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Purification and Properties of Glucose Isomerase of Alkalophilic *Bacillus* sp.

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호알칼리성 *Bacillus* sp.가 생성하는 포도당 이성화효소의 정제 및 특성

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D-Glucose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) was purified from the alkalophilic *Bacillus* sp. No. 1911 by ammonium sulfate precipitation, DEAE-cellulose ion exchange chromatography followed by Sephadex G-150 gel filtration chromatography. Molecular weight of the purified enzyme was estimated to be 17,000 by gel filtration on Sephadex G-200, and SDS-polyacrylamide gel electrophoresis showed that the enzyme consisted of four identical or similar subunits with a molecular weight of 43,000. The enzyme was the most active at pH 7.5 and 65°C, and stable up to 70°C at pH 7.5 and in the range of pH 6-9 at 60°C by 30 min incubation in the presence of Co^{++}.

Many papers have been published on the glucose isomerases of the neutrophilic bacteria (1-5). However, a few studies on the glucose isomerases of the alkalophilic microorganisms were reported (6). Recently, we isolated alkalophilic bacteria producing glucose isomerase from soil according to the idea by Vedder and Horikoshi, *et al.* (7,8). The screening was conducted using a medium containing 1.0% sodium carbonate, pH 10.0. A newly isolated soil bacterium was found to have a growth optimum at pH 10.4, but glucose isomerization activity at neutral pH.

The present paper describes the purification and some properties of the glucose isomerase produced by the isolated alkalophilic bacterium.

Materials and Methods

Media

The isolation medium was consisted of (per liter) D-xylose, 5g; Bacto-peptone, 0.5g; Difco yeast extract, 1g; K_2HPO_4, 1g; $MgSO_4 \cdot 7H_2O$, 0.2g; Na_2CO_3, 10g and agar 15g in tap water, pH 10.5. D-Xylose was used as a sole carbon source for selection of glucose isomerase producing organisms. Sodium carbonate was sterilized separately and mixed with medium before placing the medium into Petri dishes. The medium for glucose isomerase producing was the same as above except for D-xylose concentration of 10g/*l* and without agar.

Isolation method of alkalophilic bacteria producing glucose isomerase

Soil suspension was serially diluted and spread on agar plates of the isolation medium. The plates were incubated at 37°C for 2days. Colonies on the agar plates were transferred to the culture medium and incubated for 24 hrs with shaking at 37°C. The cells

Key words : Glucose isomerase, alkalophilic *Bacillus* sp., enzyme property
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were harvested by centrifugation and washed with saline. Glucose isomerase activity was measured with the whole cells of the isolated bacteria. Strain No 1911 was selected from about 550 colonies.

Identification of bacteria

Microbiological properties were investigated according to the method described in 'Aerobic Sporeforming Bacteria' and 'Bergey's Manual of Systematic Bacteriology' (9,10). Media used for identification were supplemented with separately sterilized Na_2CO_3.

Enzyme purification

A *Bacillus* species thus screened was cultured by shaking reciprocally at 37°C for 24 hours. The cells were harvested by centrifugation at 6,000×g for 10 min and sonicated (20 KHz, 200W) for 3 min in an ice bath. Cell debris was discarded by centrifugation. Solid ammonium sulfate was added to the supernatant fluid in a 70% saturation.

The resulting precipitate was used as the starting material for purification of the glucose isomerase. All purification steps were carried out at 4°C.

Assay of glucose isomerase activity

The enzyme activity was assayed by measuring the amount of D-fructose resulted from D-glucose. The reaction mixtures were contained 0.5 m*l* of 1.0% D-glucose, 0.1 m*l* of the enzyme sol'n.and 0.4 m*l* of buffer sol'n.The enzyme reaction was performed at 60°C for 10 min in a water bath. The fructose formed was determined by Resorcinol method (11). One unit of enzyme activity was defined as the enzyme amount producing 1.0 μmole of fructose per min under the assay condition

Protein concentration

Protein in a crude solution was measured by the method of Lowry with bovine serum albumin as the standard (12). Purified enzyme concentration was determined spectrophotometrically at 280 nm.

Electrophoresis

Electrophoretic experiments were carried out for determination of isoelectric point and molecular weight of the enzyme. Sodium dodesyl sulfate (SDS) gel electrophoresis were carried out according to the method of Shapiro *et al.* modified by Weber and Osborn (13). The electrophoresis was carried out at 3 mA per gel (5×90 mm) for 4 hr at room temperature. Protein bands were stained with coomassie brilliant blue R-250 and destained with 25% methanol-8% acetic acid.

