp. YC-335 isolated from soil was capable of producing large amount of cyclodextrin glycosyltransferase (CGTase) in culture broth. This enzyme was successively purified 52.9 folds with 17.8 yield by ethanol precipitation, DEAE-Toyopearl column chromatography and Sephadex G-100 column chromatography. The purified enzyme have a molecular weight of approximately 75,000 estimated by SDS polyacrylamide gel electrophoresis. The optimum pH and temperature for the enzyme activity were 6.0 and 50°C, respectively. The enzyme was stable between pH 6 and 10, and up to 50°C. The thermostability of the enzyme was increased up to 60°C by the addition of 15 mM $CaCl_2$.

Cyclodextrin glycosyltransferase (α-1, 4-glucan-4-glycosyltransferase, EC 2.4.1.19, CGTase) is produced by some certain species of *Bacillus*, i.e., *B. macerans* (1, 2), *B. circulans* (3), *B. stearothermophilus* (4), *B. megaterium* (5), *B. ohbensis* (6) and alkalophilic *Bacillus* sp. (7). These enzymes catalyze the formation of cyclodextrins from starch and related carbohydrates (8, 9).

From soil collected from several areas in Korea, we succeeded in isolating a strain of alkalophilic bacterium, *Bacillus* sp. YC-335 which produce large amount of CGTase in the culture broth. In the previous paper, we reported some taxonomical characteristics of the strain, and the properties of the crude enzyme (10).

In this paper, we described the purification method and some characteristics of the CGTase.

Key words: Alkalophilic *Bacillus* sp. cyclodextrin glycosyltransferase

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Materials and Methods

Bacterial strains, media and preparation of the crude enzyme

The alkalophilic *Bacillus* sp. YC-335 was cultured at 37°C for 48-60 hr under continuous shaking in the medium containing 1.5% soluble starch, 5% corn steep liquor, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% $CaCO_3$, 1% Na_2CO_3 (pH 10.3). The crude enzyme was prepared under the same conditions as previously reported (10). The cell was removed by centrifugation and 3 volumes of cold ethanol were added to 1 volume of the culture supernatant. The mixture was stored in a cold room for overnight, and the resulting precipitate was separated by centrifugation and dried in vacuo.

Assay for enzyme activity

CGTase activity was determined by the glucoamylase method (7). One unit of enzyme activity is defined as the amount of enzyme to form 1 μ mol

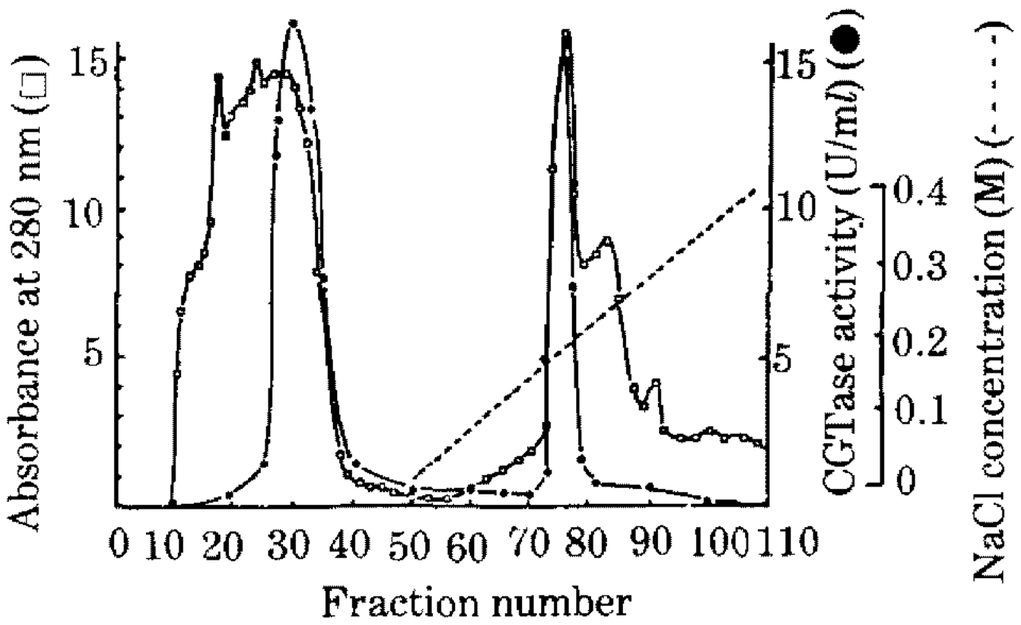

Fig. 1. DEAE-Toyopearl column chromatography (4 × 19 cm) elution volume: 10 m*l*/fraction

