J Ind Microbiol Biotechnol (2005) 32: 567–576
DOI 10.1007/s10295-005-0246-x

ENVIRONMENTAL BIOTECHNOLOGY

Tipparat Hongpattarakere · Hidenobu Komeda
Yasuhisa Asano

Purification, characterization, gene cloning and nucleotide sequencing of D-stereospecific amino acid amidase from soil bacterium: *Delftia acidovorans*

Received: 25 August 2004 / Accepted: 3 May 2005 / Published online: 23 September 2005

Abstract The D-amino acid amidase-producing bacterium was isolated from soil samples using an enrichment culture technique in medium broth containing D-phenylalanine amide as a sole source of nitrogen. The strain exhibiting the strongest activity was identified as *Delftia acidovorans* strain 16. This strain produced intracellular D-amino acid amidase constitutively. The enzyme was purified about 380-fold to homogeneity and its molecular mass was estimated to be about 50 kDa, on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme was active preferentially toward D-amino acid amides rather than their L-counterparts. It exhibited strong amino acid amidase activity toward aromatic amino acid amides including D-phenylalanine amide, D-tryptophan amide and D-tyrosine amide, yet it was not specifically active toward low-molecular-weight D-amino acid amides such as D-alanine amide, L-alanine amide and L-serine amide. Moreover, it was not specifically active toward oligopeptides. The enzyme showed maximum activity at 40°C and pH 8.5 and appeared to be very stable, with 92.5% remaining activity after the reaction was performed at 45°C for 30 min. However, it was mostly inactivated in the presence of phenylmethanesulfonyl fluoride or Cd^{2+}, Ag^{+}, Zn^{2+}, Hg^{2+} and As^{3+}. The NH_2 terminal and internal amino acid sequences of the enzyme were determined; and the gene was cloned and sequenced. The enzyme gene *damA* encodes a 466-amino-acid protein (molecular mass 49,860.46 Da); and the deduced amino acid sequence exhibits homology to the D-amino acid amidase from *Variovorax paradoxus* (67.9% identity), the amidotransferase A subunit from *Burkholderia fungorum* (50% identity) and other enantioselective amidases.

Keywords D-Amidase · D-Amino acids · Enantioselective amidase · Amino acid amides · *Delftia acidovorans*

T. Hongpattarakere (✉)
Faculty of Agro-Industry, Prince of Songkla University,
Hat Yai, 90112, Songkla, Thailand
E-mail: tipparat.h@psu.ac.th
Tel.: +66-74-286371
Fax: +66-74-445727

H. Komeda · Y. Asano
Biotechnology Research Center,
Toyama Prefectural University,
5180 Kurokawa, Kosugi 939-0398, Toyama, Japan
E-mail: asano@pu-toyama.ac.jp

Introduction

D-Amino acids are broadly useful chiral building blocks, with important applications for the production of pharmaceuticals, food additives and agrochemicals. D-*p*-Hydroxyphenylglycine and D-phenylglycine are utilized for the synthesis of penicillin derivatives. D-Cysteine and D-aspartic acid are important as side-chains of β-lactam antibiotics and D-valine is used for insecticides. D-Alanine is used as a starting material for sweetener synthesis. D-Glutamate and D-proline are used for synthetic pharmaceuticals. Although the production of D-amino acids is currently of great interest, there has been no known industrial manufacture of D-amino acids, except for D-*p*-hydroxyphenylglycine and D-phenylglycine [25].

D-Amino acid amidases are increasingly recognized to be important catalysts in the stereospecific production of D-amino acid by hydrolyzing D-amino acid amides to yield D-amino acid and ammonia. A few D-amino acid amidases have been reported so far. D-Amino acid amidase from *Arthrobacter* sp. NJ-26 was discovered to be specific to D-alaninamide [20]. A (*R*)-enantioselective amidase produced by *Comamonas acidovorans* was purified, characterized and cloned [11]. However, its stereospecificity was quite low. The strict D-stereospecific amino acid amidase from *Ochrobactrum antropi* SV 3 was investigated by Asano et al. [2] and later was cloned, purified and characterized by Komeda and Asano [13]. The enzyme was remarkably active on D-phenylalnine amide, D-tyrosine amide and D-tryptophan amide. Recently a novel D-amidase from *Variovorax paradoxus*

was isolated and characterized; and D-*tert*-leucine amide was the target substrate for the enzyme [15]. A new thermostable D-stereospecific alanine amidase from the thermophile *Brevibacillus borstelensis* BCS-1 was characterized, and the gene was cloned and sequenced. The recombinant enzyme showed strong activity toward D-alanine amide [6].

We isolated 32 bacterial strains which exhibited D-phenylalanine amide degradation at both 30°C and 45°C, from soil on the campus of the Prince of Songkla University, Thailand. The strain showing the strongest activity toward D-phenylalanine was eventually identified as *Delftia acidovorans* strain 16. This particular strain degraded D-tert-leucine amide to D-*tert*-leucine, although very slightly. D-*tert*-leucine is one of the most valuable D-amino acids with its high commercial cost of over US$ 100 per gram [24]. The D-amino acid amidase enzyme from *D. acidovorans* strain 16 was then purified and characterized. Its NH_2 terminal and internal amino acid sequences were Met^{1}-Asn-Asp-Ser-Glu^{5}-Leu-His-X-Leu-Glu^{10}-Leu-Leu-Glu-Val-Gly^{15} and Gln^{1}-Ala-Val-Glu-Gln^{5}-Ala-Leu-Ala-Val-Ala^{10}-Gln-Arg^{12}, respectively, and the gene was cloned and sequenced.

