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# L-Stereoselective amino acid amidase with broad substrate specificity from *Brevundimonas diminuta*: characterization of a new member of the leucine aminopeptidase family

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**Abstract** *Brevundimonas diminuta* TPU 5720 produces an amidase acting L-stereoselectively on phenylalaninamide. The enzyme ($LaaA_{Bd}$) was purified to electrophoretic homogeneity by ammonium sulfate fractionation and four steps of column chromatography. The final preparation gave a single band on SDS-PAGE with a molecular weight of ≈53,000. The native molecular weight of the enzyme was about 288,000 based on gel filtration chromatography, suggesting that the enzyme is active as a homohexamer. It had maximal activity at 50°C and pH 7.5. $LaaA_{Bd}$ lost its activity almost completely on dialysis against potassium phosphate buffer (pH 7.0), and the amidase activity was largely restored by the addition of $Co^{2+}$ ions. The enzyme was, however, inactivated in the presence of ethylenediaminetetraacetic acid even in the presence of $Co^{2+}$, suggesting that $LaaA_{Bd}$ is a $Co^{2+}$-dependent enzyme. $LaaA_{Bd}$ had hydrolyzing activity toward a broad range of L-amino acid amides including L-phenylalaninamide, L-glutaminamide, L-leucinamide, L-methioninamide, L-argininamide, and L-2-aminobutyric acid amide. Using information on the N-terminal amino acid sequence of the enzyme, the gene encoding $LaaA_{Bd}$ was cloned from the chromosomal DNA of the strain and sequenced. Analysis of 4,446 bp of the cloned DNA revealed the presence of seven open-reading frames (ORFs), one of which (*laaA*$_{Bd}$) encodes the amidase. $LaaA_{Bd}$ is composed of 491 amino acid residues (calculated molecular weight 51,127), and the deduced amino acid sequence exhibits significant similarity to that of ORFs encoding hypothetical cytosol aminopeptidases found in the genomes of *Caulobacter crescentus*, *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, *Mesorhizobium loti*, and *Agrobacterium tumefaciens*, and leucine aminopeptidases, PepA, from *Rickettsia prowazekii*, *Pseudomonas putida* ATCC 12633, and *Escherichia coli* K-12. The *laaA*$_{Bd}$ gene modified in the nucleotide sequence upstream from its start codon was overexpressed in an *E. coli* transformant. The activity of the recombinant $LaaA_{Bd}$ in cell-free extracts of the *E. coli* transformant was 25.9 units $mg^{-1}$ with L-phenylalaninamide as substrate, which was 50 times higher than that of *B. diminuta* TPU 5720.

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## Introduction

Enantiomerically pure amino acids and their derivatives are versatile chiral building blocks for the synthesis of pharmaceuticals, agrochemicals, and food or feed additives, and several enzymatic methods have therefore been developed to synthesize D- or L-amino acids, including the exploitation of enzymes specific for the D- or L-configuration of amino acid derivatives (Asano and Lübbehüsen 2000; Kamphuis et al. 1990; Schmid et al. 2001; Yagasaki and Ozaki 1998; Wakayama et al. 2003; Syldatk et al. 1999; Olivieri et al. 1981). We have previously isolated and characterized D-stereoselective amino acid amide hydrolases, D-aminopeptidase from *Ochrobactrum anthropi* C1-38 (Asano et al. 1989b, 1991, 1992), D-amino acid amidase from *O. anthropi* SV3 (Asano et al. 1989a; Komeda and Asano 2000; Komeda et al. 2003), and *R*-amidase from *Pseudomonas* sp. MCI 3434 (Komeda et al. 2004b) to use as catalysts for the production of D-amino acids and their derivatives from the corresponding amino acid amides. We have also discovered a novel L-stereoselective amino acid amide hydrolase, L-amino acid amidase, from *Pseudomonas azotoformans* IAM 1603 (Komeda et al. 2004a). This enzyme catalyzes the stereoselective hydrolysis of L-amino acid amide to yield L-amino acid and ammonia. The L-amino acid amidase,

named $LaaA_{Pa}$, is composed of 310 amino acid residues (molecular weight 34,514), and the deduced amino acid sequence exhibits homology to proline iminopeptidases from some bacteria. The enzyme has a relatively narrow substrate range, being active on L-prolinamide, L-alaninamide, and L-methioninamide, whereas it did not act on the peptide substrates for the proline iminopeptidases despite its sequence similarity to the peptidases. $LaaA_{Pa}$ is the first L-amino acid amidase whose primary sequence is described in the published paper.

L-Amino acid amidases are useful for the production of enantiomerically pure L-amino acids from the corresponding racemic amino acid amides, which are readily synthesized from aldehyde, hydrogen cyanide, and ammonium via aminonitrile as an intermediate (Asano and Lübbehüsen 2000). If the enzyme is used together with an amino acid amide racemase (Asano and Yamaguchi 2005) catalyzing the racemization of the remaining D-amino acid amides, the racemic amino acid amides could be completely hydrolyzed to form L-amino acids. Knowledge on the characteristics of the L-amino acid amidase is, however, limited. Besides our $LaaA_{Pa}$ from *P. azotoformans* IAM 1603, only three kinds of L-amino acid amidases have been purified from microbial species and characterized, including the enzymes from *Pseudomonas putida* ATCC 12633 (Hermes et al. 1993), *O. anthropi* NCIMB 40321 (van den Tweel et al. 1993), and *Mycobacterium neoaurum* ATCC 25795 (Hermes et al. 1994). Unlike $LaaA_{Pa}$, all of the three enzymes seem to be metal enzymes because their activities are inhibited by chelating reagents such as ethylenediaminetetraacetic acid and *o*-phenanthroline and/or activated by divalent cations.

In this study, we screened for microorganisms producing L-amino acid amidase to gather knowledge on the enzyme and its primary structure. We found in *Brevundimonas diminuta* TPU 5720 an L-stereoselective amidase hydrolyzing phenylalaninamide, which is hardly hydrolyzed by $LaaA_{Pa}$. The enzyme named $LaaA_{Bd}$ was purified and characterized. Analysis of the gene coding for the enzyme revealed that $LaaA_{Bd}$ is a new member of the leucine aminopeptidase family.

