

Identification of Isoasparaginase from *Escherichia coli* K-12 as α -aspartyl dipeptidase

Yasuhisa ASANO, Naoyuki MIZUGUCHI, Hidenobu KOMEDA, Ikuo KIRA*,

Kenzo YOKOZEKI**

Biotechnology Research Center

*On leave from Ajinomoto Co. Ltd., Kanagawa, JAPAN

**Ajinomoto Co. Ltd., Kanagawa, JAPAN

Summary

Isoasparaginase was purified from the cell-free extract of *Escherichia coli* K-12 (TPU6303) and its N-terminal amino acid sequence was determined. The molecular weight of the fraction α and fraction β , which were separated by HPLC on a TSK-GEL Octyl-80Ts column, was calculated to be about 25,000 and 27,000 by the SDS-PAGE. Twenty residues in the amino-terminal region of fraction α were identical to 20 residues of ribose-5-phosphate isomerase A from *E. coli*. Eighteen residues in the amino-terminal region of fraction β were identical to 20 residues of α -aspartyl dipeptidase (peptidase B) from *E. coli* and *Salmonella typhimurium*. Isoasparaginase was judged to be equal to aspartyl dipeptidase (PepE) (aspartyl aminopeptidase, EC 3.4.11.21). Therefore, isoasparaginase activity found in *E. coli* was identified as aspartyl dipeptidase.

Key Words: Aspartame, isoasparaginase, α -aspartyl dipeptidase (PepE), aspartyl aminopeptidase, *Escherichia coli*

1. Introduction

We have been interested in the enzyme-catalyzed synthesis of aspartame (α -L-aspartyl-L-phenylalanine methyl ester, or APM), which is a synthetic sweetener about 200 times sweeter than sucrose. The compound contains two amino acids as found at the C-terminal dipeptide of the digestive hormone gastrin, and the sweetness was discovered by chance during the synthesis of this hormone by Mazur *et al.* of G. D. Searle.¹⁾ Owing to its pleasant sweetness without a bitter aftertaste, it was well received as a low-calorie sweetener for many foodstuffs.

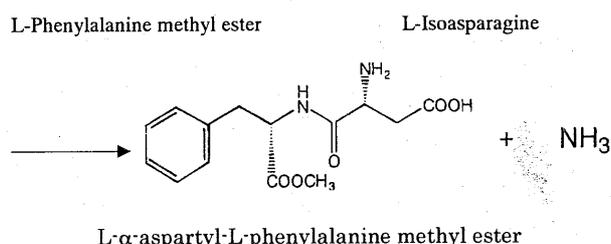
APM is industrially produced either by chemical^{2~5)} or enzymatic method. In the chemical synthesis, phenylalanine is methylated and then coupled with aspartic acid through a series of chemical reactions, and

the final product is crystallized and dried. The enzymatic method utilizes thermolysin from *Bacillus proteolysis* Rokko⁶⁾ or similar enzymes catalyzing the synthesis of Z-APM from Z-L-Asp and L-phenylalanine methyl ester. The benzyloxycarbonyl moiety of the Z-APM is removed by hydrogenation to form APM.

If there is an enzyme catalyzing the synthesis of APM from L-phenylalanine methyl ester and L-isoasparagine, it would be possible to produce APM without by-products. It was found that an enzyme from *E. coli* K-12 (TPU6303) catalyzes this reaction (Scheme 1).

In this paper, we purified and characterized isoasparaginase from *E. coli* K-12. The isoasparaginase was identified as α -aspartyl dipeptidase (PepE).





Scheme 1. Synthesis of aspartame by α -aspartyl dipeptidase

2. Materials and Methods

2.1 Bacterial strain

Escherichia coli K-12 (TPU6303) was used as a source of the enzyme. TPU: Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-0398, JAPAN.