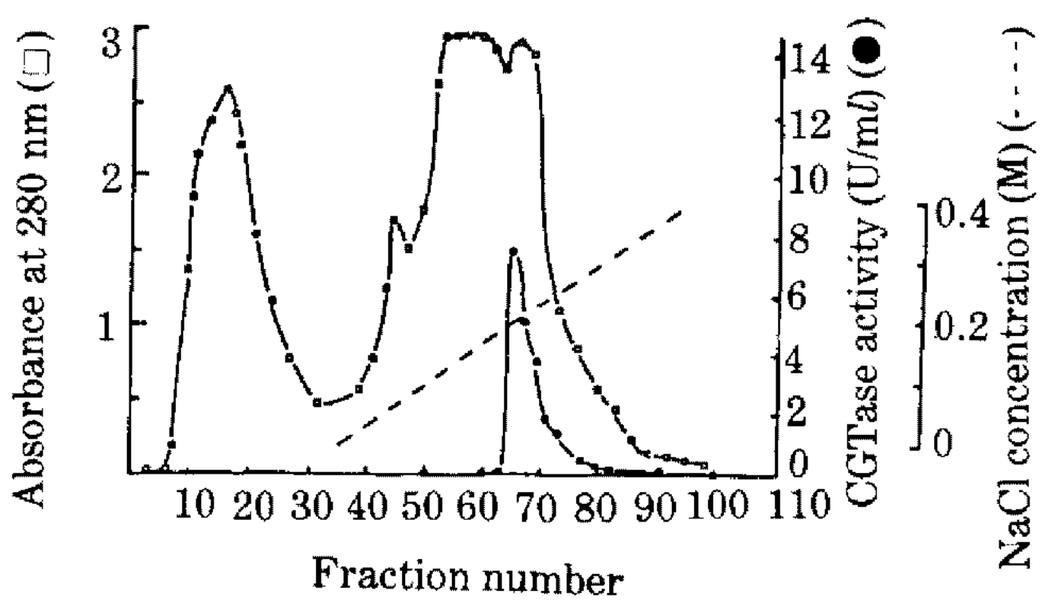

Fig. 2. DEAE-Toyopearl column rechromatography (4 × 19 cm) elution volume: 10 m*l*/fraction

of glucose per min under the conditions described previously (10).

Determination of protein

Protein concentrations were determined by the method of Lowry *et al.* (11) using bovine serum albumin as standard.

SDS-gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) were performed according to the method of Laemmli (12) with 10% polyacrylamide gel containing 0.1% SDS and Tris-glycine buffer (pH 8.5) at a constant current of 20 mA. The proteins were stained with Coomassie brilliant blue R-250.

Determination of molecular weight

The molecular weight of the enzyme was estimated by high performance liquid chromatography on a Protein-Pak I-250 gel filtration column. The standard proteins (Sigma) used for calibration were as follows; β-amylase (Mw. 200,000), alcohol dehydrogenase (Mw. 150,000), carbonic anhydrase (Mw. 29,000).

SDS-PAGE was also employed for the determination of the molecular weight of subunit. The following size marker proteins (Sigma) were used; phosphorylase B (Mw. 92,500), bovine serum albumin (Mw. 66,200), ovalbumin (Mw. 45,000), carbonic anhydrase (Mw. 31,000), soy bean trypsin inhibitor (Mw. 21,500), lysozyme (Mw. 14,400).

Purification of CGTase

All procedures were carried out at 4°C, the chromatographic procedures being described in the results and in the figure legends.

Results and Discussion

Preparation of crude enzyme

The culture conditions for *Bacillus* sp. YC-335 and the preparation of crude enzyme from the culture supernatant of the strain were described in the previous paper (7).