## Materials and methods

### Bacterial strains, plasmids, and culture conditions

*Brevundimonas diminuta* TPU 5720 (TPU, stock cultures of Toyama Prefectural University) was selected as a microorganism capable of hydrolyzing phenylalaninamide with L-stereoselectivity and used as the source of enzyme and chromosomal DNA. *Escherichia 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 plasmids. Plasmids pT7-Blue (Takara Bio Inc., Shiga, Japan), pBluescriptII SK(−) (Toyobo, Osaka, Japan), and pUC19 (Takara Bio Inc.) were used as cloning vectors. *B. diminuta* TPU 5720 was grown in TGY medium containing 5 g of Bacto tryptone (Difco), 5 g of yeast extract, 1 g of glucose, and 1 g of $K_2HPO_4$ in 1 l of distilled water, pH 7.0. Recombinant *E. coli* JM109 was cultured in Luria–Bertani medium (Sambrook et al. 1989) containing ampicillin (80 μg $ml^{-1}$). To induce expression of the gene under the control of the *lac* promoter, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM.

### Purification of the L-amino acid amidase, LaaABd, from *B. diminuta* TPU 5720

*B. diminuta* TPU 5720 was subcultured at 30°C for 24 h in a test tube containing 5 ml of TGY medium. The subculture (5 ml) was then inoculated into a 2-l Erlenmeyer flask containing 500 ml of TGY medium. The cultivation was carried out at 30°C for 24 h with reciprocal shaking at 200 rpm. All purification steps were performed at a temperature lower than 4°C. The buffer used was potassium phosphate (pH 7.0). The protein content of the eluate from column chromatography was monitored by measuring absorbance at 280 nm. Cells (70 g, wet weight) from 5 l of TGY medium were harvested by centrifugation (10,000×*g* at 4°C) and suspended in 0.1 M buffer. The cell suspension was disrupted with an ultrasonic oscillator (19 kHz insonator model 201 M, Kubota, Japan). For the removal of intact cells and cell debris, the sonicate was centrifuged at 15,000×*g* for 10 min at 4°C, and the resulting supernatant was used as the cell-free extract. To the cell-free extract was added 5% protamine sulfate, at a concentration of 13 mg of protamine sulfate to 1 g of protein. After stirring for 30 min, the precipitate formed was removed by centrifugation at 15,000×*g* for 10 min at 4°C. The resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 30–60% saturation was collected by centrifugation and dissolved in 20 mM buffer. The resulting enzyme solution was dialyzed against 15 l of the same buffer for 24 h. The dialyzed solution was applied to a column (φ 2.4×5 cm) of DEAE–Toyopearl 650 M (Tosoh Corp., 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 NaCl (0–0.5 M) in 20 mM buffer. To the active fractions was added ammonium sulfate to 30% saturation. The enzyme solution was applied to a column (φ 1.8×3 cm) of Butyl–Toyopearl 650 M (Tosoh Corp.) previously equilibrated with 20 mM buffer containing ammonium sulfate to 30% saturation. The active fractions were eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 20 mM buffer. The active fractions were combined and dialyzed against 15 l of 20 mM buffer for 24 h. The enzyme solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences K.K., Tokyo, Japan) previously equilibrated with 20 mM buffer. After the column had been washed with 30 ml of 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in 20 mM buffer using the Äkta-FPLC system (Amersham Biosciences K.K.). The active fractions were combined, dialyzed against 10 l of 20 mM buffer for 24 h,

and concentrated with Centricon-10 (Amicon, USA). The enzyme solution was applied to a Superdex 200 HR 10/30 column (Amersham Biosciences K.K.) previously equilibrated with 20 mM buffer containing 150 mM NaCl and eluted with the same buffer. The active fractions were collected and dialyzed against 10 l of 20 mM buffer for 12 h. The dialyzed enzyme was used for characterization. Amino acid sequence analysis of the amino terminus of the purified enzyme was performed at APRO Life Science Institute, Inc. (Tokushima, Japan) with a Procise 494 HT protein sequencing system.

## Enzyme assay

Activity of $LaaA_{Bd}$ was assayed with L-phenylalaninamide as a substrate. The reaction mixture (0.5 ml) contained 50 μmol potassium phosphate buffer (pH 7.0), 0.5 μmol $CoCl_2$, 20 μmol L-phenylalaninamide, and an appropriate amount of the enzyme. The reaction was performed at 30°C for 20 min and stopped by the addition of 0.1 ml of 2 N $HClO_4$. The amount of L-phenylalanine formed was determined with a Waters 600E HPLC apparatus equipped with a COSMOSIL $5C_{18}$-MS-II column (φ 0.46×15 cm) (Nacalai tesque, Kyoto, Japan) at a flow rate of 0.7 ml $min^{-1}$ using the solvent system 5 mM $H_3PO_4$/methanol (4:1, 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 L-phenylalanine $min^{-1}$ from L-phenylalaninamide under the above conditions. Protein was determined by the method of Bradford (Bradford 1976) using BSA as standard. Enzyme activity toward other amino acid amides and dipeptides was determined by measuring the production of amino acids with an amino acid analyzer (model L-8500; Hitachi, Tokyo, Japan). Amino acid amides and peptides were purchased from Bachem (Switzerland), Sigma (Tokyo, Japan), and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

## Analytical measurements

To estimate the molecular weight of the enzyme, the sample (5 μg) was subjected to HPLC on a TSK G-3000 SW column (φ 0.75×60 cm; Tosoh Corp.) at a flow rate of 0.7 ml $min^{-1}$ with 0.1 M potassium phosphate (pH 7.0) containing 0.1 M NaCl at room temperature. Absorbance of the eluate was monitored at 280 nm. The molecular weight of the enzyme was then calculated from the relative mobility compared with that of the standard proteins glutamate dehydrogenase (290,000), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000), and cytochrome *c* (12,400) (products of Oriental Yeast Co., Osaka, Japan). SDS-PAGE analysis was performed by the method of Laemmli (Laemmli 1970). Proteins were stained with Brilliant blue G and destained in ethanol/acetic acid/water (3:1:6, by volume).