Materials and methods

Materials

DEAE-Toyopearl 650M, butyl-Toyopearl 650M, ether Toyopearl and a Toso G-3000 SW column were supplied by Tosoh Corp. (Tokyo, Japan). A HPLC column (Cosmosil C18, 5C18-MS, 4.6 mm diam., 150 mm long) was obtained from Nakalai Tesque (Kyoto, Japan), and a Sumichiral OA-500 column (4.6 mm diam, 150 mm long) was supplied from Sumika Analysis Service (Osaka, Japan). MonoQ HR10/10, MonoQ HR5/5 and Superdex 200 HR10/35 columns equipped with a FPLC control system and data analysis software (Unicorn ver. 4.00.16)) were purchased from Pharmacia (Uppsala, Sweden). Marker proteins for molecular weight determination were from Amersham Pharmacia Biotech (N.J., USA). Coomassie brilliant blue G was obtained from Tokyo Kasei (Tokyo, Japan). Microcon (for ultrafiltration of 0.5-ml samples) was purchased from Amicon, Millipore Corp. (Mass., USA).

Protamine sulfate from salmon supplied by Nacalai Tesque (Tokyo, Japan). All amino acid amides, amino acids and oligopepetides were either purchased from Bachem (Bubendorf, Switzerland) or synthesized as described by Asano et al. [3, 4]. The amino acid amides included D-phenylalanine amide, D-norvaline amide, D-methionine amide, D-tryptophan amide, D-leucine amide, D-*n*-leucine amide, D-tyrosine amide, D-serine amide, D-threonine amide, D *allo*-threonine amide, D-proline amide, D-isoleusine amide, D-histidine amide, D-lysine amide, D-*tert*-leucine amide, D-aspartic acid amide, D-alanine amide, D-glutamic acid amide, D-glutamine amide, D-glycine amide, D-valine amide, L-leucine amide, L-phenylalanine amide, L-tyrosine amide, L-norvaline amide, L-alanine amide, L-asparagine amide, L-glutamic acid amide, L-lysine amide, L-proline amide and L-serine amide. The amino acids included D-phenyl- alanine and D-*tert*-leucine; and the oligopepetides included D-$phenylalanine_4$, D-$phenylalanine_3$, D-phenyl- $alanine_2$, D-phenylalanyl-L-phenylalanine, L-phenylalanyl-D-phenylalanine, and L-$phenylalanine_2$.

Bacterial strains, plasmids and culture conditions

D. acidovorans strain 16 previously isolated from soil by an enrichment culture technique was used as a source of chromosomal DNA. *E. coli* XL1-Blue MR was used as a host for the recombinant plasmids. Plasmid pBluescriptII SK(−) (Toyobo, Osaka, Japan) was used as a cloning vector. *D. acidovorans* strain 16 was grown in TGY broth (containing 5 g tryptone, 5 g yeast extract, 1 g glucose, 1 g K_2HPO_4 in 1 L distilled water, pH 7.0). Recombinant *E. coli* XL1-Blue MR was cultured at 37°C on Luria–Bertani medium containing 80 μg mL^{-1} ampicillin. To induce the gene under the control of the *lac* promoter, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM.

Enzymes and materials

Restriction endonucleases, DNA ligation kit ver. 2 and Deletion kit for kilo sequencing were purchased from Takara Shuzo (Kyoto, Japan). Alkaline phosphatase from shrimp was purchased from Boehringer–Mannheim (Tokyo, Japan). D-Amino acid oxidase from porcine kidney was obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Horseradish peroxidase was from Toyobo. QIAquick gel extraction kit was purchased from QIAGEN Sciences (Md., USA) and Invisorb spin plasmid mini kit was bought from Invitek (Berlin, Germany).

Isolation of D-amino acid amidase producers

Microorganisms possessing an ability to utilize D-phenylalanine amide as nitrogen source, were isolated from 54 soil samples taken from various locations around the Prince of Songkla University campus in southern Thailand by an enrichment culture technique [3]. Each 1-g soil sample was added into 2 mL enrichment broth (containing 5 g glycerol, 2 g D-phenylalanine amide HCl, 2 g K_2HPO_4, 1 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.5 g yeast extract, 0.02 μg biotin, 4 μg calcium pantothenate, 20 μg inositol, 4 μg nicotinic acid, 4 μg thiamine HCl, 2 μg pyridoxine HCl, 2 μg *p*-aminobenzoic acid, 2 μg riboflavin, 1 μg folic acid in 1 L distilled water, pH 7.0). D-Phenylalanine amide HCl was filter-sterilized and added to the autoclaved- medium broth. Soil samples

were aerobically incubated with the medium at 30°C and 45°C, 250 rpm for 24 h. About 10 μL of the culture were then transferred into 2 mL fresh medium and was further incubated at 30°C and 45°C for 1 week. The formation of D-phenylalanine in the culture broth was determined daily by thin layer chromatography.

Enzyme assay

The activity of D-amidase was routinely assayed at 30°C by measuring the production of D-phenylalanine from D-phenylalanine amide. The reaction mixture contained 195 μL Tris-HCl (100 mM, pH 8.0), 200 μL D-phenylalanine amide (100 mM) dissolved in 100 mM Tris–HCl (pH 8.0) and 5 μl enzyme solution. The reaction was performed at 30°C for 10 min and then was terminated by adding 200 μL reaction mixture to 40 μL $HClO_4$ (2 N). D-Phenylalanine was determined by high performance liquid chromatography through a Cosmosil C18 column equipped with a Sugai U620 column heater (30°C), a pump (Shimazu LC-10AVP) and a 717 plus Autosampler system controller (Waters). The substrate and product were eluted isocratically by a mobile phase containing 500 mL methanol, 2 L milliQ water and 684 μL phosphoric acid. Both phenylalanine and phenylalanine amide were detected by a UV–Vis detector at 254 nm wavelength; and the chromatogram was recorded on a Shimadzu C-R6A Chromatopac recorder. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis reaction to form 1 μmol min^{-1}D-phenylalanine.