DEAE Toyopearl column chromatography

The crude enzyme (27,382 mg) was dissolved in 0.05 M acetate buffer (pH 6) and then applied to DEAE Toyopearl 650 M column (4 × 19 cm) previously equilibrated with 0.05 M acetate buffer (pH 6). The unadsorbed fraction of proteins which contains excess CGTase, was eluted with the same buffer and the adsorbed proteins were eluted with a linear gradient of sodium chloride from 0 to 0.4 M in the same buffer. The elution profile is shown in Fig. 1. The two peaks (fraction I and II) of enzyme activity were obtained and both fractions were combined and then applied to DEAE Toyopearl 650 M column under the same conditions. Fig. 2 shows the second chromatography, and a single sharp peak containing the CGTase was obtained. Active fractions eluted from the column, were collected (120 m*l*) and concentrated to a final concentration of 45 mg/m*l* by ultrafiltration using Avantec kit (UHP-43, Toyo).

Gel filtration column chromatography

The concentrated enzyme solution was applied to gel filtration on Sephdex G-100 column (1.8 × 75 cm), equilibrated with 0.05 M acetate buffer (pH 6) containing 0.2 M NaCl. The elution patterns thus obtained is shown in Fig. 3. The fractions from No.

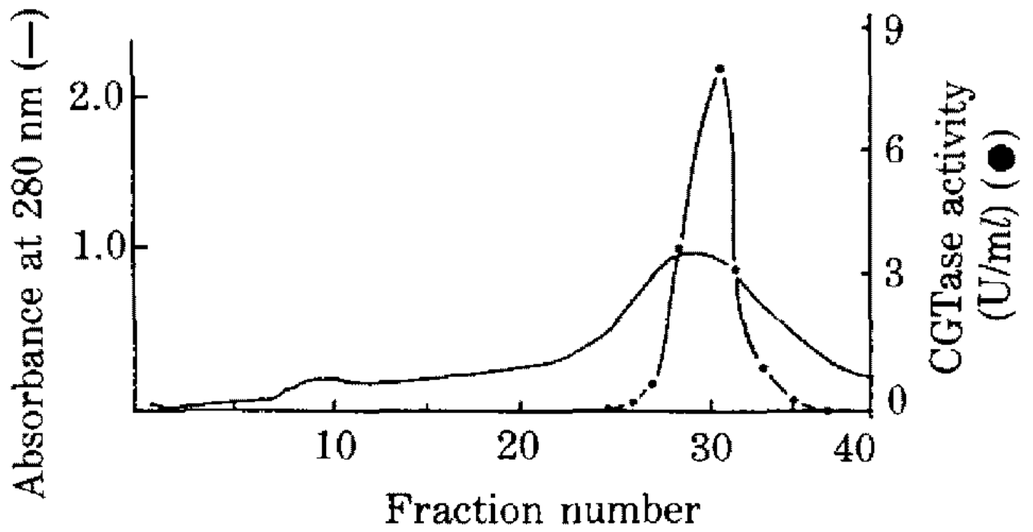

Fig. 3. Sephadex G-100 gel filtration rechromatography (1.8×75 cm) elution volume: 5 m*l*/fraction

