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Alteration of substrate specificity of aspartase by directed evolution

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Abstract

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1), which catalyzes the reversible deamination of L-aspartic acid to yield fumaric acid and ammonia, is highly selective towards L-aspartic acid. We screened for enzyme variants with altered substrate specificity by a directed evolution method. Random mutagenesis was performed on an *Escherichia coli* aspartase gene (*aspA*) by error-prone PCR to construct a mutant library. The mutant library was introduced to *E. coli* and the transformants were screened for production of fumaric acid-mono amide from L-aspartic acid-α-amide. Through the screening, one mutant, MA2100, catalyzing deamination of L-aspartic acid-α-amide was achieved. Gene analysis of the MA2100 mutant indicated that the mutated enzyme had a K327N mutation. The characteristics of the mutated enzyme were examined. The optimum pH values for the L-aspartic acid and L-aspartic acid-α-amide of the mutated enzyme were pH 8.5 and 6.0, respectively. The K_m value and V_{max} value for the L-aspartic acid of the mutated enzyme were 28.3 mM and 0.26 U/mg, respectively. The K_m value and V_{max} value for the L-aspartic acid-α-amide of the mutated enzyme were 1450 mM and 0.47 U/mg, respectively. This is the first report describing the alteration of the substrate specificity of aspartase, an industrially important enzyme.

Keywords: Aspartase; Directed evolution; Alteration of substrate specificity

1. Introduction

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartic acid to yield fumaric acid and ammonia (Fig. 1). It is one of the most important industrial enzymes and has been used to produce L-aspartic acid on a large scale [1]. The enzyme is among the most specific enzymes known, with extensive studies over the years failing to identify any alternative amino acid substrates that can replace L-aspartic acid. The enzyme has no activity with either D-aspartic acid or crotonic acid [2], and earlier work showed that glycine, alanine, glutamine, maleic acid, and glutaconic acid were not substrates [3]. Also, L-cysteic acid, diaminosuccinic acid, leucine, mesaconic acid, aconitic acid, sorbic acid, and the diamine or mono- or diethyl esters of fumaric acid have all failed to show turnover with this highly specific enzyme [4,5]. While the specificity for L-aspartic acid had been clearly demonstrated, L-aspartic acid-β-semialdehyde was identified as the second amino acid recognized by the enzyme as a mechanism-based inactivator [6].

Chemical modification and pH profile studies suggested the potential importance of lysine residues in the activity of aspartase. Of the 26 lysines that are present in *Escherichia coli* aspartase, only three are conserved among the aspartases that have been sequenced, and these lysines are also extensively conserved throughout the extended homologous family of fumarases and lyases [7–10]. During the process of identifying and examining the possible involvement of a critical lysine, a mutant was constructed to replace the most highly conserved lysine with arginine [11]. The mutant (K327R) was found to have a very low catalytic activity, and this lysine has now been proposed as a functionally important amino acid at the active site of aspartase [12]. Furthermore, an examination of the recently determined high resolution structure of aspartase shows that Lys-327 is in the active site cavity [13]. However, amino acid residues involved in

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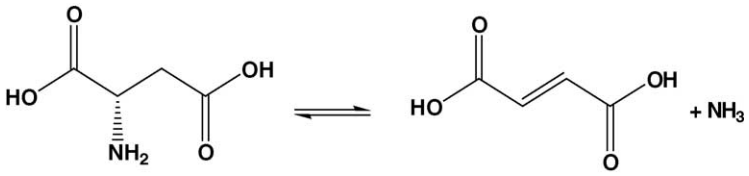

Fig. 1. The reversible deamination of L-aspartic acid catalyzed by aspartase.

the substrate recognition of aspartase have not been determined because the structure of the enzyme–substrate complex has not been solved.

Studying how to alter the substrate specificity of an existing enzyme is useful because it provides not only efficient catalysts with designed substrate specificity, but also valuable information on the mechanism of substrate recognition. In the past few years, directed evolution methods have been successfully applied to improve the properties of several enzymes [15–20].