2.2 Materials

DEAE-Toyopearl 650M, Butyl-Toyopearl 650M and TSK-gel Octyl-80Ts column were purchased from Tosoh (Tokyo, Japan). Mono-Q HR5/5, SuperdexTM200 HR10/30 and the molecular weight marker proteins for SDS-PAGE were purchased from Pharmacia (Uppsala, Sweden). Gigapaito was purchased from Seikagaku Kogyo (Tokyo, Japan). Kieselgel 60 F²⁵⁴ TLC plate was purchased from Merck (Darmstadt, Germany). Centriprep10 was purchased from Amicon (Beverly, USA). Protein assay kit was purchased from Bio Rad (California, USA). NGP- 520L for Native-PAGE was purchased from ATTO (Tokyo, Japan). Polypepton was from Nippon Seiyaku (Tokyo, Japan). The molecular weight marker proteins for Native-PAGE were purchased from Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan). All other chemicals were purchased from commercial sources and used without further purification.

2.3 Preparation of L-isoasparagine (L-isoAsn)

L-IsoAsn was synthesized as shown in Scheme 2. Firstly, Z-L-aspartyl anhydride (Z-L-Asp-anhydride) was synthesized from Z-L-aspartic acid (Z-L-Asp) and acetic anhydride. Next, Z-L-Asp-anhydride was reacted with NH₃ to form Z-L-isoasparagine (Z-L-isoAsn). Finally,

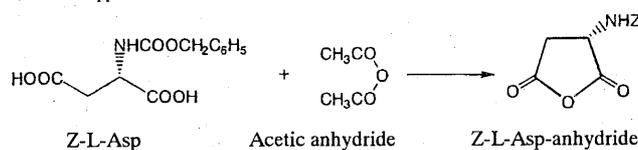
benzyloxycarbonyl moiety of Z-L-isoAsn was removed by hydrogenation, to yield L-isoasparagine (L-isoAsn).

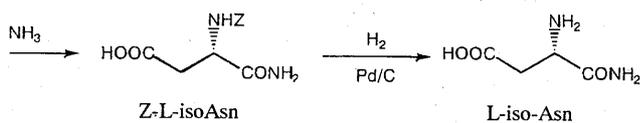
Synthesis of N-Benzyloxycarbonyl-L-aspartic acid anhydride

Acetic acid anhydride (176.7 mmol) was added to Z-L-Asp (36.3 mmol) and then stirred at 50°C for 12 hr. The reaction mixture was cooled to room temperature and then a white residue was crystallized from ether/petroleum ether = 1/2. The residue was dissolved in ethyl acetate and Z-Asp-anhydride was recrystallized from ether/petroleum ether = 1/2 to give colorless crystals (4.73g, 52.3%). ¹H-NMR (DMSO-*d*₆) δ_{ppm} 8.14 (d, 1H, *J*=7.8 Hz), 7.31 ~ 7.38 (m, 5H), 5.06 (s, 2H), 4.67 ~ 4.73 (m, 1H), 3.27 (dd, 1H, *J*=6.1, 10.0 Hz), 2.92 (dd, 1H, *J*=6.5, 6.4 Hz). ¹³C-NMR (DMSO-*d*₆) δ_{ppm} 172.2, 169.8, 155.9, 136.3, 128.4, 128.1, 127.9, 66.25, 50.42, 34.75

Synthesis of L-isoAsn

Twenty nine % ammonia water (56.2 mmol) was added to Z-L-Asp anhydride (8.0 mmol) in dry THF (55.6 ml) and then the reaction mixture was stirred for 12 hr at room temperature. The reaction mixture was evaporated to dryness to give a white residue. Twenty five % methanol (30 ml) was added to the residue, and then hydrogenated in the presence of 10% Pd/C atmospheric pressure at room temperature for 24 hr. The mixture was filtered and the filtrate was evaporated to dryness to give a white residue. The residue was dissolved in water (30 ml) and the pH was adjusted to 5.4 with formic acid. Ethanol (60 ml) was added to the mixture and then the mixture was left at 4°C for 4 hr. The resulting crystals were collected and L-isoAsn was recrystallized from ethanol to give colorless crystals (0.22 g, 21.3 %). ¹H-NMR (D₂O) δ_{ppm} 4.09 ~ 4.13 (m, 1H), 2.58 ~ 2.73 (m, 2H), ¹³C-NMR (D₂O) δ_{ppm} 176.9, 172.7, 51.4, 37.9





Scheme 2. Synthesis of L-isoAsn

2.4 Media

Medium I contained 5 g glucose, 5 g ammonium sulfate, 10 g yeast extract and 10 g Polypepton/l distilled water (pH 7.0). Medium II contained 5 g glycerol, 5 g ammonium sulfate, 1 g KH_2PO_4 , 3 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 10 g yeast extract and 10 g Polypepton/l tap water (pH 7.0).