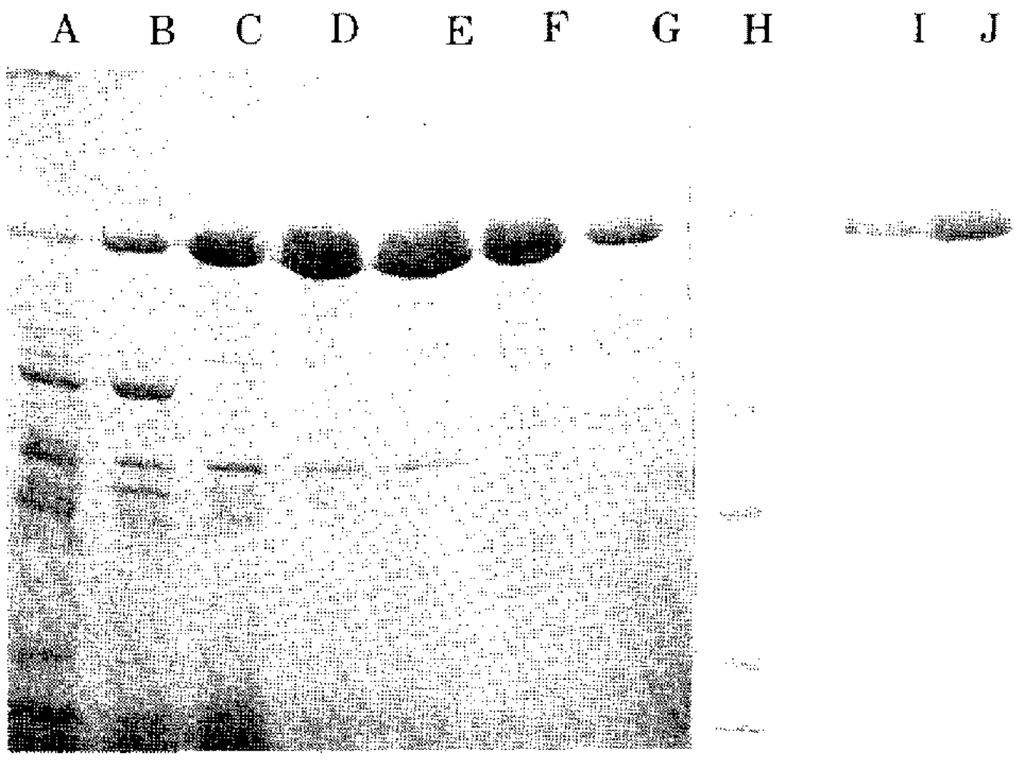

Fig. 4. Analysis of the CGTase on a SDS-PAGE.
A : Culture supernatant of *Bacillus* sp. YC-335
B : After ethanol precipitation
C : After 1st DEAE column chromatography
D : After 2nd DEAE column chromatography
E : After 1st gel filtration
F : After 2nd gel filtration
G : After 3rd gel filtration
H : Standard protein molecular weight markers
I, J : After hydroxylapatite column chromatography

27-33 were collected and concentrated by ultrafiltration. The concentrated enzyme solution was applied again to the same column under the same conditions, thus the CGTase was obtained as a single peak (data not shown). The results of the purification are summarized in Table 1. The enzyme preparation of the final stage was purified about 52.9 folds with 17.8 yield, and the specific activity was 10.53 (unit/mg of protein). The purified CGTase was found to give a single band on SDS-polyacrylamide gel electrophoresis (Fig. 4).

Estimation of molecular weight

The molecular weight of the CGTase was estimated as 75,000 by gel filtration (Fig. 5A). The molecular weight of the CGTase was estimated to be 75,000 by SDS-PAGE (Fig. 5B). These results suggest that CGTase produced from alkalophilic *Bacillus* sp. is composed of single peptide with a molecular weight of 75,000, which is different from the result that the molecular weights of acid, neutral and alkaline CGTase from *Bacillus* sp. ATCC21783 were 85,000-88,000 daltons (9, 13, 14).

Effect of pH on activity and stability

The effect of pH on activity of purified CGTase was determined by glucoamylase method (6). As shown in Fig. 6A, its activity was highest at pH 6. The enzyme was dissolved in various buffer solutions and heated at 50°C for 30 min. After the solution of enzyme was brought to pH 6, the residual activity was measured under the standard assay method. The CGTase was stable at the range of pH 6-10 under the tested conditions (Fig. 6B).

Table 1. Purification of the CGTase.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	27382.20	5448.00	0.19	100.0	1.0
Ethanol precipitation	2987.30	4087.00	1.37	75.0	6.9
DEAE-Toyopearl (1st)					
Fraction I	477.30	1820.00	3.81	33.4	
Fraction II	71.01	311.00	4.38	5.7	
Total	548.31	2131.50	3.89	39.1	19.5
DEAE-Toyopearl (2nd)	190.24	1137.24	5.98	20.9	30.1
Gel filtration (1st)	135.71	987.95	7.28	18.1	36.6
Gel filtration (2nd)	92.90	970.08	10.53	17.8	52.9