Here, to alter the substrate specificity of aspartase from *E. coli*, we used the directed evolution method. The *aspA* gene encoding aspartase was randomly mutagenized by error-prone PCR and then the mutated enzymes showing activity for L-aspartate-α-amide were screened. Through the screening, one mutated enzyme, MA2100, was obtained, and we identified the mutation site by sequencing the gene. The mutated enzyme was produced in *E. coli*, purified and characterized. This is the first report describing the alteration of the substrate specificity of aspartase, an industrially important enzyme.

2. Materials and methods

2.1. Materials, enzymes and chemicals

Restriction endonucleases, T4 DNA ligase and Mutan-Super Express Km were purchased from Takara Shuzo (Kyoto, Japan). *Taq* DNA polymerase was purchased from Boehringer-Mannheim (Tokyo, Japan). The protein assay kit was purchased from Bio-Rad (Hercules, USA) and the molecular mass markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Daiich Pure Chemicals Co., Ltd. (Kyoto, Japan). The Sure Clone Ligation kit was purchased from Pharmacia (Uppsala, Sweden) and the QIAexpress Type IV kit was purchased from QIAGEN (Hilden, Germany). L-aspartate-α-amide and fumaric acid-mono amide were purchased from Kokusan Chemicals (Tokyo, Japan). All other chemicals used were commercially available and were of analytical grade.

2.2. Strains, plasmids, and culture conditions

E. coli K-12 was used as the DNA donor. *E. coli* JM109 was used as the host strain for DNA manipulation and expression. *E. coli* MV1184 was used as the host strain for site directed mutagenesis. Plasmids, pUC18, pHSG298 and pKF18k (Takara Shuzo, Kyoto, Japan) were used as vectors for *E. coli*. A plasmid, pASPA, containing aspartase gene *aspA* of *E. coli* K-12 was used as a template in PCR mutagenesis for directed evolution. Luria–Bertani (LB) medium [21] was used for the culture of *E. coli*, which was grown aerobically for 16 h at 37 °C with reciprocal shaking in a 500-ml shaking flask containing 50 ml of LB medium. For the selection of *E. coli* transformants, ampicillin (100 μg/ml) or kanamycin (100 μg/ml) was added to the medium.

2.3. General DNA manipulation

All basic recombinant DNA procedures, such as isolation and purification of DNA, restriction enzyme digestion, ligation of DNA, and transformation of *E. coli*, were performed as described by Sambrook et al. [21]. DNA was sequenced by the dideoxynucleotide chain termination method with a Dye Terminator Cycle sequencing kit (Perkin-Elmer, Norfolk, CT) and a DNA sequencer (model 373A, Perkin-Elmer).

2.4. Enzyme assay

The enzyme activity for L-aspartic acid of wild-type aspartase was assayed by monitoring the rate of the formation of fumaric acid at 240 nm ($E_{240} = 2.53\ mM^{-1}/cm$). The standard reaction mixture contained 100 mM Tris/HCl buffer (pH 8.5), 100 mM L-aspartic acid, 2 mM $MgCl_2$ and an appropriate amount of the enzyme in a total volume of 1 ml. The reaction mixture was incubated for 1 min at 30 °C. The enzyme activity for L-aspartic acid of mutated aspartase was assayed in a standard reaction mixture containing 100 mM Tris/HCl buffer (pH8.5), 100 mM L-aspartic acid, 2 mM $MgCl_2$ and an appropriate amount of the enzyme in a total volume of 100 μl. The reaction was performed at 30 °C for 30 min and stopped by the addition of 0.9 ml of 1.7% H_3PO_4 solution. The enzyme activity for L-aspartic acid-α-amide was assayed in a standard reaction mixture containing 100 mM HEPES buffer (pH 7.0), 100 mM L-aspartic acid-α-amide, 2 mM $MgCl_2$ and an appropriate amount of the enzyme in a total volume of 100 μl. The reaction was performed at 30 °C for 30 min and stopped by the addition of 0.9 ml of 1.7% H_3PO_4 solution.

The amount of fumaric acid or fumaric acid-mono amide formed in the reaction mixture was determined with a HITACHI HPLC apparatus equipped with an Inertsil ODS-2 column (4.6 × 250 mm; GL Science, Tokyo, Japan) at a flow rate of 0.5 ml/min, using a solvent system 5 mM potassium phosphate buffer (pH 3.0): acetonitorile (90:10, v/v). Absorbance of the eluate was monitored at 254 nm. One

unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of fumaric acid or fumaric acid-mono amide per min under the above conditions. A steady-state kinetic constant was determined with a substrate concentration ranging from 0.1 to 500 mM by using a Lineweaver–Burk plot.

