Cloning of isoasparaginase (α-aspartyl dipeptidase) gene from *Escherichia coli* K-12 and overexpression of the gene in *E. coli*

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Summary

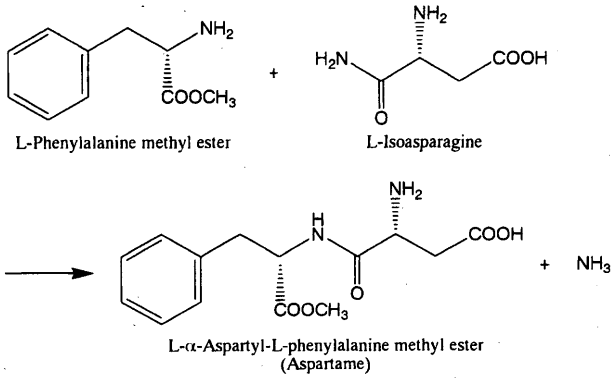
Isoasparaginase from *E. coli* K-12, which catalyzes the hydrolysis of L-isoasparagine to form L-aspartic acid and ammonia, was previously identified as α-aspartyl dipeptidase. The gene encoding the isoasparaginase (α-aspartyl dipeptidase, PepE) was cloned from the chromosomal DNA of *E. coli* K-12. The *pepE* gene modified in the nucleotide sequence upstream from its start codon was overexpressed in *E. coli* JM109. The activity of the recombinant PepE enzyme in cell-free extracts of *E. coli* JM109 harboring *pepE*-expression plasmid was 2.92 units•mg^{-1} with L-aspartic acid *p*-nitroanilide as substrate, which was 2100 times higher than that of *E. coli* host. This enzyme was purified to electrophoretic homogeneity by ammonium sulfate fractionation and four column chromatography steps. It had maximal activity at 50°C and pH 8.0. Enzymatic synthesis of aspartame was attempted using the purified PepE with L-isoasparagine and L-phenylalanine methyl ester as substrates.

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

1. Introduction

We have been interested in the enzyme-catalyzed synthesis of aspartame (L-α-aspartyl-L-phenylalanine methyl ester), a synthetic sweetener which is about 200 times sweeter than sucrose. Owing to its pleasant sweetness without a bitter aftertaste, it was well received as a low-calorie sweetener (4 kcal/g) for many foodstuffs. Aspartame is industrially produced either by chemical[1~4] or enzymatic[5] 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* Rokko or similar enzymes catalyzing the synthesis of *Z*-aspartame from *Z*-L-aspartic acid and L-phenylalanine methyl ester. The benzyloxycarbonyl moiety of the *Z*-aspartame is removed by hydrogenation to form aspartame.

We expected that isoasparaginase which hydrolyzes α-amide group of L-isoasparagine to form L-aspartic acid and ammonia may catalyze the synthesis of aspartame from L-isoasparagine and L-phenylalanine methyl ester by kinetically-controlled peptide synthesis (Scheme 1). We purified isoasparaginase activity from the cell-free extract of *E. coli* K-12 (TPU6303) and its N-terminal amino acid sequence was determined. As a result, the sequence of isoasparaginase showed strong homology to those of α-aspartyl dipeptidases (PepE) from *E. coli*[6] and *Salmonella enterica* serovar Typhimurium.[7] We thus identified the isoasparaginase as α-aspartyl dipeptidase (PepE).[8] The *PepE* from *S. enterica* serovar Typhimurium was shown to be a serine peptidase having substrate specificity for aspartyl dipeptides.[9] The PepE in *E. coli* has not been characterized yet. In this study, the gene coding for the enzyme was isolated from *E. coli* K-12 and expressed in *E. coli* JM109 host. The recombinant PepE was purified, characterized, and used for the enzymatic synthesis of aspartame.