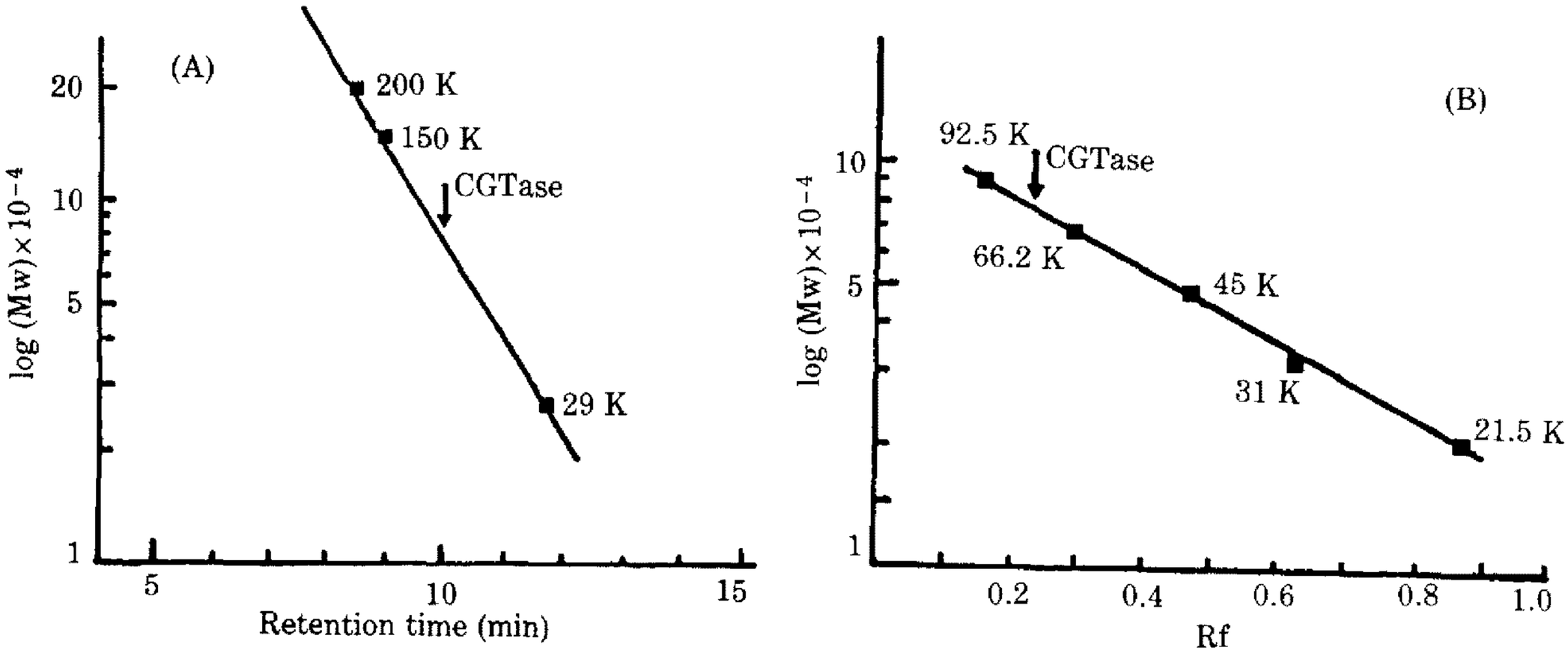

Fig. 5. Molecular weight determination CGTase by gel filtration (A) and SDS-PAGE (B)

