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Identification of a Bacterium which Produced D-Glucose Isomerase and Partial Purification on the Enzyme

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포도糖 異性化酵素 生産菌의 同定 및 그 酵素의 部分精製

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Abstract

A microorganism which produced D-glucose isomerase was identified to be similar to *Streptomyces antibioticus* on the morphological, cultural and physiological characteristics except the spore chain and the utilization of sucrose. D-xylose grown cells of *Streptomyces* sp. strain K-17 were disrupted by grinding with sea sand. D-glucose isomerase was partially purified with the fractionation by ammonium sulfate, Mn-treatment, DEAE-cellulose column chromatography, DEAE-sephadex (A-50) column chromatography and gel filtration of sephadex G-200. The enzyme was purified about 380 fold with 25% recovery.

Introduction

D-Glucose isomerase from sonic extracts and washed, lyophilized cells of D-xylose grown *Pseudomonase hydrophila* (N. R. C. 491 and 492) was initially reported by Marshall and Kooi[1]. Although the addition of arsenate was required for the enzyme reaction, the significance of the isomerization of D-glucose to D-fructose as well as D-xylose to D-xylulose encouraged the practice of D-glucose isomerase for the development of the sweetners.

After the discovery in 1957 of a D-glucose isomerase from *Pseudomonas hydrophila*[1], other bacterial sources for the enzyme, including *Aerobacter cioacae*[2~4], *Bacillus megaterium*[5], *Lactobacillus brevis*[6~7], *Escherichia intermedia*[8], *Aerobacter aerogenes*[9], *Brevibacterium pentose-aminoacidicum*[10], *Paracolobacterium aerogenoides*[11,12] and *Bacillus coagulans*[13~16] have been described. The isomerases of *Lactobacillus brevis*[7] and *Bacillus coagulans*[15,16] have been successfully purified and crystalized.

D-glucose isomerase of *Streptomyces* species was initially reported by Tsumura and Sato[17~19]. The enzyme required Mg^{2+} and Co^{2+} for the isomerization, was stable to high temperature and converted 52% of D-glucose to D-fructose at 70°C. The enzymes of *Streptomyces species* were better than those of other bacteria for the sugar industry. Takasaki[20~23] isolated *Streptomyces* sp. strain YT-No. 5, later identified to be *Streptomyces albus*[23],

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which could produce D-glucose isomerase on xylobiose, xylan or xylan-containing material instead of xylose as an inducer. These discoveries provided a way for the economical production of D-glucose isomerase. The optimal temperature and pH for the enzyme reaction were 70°C and pH 7.0 to 7.5, respectively. The enzyme required Mg^{2+} and Co^{2+} as activator and isomerized 50% of D-glucose to D-fructose.

In the previous papers[24~26] the properties of D-glucose isomerase of *Streptomyces* sp. strain K-17, which was isolated from soils, was reported. The values of pH and temperature for the maximum activity of the isomerase were 7.2 and 75°C, respectively. The D-glucose isomerase of *Streptomyces* sp. strain K-17, which required D-xylose as an inducer, was activated by Mg^{2+} and protected at high temperature by Co^{2+}. The isomerase was inhibited by the ethylenediaminetetraacetic acid (EDTA) and converted D-glucose and D-xylose to the corresponding ketose. The value of *Km* for the isomerization of D-glucose was estimated to be 7.2×10^{-1}M[27]. Sanchez and Smiley[28], Strandberg and Smiley[29] and Chen[30] have described the isomerase of *S. albus*, *S. phaeochromogenes* and *S. flavogriseus*, respectively.

Takasaki[22,23] reported that D-glucose isomerase from *S. albus* was purified with the extraction by autolysin, fractionation with acetone, DEAE-cellulose column chromatography and DEAE-sephadex (A-50) column chromatography to homogeneity as determined both by sedimentation and electrophoresis. The purified enzyme could convert D-xylose to the corresponding ketose as well as D-glucose to fructose, but D-ribose was not converted. Then the crystalized enzymes of *Lactobacillus brevis*[7] and *Bacillus coagulans*[16] catalyzed the isomerization of D-xylose, D-glucose and D-ribose.

The purification procedure of D-glucose isomerase and identification of the bacteria are reported in this paper.