2.5 Cultivation condition

E. coli K-12, subcultured on a medium I agar plate, was inoculated into a test tube containing 5 ml of a medium II, and aerobically cultivated at 30°C for 16 hr with reciprocal shaking. Then, 5 ml of the culture was transferred to a 2 l Sakaguchi flask containing 300 ml of the same fresh medium and aerobically cultivated at 30°C for 16 hr with reciprocal shaking. The cells were harvested by centrifugation at $6,000 \times g$ at 4°C for 10 min, then washed with 0.85% NaCl solution.

2.6 Enzyme assay

Isoasparaginase activity was qualitatively determined by measuring the formation of L-aspartic acid. The reaction mixture (0.1 ml) consisted of 10 μl of 100 mM L-isoAsn in 200 mM acetate buffer (pH 6.0) and 10 μl of the enzyme solution. The reaction was carried out at 30°C for 1 to 2 hr and then 1 μl of the reaction solution was spotted onto a TLC plate. L-IsoAsn and L-Asp were separated by using a solvent system of *n*-propanol / ammonia / water (8:1:3 by volume) and were determined by spraying ninhydrin reagent.

Isoasparaginase activity was quantitatively determined by measuring formation of α -aspartyl hydroxamate. The reaction mixture (0.5 ml) consisted of 375 μl of 66.7 mM L-isoAsn, 66.7 mM $\text{NH}_2\text{OH} \cdot \text{HCl}$ in 200 mM acetate buffer (pH 6.0) and 125 μl of the enzyme solution. The reaction

was carried out at 30°C for 0.5 to 2 hr and then stopped by adding 750 μl of the acid mixture (100 g FeCl_3 and 33 g trichloroacetic acid/1 0.7 M HCl). The amount of α -aspartyl hydroxamate formed in the reaction mixture was determined spectrophotometrically by measuring the absorbance at 515 nm of the solution. One unit of isoasparaginase activity was defined as the amount of enzyme which catalyzes the formation of α -aspartyl hydroxamate at a rate of 1 $\mu\text{mol}/\text{min}$ under the standard assay conditions.

Isoasparaginase activity was also quantitatively determined by measuring formation of *p*-nitroaniline from L-aspartic acid *p*-nitroanilide. The reaction mixture consisted of 30 μl of 33.3 mM L-aspartic acid *p*-nitroanilide in 30 μl DMSO, 20 mM potassium phosphate buffer (pH 7.0) and 500 μl of the enzyme solution. The reaction was carried out at 30°C for 10 to 30 min. The amount of *p*-nitroaniline formed in the reaction mixture was determined spectrophotometrically by measuring the absorbance at 405 nm of the solution. ($\epsilon = 9.82 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$)⁷⁾ One unit of isoasparaginase activity was defined as the amount of enzyme which catalyzes the formation of *p*-nitroaniline at a rate of 1 $\mu\text{mol}/\text{min}$ under the standard assay conditions.

2.7 Protein determination

The protein concentration was determined by method of Bradford⁸⁾ with bovine serum albumin as the standard by using protein assay kit. The specific activity was expressed in units/mg.

2.8 Purification of isoasparaginase from *E. coli* K-12

The enzyme was purified from cell-free extracts of *E. coli* K-12 cells. All purification steps were performed at 0 to 4°C. Centrifugation was carried out at $6,000 \times g$ for 10 min.

Preparation of cell-free extract

One hundred twenty seven g of washed cells obtained

from a 6 l culture broth were suspended in 1.26 l of 0.85% NaCl solution and then disrupted with an ultrasonic oscillator at 0°C for 15 min. The cell debris was removed by centrifugation. The supernatant solution was used as the cell-free extract.

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the cell-free extract to 30% saturation. The pH was maintained at pH 7.0 with 1 M NH₄OH. After stirring for 30 min, the precipitate was removed by centrifugation and the supernatant solution further saturated with ammonium sulfate to 60% saturation. The suspension was centrifuged and the pellet was dissolved in 20 mM Tris-HCl buffer (pH 8.3), then dialyzed against the same buffer.