## Cloning of the *B. diminuta* TPU 5720 L-amino acid amidase gene ($laaA_{Bd}$)

For routine work with recombinant DNA, established protocols were used (Sambrook et al. 1989). Restriction endonucleases were purchased from Takara Bio Inc. Alkaline phosphatase from shrimp and *Taq* DNA polymerase were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chromosomal DNA was prepared from *B. diminuta* TPU 5720 by the method of Misawa et al. (Misawa et al. 1990). Oligonucleotide primers were synthesized on the basis of the N-terminal amino acid sequence. The amino- acid sequence Met-Lys-Ile-Glu-Phe-Val-Ala was used to model the oligodeoxynucleotide pool 5′-AT GAAGATCGAGTTCGTSGC-3′ (sense strand) and Ala-Leu-Ala-Gly-Thr-Gly-Pro to model 5′-GGSCCSGTSCCS GCSARSGC-3′ (antisense strand) (S=C or G; R=A or G). The reaction mixture for the PCR contained 50 μl of *Taq* buffer with 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, the sense and antisense primers each at 1 μM, 2 U of *Taq* DNA polymerase, and 0.5 μg of chromosomal DNA from *B. diminuta* TPU 5720 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 55°C for 30 s, and an elongation step at 72°C for 30 s. The PCR product (89 bp) was cloned into pT7-Blue vector in *E. coli* JM109 and was used as a probe for the amidase-encoding gene, $laaA_{Bd}$, of *B. diminuta* TPU 5720. Chromosomal DNA of the strain was completely digested with *Pst*I. Southern hybridization showed an approximately 4.5-kbp band from *Pst*I digestion that hybridized with the probe. DNA fragments of 4.0–5.0 kbp from *Pst*I digestion were recovered from 0.7% (w/v) agarose gel by using a QIAquick gel extraction kit from QIAGEN (Tokyo, Japan) and ligated into *Pst*I-digested and alkaline phosphatase-treated pBluescriptII SK(−) using Ligation Kit ver. 2 (Takara Bio Inc.). *E. coli* JM109 was transformed with recombinant plasmid DNA and screened for the presence of the $laaA_{Bd}$ gene by colony hybridization with the probe. A positive *E. coli* transformant carried a plasmid, designated pBD2.

## DNA sequence analysis

An automatic plasmid isolation system PI-100 from Kurabo (Osaka, Japan) was used to prepare the double-stranded DNAs for sequencing. The plasmid pBD2 was used as a sequencing template. Nested unidirectional deletions were generated with the Kilo-Sequence deletion kit (Takara Bio Inc.). Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method (Sanger et al. 1977) 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

4,000 L (Li-cor, Lincoln, NE, USA). Both strands of DNA were completely sequenced. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB205151. Amino acid sequences were compared with the BLAST program (Altschul et al. 1997).

### Expression of the *laaA*$_{Bd}$ gene in *E. coli*

A modified DNA fragment coding for LaaA$_{Bd}$ was obtained by PCR using an Expand high fidelity$^{PLUS}$ PCR system from Roche Diagnostics GmbH. The reaction mixture for the PCR contained 50 μl of Expand high fidelity$^{PLUS}$ buffer with 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, a sense and an antisense primer each at 1 μM concentration, 2.5 U of Expand high fidelity$^{PLUS}$ PCR system enzyme mix, and 200 ng of plasmid pBD2 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 55°C for 30 s, and an elongation step at 72°C for 2 min. The sense primer 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 pUC19, and spanned positions 398–424 in the sequence of Genbank accession number AB205151. The antisense primer contained an *Xba*I site (underlined sequence) and corresponded to the sequence from 1918 to 1943. The two primers were as follows: sense primer, 5′-GCATGAAAGCTTTAAGGAGGAAtagTTTATGAAAA TCGAATTCGTCGCTTCG-3′; antisense primer, 5′-CTC CAGGTTCTAGAACCAGATTTCCG-3′. The amplified PCR product was digested with *Hin*dIII and *Xba*I, separated by agarose-gel electrophoresis, and then purified with a QIAquick gel extraction kit. The amplified DNA was inserted downstream of the *lac* promoter in pUC19, yielding pBD3, and then used to transform *E. coli* JM109 cells.

## Results

### Purification of the L-amino acid amidase, LaaA$_{Bd}$, from *B. diminuta* TPU 5720

An amidase acting on L-phenylalaninamide was detected in *B. diminuta* TPU 5720. HPLC analysis of the reaction mixture consisting of cells of the strain and L- or D-phenylalaninamide showed that the cells hydrolyzed L-phenylalaninamide much more efficiently than D-phenylalaninamide (Fig. 1). However, a trace amount of D-phenylalanine was also formed in the reaction mixture, which would result in a somewhat small enantiomeric excess (ee). To investigate whether the two activities are located at two different enzymes and evaluate the L-stereoselectivity of the hydrolytic activity toward L-phenylalaninamide, we tried to purify the L-amino acid amidase, named LaaA$_{Bd}$, from the cell-free extract of the strain. The enzyme, however, lost its activity on dialysis against 20 mM potassium phosphate buffer after ammonium sulfate fractionation. The effect of various metal ions and organic compounds such as dithiothreitol, 2-mercaptoethanol, ethylenediaminetetraacetic acid, *N*-ethylmaleimide, and glycerol on the enzyme activity was investigated, and $Co^{2+}$ ion at 1 mM was found to largely restore the activity. The enzyme was then purified with a recovery of 1.1% by ammonium sulfate fractionation and DEAE–Toyopearl, Butyl–Toyopearl, MonoQ, and Superdex column chromatographies (Table 1), in which $CoCl_2$ was added to the reaction mixture for determining the enzyme activity. Enhancement of the amidase activity of the enzyme during the DEAE–Toyopearl chromatography was usually observed in the three individual purifications, raising the possibility of the presence of an inhibitor against the enzyme in the soluble fraction. The final preparation gave a single band on SDS-PAGE with a molecular weight of ≈53,000 (lane 1 in Fig. 2). The molecular weight of the native enzyme was about 288,000 according to gel-filtration chromatography, indicating that the enzyme is