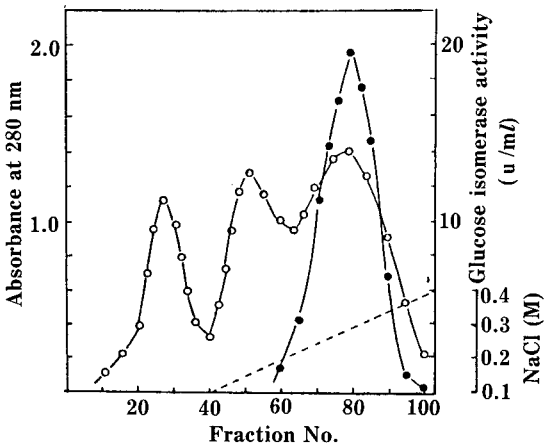

Fig. 1. Column chromatogram on DEAE-cellulose of the glucose isomerase obtained from ammonium sulfate fractions.

●-● : glucose isomerase activity
○-○ : absorbance ---: NaCl concentration

Results and Discussion

Characterization of strain No. 1911

The isolated strain No. 1911 was an aerobic, sporeforming, gram positive, motile and rod shaped bacterium with peritrichous flagella. It grew well in alkaline media rather than neutral one such as nutrient agar. The optimal pH for growth was about 10.4. From the morphological and cultural characteristics of the strain, we identified the bacterium to be *Bacillus* sp. Further taxonomical and biochemical data on this strain will be reported elsewhere.

Purification and crystallization of the enzyme

The ammonium sulfate precipitate of the crude enzyme solution was suspended in 0.05 M phosphate buffer (pH 7.2). The supernatant fluid was dialized against distilled water for 24hr and loaded on a DEAE-cellulose column (1.50×30 cm) which was equilibrated with 0.05 M phosphate buffer (pH 7.2) containing 0.1 M NaCl. After the column was successively washed with the same buffer as above, the enzyme was eluted with a linear gradient increase in

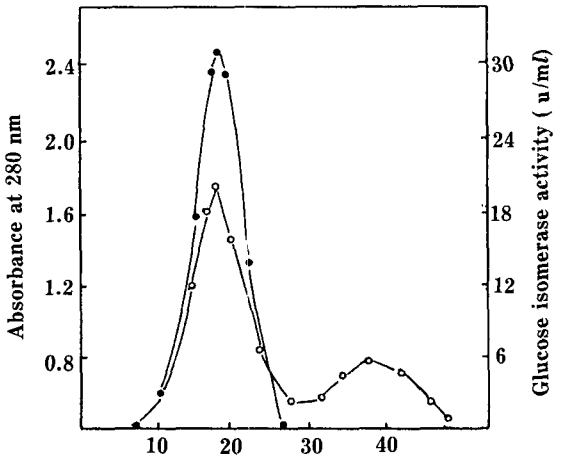

Fig. 2. Gel filtration chromatography on Sephadex G-150 of the enzyme obtained from DEAE-cellulose column chromatography.
●-●: glucose isomerase ○-○: absorbance

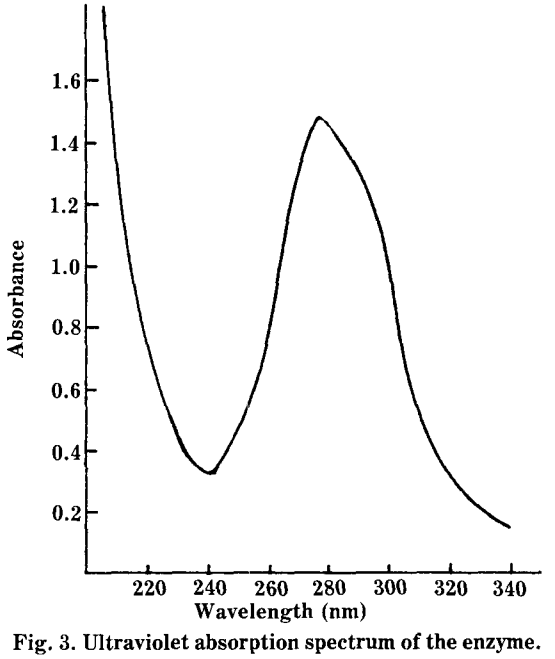

Fig. 3. Ultraviolet absorption spectrum of the enzyme. The enzyme (650μg) was dissolved in 1 m*l* of 0.05 M phosphate buffer solution (pH 7.2).