First DEAE-Toyopearl column chromatography

The dialyzed enzyme solution was applied to a DEAE-Toyopearl column (ϕ 4.2 cm \times 21.7 cm), which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.3). The column was washed with 600 ml of the same buffer, then the enzyme was eluted with a linear gradient of 0 to 200 mM NaCl in the same buffer. Fractions of 12 ml were collected and the active fractions combined were used for further purification.

Butyl-Toyopearl column chromatography

Solid ammonium sulfate was added to bring the enzyme solution to 30% saturation. The solution was then applied to a Butyl-Toyopearl column (ϕ 4.2 cm \times 21.7 cm), which had been equilibrated with 20 mM Tris-HCl (pH 8.3) containing 30% saturated ammonium sulfate. The column was washed with 600 ml of the same buffer, then the enzyme eluted with 20 mM Tris-HCl buffer (pH 8.3) with a decreasing gradient of a 30 to 0% saturation ammonium sulfate. Fractions of 10 ml were collected and the active fractions were combined, then dialyzed against 20mM Tris-HCl buffer (pH 8.3).

Second DEAE-Toyopearl column chromatography

The dialyzed enzyme solution was applied to a

DEAE-Toyopearl column (ϕ 3.2 cm \times 25 cm), which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.3). After washing the column with 400 ml of the same buffer, the enzyme was eluted with a linear gradient of 0 to 200 mM NaCl in the same buffer. Fractions of 8 ml were collected and the active fractions were combined, then dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

Gigapite column chromatography

The dialyzed enzyme solution was concentrated to 20 ml from 136 ml by using Centriprep10 (Amicon, USA). The concentrated enzyme solution applied to a Gigapite column (ϕ 2.4 cm \times 4.4 cm), which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer. Fractions of 2 ml were collected and the active fractions were combined, then dialyzed against 20 mM Tris-HCl buffer (pH 8.3).

Third DEAE-Toyopearl column chromatography

The dialyzed enzyme solution was applied to a DEAE-Toyopearl column (ϕ 2.4 cm \times 4.4 cm), which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.3). After washing the column with 40 ml of the same buffer, the enzyme was eluted with a linear gradient of 0 to 200 mM NaCl in the same buffer. Fractions of 2 ml were collected and the active fractions were combined, then dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

Mono-Q column chromatography

The dialyzed enzyme solution was concentrated to 5 ml from 24 ml by Centriprep. The concentrated enzyme solution was subjected to FPLC on a Mono-Q column (ϕ 5 mm \times 5 cm) and eluted with a linear gradient of 0 to 200 mM NaCl in 10 mM potassium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The active fractions were combined.

Superdex™200 Column chromatography

The enzyme solution was concentrated to 4 ml from 15.6 ml by Centriprep. The concentrated enzyme solution was subjected to FPLC on a Superdex™200 column ($\phi 10$ mm \times 30 cm) and eluted with a 20 mM potassium phosphate buffer (pH 7.0) containing 200 mM NaCl at a flow rate of 0.4 ml/min. The active fractions were combined.

TSK-GEL Octyl-80Ts column chromatography

The enzyme solution was incubated in 5.94 M guanidine hydrochloride at 30°C for 30 min. Then the enzyme solution was subjected to HPLC on a TSK-GEL Octyl-80Ts column ($\phi 4.6$ mm \times 15 cm) and eluted with a linear gradient of 0 to 80% acetonitrile at a flow rate of 0.5 ml/min. Trifluoroacetic acid (0.1%) was always present in the running solution.

2.9 Analytical methods for isoasparaginase

Native-PAGE was performed by using NGP-520L polyacrylamide gel with Tris/glycine buffer, according to the method of Davis.⁹⁾ SDS-PAGE was performed in 12.5% polyacrylamide gels with Tris/glycine buffer, according to the method of Laemmli.¹⁰⁾ The gels were stained with Coomassie brilliant blue R-250 and destained with methanol / acetic acid / H₂O (3:1:6 by vol.). The relative molecular weight of the subunit was determined from the relative mobilities of standard proteins.