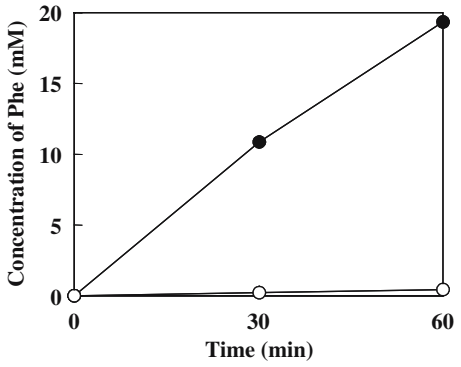


**Fig. 1** Hydrolysis of L- or D-phenylalaninemide by cells of *B. diminuta* TPU 5720. *B. diminuta* TPU 5720 was cultivated in 500 ml of TGY medium for 24 h at 30°C. The cells were then harvested, washed with 0.9% NaCl, and suspended in 8.2 ml of 0.1 M of potassium phosphate (pH 7.0). The reaction mixture contained 0.2 M of L- or D-phenylalaninemide hydrochloride, 0.1 M of potassium phosphate (pH 7.0), and 40 μl of the cell suspension in a total volume of 1 ml and was incubated at 30°C. The reaction was stopped at a specific point in time, and the concentration of phenylalanine formed was determined using HPLC as described in Materials and methods. • L-phenylalanine; ○ D-phenylalanine

**Table 1** Purification of the L-amino acid amidase from *B. diminuta* TPU5720

| Step | Total protein (mg) | Total[a] activity (U) | Specific[a] activity (U/mg) | Yield (%) |
|---|---|---|---|---|
| Cell-free extract | 5,730 | 2,940 | 0.51 | 100 |
| Ammonium sulfate (30–60%) | 348 | 335 | 0.96 | 11.4 |
| DEAE–Toyopearl | 89.1 | 434 | 4.86 | 14.8 |
| Butyl–Toyopearl | 7.43 | 139 | 18.7 | 4.7 |
| MonoQ HR10/10 | 3.94 | 81 | 20.7 | 2.8 |
| Superdex HR10/30 | 1.08 | 32 | 29.6 | 1.1 |

[a]L-Phenylalaninamide was used as a substrate

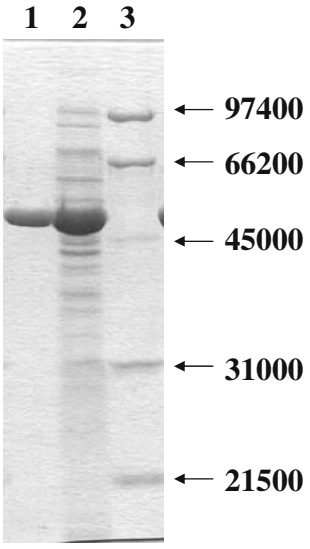


**Fig. 2** SDS-polyacrylamide slab gel electrophoresis. Lane 1, $LaaA_{Bd}$ purified from *B. diminuta* TPU 5720 (5 μg); lane 2, cell-free extract (25 μg) of *E. coli* JM109 harboring pBD3; lane 3, molecular-weight standards [phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and trypsin inhibitor (21,500)]

active in a hexameric form. The purified $LaaA_{Bd}$ was subjected to N-terminal amino acid sequencing, yielding the following result: MKIEFVASSGAAEILALLVHEGRALA GTGP.

## Effects of pH and temperature and stability

The optimal pH for the activity of the enzyme was measured using the following buffers (final concentration 100 mM): acetic acid/sodium acetate (pH 3.0–6.0), potassium phosphate (pH 6.5–9.0), Tris/HCl (pH 7.5–9.0), and $NaHCO_3/Na_2CO_3$ (pH 10.0–11.0). The enzyme showed maximum activity at pH 7.5. The enzyme reaction was carried out at various temperatures for 10 min in 100 mM potassium phosphate (pH 7.0), and enzyme activity was found to be maximal at 50°C (Fig. 3). At 30°C, the enzymatic activity corresponded to 57% of that measured at 50°C, whereas at 80°C, the enzyme retained 40% of its activity on the substrate.

The purified enzyme could be stored without noticeable loss of activity for more than 3 months on ice in 20 mM potassium phosphate buffer (pH 7.0). The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 20 min in 100 mM potassium phosphate (pH 7.0), a sample of the enzyme solution was taken, and the activity was assayed with L-phenylalaninamide as a substrate under standard conditions. It exhibited the following levels of activity: 70°C, 0%; 65°C, 29%; 60°C, 86%; 55°C, 96%; 50°C, 100%; 45°C, 100%.

## Effects of metal ions and inhibitors

During the purification of $LaaA_{Bd}$, we found that $Co^{2+}$ ion can restore the activity of the enzyme which had been inactivated by dialysis against potassium phosphate buffer. Various metal ions were again investigated for their effects on the activity of the purified enzyme. We measured the enzyme activity under standard conditions after incubation at 30°C for 10 min with various metal ions at 1 mM. The enzyme activity was detected in the presence of $CoCl_2$ (100%), $NiCl_2$ (20%), $MnSO_4$ (7.9%), $MnCl_2$ (5.0%), and $MgSO_4$ (4.5%). The relative enzyme activity was only 0.3% without any metal ions. However, addition of $CoCl_2$ to the culture medium of *B. diminuta* TPU 5720 affected neither growth of the strain nor $LaaA_{Bd}$ activity of the cells toward L-phenylalaninamide (data not shown).

The effect of inhibitors (1 mM) was also investigated in the presence of 1 mM of $CoCl_2$. The enzyme was completely inhibited by ethylenediaminetetraacetic acid, suggesting that it is a $Co^{2+}$-dependent enzyme. Other chelating reagents, e.g., *o*-phenanthroline, 8-hydroxyquinoline, and α,α′-dipyridyl had, however, no significant effect on the enzyme. Carbonyl reagents such as hydroxylamine, hydrazine, phenylhydrazine, D,L-penicillamine, and D-cycloserine were not inhibitory toward the enzyme. Furthermore, the enzyme was not inhibited by thiol reagents such as *p*-chloromercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid), iodoacetic acid, and *N*-ethylmaleimide. A serine protease inhibitor, phenylmethanesulfonyl fluoride; a serine/cysteine protease inhibitor, leupeptine; and an aspartic protease inhibitor, pepstatin, did not influence the activity.