2.5. Protein assays

The protein concentration was assayed by the method of Bradford [22] using a dye reagent concentrate (Bio-Rad laboratories, Hercules, USA) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [23], with a 5–20% gradient acrylamide gel. Proteins were stained with Coomassie brilliant blue R-250.

2.6. Cloning of the aspartase gene (aspA)

Amplification of a DNA fragment containing the *E. coli aspA* gene was carried out with 30 cycles of PCR using *E. coli* K-12 chromosonal DNA as the template and two specific primers, PR1 (5′-ATA ATC GTC GGT CGA AAA AC) and PR2 (5′-TTT AAG TTA CTG CTC ACA AG). These primers were designed on the basis of the nucleotide sequence of the asapartase gene from *E. coli* K-12, which was submitted to the DDBJ/EMBL/GenBank nucleotides sequence databases with the accession number X04066. Each cycle was carried out at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The amplified PCR product was inserted into the *Sma*I site of pUC18 by using a Sureclone ligation kit (Pharmacia). *E. coli* JM 109 transformants were grown on LB plates containing 100 μg/ml ampicillin, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 16 h. Some white colonies were chosen and cultured in 4 ml of LB medium containing 100 μg/ml ampicillin and 1 mM IPTG at 37 °C for 16 h. Cells from 4 ml of culture broth were harvested by centrifugation at 10,000 × *g* for 10 min. Washed cells were suspended in 1 ml of 100 mM HEPES-NaOH buffer (pH 7.0) and disrupted with a sonicator (Bioruptor UCW-201) at 4 °C for 10 min. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was used as the cell-free extract. Aspartase activity of the cell-free extract was measured as described above. Recombinants that exhibited higher aspartase activity than that of the host (*E. coli* JM 109) were selected. Plasmid, pASPA-containing *aspA* of *E. coli* was purified from these recombinants by using a QIAGEN Plasmid Kit.

2.7. Error-prone PCR for random mutagenesis and screening of mutants

Random mutagenesis of the *aspA* gene was performed by error-prone PCR according to the modified method of Cadwell and Joyce [24,25]. The reaction mixture for the error-prone PCR contained in 100 μl 10 mM Tris/HCl buffer (pH 8.3), 50 mM KCl, 7 mM $MgCl_2$, 0.2 mM each dATP, dGTP and dCTP, 1 mM dTTP, 30 pmol each M13 primer RV and M13 primer M4 (Takara Shuzo), 5 U of *Taq* DNA polymerase and 10 ng plasmid pASPA as template DNA. PCR was carried out with programs of 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The amplified PCR product was desalinated with a QIAquick PCR purification kit (QIAGEN) and digested with *Sac*I and *Sph*I. A mixture of *Sac*I–*Sph*I fragments, including the mutagenized *aspA* gene was separated by agarose-gel electrophoresis and, then purified with a QIAquick™ gel extraction kit (QIAGEN). The amplified DNA was inserted downstream of the *lac* promoter in pHSG298 to generate a mutant library.

The constructed mutant library was transformed with *E. coli* AJ11103-57. Each transformant (total 3000 transformants) was isolated and cultivated in LB medium containing 100 μg/ml kanamycin and 1 mM IPTG at 37 °C for 16 h. The deamination reaction of L-aspartic acid-α-amide was carried out with cell-free extracts from each of the clones. The reaction mixture contained 100 mM MOPS/NaOH buffer (pH7.0), 100 mM L-aspartic acid-α-amide, 2 mM $MgCl_2$ and cell-free extract (20 μl) in a total volume of 40 μl. After incubation for 16 h at 30 °C, the formation of fumaric acid-mono amide was detected by thin-layer chromatography (TLC). A volume of 1 μl of the reaction mixture was spotted onto silica gel TLC plates (Silicagel $60F_{254}$; Merck, Rahway, USA) and developed with butyl alcohol/acetic acid/water = 4/1/2 (v/v/v). The fumaric acid-mono amide formed was detected at UV 254 nm. A transformant exhibited the activity for L-aspartic acid-α-amide was selected and plasmid, pMA2100 containing the mutated *aspA* gene was purified from the recombinant.

