Enhancement of the thermostability and catalytic activity of D-stereospecific amino-acid amidase from *Ochrobactrum anthropi* SV3 by directed evolution

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Abstract

D-Amino-acid amidases, which catalyze the stereospecific hydrolysis of D-amino-acid amide to yield D-amino acid and ammonia, have attracted increasing attention as catalysts for stereospecific production of D-amino acids. We screened for the enzyme variants with improved thermostability generated by a directed evolution method with the goal of the application of evolved enzyme to the production of D-amino acids. Random mutagenesis by error-prone PCR and a filter-based screening was repeated twice, and as a result the most thermostable mutant BFB40 was obtained. Gene analysis of the BFB40 mutant indicated that the mutant enzyme had K278 M and E303 V mutations. To compare the enzyme characteristics with the wild-type enzyme, the mutant enzyme, BFB40, was purified from the *Escherichia coli* (*E. coli*) transformant. Both the thermostability and apparent optimum temperature of the BFB40 were shifted upward by 5 °C compared with those of the wild-type enzyme. The apparent K_m value for D-phenylalaninamide of BFB40 enzyme was almost the same with that of the wild-type enzyme, whereas V_{max} value was enhanced about three-fold. Almost complete hydrolysis of D-phenylalaninamide was achieved in 2 h from 1.0 M of racemic phenylalaninamide–HCl using the cells of *E. coli* transformant expressing BFB40 enzyme, the conversion of which was 1.7-fold higher than the case using cells expressing wild-type enzyme after the same reaction time. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: D-Amino-acid amidase; *Ochrobactrum anthropi*; Random mutation; Thermostability; D-Phenylalanine

1. Introduction

Optically active D-amino acids are important intermediates for a number of fine chemicals such as pharmaceuticals, agrochemicals, and food additives, and several attempts have been made to synthesize D-amino acids enzymatically, including the exploitation of enzymes specific for the D-configuration of amino acid derivatives [1–4]. We have previously isolated and characterized D-stereospecific hydrolases, D-aminopeptidase [5,6] and alkaline D-peptidase [7], from *Ochrobactrum anthropi* C1-38 and *Bacillus cereus* DF4-B, respectively, to use as catalysts for the production of D-amino acids and their derivatives [8–11]. We have also discovered a novel D-specific enzyme, a D-amino-acid amidase, from a soil bacterium *O. anthropi* SV3 [12]. This enzyme catalyzes the stereospecific hydrolysis of D-amino-acid amide to yield D-amino acid and ammonia. The primary structural and biochemical analyses of the enzyme have been carried out in some detail [13]. The D-amino-acid amidase is composed of 363 amino-acid residues

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(molecular mass, 40082 Da), and the deduced amino-acid sequence exhibits homology to D-aminopeptidase from *O. anthropi* C1-38, alkaline D-peptidase from *B. cereus* DF4-B, DD-peptidase from *Streptomyces* R61 [14], and other penicillin recognizing proteins. The enzyme contains the typical SXXK, YXN, and H(K)XG active-site motifs identified in the penicillin-binding proteins and β-lactamases [15]. We proposed that the D-amino-acid amidase as well as D-aminopeptidase and alkaline D-peptidase is a new member of the family of penicillin-recognizing enzymes [16].

D-Alaninamide amidase from *Arthrobacter* sp. NJ-26 showed a high D-stereospecificity towards alaninamide and was used for the production of D-alanine; by the whole cell reaction, 210 g l^{-1} (2.4 M) of DL-alaninamide was resolved to give 105 g l^{-1} of D-alanine with an optical purity of more than 99% e.e. [17]. D-Aminopeptidase from *O. anthropi* C1-38 also acted on D-alaninamide as well as D-alanine-containing peptides [5]. Recombinant *Escherichia coli* (*E. coli*) cells harboring D-aminopeptidase gene was applicable for the D-alanine production; a complete hydrolysis of D-alaninamide was achieved from 5.0 M of DL-alaninamide [8]. However, these two enzymes have rather narrow substrate specificity as to the D-stereospecific hydrolysis of amino-acid amides. They preferentially acted on low-molecular weight D-amino-acid amides but could hardly hydrolyze D-amino-acid amides with bulky side chains, such as D-phenylalaninamide and D-leucinamide. On the other hand, D-amino-acid amidase from *O. anthropi* SV3 could accept a broad range of substrates indicating its utility for the production of a wide variety of D-amino acids. At present only the D-amino-acid amidase is known to show an efficient hydrolytic activity with high stereospecificity on D-phenylalaninamide, D-tyrosinamide, D-tryptophanamide, and D-leucinamide and, therefore, it is expected to be applicable as a catalyst for the industrial production of these corresponding D-amino acids. D-phenylalanine is useful as a synthetic intermediate for thrombin inhibitor [18]. Although, other research groups have reported the bacterial hydrolases acting on peptides containing D-amino acids, such as VanX DD-dipeptidase from *Enterococcus faecium* BM4147 [19], DmpA aminopeptidase from *O. anthropi* LMG7991 [20], and DdpA D-aminopeptidase from *Bacillus subtilis* [21], these enzymes have never been used