## Substrate specificity

To study the substrate specificity, the purified $LaaA_{Bd}$ was used to hydrolyze various amino acid amides and peptides, and the activity was assayed (Table 2). Besides L-phenylalaninamide, the enzyme acted on a broad range of L-amino acid amides including L-2-aminobutyric acid amide, L-glutaminamide, L-leucinamide, L-methioninamide, and L-argininamide. Among the D counterparts of the above substrates, D-2-aminobutyric acid amide, D-glutaminamide, D-leucinamide, and D-methioninamide were inert as substrates for the enzyme. $LaaA_{Bd}$ showed L-stereoselectivity depending on the amino acid amide. The apparent *K*m values for L-phenylalaninamide, L-tryptophanamide, and L-tyrosinam-

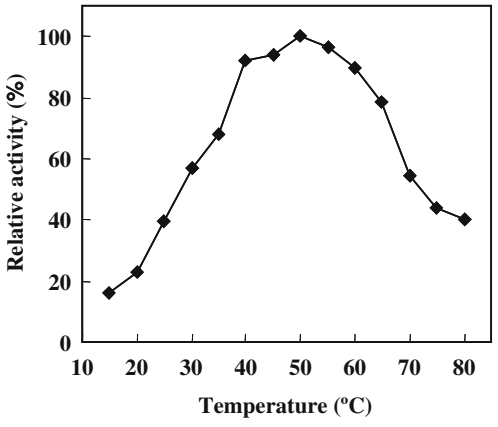


**Fig. 3** Effect of temperature on the activity of $LaaA_{Bd}$. Reactions were carried out at various temperatures as described in Materials and methods

**Table 2** Substrate specificity of purified $LaaA_{Bd}$

| Substrate | Relative activity (%) | Substrate | Relative activity (%) |
|---|---|---|---|
| L-$PheNH_2$ | 100 | D-$PheNH_2$ | 2.2 |
| L-$AlaNH_2$ | 3.3 | D-$AlaNH_2$ | 1.7 |
| L-$ValNH_2$ | 17 | D-$ValNH_2$ | ND |
| L-$LeuNH_2$ | 89 | D-$LeuNH_2$ | ND |
| L-*tert*-$LeuNH_2$ | 0.2 | D-*tert*-$LeuNH_2$ | ND |
| L-$IleNH_2$ | 29 | | |
| L-$ProNH_2$ | 2.2 | D-$ProNH_2$ | 2.2 |
| L-$TyrNH_2$ | 37 | D-$TyrNH_2$ | ND |
| L-$TrpNH_2$ | 41 | | |
| L-$SerNH_2$ | 2.8 | | |
| L-$ThrNH_2$ | 14 | | |
| L-$MetNH_2$ | 82 | D-$MetNH_2$ | ND |
| L-$AsnNH_2$ | 11 | | |
| L-$GlnNH_2$ | 91 | D-$GlnNH_2$ | ND |
| | | D-$AspNH_2$ | 0.9 |
| L-$GluNH_2$ | 11 | D-$GluNH_2$ | 2.6 |
| L-$LysNH_2$ | 18 | D-$LysNH_2$ | 2.5 |
| L-$ArgNH_2$ | 78 | D-$ArgNH_2$ | 1.9 |
| L-$HisNH_2$ | 16 | | |
| L-$ABANH_2$ | 96 | D-$ABANH_2$ | ND |
| $GlyNH_2$ | ND | | |
| β-$AlaNH_2$ | ND | | |
| (L-Phe)$_2$ | 165 | D-Phe-L-Phe | 12 |
| L-Phe-D-Phe | ND | (D-Phe)$_2$ | ND |
| L-Ala-Gly | 54 | D-Ala-Gly | ND |
| Gly-Gly | 2.5 | | |

The activity for L-phenylalaninamide (L-$PheNH_2$) corresponding to 29.6 U $mg^{-1}$ was taken as 100%
*ABANH*$_2$ 2-Aminobutyric acid amide, *ND* not detectable

ide were 15, 17, and 20 mM, whereas *V*max values for the substrates were 36, 17, and 28 U $mg^{-1}$, respectively. Some dipeptides were also efficiently hydrolyzed by the enzyme, suggesting that $LaaA_{Bd}$ could play a physiological role as a dipeptidase in *B. diminuta* TPU 5720.

## Cloning of the L-amino acid amidase gene, $laaA_{Bd}$

The oligonucleotide primers used for cloning of the $laaA_{Bd}$ gene by PCR were designed on the basis of the N-terminal amino acid sequence of the $LaaA_{Bd}$ purified from *B. diminuta* TPU 5720. PCR with the primers and the chromosomal DNA prepared from the strain yielded an amplified 89-bp DNA. Nucleotide sequencing of the DNA fragment revealed that the fragment contained the region encoding the N-terminal amino acid sequence of the purified amidase. Using Southern hybridization with the 89-bp probe, a 4.5-kb *Pst*I signal was obtained. From a genomic *Pst*I DNA library in *E. coli* JM109, a clone containing a plasmid that carried a 4.5-kb insert could be isolated. The plasmid, named pBD2, was used to generate nested deletion plasmids for the determination of the nucleotide sequence. The nucleotide sequence determined was found to be 4,446 bp long, and seven open-reading frames (ORFs) designated ORF1, ORF2, ORF3, ORF4, ORF5, ORF6, and ORF7 were present in this region. ORF1, ORF6, and ORF7 would be transcribed in the opposite direction to the other ORFs (Fig. 4).

