



Purification, characterization, and primary structure of a novel *N*-acyl-D-amino acid amidohydrolase from *Microbacterium natoriense* TNJL143-2

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A novel *N*-acyl-D-amino acid amidohydrolase (DAA) was purified from the cells of a novel species of the genus *Microbacterium*. The purified enzyme, termed AcyM, was a monomeric protein with an apparent molecular weight of 56,000. It acted on *N*-acylated hydrophobic D-amino acids with the highest preference for *N*-acetyl-D-phenylalanine (NADF). Optimum temperature and pH for the hydrolysis of NADF were 45°C and pH 8.5, respectively. The k_{cat} and K_m values for NADF were 41 s⁻¹ and 2.5 mM at 37°C and pH 8.0, although the enzyme activity was inhibited by high concentrations of NADF. Although many known DAAs are inhibited by 1 mM EDTA, AcyM displayed a 65% level of its full activity even in the presence of 20 mM EDTA. Based on partial amino acid sequences of the purified enzyme, the full-length *AcyM* gene was cloned and sequenced. It encoded a protein of 495 amino acids with a relatively low sequence similarity to a DAA from *Alcaligenes faecalis* DA1 (termed AFD), a binuclear zinc enzyme of the α/β -barrel amidohydrolase superfamily. The unique cysteine residue that serves as a ligand to the active-site zinc ions in AFD and other DAAs was not conserved in AcyM and was replaced by alanine. AcyM was the most closely related to a DAA of *Gluconobacter oxydans* (termed Gox1177) and phylogenetically distant from AFD and all other DAAs that have been biochemically characterized thus far. AcyM, along with Gox1177, appears to represent a new phylogenetic subcluster of DAAs.

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[Key words: *N*-Acyl-D-amino acid amidohydrolase; D-Amino acids; D-Aminoacylase; α/β -Barrel amidohydrolase superfamily; D-Phenylalanine; *Microbacterium natoriense*; Optical resolution]

D-Amino acids, optical isomers of L-amino acids, are widely found in bacteria and eukaryotes as a free form or a bound form such as peptidoglycan (1). In recent years, much attention has been focused on natural D-amino acid-containing compounds because of their potential applications as novel drugs. For example, amphibian skin peptides containing D-alanine have a morphine-like activity (2), and their D-serine derivatives are reported to be applicable to therapy for schizophrenia (3). In current pharmaceutical industries, D-amino acids are used as synthetic precursors for a wide variety of antibiotics, antivirals, agrochemicals, and therapeutic drugs (4). Unlike L-amino acids that can be produced by microbial fermentation (5), industrial production of D-amino acids has been attained mainly by enzymatic methods that include the optical resolution of DL-amino acids by *N*-acyl-D-amino acid amidohydrolase (DAA) (EC3.5.1.81) (4,5). DAA catalyzes the enantioselective hydrolysis of *N*-acyl-D-amino acid to produce D-amino acid and fatty acid. Thus far, DAA activities have been identified in a variety of microorganisms including bacteria [e.g., *Alcaligenes* (6–12), *Pseudomonas* (13,14),

Sebekia (Tokuyama, S., US Patent, 6030823, 2000), *Variovorax* (15), *Stenotrophomonas* (16), *Amycolatopsis* (Tokuyama, S., European Patent, 60,950,706, A2, 2000), *Streptomyces* (17,18), *Bordetella* (18), *Gluconobacter* (18), and *Deffluvibacter* (19), *Methylobacterium*, and *Nocardioides* (Osabe, M. et al., Japanese Unexamined Patent Application Publication, JP-A-Hei-2002-320491A)] and fungi [e.g., *Trichoderma* (20)]. Among them, a DAA from the *Alcaligenes xylosoxydans* subsp. *xylosoxydans* strain A-6 has been used industrially for the manufacture of neutral D-amino acids (4).

DAAs are zinc-containing enzymes that belong to the α/β -barrel amidohydrolase superfamily (4,21,22). The first crystal structure of DAA was obtained with an enzyme (termed here AFD) from the *A. faecalis* strain DA1, a closely related species of strain A-6 (see above), which contains 2 zinc ions in the active site (23). The spatial arrangement of zinc-binding amino acid residues of AFD was similar to those of other enzymes belonging to the superfamily (such as phosphotriesterase, adenosine deaminase, acetyl glucosamine-6-phosphate deacetylase, and renal dipeptidase), among which AFD uniquely utilizes a Cys residue as one of its ligands to zinc ions, in addition to His residues that are widely identified as ligands to zinc ions (23). Recent bioinformatic analyses of approximately 250 DAA-like sequences from archaea, bacteria, and

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eukaryota showed that these sequences could be partitioned into 4 distinct phylogenetic clusters (Clusters 1–4) (18). All but one of the sequences that have been biochemically established as DAA were assigned exclusively to a single cluster. The only exception was a DAA from *Gluconobacter oxydans* (Gox1177), which was found in a separate cluster (18). The partitioning of DAAs into these 2 clusters appeared to have no correlation with the reaction and substrate specificities of the enzymes.