Analytical methods

Protein content was determined by a BioRad assay using BSA as standard or by measuring absorbance at 280 nm. Polyacrylamide gel electrophoresis was carried out in 12% polyacrylamide gel with running buffer (containing 0.25 M Tris-HCl, 2 M glycine, 1% SDS) at 30 mA. Protein bands were stained with Coomassie brilliant blue G and destained in methanol/acetic acid/water (3:1:6 by vol.) before drying by ATTO Rapidry mini-system at 65°C for 3 h. The gel was stained and destained with the dye and solution previously mentioned. The destained gel was then dried at 40°C for 3 h.

Purification of D-amino acid amidase from bacterial culture

D. acidovorans strain 16 was cultivated aerobically at 30°C for 24 h in 10 L TGY broth. All purification steps were performed at a temperature lower than 5°C; and 10 mM Tris-HCl (pH 8.0) was used throughout the process.

Bacterial cells cultivated in 10 L culture broth were separated and washed twice with 0.9 % saline solution. Washed cells were then suspended in 10 mM Tris-HCl and were disrupted by sonication for 10 min (19 kHz, Insonator 201M; Kobuta, Tokyo, Japan). Cell debris was removed by centrifugation at 15,000 *g* for 20 min at 4°C. Protamine sulfate (5% in water) was added at a concentration of 20 μL mL^{-1} cell-free extract and stirred for 30 min before removal of the precipitant by centrifugation at 15,000 *g* for 20 min. The obtained supernatant was then fractionated with ammonium sulfate powder. The precipitate obtained at 20–40% saturation was separated by centrifugation at 8000 *g* for 20 min, dissolved in 0.1 M Tris-HCl (pH 8.0) and dialyzed against 5 L Tris-HCl (10 mM) for 24 h. The dialyzed solution was further applied to a DEAE-Toyopearl 650 M column (2.5 mm diam., 10.5 cm long) equilibrated with 10 mM Tris-HCl. After sample application, the column was washed thoroughly with 10 mM Tris-HCl, followed by an elution step with a linear gradient of 250 mL NaCl (500 mM) in 10 mM Tris–HCl and 250 mL Tris-HCl buffer (10 mM). The active fraction, collected and pooled, was further saturated to 5 M NaCl and was subjected to an ether Toyopearl column and subsequently to a butyl Toyopearl column. The active fractions were pooled and concentrated using Microcon and then passed through phenyl Superose equipped with a FPLC system. The elution was achieved by a linear gradient beginning with 5 M NaCl in 10 mM Tris-HCl buffer. The active fractions were collected and determined for enzyme activity, then combined, concentrated and washed to remove excessive salt before being applied to a MonoQ HR5/5 column equipped with a FPLC system. The column was equilibrated with 10 mM Tris-HCl before sample application and the elution was performed with a linear gradient of 500 mM NaCl in 10 mM Tris-HCl buffer for 1 h at the flow rate of 1 ml min^{-1}. Fractions containing protein was detected by UV–Vis detector at 280 nm. The active fractions were pooled and concentrated using Microcon spinning at 15,000 rpm. The concentrated sample was passed through a Superdex 200HR10/35 column equilibrated with 150 mM NaCl in 10 mM Tris-HCl. Isocratic elution with the same buffer was achieved by a FPLC system at the flow rate of 0.5 ml min^{-1}.

Substrate specificity

Enzyme activity toward various amino acid amides and oligopeptides was determined by either the production of amino acids or the formation of ammonia. Quantitative analysis of D-phenylalanine and L-phenylalanine was estimated by HPLC as mentioned above. D-*tert*-leucine was evaluated by HPLC on Sumichiral OA-5000 with a UV–Vis detector at 254 nm. The amino acid was eluted with 2 mM $CuSO_4$/isopropanol (95:5 by vol.) at the flow rate of 1 ml min^{-1}. Other amino acids were quantitatively assayed for ammonia content by the

phenol/hypochlorite method, using Conway microdiffusion apparatus [10].

Determination of pH and temperature optima

The enzyme reaction was performed in various buffers as follows: 0.1 M acetate buffer (pH 4.0, 4.5, 5.0, 5.5, 6.0), 0.1 M potassium-phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, 7.8, 8.5), 0.1 M Tris-HCl buffer (pH 7.5, 8.0, 8.5, 9.0, 9.5), and 0.1 M sodium bicarbonate buffer (pH 8.7, 10.2, 10.7, 11.3). D-Phenylalanine amide dissolved in water was used as substrate for the enzyme reaction assay.

Also, the reaction mixture was conducted in 0.1 M Tris-HCl, pH 8.0, at various temperatures in the presence of D-phenylalanine amide as a substrate.

Cloning of the D-amino acid amidase gene (*damA*) from *D. acidovorans* strain 16

For routine work with recombinant DNA, established protocols were used [21]. Chromosomal DNA was prepared from *D. acidovorans* strain 16 by the method of Misawa et al. [18] and partially digested with *Sau*3AI. The DNA fragments of 2–10 kbp in size were isolated from the agarose gel using the QIAQuick gel extraction kit. The isolated DNA fragments were then ligated into *Bam*HI-digested and alkaline phosphatase-treated pBluescriptII SK(−), using DNA ligation kit ver. 2. *E. coli* XL1-Blue MR was transformed with the recombinant plasmid DNA by the method of Inoue et al. [12] and ampicillin-resistant transformants were selected on Luria–Bertani agar containing ampicillin. Visualization of the D-amino acid amidase activity expressed in the transformants was carried out according to Komeda and Asano [13]. Recombinant *E. coli* colonies on the plate were transferred to a cellulose nitrate membrane filter, which was thereafter floated on the surface of 1 mL lysozyme solution (10 mg mL^{-1}) containing 10 µmol EDTA (pH 6.0) and incubated at 30°C for 30 min. The membrane filter was transferred onto a filter paper to remove the residual lysozyme solution and then put on a dry tray. The membrane filter on the tray was frozen at −20°C for 10 min and thawed at room temperature for another 10 min. The steps of freezing and thawing were repeated three times to destroy the spheroplasts. The membrane filter was then floated in 2 mL reaction mixture (60 µmol potassium phosphate buffer, pH 7.4, 6 mg D-phenylalanine amide, 5 mg phenol, 1.6 units D-amino acid oxidase, 1.4 units peroxidase, 0.2 mg 4-aminoantipyrine) and was incubated at 37°C for 20–30 min. The colony corresponding to a developed pink–red colored spot was picked up as a positive clone. The plasmids carried by the positive *E. coli* transformant were isolated by an automatic plasmid isolation system (Kurabo, Osaka, Japan) and the size of the inserted DNA fragment was determined. The positive *E. coli* transformant carried a 5.7-kb plasmid, designated pDac1.