The amino acid sequence deduced from ORF2 (positions 401–1876) contained the N-terminal amino acid sequence determined by peptide sequencing, indicating that ORF2 codes for $LaaA_{Bd}$. ORF2 was therefore designated $laaA_{Bd}$. The structural gene consists of 1,473 bp and codes for a protein of 491 amino acids. Molecular weight was calculated to be 51,127, which is in good agreement with the value estimated from the SDS-PAGE of the purified enzyme. A potential ribosome-binding site (GGAG) was located just seven nucleotides upstream from the start codon ATG. Alignment using the BLAST program showed that the deduced primary structure of $LaaA_{Bd}$ is similar to that of hypothetical cytosol aminopeptidases found in the genomes of various microorganisms such as *Caulobacter crescentus* (65% identical over 495 amino acids; Nierman et al. 2001), *Bradyrhizobium japonicum* (55% identical over 498 amino acids; Kaneko et al. 2002), *Rhodopseudomonas palustris* (51% identical over 495 amino acids; Larimer et al. 2004), *Mesorhizobium loti* (57% identical over 459 amino acids; Kaneko et al. 2000), and *Agrobacterium tumefaciens* (53% identical over 498 amino acids; Goodner et al. 2001) and characterized leucine aminopeptidases, PepA, from *Rickettsia prowazekii* (44% identical over 501 amino acids; Wood et al. 1993), *P. putida* ATCC 12633 (40% identical over 490 amino acids; EMBL accession number AJ010261), and *E. coli* K-12 (37% identical over 496 amino acids; EMBL accession number AY283772). Figure 5 shows the alignment of the primary structures of $LaaA_{Bd}$ from *B. diminuta* TPU 5720, the

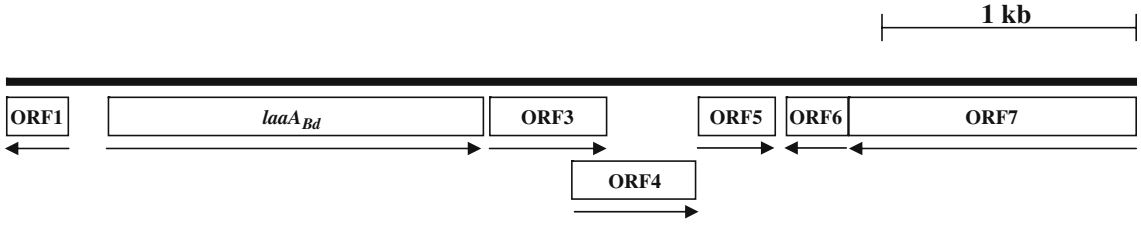


**Fig. 4** Organization of the genes flanking $laaA_{Bd}$ in the *B. diminuta* TPU 5720 genome. Directions of transcription are indicated with *arrows*

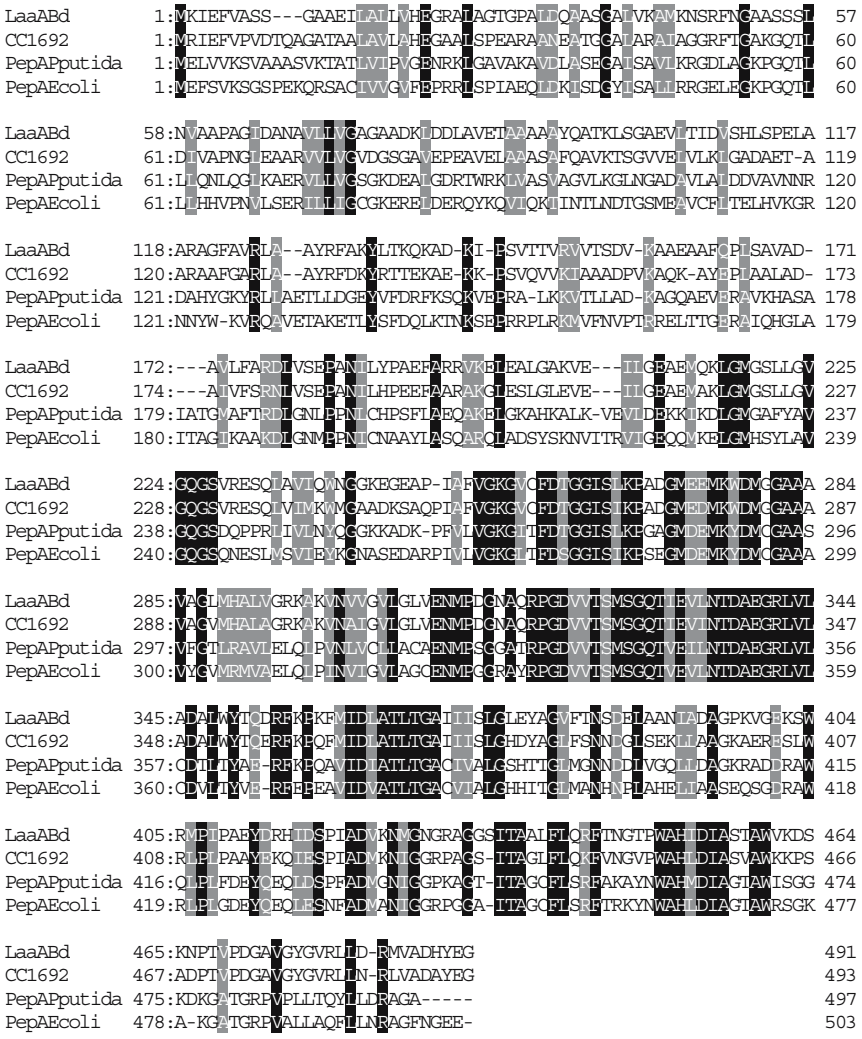


**Fig. 5** Comparison of the amino acid sequences of $LaaA_{Bd}$ from *B. diminuta* TPU 5720 (*LaaABd*), the hypothetical aminopeptidase from *C. crescentus* (*CC1692*), L-aminopeptidase from *P. putida* ATCC12633 (*PepAPputida*), and Leucine aminopeptidase from *E. coli* K-12 (*PepAEcoli*). *Dashed lines* indicate the gaps introduced for better alignment. Identical and conserved amino acids among the sequences are marked in *black* and in *gray*, respectively

hypothetical aminopeptidase, CC1692 from *C. crescentus*, and PepA from *P. putida* ATCC 12633 and *E. coli* K-12. The level of identity is higher in the carboxy half of these proteins.