Materials and Methods

Microorganism and culture

Streptomyces sp. strain K-17, which produced high activity of D-glucose isomerase as described in the previous papers[24~27], was isolated from soil. The bacteria were maintained on the agar slant medium shown in Table 1. One loop of the bacteria from the agar slant cultivated for 7 days at 30°C was inoculated to 100m*l* of liquid medium shown in Table 1 in 500m*l* shaking flask (sakaguchi flask). The bacteria were incubated at 30°C for 26 hr on the shaker.

Table 1. Composition of the media.

Medium for agar slant	
bran extract*	30 % (v/v)
nutrient broth	0.8 %
glucose	0.2 %
agar	2.0 %
Liquid medium	
D-xylose	1.0 %
peptone	1.0 %
K_2HPO_4	0.05%
$MgSO_4 \cdot 7H_2O$	0.05%
NaCl	0.1 %
Basal salt mixture	
K_2HPO_4	0.05%
$MgSO_4 \cdot 7H_2O$	0.05%
NaCl	0.1 %

*Hundred g of wheat bran in 1*l* of tap water which was adjusted to pH 2.0 with N-HCl. were boiled for one hour. After cooling. the broth was neutralized with N-NaOH. The filtrate was added to the medium.

Identification of the bacteria

Identification procedures were carried out according to the method of Shirling and Gottlieb[31] and Nonomura[32]. Hydrolysis of the starch was determined on inorganic salts-starch agar plate after the incubation at 30°C for 4 days. Hydrolysed starch appears as a clear zone formed when Lugol solution was sprayed on the above agar plate. Casein and gelatin hydrolysis were observed on the nutrient agar plates added 10% (v/v) of skim milk and 0.4% of gelatin respectively. The bacteria were inoculated by streaking to the agar plate mentioned above and observed at 4, 7 and 10 days. Hydrolysed casein and gelatin appears clears clear zone by developing with 15% $HgCl_2$ solution in

N-HCl. One m*l* of 3% H_2O_2 was added to 5m*l* of the culture broth incubated on the liquid medium shown Table 1 to determine catalase activity. If the bacteria produced catalase, oxygen gases were formed from the culture broth. To observe growth inhibition by streptomycin, the bacteria were incubased on the glycerol asparagine agar slant medium containing 10, 20, 50, 100μg of streptomycin per m*l* of the medium at 30°C for 2 weeks.

Morpholoical characteristics of the aerial mycelium were observed with an optical microscope from the slide culture according to the general procedure. Spore surfaces were observed with electron microscope Hitachi model HU-11C. The suspension of the spores from the glucose asparagine agar slant incubated at 30°C for 2 weeks. in 2% phosphotangstic acid, pH 7.2, was smeared on the grid coated with folmbar stood for one minute at room temperature and was dried.

Assay of enzyme activity

D-Glucose isomerase was determined as described in the previous paper[24] by measuring the amount of D-fructose converted from D-glucose. The method of cystein carbazole sulfuric acid[33] was used for the assay of fructose. The standard reaction mixture was composed of 1 m*l* of 0.2M glucose solution containing 2×10^{-3}M $Co(Ac)_2$ and 2×10^{-3} M $MgSO_4$, 0.5 m*l* of 0.2M phosphate buffer solution, pH 7.2, and 0.5m*l* of the enzyme solution. The reaction was intiated by the addition of the enzyme solution and the reaction mixture was incubated at 70°C for 60 min. One unit of the activity was defined as the enzyme quantity which converted to 1 mg fructose per m*l* of reaction mixture per min.

Protein concentrations

The amounts of protein were spectrophotometrically estimated from the absorbance at 280nm with Shimazu double beam spectrophotometer model UV -200.

Electrophoresis

The assay of homogeneity was made by polyacrylamide gel electrophoresis. Samples were run in discontinuous 7.0% gels with the method of Davis[34] that stacks at pH 8.9 (tris-HCl buffer) and runs at pH 8.3 (tris-glycine buffer) with a constant current of 2 mA per tube for 3 hr. About 100 to 200μg of protein were applied to a gel tube. Gels were stained in amide black 10-B and destaine delectrophoretically in 7% acetic acid at the constant current of 7 mA per gel.

Reagent

D-xylose, D-glucose and yeast extract from Wako pure chemical Co. and other sugars from Sigma Co. for identification of the bacteria were used. The reagents for the polyacrylamide electrophoresis were obtained for Nakarai Co.