DNA sequence analysis

The Invisorb spin plasmid mini kit was used to prepare double-stranded DNAs for sequencing. pDac1 was used as a sequencing template. Nested unidirectional deletions were generated with the Kilo sequence deletion kit. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method [22] with M13 forward and reverse oligonucleotides as primers. Sequencing reactions were carried out with a Thermo sequenase cycle sequencing kit and dNTP mixture with DNA Thermo sequenase DNA polymerase with pyrophosphatase (Pharmacia Biotech, Tokyo, Japan). The reaction mixtures were run on a 4000L DNA sequencer (Li-cor, Lincoln, Neb., USA). Both strands of DNA were sequenced. The nucleotide sequence data reported in this paper are in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB154822. Amino acid sequences were compared using the Blast program [1].

Results

Isolation of microorganisms

Thirty-two strains exhibiting D-phenylalanine amide degradation were isolated at 30°C and 45°C by an enrichment culture technique. However, only strain 16 showed the degradation of D-*tert*-leucine amide to D-*tert*-leucine in the whole-cell reaction assay. This particular strain also exhibited the strongest activity toward D-phenylalanine amide. Strain characterization revealed that it was *D. acidovorans* (Table 1).

The activity of D-amino acid amidase was detected only in the supernatant of the sonicated cell-free extract and no activity was found in the supernantant of the culture broth, indicating that the enzyme was intracellularly produced. However, *D. acidovorans*strain 16 was able to produce D-amino acid amidase constitutively, as the enzyme activity was detected from cells grown in TGY broth (absence of D-phenylalanine amide). *D. acidovorans* was isolated from soil in Japan and was reported by Asano et al. [5] for its D-peptidase activity on octameric D-peptides. However there was no such report regarding the D-amino acid amidase activity which was produced by *D. acidovorans* strain 16 isolated from soil in Thailand.

Purification of D-amino acid amidase from *D. acidovorans* strain 16

D-Amino acid amidase was purified from 10 L culture broth of *D. acidovorans* strain 16 with a recovery of

Table 1 Morphological and biochemical characteristics of strain 16 isolated from soil sample

Morphological characteristics	Biochemical characteristics
Rod shape with dimensions of 0.5–0.6 μm × 1.5–2.0 μm	Esculin and gelatin hydrolysis: negative Nitrate reduction: positive Indole formation: negative
Gram-negative	Catalase and oxidase: positive Cytochrome oxidase: positive
Non-spore forming	Urease and arginine dihydrolase: negative Hugh–Leifson reaction (glucose): negative
Motile	Carbon assimilation
Colony appearance on nutrient agar: smooth, entire, low convex, round, pale yellow and glossy	D-Manitol, gluconate: positive Caprylate, adipate, phenylacetate, DL-malate: negative L-Arabinose, D-mannose: negative N-Acetyl-D-glucosamine, maltose, citrate, glucose: positive

0.22%, by ammonium sulfate fractionation, DEAE-Toyopearl, ether-Toyopearl, butyl-Toyopearl, FPLC phenyl superose, FPLC MonoQ and FPLC Superdex 200 column chromatography. The final step with FPLC Superdex 200 chromatography provided a single band on SDS/PAGE (Fig. 1) corresponding to the only peak on the chromatogram which exhibited the activity toward D-phenylalanine amide. The molecular mass was estimated by SDS/PAGE to be about 50 kDa. The native molecular mass of the enzyme was then calculated from the relative mobility, compared with those of the standard proteins glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome C (12.4 kDa; products of Oriental Yeast Co., Tokyo, Japan). However, the molecular mass of the native enzyme could not be drawn by high-pressure liquid chromatography performed on a Toso G-3000 SW column due to its high hydrophobicity. This highly hydrophobic property could explain the significant loss of enzyme activity in the process of enzyme concentration using an ultrafiltration membrane when the enzyme was highly homogeneous in the presence of a high salt concentration. The strong activity was eventually found when a piece of

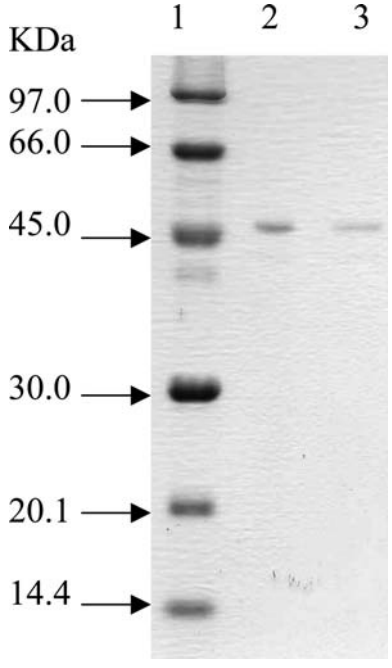

Fig. 1 SDS/polyacrylamide slab gel electrophoresis of D-amino acid amidase enzyme from *D. acidovorans* strain 16. *Lane 1* Molecular mass standards: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). *Lanes 2, 3* Purified D-amino acid amidase from *D. acidovorans* strain 16