As summarized in Table 3, the deduced amino acid sequences of the other ORFs, ORF1 (positions 1–243; complementary), ORF3 (positions 1902–2363, 153 amino acids with a molecular weight of 17,481), ORF4 (positions 2225–2704, 159 amino acids with a molecular weight of 16,996), ORF5 (positions 2722–3024, 100 amino acid residues with a molecular weight of 10,454), ORF6 (positions 3074–3316; complementary, 80 amino acids with a molecular weight of 9,200), and ORF7 (positions 3316–4446; complementary), showed significant similarity with a probable transmembrane protein, a probable DNA polymerase III chi subunit, three hypothetical proteins of unknown function, and a probable ABC transporter ATP-binding protein, respectively. Comparison of the deduced amino acid sequences of ORF1 and ORF7 with their homologous genes indicated that ORF1 lacks a 3′ terminus, probably coding for 298 amino acid residues, and ORF7 lacks a 5′ terminus, probably coding for 245 amino acid residues. ORF4 and ORF5 showed similarity with the same sequence, CC1694 from *C. crescentus*, indicating that homologous genes, ORF4 and ORF5, are located in tandem in the *B. diminuta* genome. The seven proteins encoded in the 4,446-bp region of *B. diminuta* TPU 5720 showed 33–71% identity with those encoded in the *C. crescentus* genome (Nierman et al. 2001).

## Production of $LaaA_{Bd}$ in *E. coli*

To express the $laaA_{Bd}$ gene in *E. coli*, we modified the sequence upstream from the ATG start codon by PCR with plasmid pBD2 as a template as described in Materials and methods. The resultant plasmid, pBD3, in which the $laaA_{Bd}$ gene was under the control of the *lac* promoter of pUC19, was introduced into *E. coli* JM109 cells. When *E. coli* JM109 harboring pBD3 was cultivated in Luria–Bertani medium supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside for 14 h at 37°C, the level of $LaaA_{Bd}$ activity in the supernatant of the sonicated cell-free extracts of the transformant was 25.9 units $mg^{-1}$ with L-phenylalaninamide as substrate, which is 50 times higher than that of *B. diminuta* TPU 5720. A large amount of a protein

**Table 3** Homology search analysis of seven ORFs in 4,446-bp-long DNA region of *B. diminuta* TPU 5720

| ORF | Function | Homology | | | |
|---|---|---|---|---|---|
| | | Amino acid identity (%) | Name of ORF | Source | Accession number |
| ORF1 | Probable transmembrane protein | 52 | CC1691 | *C. crescentus* | H87458 |
| | | 33 | SMc00584 | *S. meliloti* | Q92QY8 |
| ORF2 (*laaA*$_{Bd}$) | L-Amino acid amidase | 65 | CC1692 | *C. crescentus* | Q9A7M9 |
| | | 55 | PepA | *B. japonicum* | BA000040 |
| | | 51 | LAP | *R. palustris* | Q6N5B9 |
| | | 57 | LAP | *M. loti* | Q984S1 |
| | | 53 | LAP | *A. tumefaciens* | B97495 |
| | | 44 | PepA | *R. prowazekii* | P27888 |
| | | 40 | PepA | *P. putida* | O86436 |
| | | 37 | PepA | *E. coli* | Q7X4V5 |
| ORF3 | Probable DNA polymerase III chi subunit | 54 | CC1693 | *C. crescentus* | B87459 |
| | | 48 | AGR_C_2056 | *A. tumefaciens* | C97495 |
| | | 46 | SMc00586 | *S. meliloti* | Q92QY6 |
| ORF4 | Unknown | 36 | CC1694 | *C. crescentus* | C87459 |
| ORF5 | Unknown | 33 | CC1694 | *C. crescentus* | C87459 |
| ORF6 | Unknown | 30 | VPA0848 | *V. parahaemolyticus* | Q87HV8 |
| | | 34 | VFA0449 | *V. fischeri* | Q5E0C7 |
| | | 36 | CC0773 | *C. crescentus* | B87345 |
| ORF7 | Probable ABC transporter ATP-binding protein | 71 | CC1698 | *C. crescentus* | G87459 |
| | | 70 | GOX2143 | *G. oxydans* | Q5FP17 |

*C. crescentus*, *Caulobacter crescentus* CB15; *S. meliloti*, *Sinorhizobium meliloti*1021; *B. japonicum*, *Bradyrhizobium japonicum* USDA110; *R. palustris*, *Rhodopseudomonas palustris* CGA009; *M. loti*, *Mesorhizobium loti* MAFF303099; *A. tumefaciens*, *Agrobacterium tumefaciens* str. C58; *V. fischeri*, *Vibrio fischeri* ES114; *G. oxydans*, *Gluconobacter oxydans* 621H

corresponding to LaaA$_{Bd}$ purified from *B. diminuta* TPU 5720 was present in the cell-free extract (lane 2 in Fig. 2).

## Discussion

In this study, we purified an L-stereoselective amino acid amidase, LaaA$_{Bd}$, acting on L-phenylalaninamide from *B. diminuta* TPU 5720, investigated its characteristics, and cloned the gene, *laaA*$_{Bd}$, coding for the enzyme. Among the bacterial L-amino acid amidases previously reported (Table 4), L-aminopeptidase from *P. putida* ATCC 12633 (Hermes et al. 1993) was found to be most similar to LaaA$_{Bd}$ with respect to the molecular weight of the subunit, inhibition by EDTA, activation by $Co^{2+}$ or $Mn^{2+}$, and some substrate specificity acting not only on L-amino acid amides but also on dipeptides. Homology search analysis revealed that the amino acid sequence deduced from *laaA*$_{Bd}$ showed significant similarity to that of the *P. putida* enzyme as well as to sequences of hypothetical cytosol aminopeptidases found in the genomes of various microorganisms and functionally characterized leucine aminopeptidases (PepA) from *R. prowazekii* and *E. coli*, clearly showing that LaaA$_{Bd}$ should be categorized into the PepA family protein. The primary sequence of L-aminopeptidase from *P. putida* ATCC 12633 showing sequence similarity with bacterial leucine aminopeptidases, PepA, has been submitted to the EMBL/GenBank/DDBJ databases without publication. The number of subunits in the native form of the enzyme, optimum pH, optimum temperature and effects of a thiol reagent, *p*-chloromercuribenzoic acid, and a serine protease inhibitor, phenylmethylsulfonyl fluoride, for LaaA$_{Bd}$ were, however, different from those described for the *P. putida* enzyme, possibly due to the relatively low sequence identity between the two enzymes (40%). Dependence on divalent cations of LaaA$_{Bd}$ was also different from that of the *P. putida* enzyme: LaaA$_{Bd}$ lost its activity almost completely (0.3%) on dialysis against potassium phosphate buffer, and the amidase activity was restored by the addition of $Co^{2+}$ (100%), $Ni^{2+}$ (20%), $Mn^{2+}$ (7.9%), and so on, whereas the enzyme activity of *P. putida* PepA was reported to be stimulated by $Mg^{2+}$, $Co^{2+}$ (2- to 3-fold), and $Mn^{2+}$ (12-fold) (Hermes et al. 1993).