Results and Discussion

1. Identification of the microorganism tested

Streptomyces sp. strain K-17 used in this study was identified by Bergey's mannual of determinative bacteriology 8th edition[35] and Method of Nonomura[32].

Characteristics of morphology and growth

The colony form of *Streptomyces* sp. strain K-17 was observed to be velvety on the oat meal agar, glucose asparagine agar slant and plate (Fig. 1). The form of the spore chain in the slide culture on the glucose asparagine agar medium was retinaculum apertum and the spore surface observed under the electron microscope was smooth type (Fig. 2).

The mass color of the sporulating aerial surface appeared grey on the oat meal agar, the inorganic salt-starch agar, the glycerolasparagine agar slant

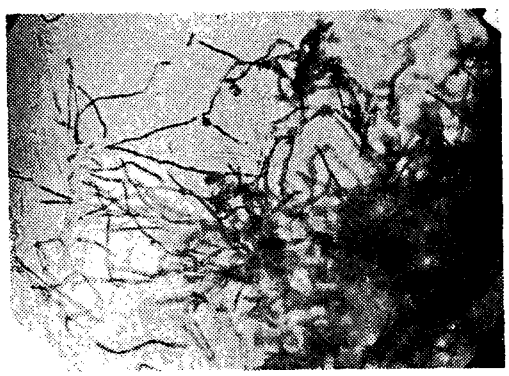

Fig. 1. Spore chain of *Streptomyces* sp. strain K-17. Note, setinaculum-apertum.

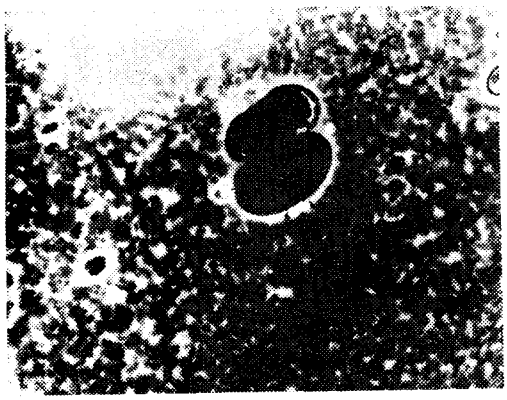

Fig 2. Electron photomicrograph of spore surface of *Streptomyces* sp. strain K-17. Note, smooth surface.

and plate, and potato plug. The reverse side colors and the soluble colors other than the melanoid pigment were not distinctive. The production of the melanoid pigment was observed on the peptone yeast extract iron agar, tyrosine agar slant and plate, and potato plug after 2 days. The characteristics of growth in the various media were summarized in Table 2.

Physiological and biochemical characteristics

Streptomyces sp. strain K-17 could hydrolyse starch strongly, not casein and gelatin, and produce catalase. The growth of the bacteria containing 10μg per m*l* of streptomycin. The bacteria could

Table 2. Morphological and cultural characteristics of *Streptomyces* sp. stain K-17.

Morphological characteristics		
colony form	velvety	
spore chain	retinaculum-apertum	
spore surface	smooth type	
aerial mass color	grey	
melanoid pigment	positive	
reverse side color	not distinctive	
other soluble color	not distinctive	
Cultural characteristics		
oat meal agar	excellent growth	grey
glycel asparagine agar	excellent growth	grey
inorganic salt agar	excellent growth	grey
nutrient agar	poor growth	grey
Czapek agar	fair growth	grey
potato plug	good gowth	grey

Table 3. Physiological and biochemical characteristics of *Streptomyces* sp. strain K-17.

Physiological	Characteristcs
Starch hydrolysis	+
Gelatin hydrolysis	−
Casein hydrolysis	−
Catalase production	+
Streptomycin inhibition	+
NaCl tolerance	≧7%, but<9%

+, positive; −, negative.

Table 4. Utilization of carbon compounds. Growth was checked after 14 days at 30°C.

Carbon compounds (1%)	Growth
no carbon	−
D-glucose	+
D-xylose	+
L-arabinose	+
L-rhamnose	+
D-fructose	+
D-galactose	+
raffinose	−
D-mannitol	+
i-inositol	+
Salicin	−
Sucrose	±
Starch	+

+, utilized; −, not utilized;
±, slightly utilized.

grow in the medium added 7% NaCl weakly, but not more than 9% NaCl (Table 3). Salicin and raffinose were not assimilated as a carbon source and the utilization of sucrose was doubtful whereas other other sugarswere utilized (Table 4).