2.8. Saturation mutagenesis

To prepare various kinds of mutated aspartases with an amino acid substitution at position 327, site-directed mutagenesis was performed using a Mutan-Super Express Km kit (Takara Shuzo). The pASPA was digested with *Sac*I and *Sph*I. A mixture of *Sac*I–*Sph*I fragments, including the *aspA* gene, was purified and inserted into the *Sac*I–*Sph*I sites in plasmid pKF18k to construct the recombinant plasmid, pKFASPA.

The target mutation was introduced into plasmid pKFASPA using PCR with the selection primer (Takara Shuzo) and the following mutagenic primer. Eighteen types of mutagenic primers were synthesized: MUTAla: 5′-P-ATG CCA GCT GCG GTA AAC-3′, MUTArg: 5′-P-ATG CCA GCT GCG GTA AAC-3′, MUTAsp: 5′-P-ATG CCA GCT GCG GTA AAC-3′, MUTCys: 5′-P-ATG CCA GCT GCG GTA AAC-3′, MUTGln: 5′-P-ATG CCA GCT GCG GTA AAC-3′, MUTGlu: 5′-P-ATG CCA GCT GCG GTA AAC-3′,

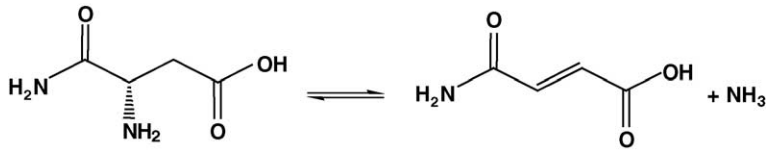

Fig. 2. The reversible deamination of L-aspartic acid-α-amide catalyzed by mutated aspartase.

MUTGly: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTHis: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTIle: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTLeu: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTMet: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTPhe: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTPro: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTSer: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTThr: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTTrp: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTTyr: 5′-P-ATG CCA GCT GCG GTA AAC-3′,
MUTVal 5′-P-ATG CCA GCT GCG GTA AAC-3′.

PCR was carried out with programs of 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. The amplified PCR product was transformed with *E. coli* MV1184. In principle, only a plasmid carrying the *aspA* gene inserted with the target mutation could be selectively reproduced. The mutation on the *aspA* gene was confirmed by sequencing.

2.9. Expression of the His-tagged wild-type aspartase and the His-tagged mutated aspartase

To express the *N*-terminal-His-tagged wild-type aspartase and the *N*-terminal-His-tagged mutated aspartase, the expression vectors for these enzymes were constructed using a QIAexpress Type IV Kit (QIAGEN). Amplification of a DNA fragment containing the wild-type *aspA* gene or the mutated *aspA* gene was carried out by PCR using a plasmid, pASPA or pMASPA, as the template and two specific primers, PR1 (5′-CGG GAT CCA TGT CAA ACA ACA TTC GTA TC) and PR2 (5′-CCC AAG CTT TTA CTG TTC GCT TTC ATC AG). PCR was carried out with programs of 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The amplified PCR product was desalinated and digested with *Bam*HI and *Hin*dIII. A mixture of *Bam*HI–*Hin*dIII fragments, including the wild-type *aspA* gene or the mutated *aspA* gene, was purified as described above. The amplified DNA was inserted into the *Bam*HI/*Hin*dIII sites of the expression vector (pQE30).

Each His-tagged aspartase was purified from harvested cells of *E. coli* JM 109 transformants expressing wild-type or mutated aspartase by using an Ni-NTA (nickel-nitrilotriacetic acid) column according to the manual. The purity of the recovered samples was checked by SDS-PAGE.