NaCl at flow rate of 36 m*l*/hr. A linear gradient was obtained by using 400 m*l* each of 0.1 M and 0.4 M NaCl in 0.05 M phosphate buffer (pH 7.2). 5 m*l* fractions were collected and each fraction was assayed. A typical chromatogram is shown in Fig. 1. The active fractions were collected and passed

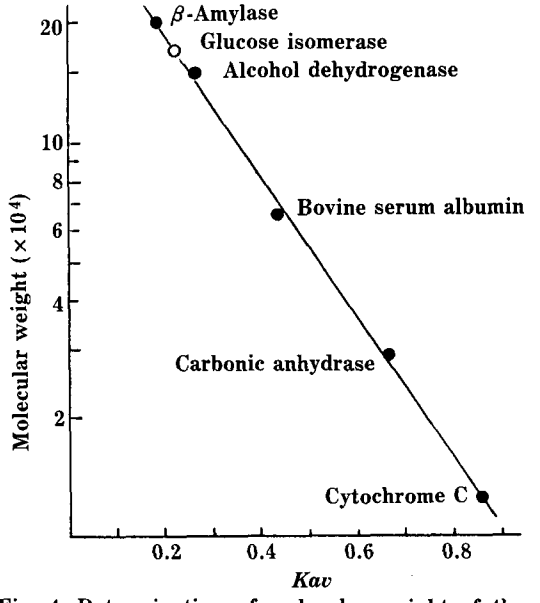

Fig. 4. Determination of molecular weight of the glucose isomerase by Sephadex G-200 gel filtration.

through a Sephadex G-150 column (5.0×60 cm) equilibrated with 0.05 M phosphate buffer (pH 7.2). The typical elution profile is shown in Fig. 2. The glucose isomerase was purified about 24 fold by these treatments. The results are shown in Table 1. The following experiments were carried out by using this preparation.

Molecular weight and isoelectric point

The purified enzyme showed a typical absorption spectrum of protein as shown in Fig. 3. The ratio of absorption at 280 nm to 260 nm was about 1.8. This value indicates the absence of nucleic acid. The enzyme gave a single band protein by disc gel elec-

Table 1. Summary of purification of the glucose isomerase from *Bacillus* sp. No. 1911.

Preparation	Total volume (m*l*)	Total activity (u)	Total protein (mg)	Specific activity (u/mg·prot.)	Recovery (%)
Sonic extract	500	8400	7500	1.12	100
$(NH_4)_2SO_4$ fractionation	175	6720	1976	3.40	80
DEAE-Cellulose	150	4620	216	21.38	55
Sephadex G-150	50	2520	96	26.25	30

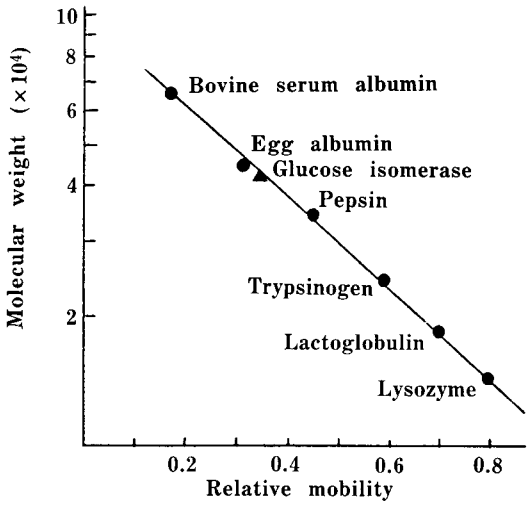

Fig. 5. Determination of molecular weight of subunits of the enzyme by SDS-polyacrylamide gel electrophoresis.