for the production of D-amino acids in industrial application.

In the past few years, directed evolution method has been successfully applied in improving the properties of several enzymes for industrial application [22–24]. Here, in order for the D-amino-acid amidase from *O. anthropi* SV3 to be more useful in industrial application, we tried to obtain a mutant enzyme with suitable properties such as sufficient thermostability, catalytic activity, and productivity for the efficient production of D-amino acids by the directed evolution method. Since it seemed to be difficult to screen for the mutant enzymes with improved catalytic activity or productivity, the thermostability of the enzyme was compared with that of the wild-type enzyme in this screening. The *daaA* gene encoding the enzyme was randomly mutagenized by error-prone PCR and, then thermostabilized enzymes were screened. After two cycles of the random mutagenesis and screening, the most improved enzyme variant, BFB40, was obtained. We identified the mutation sites by sequencing the gene encoding the mutant enzyme. The mutant enzyme was produced in *E. coli*, purified and characterized. We also developed an efficient synthetic method for D-phenylalanine by kinetic resolution of racemic D-phenylalaninamide utilizing *E. coli* cells expressing BFB40 enzyme.

2. Experimental

2.1. Bacterial strain, plasmids, and culture condition

E. coli JM109 was used as a host for the recombinant plasmids. Plasmid, pDAA containing D-amino-acid amidase gene *daaA* of *O. anthropi* SV3 [13] was used as a template in PCR mutagenesis for directed evolution. Plasmid, pUC19 (Takara Shuzo, Kyoto, Japan) was used as a cloning vector. Recombinant *E. coli* JM109 was cultured at 37 °C on Luria-Bertani medium [25] containing 80 μg ml^{-1} of ampicillin. To induce the gene under the control of the *lac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM.

2.2. Materials, enzymes, and chemicals

Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo. Alkaline phosphatase

from shrimp and *Taq* DNA polymerase were purchased from Boehringer-Mannheim (Tokyo, Japan). D-Amino-acid oxidase from porcine kidney was obtained from Sigma (St. Louis, MO, USA). Horseradish peroxidase was from Toyobo (Osaka, Japan). D-Phenylalaninamide–HCl and L-phenylalaninamide–HCl were synthesized as described previously [5]. Racemic phenylalaninamide–HCl was provided by Mitsubishi Rayon Co. Ltd. (Kanagawa, Japan). DEAE-Toyopearl 650 M and Butyl-Toyopearl 650 M were purchased from Tosoh Corp. (Tokyo, Japan). Superdex 200 HR 10/30 was from Pharmacia (Uppsala, Sweden).

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

For routine work with recombinant DNA, established protocols were used [25]. Random mutagenesis of the *daaA* gene was performed by error-prone PCR according to the method of Cadwell and Joyce [26,27]. The reaction mixture for the error-prone PCR contained in 50 μl 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.2 mM each dATP and dGTP, 1 mM each dCTP and dTTP, a sense and an anti-sense primer each at 1 μM concentration, 2.5 U Taq DNA polymerase and 500 ng plasmid pDAA as template DNA. 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 55 °C for 30 s and an elongation step at 72 °C for 2 min. Nucleotide sequences of the two primers used were described previously [13]. PCR was carried out on an MJ Research (Watertown, MA, USA) thermal cycler, PTC-200. The amplified PCR product was digested with *Hin*dIII and *Xba*I, separated by agarose-gel electrophoresis and, then purified with QIAquick™ gel extraction kit from QIAGEN (Tokyo, Japan). The amplified DNA was inserted downstream of the *lac* promoter in pUC19 to generate a mutant library. *E. coli* JM109 was transformed with the constructed mutant library by the method of Inoue et al. [28], and ampicillin-resistant transformants were selected. Visualization of the thermostable D-amino-acid amidase activity expressed in the transformants was carried out essentially as described previously [13]. Recombinant *E. coli* cells harboring the randomly mutagenized D-amino-acid amidase genes