Scheme 1. Synthesis of aspartame by α-aspartyl dipeptidase (PepE)

2. Materials and Methods

2.1 Bacterial strains, plasmid, and culture conditions

Escherichia coli K-12 (TPU6303) was used as the source of chromosomal DNA. *E. coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) was used as a host for the recombinant plasmid. Plasmid pUC119 (Takara Shuzo, Kyoto, Japan) was used as a cloning vector. *E. coli* K-12 was cultivated at 30°C on Medium I containing 5 g glycerol, 5 g ammonium sulfate, 1 g KH_2PO_4, 3 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnCl_2 \cdot 4H_2O$, 10 g yeast extract and 10 g Polypepton in 1 litre distilled water, pH 7.0. Recombinant *E. coli* JM109 was cultured at 37°C on Luria-Bertani medium[10] containing 80 μg•ml^{-1} of ampicillin. To induce the gene under the control of the *lac* promoter, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM.

2.2 Cloning of the *E. coli* K-12 *pepE* gene and expression of the gene in *E. coli* JM109

For routine work with recombinant DNA, established protocols were used.[10] Restriction endonucleases were purchased from Takara Shuzo and alkaline phosphatase from shrimp was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chromosomal DNA was prepared from *E. coli* K-12 by the method of Misawa et al.[3] Oligonucleotide primers were synthesized on the basis of the nucleotide sequences of the flanking region of *pepE* gene (Genbank accession number P32666). The sense primer (TOY-1) containing an *Sph*I-recognition site (underlined sequence) corresponded to the 26-bp upstream region from the ATG start codon of *pepE*. The antisense primer (TOY-2) containing an *Stu*I site (underlined sequence) corresponded to the 63-bp downstream region from the TAA stop codon of the gene. The two primers were as follows: TOY-1, 5'-CAGTGGTAGC ATGCCTCCCG-3'; TOY-2, 5'-AATCCTGTAGGCCTG ATAAG-3'. The reaction mixture (100 μl) for the PCR contained 10 mM Tris/HCl, pH 8.85, 25 mM KCl, 2 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, each dNTP at a concentration of 0.2 mM, TOY-1 and TOY-2 primers each at 1 μM concentration, 5 U *Pwo* DNA polymerase and 160 ng of chromosomal DNA from E. *coli* K-12 as a template. Thirty cycles were performed, each consisting of a denaturing step at 94°C for 30 s (first cycle 2 min 30 s), an annealing step at 61°C for 30 s, and an elongation step at 72°C for 30 s. The amplified PCR product was digested with *Sph*I and *Stu*I, separated by agarose-gel electrophoresis, and then purified with QIAquick™ gel extraction kit. The amplified DNA was ligated into *Sph*I and *Hin*cII-digested and alkaline phosphatase-treated pUC119 using Ligation Kit ver. 2 from Takara Shuzo to yield pPEPE9, and then used to transform *E. coli* JM109 cells.[12]

A modified DNA fragment coding for the PepE was also obtained by PCR as described above. The sense primer (TOY-3) contained an *Hin*dIII-recognition site (underlined sequence), a ribosome-binding site (double underlined sequence), a TAG stop codon (lowercase letters) in-frame with the *lacZ* gene in pUC119. TOY-2 was used as an antisense primer. The nucleotide sequence of TOY-3 was 5' –CCGTGAAAGCTTTAAGGAGGAA tagGTAATGGAACTGCTTTTATTG-3'. Thirty cycles were performed, each consisting of a denaturing step at 94°C for 30 s (first cycle 2 min 30 s), an annealing step at 48°C for 30 s, and an elongation step at 72°C for 2 min. The amplified PCR product was digested with *Hin*dIII and *Stu*I, separated by agarose-gel electrophoresis, and then purified with QIAquick™ gel extraction kit. The amplified DNA was ligated into *Hin*dIII and *Hin*cII-digested and alkaline phosphatase-treated pUC119 to yield pPEPE9-N, and then used to transform *E. coli* JM109 cells.

Nucleotide sequences of the *pepE* gene in pPEPE9 and pPEPE9-N were determined to verify the introduction of no mutations into the gene during the amplification by PCR. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method[13] with M13 forward and reverse oligonucleotides as primers. Sequencing reactions were carried out with a Thermo Sequenase™ cycle sequencing kit and dNTP mixture with 7-deaza-dGTP from Amersham Biosciences K.K., and the reaction mixtures were run on a DNA sequencer 4000L (Li-cor, Lincoln, NE, USA). Both strands of DNA were completely sequenced.