trophoresis. An estimation of molecular weight of the enzyme was made by using a calibrated Sephadex G-200 column (1.6×80 cm) equilibrated with 0.05 M phosphate buffer (pH 7.2) containing 0.1 M NaCl. Molecular weight of the enzyme was estimated as 170,000 as shown in Fig. 4. The enzyme was easily dissociated into subunits with incubation of 1% SDS and 1% 2-mercaptoethanol at pH 7.0, 37°C for 2 hrs. Electrophoresis of the SDS-treated enzyme preparation on SDS-polyacrylamide gel (7%) revealed one band with an identical Rf value (0.35) for all cases. The moleuclar weight of the dissociated protein was estimated as 43,000 as shown in Fig. 5. Therefore, the glucose isomerase was concluded to be consisting of four identical or very similar subunits of 43,000 with a tetrameric structure. This molecular property was very close to those of many other reports (14-18). The isoelectric point (pI) of the enzyme was measured by electrofocusing method using slab gel (130×150×1.5 mm). The pH range of the gel was 3.55 and 8.55. The mobility of the enzyme was almost same or slightly slow than Sigma carbonic anhydrase B from human erythrocytes (pI; 6.57). From this result we calculated the pI of the enzyme as 6.40 (data not shown).

Effect of pH on activity and stability of the enzyme

The enzyme was dissolved in various buffer solutions and the activity was measured at 60°C. As shown in Fig. 6, the enzyme was the most active at a pH range between 7.2 and 7.5. The optimal pH for growth of *Bacillus* sp. No. 1911 was about 10.4. But the optimal pH of the glucose isomerase was neutral,

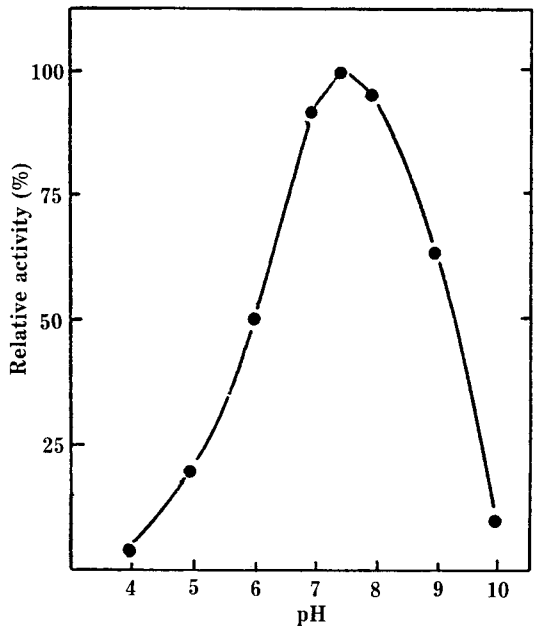

Fig. 6. Effect of pH on the enzyme activity.
Buffer systems used were citrate-phosphate (0.05 M, pH 4-5); phosphate (0.02 M, pH 6-8) and carbonate-bicarbonate (0.02 M, pH 9-10.5).

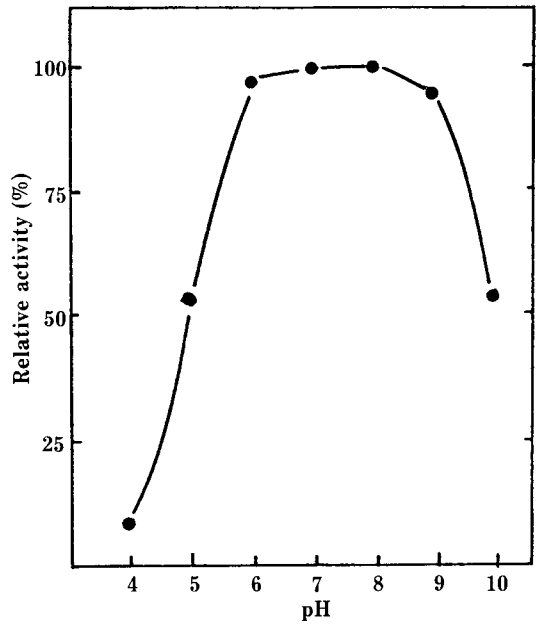

Fig. 7. pH stability of the enzyme.
Buffer systems used were same as Fig. 6.