We previously isolated a novel DAA-producing bacterium, *Microbacterium natoriense* strain TNJL143-2 (24). The enzymatic properties and phylogenetics of DAA from this bacterium were of great interest because there have been no reports of DAA from this bacterial species. In the present study, we purified and biochemically characterized this DAA and cloned the gene encoding the enzyme, which is referred to herein as AcyM. The DAA purified from the cells of strain TNJL143-2 displayed the highest preference for *N*-acetyl-D-phenylalanine, and, unlike many other DAAs, its enzymatic activity was virtually insensitive to EDTA. AcyM displayed a 41% sequence identity to Gox1177 and only low sequence similarities to all other DAAs that have been biochemically characterized thus far. The conserved zinc-binding Cys residue was replaced by alanine in AcyM. Phylogenetic analyses suggested that AcyM, along with Gox1177, appears to represent a new phylogenetic subcluster of DAAs.

MATERIALS AND METHODS

Materials *N*-Acetyl-D-phenylalanine (NADF), *N*-acetyl-DL-phenylalanine, *N*-acetyl-D-leucine, *N*-acetyl-D-valine, *N*-acetyl-D-methionine, *N*-acetyl-D-tryptophan, and *N*-acetyl-L-amino acids were purchased from Tokyo Chemical Industry, Tokyo, Japan. Restriction enzymes were purchased from Takara Bio, Shiga, Japan, and from Toyobo, Osaka, Japan. All other chemicals used were of analytical grade.

Bacterial strains *M. natoriense* TNJL143-2 nov. (24) is available from the Japan Collection of Microorganisms, Wako, Saitama, Japan (JCM 12611^T), and from the American Type Culture Collection, Manassas, VA, USA (BAA-1032^T).

Enzyme assay The standard assay system consisted of 2 μmol of NADF, 2 μmol of Hepes-NaOH, pH 7.0, and enzyme in a final volume of 200 μL. The reaction mixture without the enzyme was previously incubated at 37°C, and the reaction was started with the addition of the enzyme. The enzyme was replaced with water for the blank. Incubation was carried out at 37°C for 60 min. The reaction was terminated by heating the mixture to 100°C and maintaining that for 5 min. Next, 50 μL of the mixture was combined with 50 μL of a 19:1 mixture of solutions A and B, where solution A consisted of 30 mM potassium phosphate, pH 7.4, 0.25% (w/v) phenol, and 2.4 units/ml of D-amino acid oxidase, and solution B consisted of 70 units/ml horseradish peroxidase and 1% 4-aminoantipyrine. The mixture was allowed to stand at room temperature for 20 min. To the mixture, 100 μL of H₂O was added, and the absorbance at 505 nm was measured using a SpectraMax 340PC/SoftMax Pro spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA). A single unit of enzyme was defined as the amount that produces 1 μmol of D-phenylalanine per min under these assay conditions. The specific activity was expressed as units per mg of protein. Protein concentration was determined using Bradford's method (25) with bovine serum albumin as the standard protein. The kinetic parameters were determined by fitting of the initial velocity data to a Michaelis–Menten equation by non-linear regression analysis (26).

Purification of DAA The cells of strain TNJL143-2 were grown at 30°C for 30 h in 100 ml of medium (pH 7.0) containing 0.01% NADF, 0.5% yeast extract, 0.5% NaCl, 0.1% KH₂PO₄, and 0.00005% MgSO₄·7H₂O. The culture was then inoculated to 3 L of the medium, where NADF was replaced by *N*-acetyl-DL-phenylalanine. The cells were grown at 30°C for 36 h in a 5-L jar fermentor (Mitsuiwa Co., Osaka, Japan) with an agitation rate of 250 rpm and an aeration rate of 1 volume/volume/min. The cells were collected following centrifugation at 6000 rpm and 4°C for 20 min and being washed 3 times with 0.01 M Hepes-NaOH buffer, pH 7.0.

Purification of DAA from the crude extract was completed at 0–5°C, unless otherwise stated, as follows. Buffer A was 0.01 M Hepes-NaOH, pH 7.0. The cells (111 g, wet weight) were suspended in 200 ml of buffer A, and disrupted at 4°C by using a Multi-beads shocker model MBS200 (Yasui Kikai, Osaka, Japan). Polyethyleneimine was added to the mixture for a final concentration of 0.12%, and the mixture was allowed to stand at 4°C for 30 min followed by centrifugation at 8000 ×g for 20 min. The enzyme solution (390 ml) was applied to a column (3 × 20 cm) of DEAE-Toyopearl 650 M (Tosoh, Tokyo, Japan) equilibrated with buffer A. The column was washed with the same buffer. The enzyme was eluted with a linear gradient of 0–1.0 M NaCl in buffer A. The active fractions were combined and dialyzed against buffer A. The enzyme solution was subjected to re-chromatography on DEAE-Toyopearl 650 M in essentially the same manner as described above. The active fractions were combined. To the enzyme solution, ammonium sulfate was slowly added to 20% saturation. The enzyme solution was then subjected to fast