2.10 Amino terminal amino acid sequencing

To sequence NH₂-terminal amino acids, the enzyme sample was covalently bound to Sequetlon-AA and -DITC membranes using the procedures supplied by the manufacturer. Sequetlon-AA and -DITC membranes are made of a PVDF (polyvinylidene difluoride) matrix that have been derivatized with aryl amine and 1,4-phenylene diisothiocyanate groups, which react with both the C-terminal and side-chain carboxyl groups, and both N-terminal and side-chain amino groups of the enzyme, respectively. The enzyme samples on the membranes were analyzed with an automatic protein sequencer 6625

Prosequencer (Milligen/Biosearch) using phenylthiohydantoin method.¹¹⁾

3. RESULTS

3.1 Purification of the isoasparaginase from *E. coli* K-12 (TPU6303)

Isoasparaginase was purified by chromatographies on DEAE-Toyopearl, Butyl-Toyopearl, Gigapite, Mono-Q, Superdex™200, and TSK-GEL Octyl-80Ts.

The enzyme solution purified by Mono-Q column chromatography was electrophoresed on polyacrylamide gel. Two bands were observed on the gel. The molecular weight of these proteins was calculated to be about 42,000 and 46,000 respectively. The isoasparaginase was not purified to homogeneity on electrophoresis but highly purified by a 9 step procedure, as described in Materials and Methods.

A summary of the purification procedures for the isoasparaginase is shown in Table 1. The enzyme was partially purified about 2,500-fold and recovered yield was 1.2% from the cell-free extract. The enzyme purified by an 8 step procedure was separated to fraction α and fraction β by TSK-GEL Octyl-80Ts column chromatography.

3.2 Molecular weight

The molecular weight of the fraction α and fraction β , which were separated by HPLC on a TSK-GEL Octyl-80Ts column, was calculated to be about 25,000 and 27,000 by the SDS-PAGE.

3.3 Amino-terminal sequence

Amino-terminal sequence of fraction α and β which were separated by HPLC on a TSK-GEL Octyl-80Ts, were determined. Twenty residues in the amino-terminal region of fraction α were identical to 20 residues of ribose-5-phosphate isomerase A from *E. coli* (Figure 1). Eighteen residues in the amino-terminal region of fraction β were identical to 20 residues of α -aspartyl dipeptidase

(peptidase B) from *E. coli*⁷⁾ and *Salmonella typhimurium*¹²⁾ (Figure 2). The results suggested that the fraction α was ribose-5-phosphate isomerase A and the fraction β was α -aspartyl dipeptidase.

(Fraction- α)

MTQDELKKA VGWAALQYVQP

MTQDELKKA VGWAALQYVQP

(Ribose-5-phosphate isomerase A from *E. coli*)

Figure 1. Comparison of amino-terminal sequence of fraction α with that of ribose-5-phosphate. The identical amino acid residues between fraction α and ribose-5-phosphate are shown as *.

(Fraction- β)

MELLLLSNSNSTLPGKAXLEHA

***** ** *

MELLLLSNSNSTLPGKAWLEHA

(α -aspartyl dipeptidase (peptidase E) from *E. coli*)

MELLLLSNSNSTLPGKAWLEHA

(α -aspartyl dipeptidase (peptidase E) from *S. typhimurium*)

Figure 2. Comparison of amino-terminal sequence of fraction β with that of α -aspartyl dipeptidase. The identical amino acid residues between fraction β and α -aspartyl dipeptidase were shown as *. The amino acids, which were not detected, are shown as X.

4. Discussion

The authors expected that isoasparaginase which acts on α -amide group of isoasparagine may catalyze the synthesis of aspartame. The isoasparaginase was purified from the cell-free extract of *E. coli* K-12 (TPU6303) and its *N*-terminal amino acids were sequenced. As a result, isoasparaginase showed strong homology to α -aspartyl dipeptidase (PepE) from *E. coli* and *S. typhimurium*, and ribose-5-phosphate isomerase A from *E. coli*. PepE

liberates L-aspartate upon hydrolysis from peptides containing aspartic acid residues in *N*-terminal amino acids. We thus identified the isoasparaginase as α -aspartyl dipeptidase (PepE) (aspartyl aminopeptidase (EC 3.4.11.21)).¹²⁾ Cloning and high expression in *E. coli* of the gene for the enzyme is in progress.