2.3 Enzyme assay

PepE activity was 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 amount of *p*-nitroaniline formed in the reaction mixture was determined spectrophotometrically by measuring the absorbance at 405 nm of the solution (ε = 9.82 x 10^3 $M^{-1}cm^{-1}$).[7] One unit of PepE activity was defined as the amount of enzyme which catalyzes the formation of *p*-nitroaniline at a rate of 1 μmol/min under the standard assay conditions. The protein concentration was determined by the method of Bradford[14] with bovine serum albumin as the standard by using protein assay kit from Bio-rad (Tokyo, Japan). The specific activity was expressed in units/mg.

2.4 Purification of the PepE from *E. coli* transformant

E. coli JM109 harboring pPEPE9-N was subcultured at 37°C for 12 h in a test tube containing 5 mL Luria-Bertani medium supplemented with ampicillin. The subculture (1 mL) was then inoculated into a 2-L Sakaguchi flask containing 400 mL Luria-Bertani medium supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside. After a 12-h incubation at 37°C with rotary shaking, the cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and washed with 0.85% (w/v) NaCl. All the purification procedures were performed at a temperature lower than 5°C. The buffer used throughout this purification was Tris/HCl buffer (pH 8.3), unless otherwise noted. Washed cells (30 g) from 2 L culture were suspended in 100 mM buffer and disrupted by sonication for 10 min. For the removal of intact cells and cell debris, the sonicate was centrifuged at 15,000 × *g* for 20 min at 4°C to yield supernatant as a cell-free extract. Solid ammonium sulfate was added to the cell-free extract to 30% saturation. The pH was maintained at pH 7.0 with 1 M NH_4OH. After stirring for 30 min, the precipitate was removed by centrifugation and the supernatant solution was further saturated with ammonium sulfate to 60% saturation. The suspension was centrifuged and the pellet was dissolved in 20 mM buffer, then dialyzed against the same buffer. The dialyzed solution was applied to a column (3 x 20 cm) of DEAE-Toyopearl 650 M (Tosoh, Tokyo, Japan) previously equilibrated with 20 mM buffer. After the column had been washed thoroughly with 20 mM buffer, the enzyme was eluted with a linear gradient of 0 to 200 mM NaCl. The active fractions were then brought to 30% ammonium sulfate saturation and added to a column (3 x 15 cm) of Butyl-Toyopearl 650 M equilibrated with 20 mM buffer 30% saturated with ammonium sulfate. After the column had been washed with the same buffer, the enzyme was eluted with 20 mM buffer with a decreasing gradient of 30 to 0% saturation with ammonium sulfate. The active fractions were combined, dialyzed against 20 mM buffer, and concentrated to 4 ml from 84 ml by Centriprep (Amicon, Japan). The concentrated enzyme solution was subjected to FPLC on a MonoQ HR5/5 column and eluted with a linear gradient of 0 to 200 mM NaCl in 20 mM potassium phosphate buffer (pH 7.0). The active fractions were combined, then dialyzed against 20 mM potassium phosphate buffer (pH 7.0). The enzyme solution was concentrated to 3.2 ml from 46 ml by Centriprep and subjected to FPLC on a Superdex 200 HR10/30 column 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.

Purity of the enzyme was verified with SDS/PAGE analysis performed by the method of Laemmli.[15] Proteins were stained with Brilliant blue R-250 and destained in ethanol/acetic acid/water (3:1:6, by vol.).

2.5 Synthesis of aspartame by PepE

L-Isoasparagine and L-phenylalanine methyl ester were dissolved at 50 mM to 500 mM in 490 μl buffer and was mixed with 0.001 to 1 units of the purified PepE in 10 μl of 20 mM potassium phosphate buffer (pH 7.0). The reaction temperatures were 30 or 37°C and the reaction pH was 5 to 10. The reaction times were 0.5 to 24 hr. The 0.5 ml of the reaction mixture was taken at the appropriate times, and mixed with 0.5 ml of methanol to stop the reaction. Then, this mxture was analyzed by HPLC with an ODS column (0.5 x 20 cm, ODS-2) with a solvent system of acetonitrile/water (20/80; v/v) adjusted to pH 3.0 with phosphoric acid.