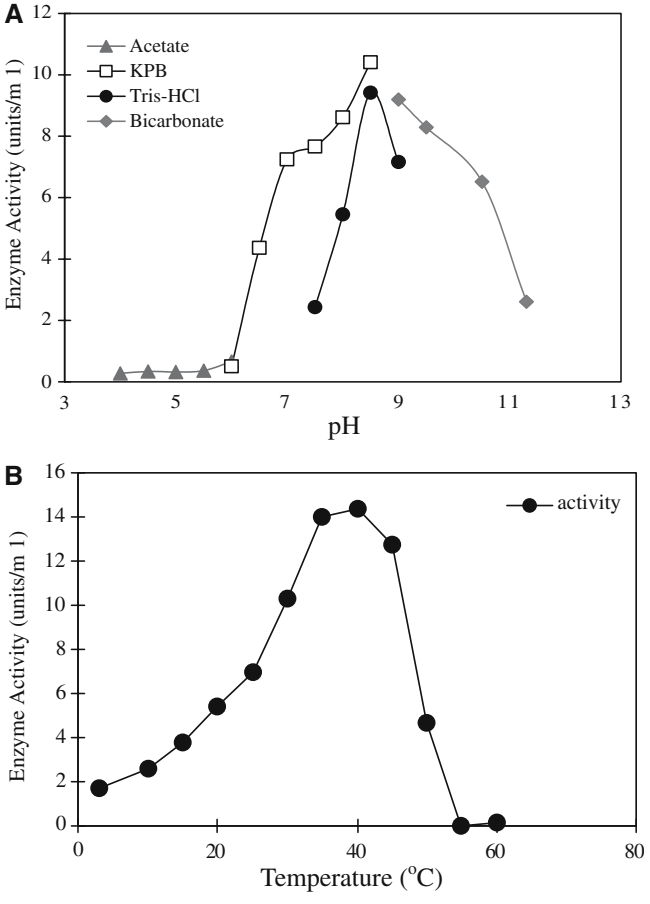

Fig. 2 Effects of pH (**a**) and temperature (**b**) on the activity of D-amino acid amidase from *D. acidovorans* strain 16

membrane filter was added to the reaction mixture. Moreover, the enzyme was eluted in the washing step with water after the elution gradient was over, when hydrophobic chromatography such as butyl Toyopearl and Superose was applied. The phenomenon indicated the highly hydrophobic property of the enzyme.

Effects of pH and temperature

The optimum pH for the activity of this enzyme was measured in the buffers described above. The enzyme showed maximum activity at pH 8.5, as shown in Fig. 2a. The enzyme reaction was carried out at various temperatures in 0.1 M Tris-HCl buffer, pH 8.0; and maximum activity was exhibited at 40°C (Fig. 2b). About 100% and 93% activity remained after incubation at 40°C and 45°C, respectively, in 0.1 M Tris-HCl, pH 8.0, for 30 min.

Effects of metal ions and inhibitors

The enzyme activity was measured after the enzyme was pre-incubated at 30°C for 10 min with various compounds at a final concentration of 1 mM unless otherwise noted. Only 3–26% inhibition was observed when the enzyme was incubated with $K_4Fe(CN)_6$, CsCl, Tl_2SO_4, $Co(NH_3)_6Cl_3$, LiBr, $MnSO_4$, $K_3Fe(CN)_6$ and CuCl. Other inorganic compounds, such as H_2BO_3, $MnCl_2$, Na_2MoO_4, LiCl, $CaCl_2$, $BaCl_2$, $MgSO_4$, KCl, $PbCl_2$, $CoCl_2$, $FeC_6H_5O_7{\cdot}nH_2O$, KI, $CrCl_3$, RbCl, NaCl, $(NH_4)_6Mo_7O_{24}$ and $MgCl_2$, did not influence the enzyme activity (data not shown). The enzyme activity was inhibited 42–55% by Fe^{3+}, Sn^{2+}, Cu^{2+}, Al^{3+} and Ni^{2+}; and 91–97% by As^{3+}, Zn^{2+}, Hg^{2+} and Ag^{+}. The activity was completely lost in the presence of Cd^{2+}.

Among all inhibitors, phenylmethanesulfonyl showed a strongly inhibitory effect on the enzyme activity (Table 2), indicating that the amino acid serine may be involved in the enzyme active site. Carbonyl reagents such as hydroxylamine, phenylhydrazine, D,L-penicillamine and D-cycloserine did not inactivate the enzyme activity, neither did the chelating agents like EDTA, 8-quinolinol and bipyridyl. This phenomenon indicated that *D. acidovorans* amidase had no requirement for metal ions since no inhibition was observed in the presence of EDTA and *O*-phenanthroline. The amidase was strongly inhibited by sulfhydryl reagents, particularly *p*-chloromercuribenzoic acid and the metal ions As^{3+}, Zn^{2+}, Hg^{2+}, Ag^{+} and Cd^{2+}, indicating that a cysteine residue is required for activity. The amidase activity was also protected and not inhibited by dithiothreitol, indicating that disulfide bonds are not critical for the enzyme activity.