were grown on Luria–Bertani agar plates containing ampicillin and, then transferred to nitrocellulose filters. The filters containing the transferred cells were laid on fresh Luria–Bertani agar plates containing ampicillin and IPTG and incubated at 37 °C for 8 h and, then the colonies on the filter were lysed as described previously [13]. After heat-treatment for 15 min at a specific temperature (45–65 °C) to select thermostable D-amino-acid amidase variants, the filter was soaked in a reaction mixture containing D-phenylalaninamide, phenol, D-amino-acid oxidase, peroxidase, and 4-aminoantipyrine [13] and stood at room temperature. A colony corresponding to developed red color was picked up as a clone expressing thermostable enzyme. The random mutagenesis and screening was repeated and, as a result two thermostable mutant enzymes, B29 and BFB40, were obtained. The *E. coli* transformants expressing the B29 and BFB40 enzymes carried mutated plasmids, designated pDAA-B29 and pDAA-BFB40, respectively.

2.4. DNA sequence analysis

An automatic plasmid isolation system (Kurabo, Osaka, Japan) was used to prepare double-stranded DNAs for sequencing. Plasmids, pDAA-B29 and pDAA-BFB40 were used as sequencing templates. Nucleotide sequencing was performed using the di-deoxynucleotide chain-termination method [29] with M13 forward and reverse oligonucleotides and two additional oligonucleotides, DAA101 and DAA102, as primers. The two primers, DAA101 and DAA102 had the respective sequences 5′-TCTGGCCGGAATGAT-CATCG-3′ and 5′-CCGACAAGGGAAACCATTCG-3′, which were designed on the basis of the nucleotide sequence of the *daaA* gene. Sequencing reactions were carried out with a Thermo Sequenase™ cycle sequencing kit and dNTP mixture with 7-deaza-dGTP from Amersham Pharmacia Biotech (Uppsala, Sweden), and the reaction mixtures were run on a DNA sequencer 4000L (Li-cor, Lincoln, NE, USA).

2.5. Purification of the wild-type and mutant (BFB40) D-amino acid amidases from E. coli transformant

E. coli JM109 harboring pDAA or pDAA-BFB40 was cultured and the cells were harvested as described previously [13]. The purification procedures were

same as that used for the purification of D-amino-acid amidase from *E. coli* transformant [13].

2.6. Enzyme assay

The standard reaction mixture (1 ml) for assaying the D-amino-acid amidase activity contained 0.1 mmol Tris/HCl buffer (pH 8.0), 20 μmol D-phenylalaninamide–HCl and an appropriate amount of the enzyme. The reaction was performed at 30 °C for 5 min and stopped by the addition of 0.2 ml 2 M HClO$_4$. The amount of D-phenylalanine formed in the reaction mixture was determined with a Waters HPLC apparatus equipped with a Mightysil RP-18 GP (Kanto Chemicals Co., Tokyo) column (4.6 mm × 150 mm) at a flow rate of 1.0 ml min^{-1}, using the solvent system methanol:5 mM H$_3$PO$_4$ (1:4 (v/v)). Absorbance of the eluate was monitored at 254 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol D-phenylalanine per minute from D-phenylalaninamide under the above conditions. Protein was determined by the method of Bradford [30] using BSA as standard. A steady-state kinetic parameter was determined with a substrate concentration ranging from 0.05 to 0.5 mM by using a Lineweaver–Burk plot.

2.7. Analytical measurements

SDS/PAGE analysis was performed by the method of Laemmli [31]. Proteins were stained with Brilliant blue G and de-stained in ethanol/acetic acid/water (3:1:6 (v/v)). The isomers of phenylalanine were analyzed by a Waters HPLC apparatus equipped with a Sumichiral OA-5000 (Sumika Chemical Analysis Service Ltd., Osaka, Japan) column (4.6 mm × 150 mm) at a flow rate of 1.0 ml min^{-1}, using the solvent system 2 mM copper sulfate/isopropanol (85:15 (v/v)). Absorbance of the eluate was monitored at 254 nm.