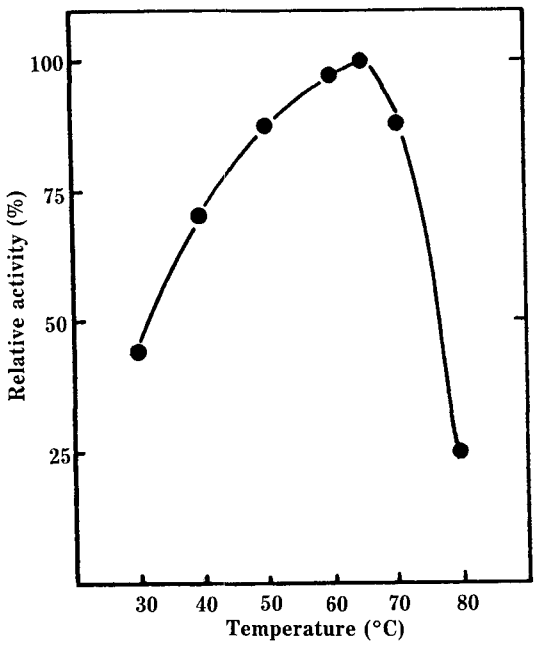

Fig. 8. Effect of temperature on the enzyme activity.

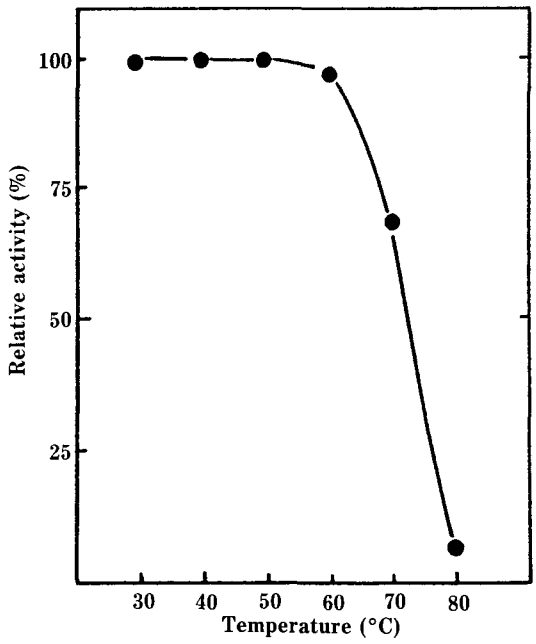

Fig. 9. Thermal stability of the enzyme.

because of the enzyme to be intracellular.

Stability of the enzyme was investigated in buffer solution of various pH values. The mixture was incubated at 70°C for 10 min, and the residual activity was determined at pH 7.5. As shown in Fig. 7, the enzyme was the most stable at pH 6.0-9.0.

Temperature optimum for activity and thermal stability

The optimum temperature was determined by varying the incubation temperature. In Fig. 8, the optimum temperature of the enzyme was 65°C. The enzyme was dissolved in 0.05 M phosphate buffer (pH 7.5) in the presence of 1.4 mM $CoSO_4.7H_2O$ at various temperatures for 30 min, and the residual activity was measured under the standard assay condition. Over ninety-five percent of the original activity was recovered after incubation at 70°C for 30 min, but the enzyme became unstable beyond this temperature (Fig. 9).

Substrate specificity of the enzyme

The enzyme was active on both D-glucose and D-xylose and slightly active on D-galactose, but not active on D-mannose, L-arabinose and D-Ribose (Table 2). Fig. 10 shows the effect of D-glucose concentration. Michaelis constant (K_m) for D-glucose was calculated to be 73 mM.

Table 2. Substrate specificity of the enzyme

Substrate (10 mM)	Ketose formed (μmoles/min)
Glucose	18.6
Xylose	12.0
Ribose	–
Lyxose	–
Arabinose	–
Mannose	–
Galactose	1.50

Reaction mixture contained 0.25 m*l* of 0.1 M phosphate buffer (pH 7.5), 0.1 m*l* of 0.1 M substrate solution and the enzyme (0.7 mg) solution containing 1.04 mM $CoSO_4$. Total volume was made up to 1 m*l* with distilled water and incubated for 10 min at 65°C. The ketoses formed were determined by the Resorcinol method.

Inhibition by sugar alcohols

Inhibition effects of sugar alcohols on the enzyme activity were examined. Among them, xylitol, sorbitol and mannitol inhibited the enzyme action. On the other hand ribitol and arabitol did not inhibit the enzyme action (Data not shown). The inhibition on this enzyme by xylitol was non-competitive and its inhibition constant was found to be 6.3 mM (Fig. 11). The inhibitions by sorbitol and mannitol were also non-competitive.