Table 2 Effects of various inhibitors (1 mM) on the activity of D-amino acid amidase from *D. acidovorans* strain 16

Compound	Relative activity (%)
None	100.0
NaF	32.8
p-Chloromercuribenzoate	10.4
Phenylmethanesulfonyl fluoride	4.7
Hydroxylamine	124.0
Phenylhydrazine	95.4
D,L-Penicillamine	118.0
D-Cycloserine	144.0
EDTA	105.0
8-Quinolinol	144.0
Bipyridyl	153.0
5,5-Dithiobis-2-nitrobenzoic acid	85.8
Iodoacetic acid	91.2
Dithiothreitol	117.0

Substrate specificity

The enzyme showed a preference in catalysis of the D-enantiomeric substrate to the L-amino acid amide (Tables 3, 4). It also exhibited the ability to catalyze a broad range of D-amino acid amides, although its stereospecificity was not high toward many amino acid amides such as phenylalanine amide, norvaline amide, methionine amide, leucine amide, tyrosine amide and isoleucine amide. However, the enzyme showed a preference toward D-enantiomers rather than L-enantiomers; and it showed strict stereospecificities toward D-tryptophan amide, D-serine amide, D-proline amide and D-lysine amide. The enzyme preferably catalyzed bulky and aromatic D-amino acid amides, for example D-phenylalanine amide, D-tyrosine amide and D-tryptophan amide; but it was not capable of catalyzing small molecules of aliphatic amino acid amides such as D-alanine amide and D-glycine amide. Moreover, the enzyme was not active on either the D-oligopeptides or L-oligopeptides tested (Tables 3, 4).

NH_2 terminal and internal amino acid sequences

The NH_2 terminal amino acid sequence of the enzyme was determined as Met^{1}-Asn-Asp-Ser-Glu^{5}-Leu-His-*X*-$Leu\text{-}Glu^{10}$-Leu-Leu-Glu-Val-Gly^{15} and its internal sequence was revealed to be Gln^{1}-Ala-Val-Glu-Gln^{5}-$Ala\text{-}Leu\text{-}Ala\text{-}Val\text{-}Ala^{10}$-Gln-$Arg^{12}$ (*X* denotes an undetermined amino acid residue).

Cloning of the D-amino acid amidase gene, *damA*

*Sau*3AI-digested DNA fragments from *D. acidovorans* strain16 were ligated into pBluescriptII SK(−) and were then transformed into either *E. coli* XL1-Blue MR or *E. coli* JM109. A few clones of *E. coli* XL1-Blue MR transformants expressed D-amino acid amidase toward D-phenylalanine amide, whereas there was no positive clone among the *E. coli* JM109 transformants. The recombinant plasmid was isolated from a positive clone

Table 3 Substrate specificity of D-amino acid amidase from *D. acidovorans* strain 16 toward D- and L-amino acid derivatives

Substrate	Relative activity (%)
D-Phenylalanine amide	100.0
D-Norvaline amide	70.6
D-Methionine amide	54.2
D-Tryptophan amide	47.6
D-Leucine amide	45.6
D-it *n*-Leucine amide	44.4
D-Tyrosine amide	41.6
D-Threonine amide	23.0
D-*allo*-Threonine amide	17.1
D-Proline amide	6.2
D-Isoleucine amide	4.5
D-Serine amide	4.1
D-Histidine amide	3.4
D-Lysine amide	2.5
D-it *tert*-Leucine amide	1.4
D-Aspartic acid amide	0.4
L-Leucine amide	28.7
L-Phenylalanine amide	12.8
L-Tyrosine amide	7.2
L-Norvaline amide	5.2
L-Methionine amide	5.0
L-Threonine amide	2.4
L-Valine amide	1.5
L-Glycine amide	0.8
L-Isoleucine amide	0.8

Table 4 Amino acid derivatives as inactive substrate for the enzyme

D-Amino acid derivatives	L-Amino acid derivatives	Peptides
D-Alanine amide	L-Alanine amide	D-Phenylalanine$_4$
D-Glutamic acid amide	L-Asparagine amide	D-Phenylalanine$_3$
D-Glutamine amide	L-Glutamic acid amide	D-Phenylalanine$_2$
D-Valine amide	L-Lysine amide	D-Phenylalanyl-L-phenylalanine
	L-Proline amide	L-Phenylalanine-D-phenyalanine
	L-Serine amide	
	L-Tryptophan amide	

(pDac1) and the size of inserted DNA was estimated to be about 3.0 kb by agarose gel electrophoresis. Overproduction of this amidase protein was observed in about the same level when the transformant was grown in Luria–Bertani broth either with or without IPTG, suggesting that a promoter for this gene does function in *E. coli*. The plasmid pDac1, containing a 3.0-kb *Sau*3AI fragment, was used to generate nested deletion plasmids for the determination of the nucleotide sequence. The nucleotide sequence determined was found to be 2,996 bp long, which is in good agreement with that determined by agarose gel electrophoresis. There were two open reading frames (ORFs), designated ORF1 and ORF2, present in this region.