References

- 1) R. H. Mazur, J. M. Schlatter, and A. H. Goldkamp (1969): Structure-taste relationships of some dipeptides, *J. Am. Chem. Soc.*, **91**, 2684-2691
- 2) Ajinomoto, *US Pat.*, 3 798 206 (1974)
- 3) Stamicarbon, *US Pat.*, 3 879 372 (1975)
- 4) Nutrasweet, *US Pat.*, 4 946 988 (1990)
- 5) Ajinomoto, *US Pat.*, 3 7860 39 (1974)
- 6) K. Oyama (1992): The industrial production of aspartame, in "Chirality in Industry", A.N. Collins, G.N. Sheldrake, and J. Crosby, Ed., 237-247
- 7) C. A. Conlin, T. M. Knox, and C. G. Miller (1994): Cloning and physical map position of an alpha-aspartyl dipeptidase gene, *pepE*, from *Escherichia coli*, *J. Bacteriol.*, **176**, 1552-1553
- 8) M. M. Bradford (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, **72**, 248-254
- 9) B. J. Davis (1964): Disc electrophoresis II. Method and application to human serum proteins, *Ann. N. Y. Acad. Sci.*, **121**, Art2, 404-427
- 10) U. K. Laemmli (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685
- 11) M. W. Hunkapiller, R. M. Hewick, W. J. Drewer, and L. E. Hood (1983): High-sensitivity sequencing with a gas-phase sequencer, *Methods Enzymol.*, **91**, 399-413
- 12) H. Zhang, J. Zhang, X. Wang, W. Yang, and J. Lu, (1998): Biochemical characterization of alpha-aspartyl dipeptidase. Cloning and expression of its gene, *Ann N. Y. Acad. Sci.*, **864**, 621-625

- 13) P. B. Nathan, S. I. Ahmad, M. Griffin (1989): peptidase (peptidase E) in *Escherichia coli* K12, *Biochem. Soc. Trans.*, **17**, 354
 Identification and characterization of an aspartate-specific

Table 1. Purification of isoasparaginase from *E. coli* K-12 (TPU6303)

Purification step	Protein (mg)	Total activity (units)	Specific activity (mU/mg)	Purification (-fold)	Recovery (%)
Cell-free extract	18,900	25.4	1.34	1	100
Ammonium sulfate fraction	8,430	3.1	2.4	1.8	79.7
DEAE-Toyopearl 1st	2,320	1.2	6.95	5.2	63.4
Butyl-Toyopearl	225	5.47	24.2	18	21.5
DEAE-Toyopearl 2nd	41.2	2	48.4	36	7.9
Gigapite	7.7	1.63	213	158	6.4
DEAE-Toyopearl 3rd	5.1	1.23	240	179	4.8
Mono-Q	0.5	0.78	1,660	1,240	
SuperdexTM200	0.09	0.31	3,390	2,530	

Escherichia coli K-12 が生産するイソアスパラギナーゼの α -アスパルチルジペプチダーゼとしての同定

浅野 泰久・水口 直之・米田 英伸・吉良 郁夫・横関 健三*

(生物工学研究センター、*味の素(株))

Escherichia coli K-12 (TPU6303)の無細胞抽出液よりイソアスパラギナーゼを精製し、そのN末端アミノ酸配列を決定した。TSK-GEL Octyl-80Ts を用いて HPLC で精製されたフラクション α およびフラクション β の分子量は、SDS-PAGE により、それぞれ約 25,000 および 27,000 と算出された。フラクション α のN末端アミノ酸配列を20残基決定し、*E. coli* のリボース-5-リン酸イソメラーゼ A と同定した。フラクション β のN末端アミノ酸配列を18残基決定し、 α -アスパルチルジペプチダーゼ(PepE) (アスパルチルアミノペプチダーゼ、EC 3.4.11.21)と同定した。以上より、*E. coli* に検出されたイソアスパラギナーゼは α -アスパルチルジペプチダーゼと同一酵素であることが明らかになった。

キーワード: アスパルテーム、イソアスパラギナーゼ、 α -アスパルチルジペプチダーゼ (PepE)、アスパルチルアミノペプチダーゼ、*Escherichia coli*