Identification

The microorganism tested, which grew well in the aerobic conditions and produced a well developed branched mycelium and spore forming linear spore chains at the end of the aerial mycelium, was a species of the genus *Streptomyces* which could be identified by colony form, morphology, biological and physiological characteristics. *Streptomyces* sp. strain K-17 was similar to *S antibioticus* except the spore chain and the utilization of sucrose

Table 5. Identification of strain tested.

	Streptomyces mutabilis	*Streptomyces antibioticus*	Test strain K-17
Aerial mass color	Gy	Gy	Gy
Melanoid pigment	0	1	1
Reverse side pigment	0	0	0
Soluble pigment	0	0	0
Spore chain	SRA	RF	RA
Spore surface	smooth	smooth	smooth
NaCl tolerance	≧10%, <13%	≧7%, <10%	≧7%, <9%
L-arabinose	+	+	+
D-xylose	+	+	+
i-inositol	+	+	+
D-mannitol	+	+	+
D-fructose	+	+	+
L-rhamnose	+	+	+
Sucrose	±	−	±
Raffinose	−	−	−
Salicin	−	−	−
D-galactose	+	+	+

Gy. grey; l, positive; O, negative; SRA, spiral and retinaculum-apertum; RF, rectus-flexibilis; RA, retinaculum-apertum; +, utilized; −, not utilized; ±, slightly utilized.

and *S. mutabilis* except the spore chain and the melanoid pigmentation. The strain was more similar to *S. antibioticus* than to *S. mutabilis*, but was not attempted to specify the species, since it appeared not to be described as known *Streptomyces* spp. (Table 5). Also *Streptomyces* sp. strain K-17 was different from *S. albus*[20,28], *S. phaeochromogenes*[17,29] and *S. flavogriseus*[30] which could produce D-glucose isomerase.

2. Purification of D-glucose isomerase

All steps were performed at 2 to 5 C unless otherwise stated.

Enzyme extraction

Cells collected with filtration from 5 liters of the cultured broth were washed with distilled water three times. The washed cells were ditrupted by grinding with 50g sea sand. Enzymes were extracted with 500m*l* distilled water from the resulting cells and the sea sand suspension. Cell debrises and sea sand were removed by the centrifugation at 2500 rpm for 20 min.

Mn-treatment

To 413 m*l* of the supernatant was added dropwisely 46 m*l* of M-$MnCl_2$, and pH was maintained at 6.0 to 6.2 by adjusting with 0.5N NaOH. The solution was allowed to stand for an additional 1 hr at room temperature and centrifuged at 10,000 rpm for 20 min. After centrifugation the precipitate was discarded.

Ammonium sulfate fractionation

Mn-treated enzyme solution was fractionated by salting out with ammonium sulfate. Almost enzyme was recovered in the fraction of 0.5 to 0.8 saturation and remarkable amounts of the protein without the activity were removed by the ammonium fractionation (Fig. 3). To 438 m*l* of the supernatant the solid ammonium sulfate was added to give a 0.5 saturation by mechanical stirring. The solution was allowed to stand for 5 hr. The precipitate was discarded by centrifugation at 10,000 rpm for 20 min, and to the supernatant the ammonium sulfate was added to give a 0.8 saturation. After standing for 5 hr the precipitate was collected, dissolved in the 0.05M phosphate buffer, pH 7.2, and dialysed for 24 hr against the same buffer. After dialysis the precipitate without the activity was removed

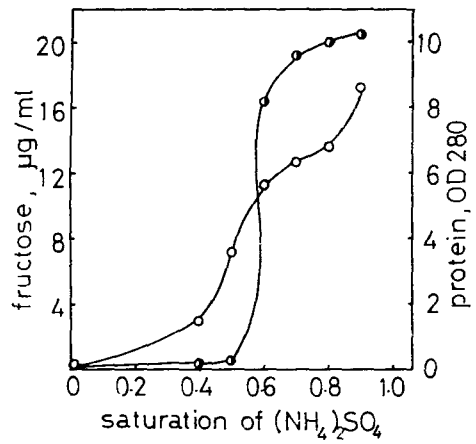

Fig. 3. Fractionation of crude enzymes by ammonium sulfate. ◐—◐. enzyme activity; ○—○, amount of protein.

by the centrifugation.