3. Results

3.1. Random mutagenesis of the D-amino-acid amidase gene, daaA, and screening for thermostable mutants

By error-prone PCR, random mutations were introduced into the *daaA* gene. *E. coli* JM109 was trans-

formed with the first-generation gene library to generate a mutant library comprising about 10,000 colonies. The library was screened after heat-treating at 45 °C for 15 min, which was found to be sufficient to inactivate the wild-type D-amino-acid amidase activity. Several colonies were found to express the enzyme variants that survived the heat treatment. Purification of these colonies and further testing by the colorimetric filter assay led to the isolation of five thermostable mutants. The thermostability of these mutants was evaluated by use of their cell suspensions that had been prepared from 12-h culture in Luria–Bertani medium containing ampicillin and IPTG. The cell suspensions were incubated at various temperatures for 15 min and, then the residual activities were assayed. As a result, the thermostability of one clone, designated as B29, was increased about 5 °C under these conditions compared to that of the *E. coli* JM109/pDAA expressing wild-type D-amino-acid amidase. However, when B29-colony on the medium plate was transferred to a nitrocellulose filter, lysed, and treated at 50 °C for 15 min, the colorimetric assay indicated that the residual D-amino-acid amidase activity was lost completely.

The plasmid containing the B29 gene (pDAA-B29) was used as a template for the second mutant library. Screening of the second generation consisting of about 40,000 colonies was carried out similarly except for the screening temperature (50 °C), and a clone that showed the enzyme activity on the filter was selected and designated as BFB40. The mutant BFB40 could show the enzyme activity even after the filter was treated at 60 °C for 15 min. Although the third round mutagenesis was performed using the plasmid containing BFB40 gene (pDAA-BFB40) as a template with the heat treatment at 65 °C, no further improved variant was obtained. We actually attempted the saturation mutagenesis on the two mutation sites of the parent BFB40 separately, by the use of oligonucleotides containing NNN codon at the respective site. However, we could not find more stable mutant than BFB40. Since *E. coli* transformant expressing BFB40 enzyme has enough stability and activity for the complete hydrolysis of D-phenylalaninamde even in the presence of high concentration of the racemic substrate (1 M) as described later, we decided that there is no need to obtain any further improved enzyme.

3.2. Characterization of the thermostable enzymes

Nucleotide sequencing of *Hin*dIII-*Xba*I fragments cloned into pDAA-B29 and pDAA-BFB40 showed that the only mutation site in *daaA* from pDAA-B29 was at Glu-303 (GAA), which was altered to Val (GTA). In the gene from pDAA-BFB40, a further mutation was observed at Lys-278 (AAG), which was altered to Met (ATG).

Each *E. coli* JM109/pDAA and *E. coli* JM109/pDAA-BFB40 was cultured in Luria–Bertani medium containing ampicillin and IPTG, and the cell-free extract was prepared from the harvested cells as described in Section 2.2. Specific activity for D-phenylalaninamide of the cell-free extract of the mutant BFB40 was 85.9 U mg per protein, which is 4.5-fold higher than that of the cell-free extract of the wild-type (19.3 U mg per protein).

The wild-type and mutant (BFB40) D-amino-acid amidases were purified from the each cell-free extract by ammonium sulfate fractionation and DEAE-Toyopearl, Butyl-Toyopearl, and FPLC Superdex 200 column chromatographies. The final preparations gave single bands on SDS–PAGE with a molecular mass of ≈40 kDa. The kinetic parameters of these enzymes in the hydrolysis reaction of D-phenylalaninamide are as shown in Table 1. The Michaelis constant (K_m) of the mutant BFB40 enzyme was 0.154 mM, which is almost the same with the value, 0.151 mM of the wild-type enzyme. The V_{max} of the mutant enzyme was much higher (2.9-fold) than that of the wild-type. When L-phenylalaninamide–HCl was used as a substrate in a reaction mixture, the mutant BFB40 enzyme as well as the wild-type enzyme did not show the hydrolytic activity toward the amino-acid amide with L-configuration, indicating that the two mutations did not affect the strict D-stereospecificity of the enzyme.

Table 1
Kinetic parameters for wild-type and BFB40 enzymes

	V_{max} (U mg^{-1})	K_m (mM)	V_{max}/K_m (U mg^{-1} mM^{-1})
Wild-type	441	0.151	2920
BFB40	1280	0.154	8310

The enzyme activity was assayed as described in Section 2.2. The initial velocities were determined, and the steady-state kinetic parameters were calculated by using a Lineweaver–Burk plot.
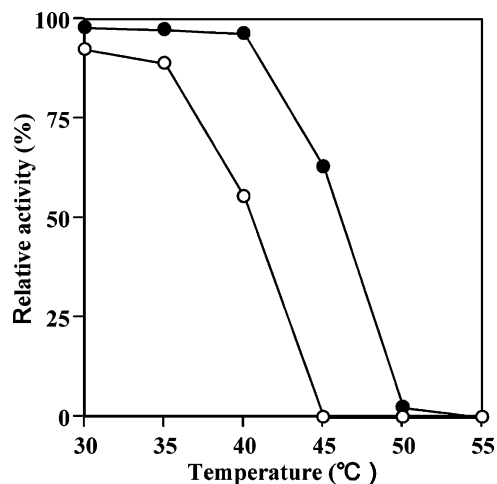