3. Results

3.1. Random mutagenesis of the aspartase gene, aspA, and screening of mutants

To alternate substrate specificity of aspartase, a random mutagenesis approach was used. By error-prone PCR, random mutations were introduced into the *aspA* aspartase gene. To construct effective reaction conditions for error-prone PCR, we examined effect of the Mn^{2+} concentration on the frequencies of mutations introduced into the *aspA* gene. No addition of Mn^{2+} to the reaction mixture caused one amino acid mutation per 478 amino acid residues (all amino acid residues of aspartase), whereas addition of 5 mM Mn^{2+} to the reaction mixture caused 10 amino acid mutations per 478 amino acid residues. In order to introduce one amino acid mutation into aspartase, we performed error-prone PCR without Mn^{2+}. Furthermore, we used *E. coli* AJ11103-57, which was a peptidaseE-deficient strain, as the host strain for screening of mutated aspartase because peptidaseE hydrolyzes L-aspartic acid-α-amide.

About 3000 transformants that overexpressed the mutated *aspA* were screened for mutants with L-aspartic acid-α-amide deamination activity. One transformant (MA2100) produced fumaric acid-mono amide and ammonia from L-aspartic acid-α-amide, as shown in Fig. 2. Nucleotide sequencing of fragments cloned into pMA2100 showed that two mutation sites were at C462T and A981T. The mutation of C462T was a silent mutation. On the other hand, a A981T mutation altered a 327Lys residue to an Asn residue.

To investigate the effect of the amino acid residue at position 327 on the substrate specificity of the enzyme, we attempted the saturation mutagenesis of the mutation site of pMA2100 as described in Section 2. However, we could not find any other mutated enzyme exhibiting deamination activity for L-aspartic acid-α-amide except for the K327N-mutated enzyme.

3.2. Optimum pH of the mutated enzyme

Since the amino acid at position 327 was related to the substrate specificity, the effect of pH on the activity of the mutated enzyme was investigated with L-aspartic acid and L-aspartic acid-α-amide. We used the wild-type and K327N-mutated N-terminal-His-tagged aspartases as the enzyme source. Fig. 3 shows the result of SDS-PAGE of purified His-

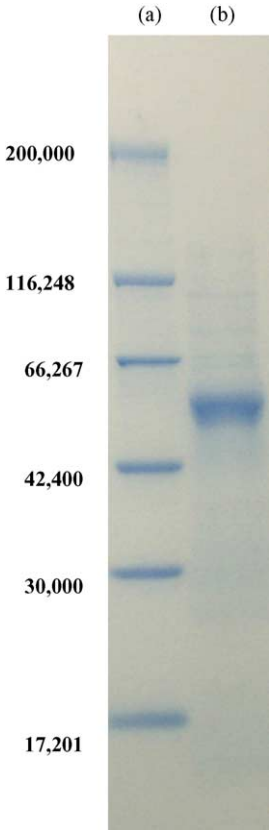

Fig. 3. SDS-PAGE of purified His-tagged-K327N-mutated enzyme. (a) Marker proteins myosine: M_W 200,000, β-galactosidase: M_W 116,248, bovine serum albumin: M_W 66,267, aldolase: M_W 42,400, carbonic anhydrolase: M_W 30,000, myoglovin: M_W 17,201. (b) His-tagged-K327N-mutated enzyme.

tagged-K327N-mutated enzyme. As shown in Fig. 4, both the wild-type enzyme and the K327N-mutated enzyme showed maximum activities for L-aspartic acid at pH 8.5. On the other hand, for L-aspartic acid-α-amide, the K327N-mutated enzyme showed maximum activity at pH 6.0 and showed no activity at pH 8.5, which was the optimum pH value for L-aspartic acid, as shown in Fig. 5. We examined the activity for L-aspartic acid-α-amide of the wild-type

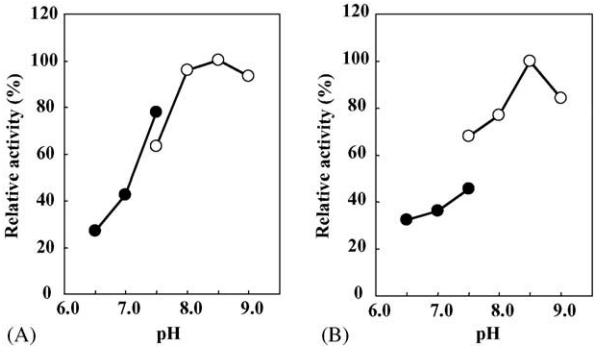

Fig. 4. Effect of pH on the activity for L-aspartic acid of the wild-type (A) and mutant enzymes (B). The enzyme activities were measured at different pH values with 0.1 M HEPES-NaOH buffer, pH 6.5–7.5 (●), Tris–HCl buffer, pH 7.5–9.0 (○).