A potential Shine/Dalgarno (GGAG) sequence was found respectively 9 nucleotides and 7 nucleotides upstream from the start codons ATG of both ORF1 and ORF2 [23]. The sequence was exactly the same as that found 6 nucleotides upstream of the enantiomer-selective amidase from *Rhodococcus* sp. [17]. According to a homology search (Blast program), the primary structure of the 253-amino-acid protein deduced from ORF1 showed 33.6% identity (over 235 amino acids) to the transcription regulator (AraC family DNA-binding domain-containing protein) from *Pseudomonas syringae* pv. *syringae* B728a (GenBank accession number ZP_00124049) and 33.3% identity (over 228 amino acids) to the transcriptional regulator (AraC family) from *Caulobacter crescentus* CB15 (GenBank NP_421202). ORF2 encoded a 466-amino-acid protein (molecular mass 49,860.46 Da) and was indicated as a D-amino acid amidase gene, due to the exact correspondence of its deduced amino acid sequence with both the NH_2 terminal and the internal amino acid sequences determined earlier with the D-amino acid amidase purified from *D. acidovorans* strain 16 (underlined in Fig. 3). Therefore ORF1 and ORF2 were designated as *damR* and *damA*, respectively. Alignment by the nr database using the BLAST program showed that the deduced primary structure of *damA* is significantly similar to those of D-amidase from *Variovorax paradoxus* (67.9% identical over 461 amino acids [15]; GenBank AX772949), Asp-tRNAAsn/Glu-tRNAGln amidotransferase A subunit and related amidases from *Burkholderia fungorum* (50% identical over 465 amino acid; GenBank ZP_00032055), enantiomer-selective amidase from *Rhodococcus* sp. (37.58% identical over 462 amino acid [17]; GenBank A41326) and amidase from *P. putida* (35.9% identical over 465 amino acid [26]; GenBank U89363). According to the alignment with previously reported enantiomer-selective amidases using the SIM program, the deduced protein was 56.5% identical to enantioselective amidase from *Comamonas acidovorans* KPO-2771-4 (over 461 amino acids [11]), 40.7% identical to amidase from *P. chlororaphis* (over 246 amino acids [19]; GenBank P27765) and 38.3% identical to an enantiomer-selective amidase from *Brevibacterium* sp. (over 243 amino acids [16]; GenBank M32282). However, no significant homology was observed with a strictly D-stereospecific amino acid amidase from *Ochrobactrum anthropi* SV3 [13]. Figure 3 shows the alignment (performed using the ClustaIW program; EMBL-EBI) of the primary structure of *damA* from *D. acidovorans* strain 16 and enantioselective amidases from *V. paradoxus*, *C. acidovorans* KPO-2771-4, *P. putida* and *Rhodococcus* sp. These five proteins display a highly significant level of homology, especially between residues 153 and 239 of the *damA* sequence. They all share the same conserved regions, including GXSSXGX (residues 153–159), GTDTGGSIR (residues 173–179), GXKPT (residues 192–196) and GR (residues 198–199), all of which except GXSSXGX are correlated to the amidase conserved motifs analyzed by Chebrou et al. [8] and Mayaux et al. [17], indicating that GGSSGG, GXDXGGSIR, GLKPT and GR are

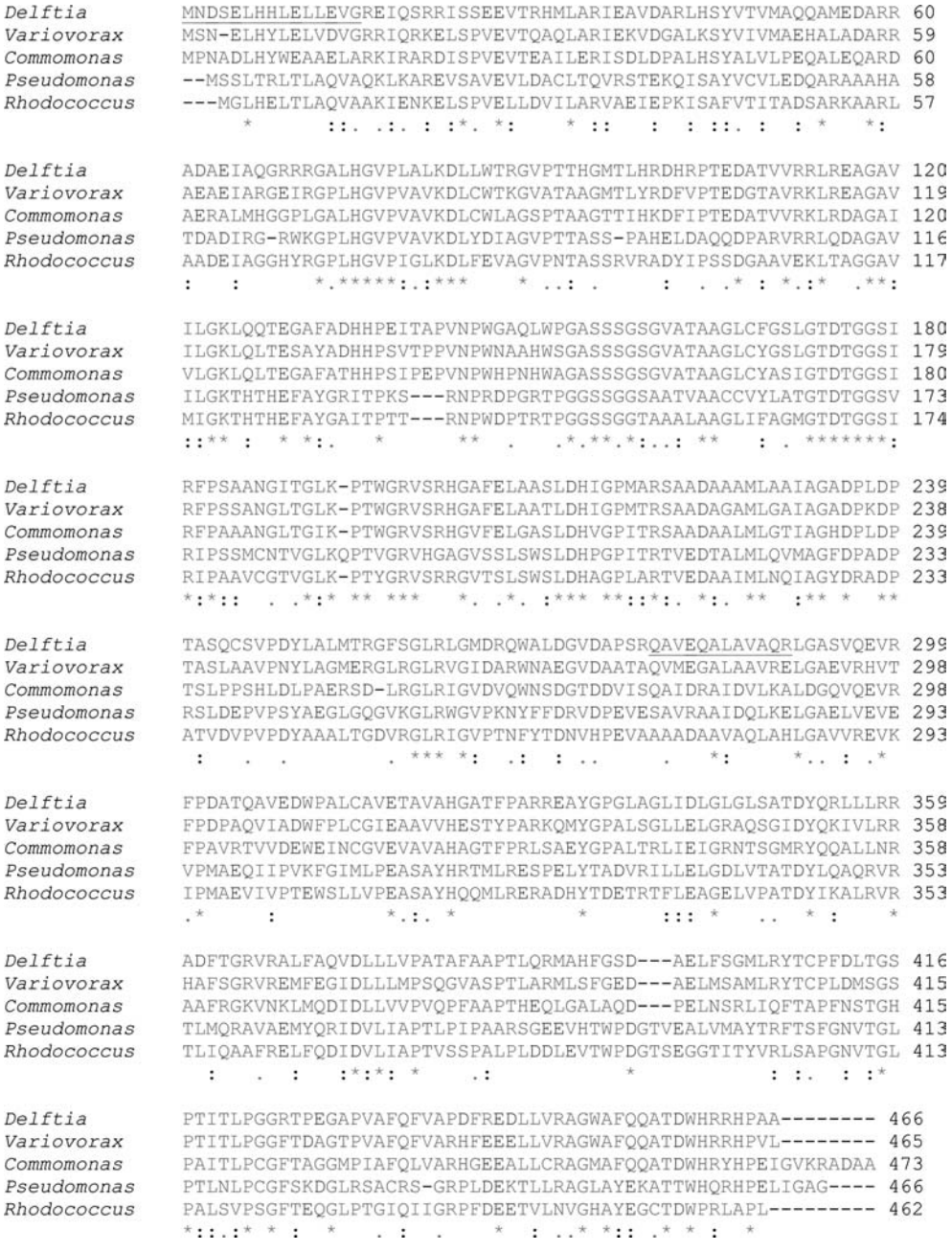

Fig. 3 Comparison of the amino acid sequences of D-amino acid amidase (*damA*) from *D. acidovorans* strain 16 and other homologous proteins from *V. paradoxus*, *C. acidovorans*, *P. putida* and *Rhodococcus* sp. Identical and conserved amino acids among the sequences are marked by *asterisks* and *dots*, respectively. *Dashed lines* indicate gaps introduced for better alignment. The *underlined regions* represent the corresponding sequences obtained from NH_2 terminal analysis of the purified amidase from *D. acidovorans* strain 16 and deduction of the inserted nucleotide sequence

amidase consensus residues. Chebrou et al. [8] compared the sequences of primary structures of 22 amidases produced by both prokaryotes and eukaryotes; and several conserved motifs were observed among this enzyme family, in that the highly conserved region was rich in glycine, serine and alanine residues, particularly the GGSSGG motif, which is known as the amidase signature. The amidases from both *D. acidovorans* and *V. paradoxus* appear to share the GASSSG motif instead of GGSSGG. However, not all amidases share the GGSSGG consensus, since the amidase from *P. aeruginosa* and *O. anthropi* does not exhibit any significant sequence homology to the amidase consensus [7, 13].