Fig. 1. Effects of temperature on the stability of the wild-type and mutant BFB40 enzymes. The remaining activities were assayed under the standard condition after the purified enzymes had been incubated at various temperatures for 5 min, and expressed as a percentage of the activity of each untreated enzymes. Symbols: (○) wild-type; (●) BFB40.

The purified enzymes were incubated at various temperatures for 5 min, and then the residual activities were assayed. The thermostability of the BFB40 enzyme was shifted upward by 5 °C compared with that of the wild-type enzyme (Fig. 1). To evaluate the effect of temperature on the enzyme activity, the enzyme reaction was carried out at various temperatures for 5 min by using the purified enzymes (Fig. 2). The mutations in BFB40 enzyme increased the specific activity more than two-fold over a wide temperature range. The specific activity of the mutant BFB40 was highest at 50 °C, increased by 5 °C compared with that of the wild-type enzyme. Considering the activity increases with temperature until the enzyme denatures, this 5 °C shift may demonstrate the increased stability of BFB40 enzyme at high temperature.

3.3. Production of D-phenylalanine by kinetic resolution of racemic DL-phenylalaninamide

D-Phenylalanine production from DL-phenylalaninamide–HCl was examined by the cell reaction using *E. coli* JM109/pDAA expressing wild-type enzyme or *E. coli* JM109/pDAA-BFB40 expressing BFB40 enzyme. Time courses of total D-amino acid activity
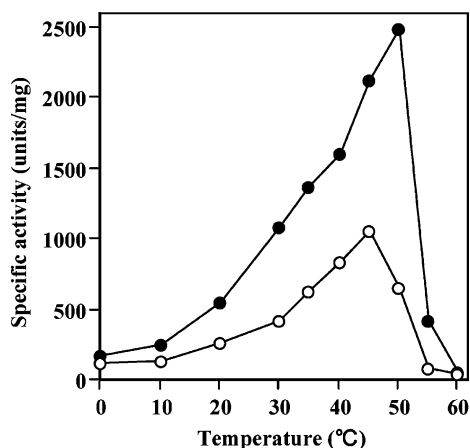
Fig. 2. Effect of temperature on the activity of the wild-type and mutant (BFB40) D-amino-acid amidase. Reactions were carried out at various temperatures. Symbols: (○) wild-type; (●) BFB40.

(U per ml culture) of the two *E. coli* transformants are as shown in Fig. 3. In the case of *E. coli* JM109/pDAA, the maximun activity (19.0 U per ml culture) was observed after 12 h-cultivation, whereas *E. coli* JM109/pDAA-BFB40 gave the activity of 55.7 U per ml culture at the same cultivation time. The cell
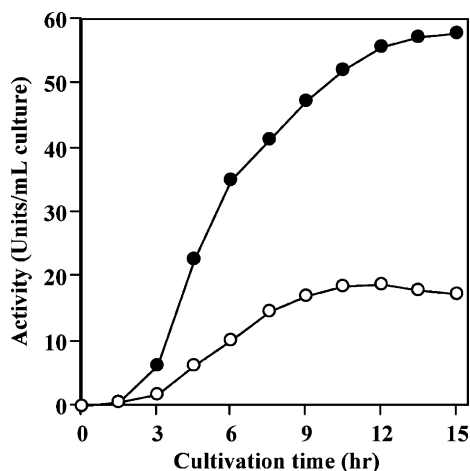

Fig. 3. Time course of D-amino-acid amidase activity during the cultivation of *E. coli* JM109/pDAA or *E. coli* JM109/pDAA-BFB40. The *E. coli* transformants were cultured in 2 ml of Luria–Bertani medium supplemented with ampicillin and IPTG. The culture broth (2 or 5 ml) of each cultivation time was used to determine the total activity (U per ml culture). Symbols: (○) wild-type; (●) BFB40.
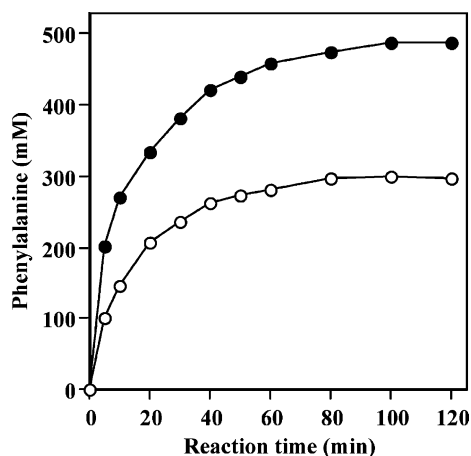