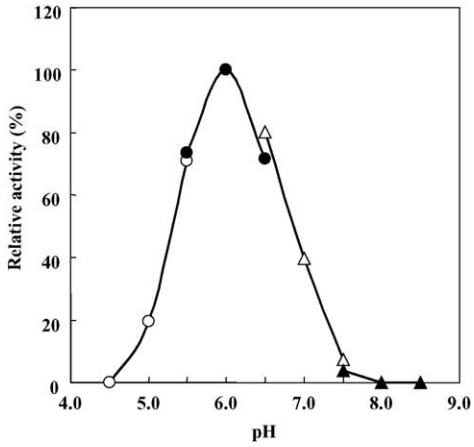

Fig. 5. Effect of pH on the activity for L-aspartic acid-α-amide of the mutant enzymes. The enzyme activities were measured at different pH values with 0.1 M sodium acetate buffer, pH 4.5–5.5 (○), MES-NaOH buffer, pH 5.5–6.5 (●), HEPES-NaOH buffer, pH 6.5–7.5 (△), Tris–HCl buffer, pH 7.5–8.5 (▲).

enzyme at different pH values; however, the wild-type enzyme exhibited no activity for L-aspartic acid-α-amide. The mutated enzyme catalyzed not only the deamination reaction of L-aspartic acid-α-amide, but also amination reaction of fumaric acid-mono amide at pH 7.0 (data not shown). When D-aspartic acid was used as a substrate in the reaction mixture, neither the K327N-mutated enzyme, nor the wild-type enzyme showed deamination activity toward the amino acid with a D-configuration, indicating that the mutation did not affect the strict L-stereospecificity of the enzyme.

3.3. Kinetic constants

The kinetic constants of the wild-type enzyme and the mutated enzyme were examined. Table 1 shows the kinetic constants of these enzymes for L-aspartic acid and L-aspartic acid-α-amide in the deamination reaction. The K_m value for L-aspartic acid of the mutated enzyme was 28.3 mM, which was much larger (14-fold) than that of the wild-type enzyme. The V_{max} for L-aspartic acid of the mutant enzyme was 0.26 U/mg, which was much lower (1–560th) than that of the wild-type enzyme. On the other hand, the K_m value for the

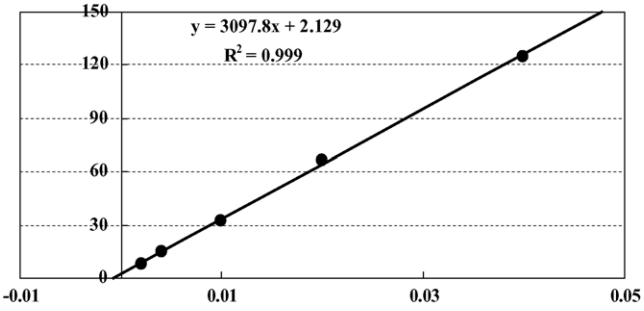

Fig. 6. A Lineweaver–Burk plot of the steady-state kinetic constant of the mutated aspartase toward to L-aspartic acid-α-amide.

Table 1
Kinetic constants of wild-type and mutant enzymes

	Substrate					
	L-Aspartic acid			L-Aspartic acid-α-amide		
	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg mM)	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg mM)
Wild-type	2.1	146	69.5	N.D.	N.D.	
Mutant	28.3	0.26	91.9×10^{-4}	1449	0.47	3.2×10^{-4}

The enzyme activities were assayed as described in Section 2. The initial velocities were determined, and the steady-state kinetic constants were calculated by using a Lineweaver–Burk plot. N.D.: not detectable.

L-aspartic acid-α-amide of the mutant enzyme was calculated to be 1450 mM (Fig. 6), which is about 50-fold larger than that for L-aspartic acid. The V_{max} for the L-aspartic acid-α-amide of the mutant enzyme was 0.47 U/mg, which was about 1.8-fold higher than that for L-aspartic acid.