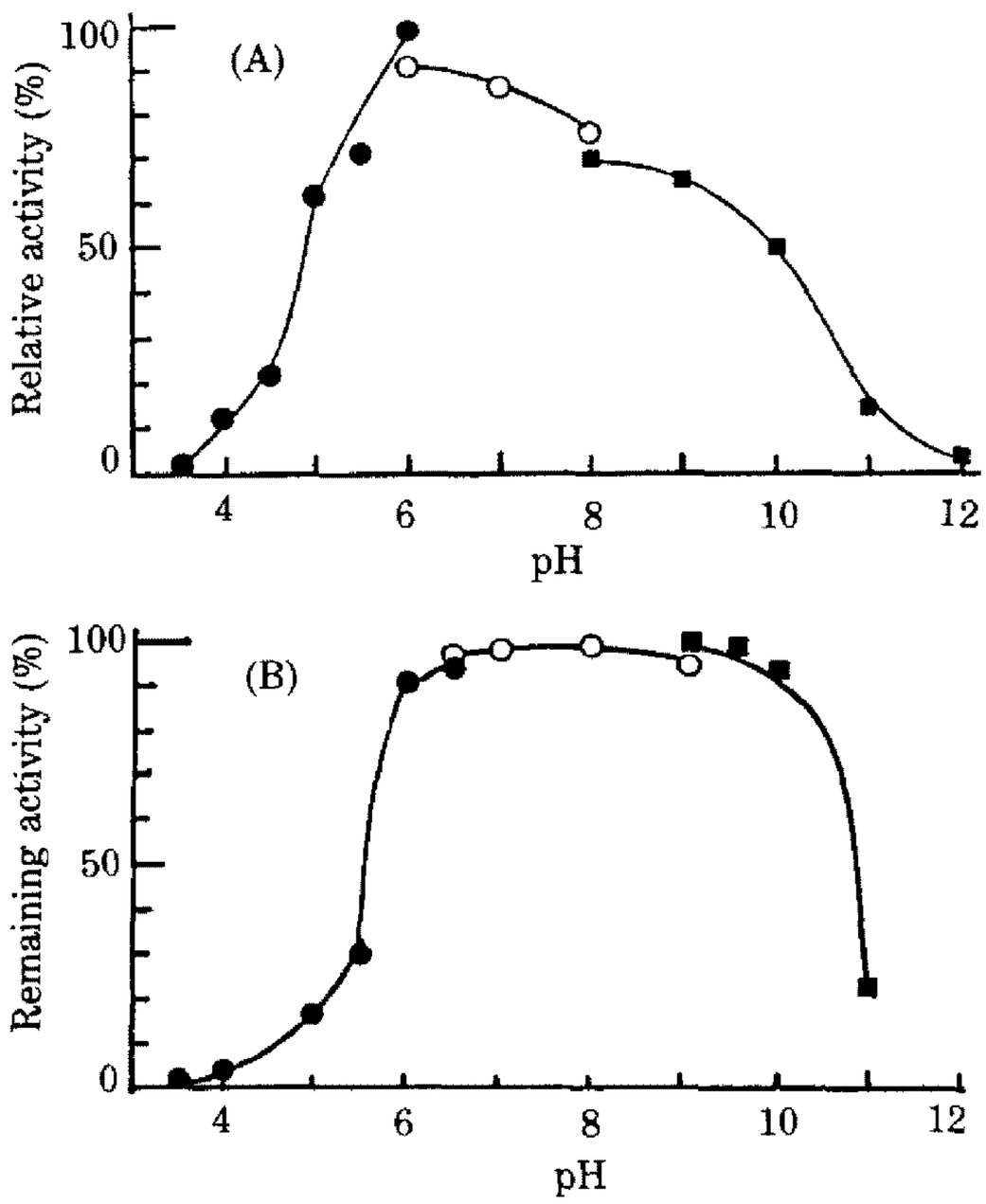

Fig. 6. Effect of pH on the activity (A) and stability (B) of the CGTase

● ; 0.05 M acetate buffer
○ ; 0.05 M phosphate buffer
■ ; 0.05 M glycine-NaOH buffer