DEAE-cellulose column chromatography

As shown in Fig. 4, 66 m*l* of the dialysed enzyme solution was applied to a 2.5×50cm DEAE-cellulose column equilibrated with 0.05M phosphate buffer, pH 7.2. The column was washed with 200 m*l* of the buffer solution at the flow rate of 100m*l* per hr. The enzyme was linearly eluted with 800 m*l* of the buffer solution increasing the concentration of KCl to 0.6M and eluted with the buffer solution containing 0.6M KCl in succession. Seven m*l* of the fractions were collected.

The peak of the enzyme emerged at about 0.5M KCl, and much proteins without the isomerase activity emerged at 0.4M and 0.6M KCl and were discarded.

DEAE-sephadex (A-50) column chromatography

The enzyme solution (84 m*l*) which pooled the active peak fractions from DEAE-cellulose column chromatography was applied to a 2.0×20cm DEAE -sephadex (A-50) column equilibrated with 0.05M phosphate buffer, pH 7.2. The column was washed with 300m*l* of the buffer solution at the flow rate

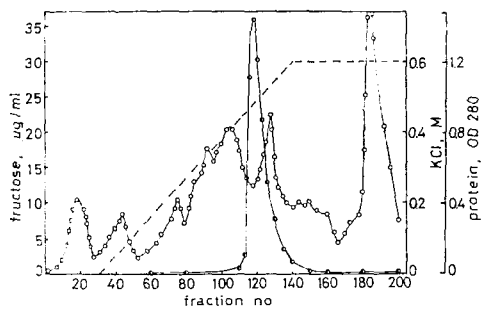

Fig. 4. Column chromatography of a glucose isomerase on DEAE-cellulose. The column (2.5×50cm) was loaded with 66 m*l* of the dialyzed enzyme and washed with 0.05M phosphate buffer, pH 7.2, at a flow rate of 100m*l* per hr. The adsorbed enzyme was eluted with a linear gradient of KCl from 0 to 0.6M and fractions of 7 m*l* were collected. ◐—◑. enzyme activity; ○—○, amount of protein; ···, concentration of KCl.

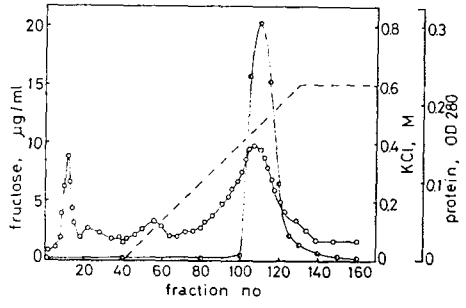

Fig. 5. Column chromatography of D-glucose isomerase on DEAE-sephadex (A-50). The column (2.0×20cm) was loaded with 84m*l* of the active fractions from DEAE -cellose column and washed with 0.05M phosphate buffer, pH 7.2, at a flow rate of 100m*l* per hr. The adsorbed enzyme was eluted with a linear gradient of KCl from 0 to 0.6M and fractions of 7m*l* were collected. ◐—◐. enzyme activity; ○—○, amount of protein; concentration of KCl

Table 6. Summary of the enzyme purification.

	Volume	Protein*	Activity			Recovery
	(m*l*)	(mg)	Total unit	unit/m*l*	Sp. activity	(%)
Crude extract	413	10160	3180.1	7.7	0.3	100
Mn-treatment	438	7446	—	—	—	—
$(NH_4)_2SO_4$ fractionation	65	4485	2574.0	39.6	0.6	81
Dialysis	66	1765	2550.9	38.7	1.4	80
DEAE-cellulose column	84	49	2125.2	25.3	43.4	67
DEAE-sephadex column	189	21	1927.8	10.2	91.8	61
Gel filtration of sephadex G-200	22	7	805.2	36.6	115.0	25

*The amount of protein was estimated from absorbance at OD 280 nm. assuming $E^{1\%}_{cm}=10$.

of 100m*l* per hr by 5 m*l* offraction volume. The enzyme was linearly eluted with 600 m*l* of the buffer solution increasing the concentration of KCl to 0.6M and eluted with the buffer solution containing 0.6M KCl in succession. The active peak fractions were combined and concentrated by ultrafiltration in a diafilter with a T-10 membrane. At that time the enzyme solution was concentrated without loss of the activity (Fig. 5).