3. Results and Discussion

3.1 Cloning of the *E. coli* K-12 *pepE* gene and expression of the gene in *E. coli* JM109

PCR with the primers, TOY-1 and TOY-2, and the chromosomal DNA prepared from *E. coli* K-12 yielded an amplified DNA with 794 bp. The DNA fragment was ligated into pUC119 to construct pPEPE9. Nucleotide sequencing of the inserted DNA fragment revealed that the *pepE* gene was amplified by *Pwo* DNA polymerase without any point mutations. The plasmid, pPEPE9, in which the *pepE* gene was under the control of the *lac* promoter of pUC119 vector, was introduced into *E. coli* JM109 cells. When *E. coli* JM109 harbouring pPEPE9 was cultivated in Luria-Bertani medium supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside for 12 h at 37°C, the level of PepE activity in the supernatant of the sonicated cell-free extracts of the transformants was 0.34 units•mg^{-1} with L-aspartic acid *p*-nitroanilide as a substrate. This value was 240 times higher than that of *E. coli* JM109 harbouring pUC119 as a control.

To enhance the expression of the *pepE* gene in *E. coli*, we improved the sequence upstream from the ATG start codon by PCR, with primers TOY-3 and TOY-2 as described in Materials and Methods. The resultant plasmid, pPEP9-N was introduced into *E. coli* JM109 cells. A protein corresponding to the predicted molecular mass of 27 kDa was produced when the *lac* promoter was induced by isopropyl-β-D-thiogalactopyranoside (data not shown). The level of PepE activity in the cell-free extracts of *E. coli* JM109 harboring pPEPE9-N was elevated to 2.92 units•mg^{-1} which was 2100 times higher than that of the control, possibly because of the presence of ribosome-binding

site which may lead an efficient translation from the *pepE* mRNA in *E. coli*.

3.2 Purification of the PepE from *E. coli* transformant

Recombinant PepE was purified from the *E. coli* JM109 harboring pPEPE9-N with a recovery of 3% by 5 steps including ammonium sulfate fractionation and DEAE-Toyopearl, Butyl-Toyopearl, MonoQ, and Superdex column chromatographies (Table 1), while it took 8 steps from the wild type strain *E. coli* K-12.[8] The final preparation gave a single band on SDS-PAGE with a molecular mass of 27 kDa (data not shown). This value is in good agreement with that of the enzyme purified as isoasparaginase from *E. coli* K-12.[8] The purified enzyme catalyzed the hydrolysis of L-aspartic acid *p*-nitroanilide to L-aspartic acid and *p*-nitroaniline at 15.0 units•mg^{-1} under the standard conditions.

Table 1. Purification of PepE from *E. coli* JM109/pPEPE9-N

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1480	840	0.57	100
$(NH_4)_2SO_4$ fractionation	835	652	0.78	78
DEAE-Toyopeal	154	504	3.28	60
Butyl-Toyopearl	59	464	7.86	55
MonoQ HR5/5	3.2	31.3	9.80	4
Superdex 200 HR26/60	1.7	25.5	15.0	3

3.3 Stability of the PepE

The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 15 min in 20 mM potassium phosphate buffer (pH 7.0), a sample of the enzyme solution was taken and the activity was assayed with L-aspartic acid *p*-nitroanilide as a substrate under the standard conditions. It exhibited the following activity: 70°C, 12%; 60°C, 46%; 50°C, 94%; 40°C, 100%; 30°C, 100%. The stability of the enzyme was also examined at various pH values. The enzyme was incubated at 30°C for 10 min in the following buffers (final concentration 20 mM): acetic acid/sodium acetate (pH 3.5-6.0), potassium phosphate (pH 6.5-8.0), Tris/HCl (pH 8.0-9.0), Na_2CO_3/$NaHCO_3$ (pH 10.0-11.0), glycine/NaCl/NaOH (pH 10.5-13.0). Then a sample of the enzyme solution was taken, and the PepE activity was assayed with L-aspartic acid *p*-nitroanilide as a substrate under the standard conditions. The enzyme was most stable in the pH range 10.0-12.0