Fig. 4. Production of D-phenylalanine from DL-phenylalaninamide–HCl by cells of *E. coli* JM109/pDAA or *E. coli* JM109/pDAA-BFB40. The reaction mixture contained 1.0 M of DL-phenylalaninamide–HCl, washed *E. coli* cells (0.1% (w/w)) prepared from 100 ml culture broth after 12-h cultivation, and 0.1 M of Tris/HCl (pH 8.0) in a total volume of 100 μl, and incubated at 30 °C. The reaction was stopped at the specific time and the concentration of phenylalanine formed was determined as described in Section 2. Symbols: (○) wild-type; (●) BFB40.

reaction with 1.0 M of DL-phenylalaninamide–HCl was carried out by using the *E. coli* cells prepared from the 12-h culture (Fig. 4). The two kinds of cells produced D-phenylalanine with high optical purity (>99.7% e.e.) at the all reaction times tested. The BFB40 mutant with enhanced thermostability and catalytic activity increased D-phenylalanine production. Especially, almost complete hydrolysis of 500 mM D-phenylalaninmide was achieved by the cell reaction of BFB40 after 2 h, whereas the concentration of D-phenylalanine reached only 300 mM with cells expressing wild-type enzyme after the same reaction time.

4. Discussion

D-Amino-acid amidase from *O. anthropi* SV3 shows extremely high stereospecificity for D-configuration and, therefore, it is expected to be applicable as a catalyst for the production of D-amino acids by kinetic resolution of racemic amino-acid amides. Here, in order for the enzyme to be more useful in industrial applications, we created a mutant D-amino-acid

amidase with improved properties by directed evolution method. From the two cycles of random mutagenesis and screening for themostabilized enzyme, the most improved enzyme, BFB40 with two amino-acid substitutions was obtained. Although improvement of the thermostability and optimum temperature of the BFB40 enzyme was only 5 °C compared with that of the wild-type enzyme, the V_{max} of the mutant enzyme was 2.9-fold higher than that of the wild-type. D-Phenylalanine (∼0.5 M) with the optical purity of more than 99.7% e.e. was obtained after 2 h from 1.0 M racemic DL-phenylalaninamide–HCl by using the *E. coli* cells expressing BFB40 enzyme, the conversion of which was 1.7-fold higher than the case using cells expressing wild-type enzyme after the same reaction time. This is the first report describing the enzymatic synthesis of D-phenylalanine by kinetic resolution of racemic phenylalaninamide.

In the screening for thermostable mutant enzyme, we obtained BFB40 mutant, which was active on the filter assay after 15 min incubation at 50 °C. The BFB40 on the filter was still active after 15 min incubation at 60 °C. On the other hand, the BFB40 enzyme purified from the cell-free extract of *E. coli* transformant was used for its characterization, and it was found to be slightly active after 5 min incubation at 50 °C. Main cause of this apparent discrepancy may be the improved catalytic activity of the BFB40 enzyme. The high catalytic activity of the mutant enzyme on the filter led to the substantial level of residual activity after the incubation at higher temperature, and explains why this mutant was isolated. These findings indicate that our screening method for thermostable mutant is also effective to obtain the mutant enzyme with improved catalytic activity.

Stabilization in BFB40 enzyme was accompanied by an increase of the optimal temperature. It is assumed that the ability of the mutant enzyme to remain folded at higher temperatures gave its higher optimal temperature. Furthermore, the specific activity of the mutant enzyme was increased ∼2-fold at all temperatures tested. Similar results were reported in recent studies in which the thermostabilities of *p*-nitrobenzyl esterase [32], mesophilic subtilisin E [33], and psychrophilic subtilisin S41 [34] were improved by directed evolution. In these three cases, dramatic increase in both thermostability and catalytic activity at a wide range of temperature were observed. Although

sometimes an enzyme is thermostabilized with a significant loss of catalytic activity [35], judging from the above reports and our result, thermostability and catalytic activity seem to be at least partially independent properties and can be optimized together in the same enzyme.

Acknowledgements

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