Gel filtration of D-glucose isomerase

Three m*l* of the enzyme solution concentrated by the ultrafiltration was applied to a 1.8×56cm sephadex G-200 column equilibrated with 0.05M

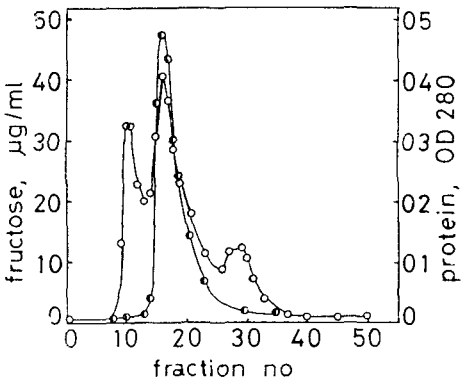

Fig. 6. Gel filtration of D-glucose isomerase with sephadex G-200. About 20mg in 3m*l* of enzyme were subjected to gel filtration on a column (1.8×56cm) of sephadex G-200 at a flow rate of 4m*l* per hr *fractionating 4ml portions of the effluent.* ◐—◐, enzyme activity; ○—○, amount of protein.

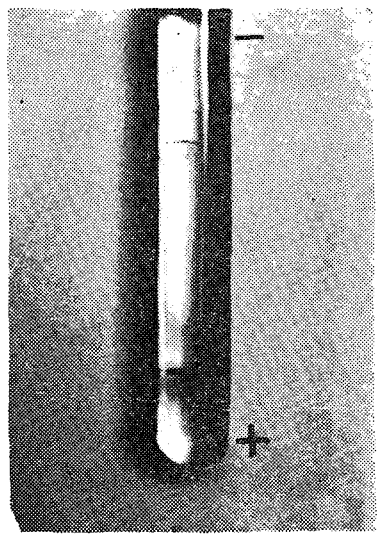

Fig. 7. Polyacrylamide gel electrophoretic pattern of D-glucose isomerase obtained from gel filtration with sephadex G-200. The disconsinous gel contained 7.0% acrylamide, stacked at pH 8.9 and run at pH 8.3 with a constant current of 2mA per tube. About 200μg of enzyme were placed in a gel tube. Gels were stained in amide black 10 B and destained electrophoretically in 7% acetic acid at constant current of 7 mA per gel.

phosphate buffer, pH 7.2. The column was operated at 4 m*l* per hr and 4 m*l* fractions were collected. When the column was washed with the same buffer, small a mounts of the proteins without the activity emerged in the front and the near of the active peak and were discarded (Fig. 6). The enzyme was then purified about 380 fold with 25% recovery. A typical purification is summarized in Table 6.

Criteria of homogeneity

About 100 to 200μg of the enzyme protein from the gel filtration of sephadex G-200 were placed in a gel tube in order to observe the homogeneity of the purified enzyme by the polyacrylamide electrcphoresis at pH 8.3. When about 100μg of the enzyme protein were placed in a gel tube, the enzymes were homogeneous with a single band, but when about 200μg of the enzyme protein were placed, a minor band appeared at the near part of anode of the main band (Fig. 7). The purification procedure of D-glucose isomerase of *Streptomyces* sp. strain K-17 was similar to that of *S. albus*[23].

要 約

토양으로부터 분리한 포도糖 異性化酵素를 강하게 生産하는 放線菌을 Bergey's manual 8版에 따라 同定한 結果 *Streptomyces antibioticus* 近緣의 菌株이었다. 本菌의 培養液으로부터 菌體를 모아서 海砂를 넣고 破碎하여 증류수로 抽出하고 Mn-處理를 하여 核蛋白質을 除去한 후 黃酸 ammonia 분획침전(0.5~0.8 飽和), 透析, DEAE-cellulose column chromatography, DEAE-sephadex (A-50) column chromatography 및 sephadex G-200에 의한 gel filtration을 거쳐 比活性度로 약 380倍, 回收率 25% 程度로 部分 精製하였다.

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