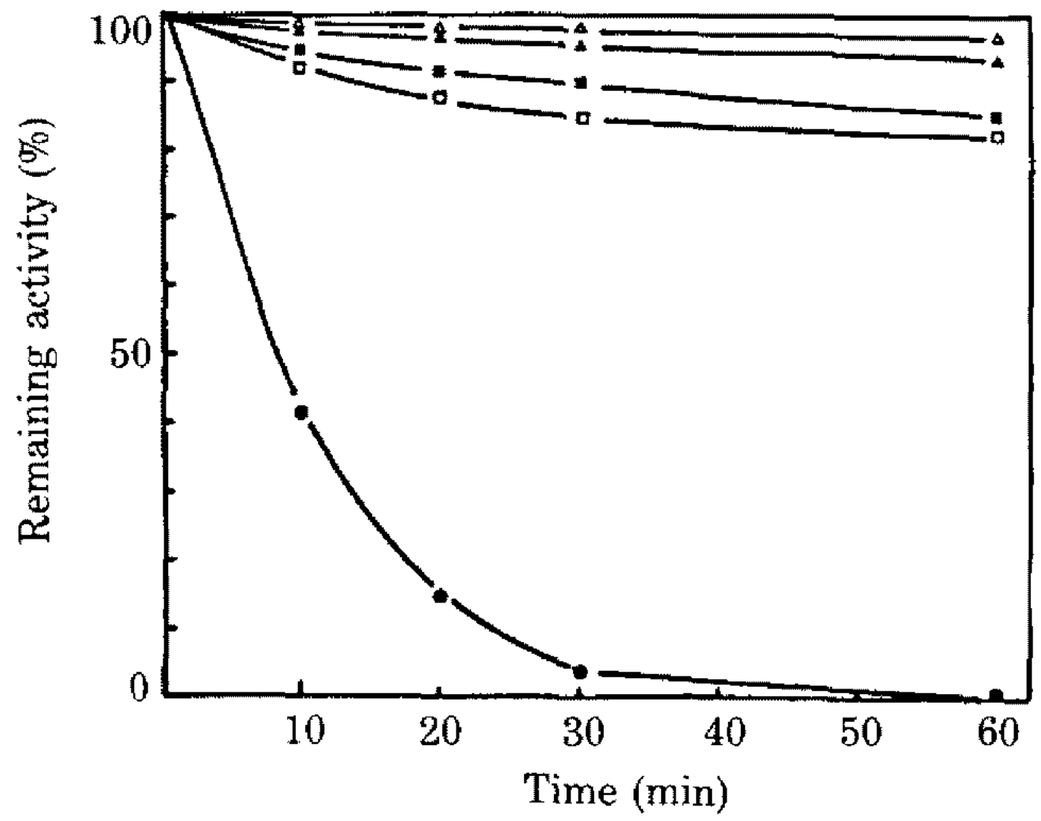

Fig. 7. Effect of $CaCl_2$ on thermal stability of the CGTase.

□ ; 5 mM $CaCl_2$
■ ; 10 mM $CaCl_2$
△ ; 15 mM $CaCl_2$
▲ ; 20 mM $CaCl_2$
*Incubated at 60°C

Effect of $CaCl_2$ on thermal stability

The enzyme in 0.05 M acetate buffer (pH 6) was incubated at 60°C in the presence of various concentration of $CaCl_2$. Residual activity was measured under the standard assay conditions. As shown in Fig. 7, the enzyme was more stable in the presence of $CaCl_2$ than in the absence of $CaCl_2$. The highest stability of enzyme activity was shown at 15 mM of $CaCl_2$ concentration.

Effect of temperature on activity and stability

The enzyme reaction was carried out at pH 6 at various temperatures. The temperature optimum for the enzyme reaction was about 50°C as shown in Fig. 8A. To check the thermal stability, the enzyme in 0.05 M acetate buffer (pH 6) was incubated at various temperatures for 30 min in the presence or