Amino acid amide racemizing activity has been discovered in α-amino-ε-caprolactam racemase from *Achromobacter obae* and demonstrated to be useful for the dynamic kinetic resolution of racemic alaninamide to produce D-alanine by a combination of the racemase and a D-stereospecific aminopeptidase, D-aminopeptidase, from *O. anthropi* C1-38 (Asano and Yamaguchi 2005; Asano et al. 1992). Among the amino acid amides that has been found to be substrates for the racemase, the purified LaaA$_{Bd}$ showed strict L-stereoselectivity toward 2-aminobutyric acid amide, leucinamide, and methioninamide (Table 2), suggesting the applicability of the combination of the racemase and LaaA$_{Bd}$ to the production of their corresponding L-amino acids by dynamic kinetic resolution, as LaaA$_{Bd}$ has become abundantly available using the DNA technique. Although L-phenylalaninamide was the best substrate for LaaA$_{Bd}$ among the amino acid amides tested for its substrate specificity, the L-stereoselectivity toward phenylalaninamide was not so strict, the enzyme being slightly active on

**Table 4** Comparison of the characteristics of $LaaA_{Bd}$ from *B. diminuta* TPU 5720 and bacterial L-amino acid amidases

| | $LaaA_{Bd}$ | $LaaA_{Pa}$ | L-Aminopeptidase (PepA) | L-Specific amidase | L-Amino amidase |
|---|---|---|---|---|---|
| Origin | *Brevundimonas diminuta* TPU 5720 | *Pseudomonas azotoformans* IAM 1603 | *Pseudomonas putida* ATCC 12633 | *Ochrobactrum anthropi* NCIMB 40321 | *Mycobacterium neoaurum* ATCC 25795 |
| Molecular weight of subunit | | | | | |
| (SDS-PAGE) | 53,000 | 34,000 | 53,000 | 36,000 | 40,000 |
| (Calculated) | 51,127 | 34,514 | 52,468 | | |
| Number of subunits | 6 | 1 | 8 | 2 | 3 or 4 |
| Optimum pH | 7.5 | 9.0 | 9.5 | 6.0–8.5 | 8.0–9.5 |
| pH stability | | 6.0–9.5 | | | |
| Optimum temperature (°C) | 50 | 45 | 40 | 70 | 50 |
| Heat stability (°C) | 60 | 45 | | 60 | 55 |
| Inhibitor | EDTA | Phenylhydrazine, *p*CMB, iodoacetate, *N*-ethylmaleimide, $Zn^{2+}$, $Ag^{+}$, $Cd^{2+}$, $Hg^{2+}$ | *p*CMB, DFP, EDTA, PMSF, *o*-phenanthroline, $Cu^{2+}$, $Ca^{2+}$ | EDTA, *o*-phenanthroline | DTT, *o*-phenanthroline, iodoacetamide |
| Activator | $Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$ | No | DTT $Mn^{2+}$, $Mg^{2+}$, $Co^{2+}$ | $Zn^{2+}$, $Mn^{2+}$, $Mg^{2+}$ | |
| Substrate Specificity | L-$PheNH_2$ L-$GlnNH_2$, L-$LeuNH_2$, L-$MetNH_2$ | L-$ProNH_2$ L-Pro-*p*NA, (*S*)-Piperidine-2-carboxamide, L-$AlaNH_2$, L-$MetNH_2$ | L-$LeuNH_2$ L-$PheglyNH_2$, L-$MetNH_2$ | L-$ProNH_2$ L-$PheNH_2$, L-$MetNH_2$, L-$PheglyNH_2$, L-$AlaNH_2$ | L-$ProNH_2$ L-$ValNH_2$, L-α-MethyL-$ValNH_2$ |
| Peptidase activity | Yes L-Phe-L-Phe | No | Yes L-Phe-L-Phe, L-Phe-L-Leu | No | No |

*pCMB* *p*-Chloromercuribenzoate, *DFP* diisopropylfluorophosphate, *EDTA* ethylenediaminetetraacetic acid, *PMSF* phenylmethylsulfonyl fluoride, *DTT* dithiothreitol

D-phenylalaninamide as observed in the case of the cell reaction (Fig. 1). On the other hand, using the cells of *E. coli* transformants expressing the α-amino-ε-caprolactam racemase and D-aminopeptidase for the dynamic kinetic resolution of racemic amino acid amides to produce D-amino acids might result in a decrease in the enantiomeric excess of the products, possibly because of the hydrolysis of L-amino acid amides in the *E. coli* hosts. As mentioned above, $LaaA_{Bd}$ showed significant sequence similarity with *E. coli* PepA which is one of the candidates for the L-stereoselective hydrolase, although there is no description about the activity of the *E. coli* PepA toward amino acid amides (Vogt 1970). The result that $LaaA_{Bd}$ activity was completely inhibited by the addition of ethylenediaminetetraacetic acid would therefore be a useful information for the dynamic kinetic resolution, as neither α-amino-ε-caprolactam racemase nor D-aminopeptidase is inhibited by the chelating reagent (Ahmed et al. 1983; Asano et al. 1989b).

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