4. Discussion

Methylaspartate ammonia-lyase (MAL) and histidine ammonia-lyase (HAL) catalyze α,β-elimination of ammonia from (S)-*threo*-(2S, 3S)-3-methylaspartic acid to yield mesaconic acid, and L-histidine to urocanic acid, respectively. The reaction mechanisms of these enzymes have been proposed on the basis of X-ray crystallographic enzyme analysis [19–30]. Aspartase catalyzes α,β-elimination of ammonia from L-aspartic acid to fumaric acid, and the three-dimensional structure of aspartase is similar to that of HAL, which has an electrophilic group 4-methylidene-imidazole-one (MIO) [31]. This suggests that aspartase has a similar reaction mechanism to that of HAL, although the MIO group has never been discovered in the structure of aspartase [30]. The reaction mechanism and active site of the enzyme have not been elucidated, since there is no information on the structure of the enzyme–substrate complex [12–15].

The directed evolution method is very useful for studying enzymes in the absence of any knowledge of their spatial structure and catalytic mechanism. The enhancement of aspartase activity from *E. coli* by the directed evolution method has been reported [32]. However, the alteration of aspartase substrate specificity has not been reported.

Here, we created a mutated aspartase with alteration of substrate specificity by a directed evolution method. From the random mutagenesis and screening for the enzyme catalyzing deamination of aspartic acid-α-amide, a mutated enzyme (MA2100) with one amino acid substitution was obtained. The mutated aspartase showed substitution of a lysine residue to an asparagine residue at position 327. The mutated enzyme, as well as the wild-type enzyme, showed the maximum activity toward L-aspartic acid at pH 8.5. On the other hand, replacement of lysine-327 with asparagine resulted in a 14-fold loss of binding affinity and a 560-fold decrease in catalysis.

Saribas et al. reported that replacement of lysine-327 with arginine resulted in a six-fold loss of binding affinity and over a 300-fold decrease in catalysis [11]. Furthermore, the X-ray crystal structure of aspartase suggested that arginine-29 would interact with the β-carboxylate of L-aspartic acid and that lysine-327 would interact with an oxygen of the α-carboxylate of L-aspartic acid [12]. The increase in the K_m value and the decrease in the V_{max} value of the K327N-mutated aspartase strongly suggested that the most likely role for lysine-327 was to participate in a hydrogen bonding interaction with the substrate. It is interesting to note that the K327N-mutated enzyme showed maximum activity at pH 6.0 for L-aspartic acid-α-amide and showed no activity at pH 8.5, which was the optimum pH value for L-aspartic acid. It is possible that the binding of the asparagine-327 residue of the mutated enzyme to an amino residue of the amide of L-aspartic acid-α-amide under alkaline conditions was weaker than that under neutral conditions. Recently, Fujii et al. solved the structure of thermostable aspartase from *Bacillus* sp. [15] and proposed that lysine-324, which corresponds to lysine-327 in *E. coli* aspartase, is probably the residue that binds one of two carboxylate groups of the substrate aspartate. They also showed a docking model of the enzyme–substrate complex, suggesting the interaction of lysine-324 with the α-carboxylate of aspartate. Our experimental results strongly suggest that lysine-327 in *E. coli* aspartase is the residue that binds the α-carboxylate of aspartate.

The K_m value for the L-aspartic acid-α-amide of the mutant enzyme was 1449 mM, which is about 50-fold larger than that for L-aspartic acid. Recently, a mutated aspartase from *E. coli* with a three amino acid substitution (N217K, T233R, V367G) was obtained [32]. The K_m value for the L-aspartic acid of the triple mutated enzyme decreased to one-fifth of that of the wild-type enzyme. From this study, Wang et al. speculated that arginine-233 (threonine residue of the wild-type) might bind to the carbonyl of α-carboxylate of L-aspartic acid. On the hypothesis that threonine-233 is an amino acid residue involved in substrate binding, it might be possible to create a mutated enzyme with higher affinity for L-aspartic acid-α-amide than that of the K327N-mutated enzyme by site directed mutagenesis of threonine-233.

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