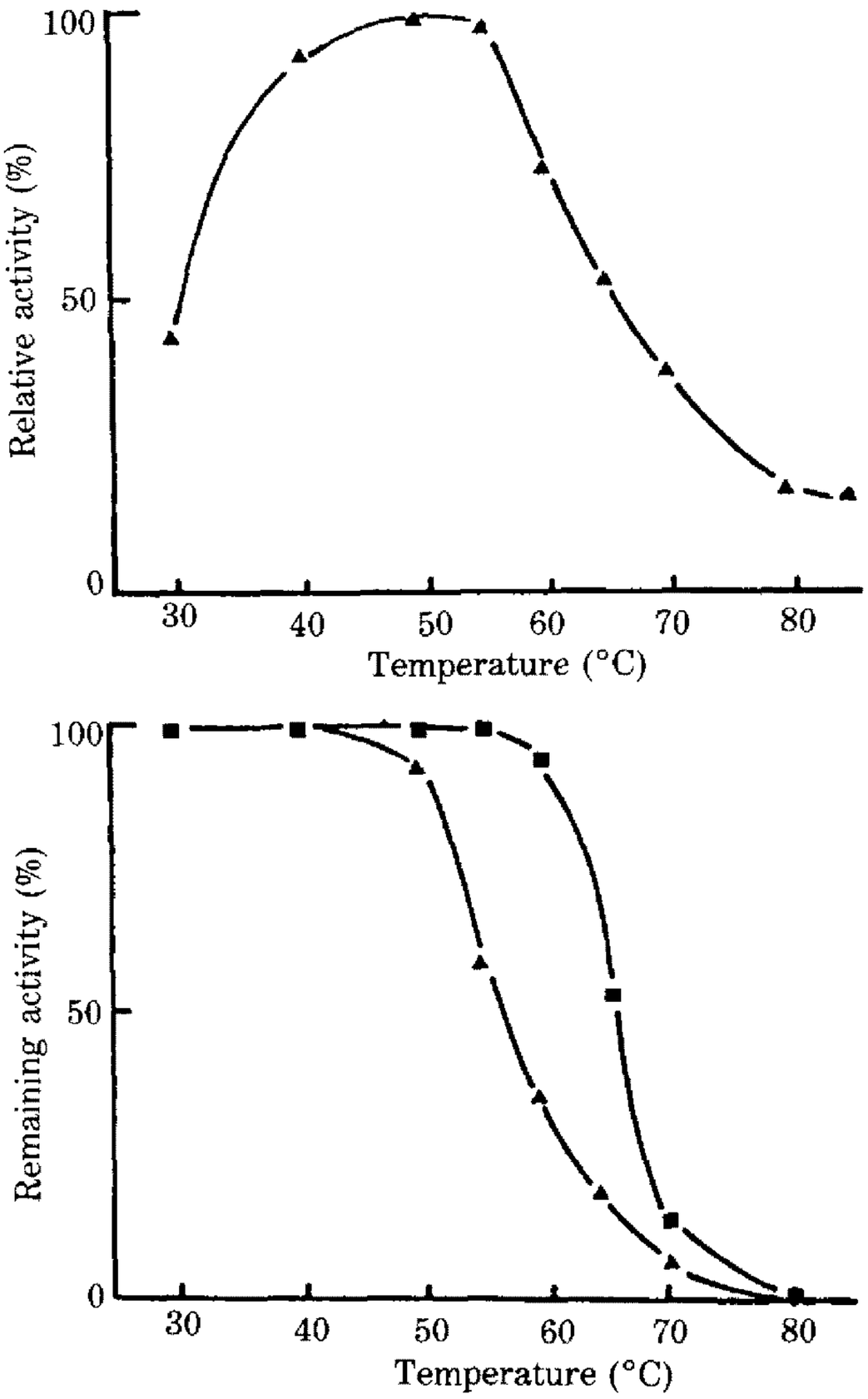

Fig. 8. Effect of temperature on the activity (A) and stability (B) of the CGTase

■; 15 mM $CaCl_2$

▲; No addition

*Incubated for 30 min at various temperature

absence of 15 mM $CaCl_2$. Residual activity was measured under the standard assay conditions. As shown in Fig. 8B, the enzyme was stable up to 50°C without any significant loss of activity, and the addition of $CaCl_2$ increased the thermal stability of the enzyme. Similar results were reported on the CGTase of alkalophilic *Bacillus* sp. ATCC21783 (13, 14).

요 약

토양에서 분리한 호알카리성 *Bacillus* sp. YC-335가 생산하는 CGTase를 ethanol 침전법, DEAE-Toyopearl column chromatography, Sephadex G-100 column chromatography 방법 등에 의해 대량 정제하였다. 이 때 수율은 17.8%이었고 52.9배의 정제도를 가진 효소단백질을 얻을 수 있었다. 정제효소는 SDS-polyacrylamide gel 전기영동에 의해 분자량이 75,000 정도인 단일 peptide의 단백질임을 확인하였고 효소의 최적활성 pH는 6.0이었으며 pH 안정성은 pH 6-10까지 안정하였다. 최적활성온도는 50℃이었으며 열안정성은 50℃까지 안정하였고 이는 15mM $CaCl_2$에 의해 열안정성이 보호되었다.

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