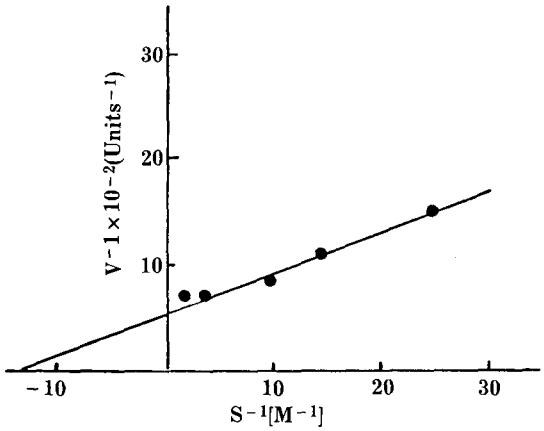

Fig. 10. Determination of K_m value for D-glucose by Lineweaver-Burk plot.

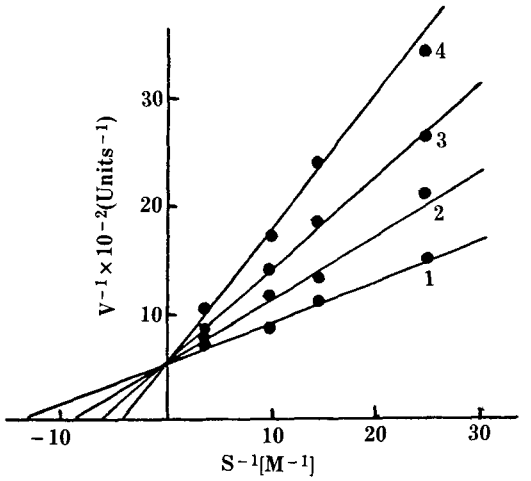

Fig. 11. Inhibition of D-glucose isomerase by xylitol.
Xylitol concentration : 1, 0 mM; 2, 5 mM
3, 10 mM; 4, 20 mM

Effect of metal ions on the enzyme activity

When the crystalline enzyme was used, the reference without metal ions showed a slight activity (about 6% of the maximum activity). But the enzyme was activated remarkably by addition of cobalt or magnesium ion to the reaction mixture (Table 3). The functional role of Co^{++} and Mg^{++} on the D-glucose isomerizing activity of the enzyme was explained in terms of the suitable changes for the isomerization which was induced at the catalytic site of the enzyme by binding Co^{++} and Mg^{++}. Many pentose isomerases from various bacteria and *Streptomyces* required Co^{++}, Mn^{++} or Mg^{++} (19). This means that these enzymes require the metalic ions such as above for its activity.

Table 3. Effect of metal ion on the enzyme activity.

Metal ion (5×10 M)	D-Fructose formed (mg/ml)	Relative activity (%)
None	0.17	5.0
$MgSO_4$	3.08	91.9
$CoSO_4$	3.35	100
$Fe_2(SO_4)_3$	0.25	7.4
$MnCl_2$	0.35	10.4
$CaCl_2$	0.58	17.3
$ZnSO_4$	0.15	4.5
$CuSO_4$	-	-
$BaCl_2$	-	-
$NiSO_4$	-	-

The enzyme was dialyzed against 0.05 M phosphate buffer (pH 7.5), containing 10 mM EDTA for 48 hr and against the same buffer without EDTA for an additional 24 hr. Reaction mixture contained 0.25 m*l* of phosphate buffer (pH 7.5), 0.1 m*l* of 1 M D-glucose, 0.1 m*l* of metal solution, 0.1 m*l* of enzyme solution (0.7 mg) and was made up to 1 m*l* with distilled water. The reaction was made at 65°C for 10 min.

요 약

토양으로부터 분리한 호알카리성 세균 *Bacillus* sp. No.1911을 파쇄하여 유안투석, DEAE-cellulose column chromatography, Sephadex G-150 gel filtration으로 포도당 이성화효소를 정제하여 효소적 특성을 조사하였다. 이 효소는 Sephadex G-200 gel filtration에서 분자량은 170,000으로 측정되었고 SDS-polyacrylamide 전기영동에서 43,000의 단일 band를 얻어, 이 효소는 4개의 동일한 subunit로 구성되었음을 나타내었다. 이 효소는 pH 7.5 및 65℃에서 최적활성을 나타내었으며 pH 7.5에서 70℃까지 안정하였고 Co^{++} 존재하에 60℃에서 30분간 가열하였을 때 pH 6-9까지는 비교적 안정되었다.

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