Discussion

A D-amino acid amidase producer was isolated from soil obtained from southern Thailand and was taxonomically identified as *D. acidovorans*. The enzyme was formed intracellularly, which was similar to most D-amidases reported so far, including those from *V. paradoxus* [15], *O. anthropi* SV3 [13], *Rhodococcus* sp. [9]. *D. acidovorans* strain 16 secreted the D-amidase constitutively, whereas most amidase producers, such as *V. paradoxus* [15], *Arthrobacter* sp. NJ-26 [20], *Rhodococcus rhodochrous* M8 [14] and *Brevibacterium* sp. [16], required inducers for their amidase production. Its

chromatographic behavior with hydrophobic resin and loss of activity during ultrafiltration using an Amicon product suggested its highly hydrophobic property. This similar phenomenon was also observed in the purification process of amidase produced by *V. paradoxus* [15].

D-Amino acid amidase from *D. acidovorans* hydrolyzed a broad range of aliphatic and aromatic amides, preferentially to D-enantiomers. It showed high activity for D-phenylalnine amide, similar to the D-amino acid amidase from *O. anthropi* [13] and the (*R*)-ketoprofen amidase from *C. acidovorans* KPO-2771-4 [11]. The purified amidase from *D. acidovorans* strain 16 is strictly D-enantioselective for certain amino acid amides such as D-tryptophan amide, D-proline amide, D-serine amide and D-lysine amide. But it is not strictly active toward D-phenyalanine amide whereas the D-amino acid amidase from *O. anthropi* was strictly active toward all D-amino acid amides tested [13]. It also exhibited slight activity to D-*tert*-leucine amide, similar to the D-amidase from *V. paradoxus* [15].

D. acidovorans strain 16 amidase has maximal activity at pH 8.5, similar to the D-stereospecific amino acid amidase from *O. anthropi* SV3 [13], *V. paradoxus* [15] and the enantioselective amidase from *C. acidovorans* [11], which have maximal activity at alkaline pH of 9.0, 7.0–8.6 and 8.5–10.0, respectively. The optimal temperature for *D. acidovorans* amidase is 40°C, whereas those of the three above are 45°C, 47–49°C and 35°C, respectively. Compared to the pH 5–8, 55–60°C of *R. rhodochrous* [14] and the pH 7.0-8.6, 50°C of *P. chlororaphis* B23 [9], the optimal pH for *D. acidovorans* amidase is more alkaline and its optimal temperature slightly lower. The amidase was completely inhibited by phenylmethanesulfonyl fluoride, suggesting the involvement of a serine residue at the active site of the enzyme, the same as D-stereospecific amino acid amidases from *O. anthropi* SV3 [13] and *V. paradoxus* [15]. Cysteine appears to play an important role for activity and structural integrity of the enzyme. This was inferred from the strong inhibition by the sulfhydryl reagent *p*-chloromercuribenzoate and the metal ions As^{3+}, Zn^{2+}, Hg^{2+}, Ag^{+} and Cd^{2+}. The enzyme has a behavior with metal ions and inhibitors similar to that from *V. paradoxus*, which also shows a highly significant homology in nucleotide and amino acid sequences. In contrast, some amidases reported to date [6, 9, 14] have shown the involvement of a thiol group at the active center of the enzyme.

The D-amino acid amidase gene from *D. acidovorans* strain 16 was cloned in *E. coli* XL1-Blue MR. The deduced amino acid sequence of ORF2 designated as *damA* contained all of the amino acid sequences determined for the amino acid terminus and the internal peptides of the purified D-amino acid amidase from *D. acidovorans* strain 16. ORF2 contained 1,404-bp nucleotide sequences encoding a 466-amino-acid protein with an estimated molecular mass of 49,860.46 Da, which is in good agreement both with the molecular mass determined by SDS-PAGE and with the amidase enzymes from *P. putida* [26] and *V. paradoxus*. However, the native molecular weight of the enzyme cannot be obtained due to its highly hydrophobic property. The D-amino acid amidases from *C. acidovorans* and *O. anthropi* appear to be monomers with a molecular mass of 50 kDa and 40 kDa, respectively, whereas that from *V. paradoxus* is not yet determined.

A comparison of the peptide sequences of five amidases shows several conserved motifs, characterized as the amidase enzyme family. Chebrou et al. [8] reported a common consensus region in the deduced amino acid sequence of the genes from various amidases. Seventeen stable positions scattered throughout the sequence were observed to conserve for the GGSS-signature of amidase, correlating to the amidase from *P. putida* and *Rhodococcus* sp., although the proteins from *D. acidovorans*, *V. paradoxus* and *C. acidovorans* have a GASSSGSG. motif. However, the GXSSXGX sequence (residues 153–159), the GTDTGGSIR sequence (residues 173–179) and the GXKPT sequence (residues 192–196), blocks of strictly conserved residues, appear to be the salient features of this consensus.

Acknowledgements The accomplishment of this work was possible due to the support from the NRCT-JSPS Core University Programme. The work described was in part financially supported by Thailand Research Funds.

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