3.4 Effects of temperature and pH on PepE activity

The enzyme reaction was carried out at various temperatures for 10 min in 20 mM potassium phosphate buffer (pH 7.0), and enzyme activity was found to be maximal at 50°C. Above 50°C, it decreased rapidly, possibly because of instability of the enzyme at the higher temperatures. The optimal pH for the activity of the enzyme was measured in the buffers described above. The enzyme showed maximum activity at pH 8.0.

3.5 Synthesis of aspartame by PepE

Alkaline conditions are usually essential for peptide bond formation in kinetically controlled systems. As shown in Table 2, an appreciable amount of aspartame was formed by PepE under some alkaline conditions. The L-aspartyl-L-phenylalanine and L-phenylalanine were also detected. It was suggested that L-aspartyl-L-phenylalanine were formed after aspartame was synthesized, by a spontaneous hydrolysis of methyl ester in alkaline pH. The equilibrium of the reaction was found to be in a favor of the degradation of aspartame. The yield was not improved even when the reaction was done under various conditions, e.g. reaction time, the amount of the substrates, reaction pH and temperature, and addition of various amount of the enzyme. Addition of various compounds such as ethanol, ether, 1,4-dioxane, *n*-propanol, etc. to the reaction mixture did not improve the yield of aspartame. At a present stage, the productivity of aspartame is still low. In order to solve this problem, it may be necessary to alter the properties of the enzyme suitable for the synthesis of aspartame by molecular evolutionary strategies such as error prone PCR and DNA shuffling.

Table 2. Enzymatic synthesis of aspartame under various conditions.

pH	Temp. (°C)	L-IsoAsn (μmol)	L-Phe-OMe (μmol)	Enzyme (Units)	APM	AP	Phe
5	30	25	250	0.01	-	-	-
7	30	25	250	0.01	-	-	-
8	30	25	250	0.01	-	-	-
10	30	25	250	0.01	trace	+	+
11	30	25	250	0.01	trace	+	+
10	30	50	250	0.01	-	-	-
10	30	100	250	0.01	-	-	-
10	30	250	250	0.01	-	-	-
10	30	25	25	0.01	-	-	-
10	30	25	50	0.01	-	-	-
10	30	25	100	0.01	-	-	-
10	37	25	250	0.01	-	-	-
10	30	25	250	0.001	trace	+	+
10	30	25	250	0.1	-	+	+
10	30	25	250	1	-	+	+

L-IsoAsn: L-Isoasparagine, L-Phe-OMe: L-phenylalanine methyl ester, APM: aspartame, AP: L-aspartyl-L-phenylalanine, Phe: L-phenylalanine. The amount of products; aspartame (0-100 μM), AP (3-5 mM), Phe (28-30 mM).

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Escherichia coli K-12 由来のイソアスパラギナーゼ遺伝子（*pepE*）のクローニングと大量発現

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L-イソアスパラギンのα-アミド基を加水分解し、アスパラギン酸を生成する反応を触媒するイソアスパラギナーゼと同一酵素と考えられるα-アスパルチルジペプチダーゼ（PepE）をコードする遺伝子、*pepE* を大腸菌 K-12 株よりクローン化した。その開始コドン上流部分を改良した *pepE* を大腸菌 JM109 株で大量発現させた場合、その無細胞抽出液の PepE 活性は L-アスパラギン酸 *p*-ニトロアニリドを基質とした場合、2.92 ユニット/ミリグラムとなった。この値はコントロールの 2100 倍である。本酵素を大腸菌形質転換体の無細胞抽出液より精製し、その酵素化学的諸性質を明らかにした。本酵素は 50℃ 及び pH 8.0 において最大活性を示した。精製酵素を用いて L-イソアスパラギンと L-フェニルアラニンメチルエステルからのアスパルテームの合成を検討した。

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)、*Escherichia coli*