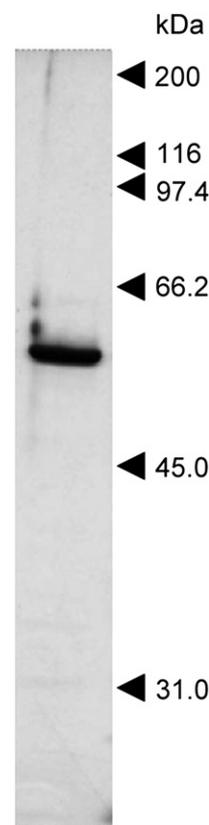


FIG. 1. SDS-PAGE of the purified AcyM.

protein liquid chromatography (FPLC) on a HiPrep Phenyl FF High-Sub column (1.6 × 10 cm, GE Healthcare Japan, Tokyo, Japan) equilibrated with buffer A containing ammonium sulfate at 20% saturation. The column was washed with the equilibration buffer. The enzyme activity was eluted at a flow rate of 1.0 ml/min with linear gradients of ammonium sulfate (20–0% saturation) and ethyleneglycol [0–60% (w/w)] in buffer A for 70 min. The active fractions were combined, dialyzed thoroughly against buffer A, and then subjected to FPLC on Mono Q HR10/10 (GE Healthcare Japan) equilibrated with the same buffer. After loading the enzyme solution into the column followed by an extensive washing of the column with buffer A, the enzyme was eluted with a linear gradient of 0–1.0 M NaCl in buffer A in 100 min at a flow rate of 0.5 ml/min. The active fractions were combined and applied to a Superdex 200 HR10/30 column (GE Healthcare Japan) equilibrated with buffer A containing 0.15 M NaCl. The enzyme activity was eluted at a flow rate of 0.5 ml/min. Sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) was carried out according to a method established by Laemmli (27). Proteins on the gels were visualized by silver staining or staining with Coomassie Brilliant Blue R-250.

Profiles of pH activity The enzymatic formation of D-phenylalanine from NADF was assayed using a standard assay system (200 μL) consisting of 2 μmol of NADF and 10 μmol of one of the following buffers: pH 4.0–5.6, sodium acetate; pH 6.0–7.4, potassium phosphate; pH 7.0–8.6, Tris-HCl; and pH 8.6–10.0, Gly-NaOH.

Stability studies To examine the temperature stability of AcyM, the enzyme was incubated in 0.1 M sodium phosphate buffer, pH 7.2, at 30–70°C. At 30 min after

TABLE 1. Purification of AcyM.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	20.4	24	1.2	1	100
DEAE-Toyopearl 650 M 1	2.3	16.4	7.2	6.1	68
DEAE-Toyopearl 650 M 2	2.3	4.6	2.1	1.8	19
HiPrep Phenyl FF High-Sub	0.34	8.0	23.7	20	33
Mono-Q	0.16	1.9	11.7	10	8
Superdex-200	0.019	0.9	46.4	39	4

this incubation, the aliquots were withdrawn and placed in tubes on ice. The remaining enzyme activity was assayed via a standard assay method.

For pH stability studies, the enzyme was incubated at 37°C for 30 min at various levels of pH (see above for buffers used, final concentrations were 0.05 M). After incubation, the remaining enzyme activity was assayed via a standard assay method.

Effect of various reagents on enzyme activity Enzyme activity was assayed under standard conditions supplemented with one of the following additives (final concentration, 1.0 mM): EDTA, CaCl₂, CdCl₂, CoCl₂, CuCl₂, FeCl₂, HgCl₂, MnCl₂, and ZnCl₂. The activity of the enzyme, assayed as above without additives, was taken to be 100%.

Protein chemical analyses To determine the N-terminal amino acid sequence of the purified enzyme, the enzyme samples were subjected to SDS-PAGE without prior boiling of the samples. Protein bands in the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by electroblotting, and the membrane was stained with Coomassie brilliant blue R-250. The stained portion of the membrane corresponding to AcyM was excised with dissecting scissors and subjected to automated Edman degradation using either a Hewlett Packard G1005A Protein Sequencer or a Procise 494 HT Protein Sequencing System. To determine the internal amino acid sequences of the enzyme, the protein in the SDS-PAGE gels was digested with TPCK trypsin (Takara Bio) at 35°C for 20 h (pH 8.5), and the resultant peptides were separated by a reversed-phase HPLC system, as described previously (28). The N-terminal amino acid sequences of the purified peptides were determined as described above.

Cloning of the DAA gene Partial nucleotide sequences of the gene coding for AcyM was obtained by PCR amplification from the chromosomal DNA of *M. natoriense* TNJL143-2 using degenerated primers, which were designed on the basis of an N-terminal amino acid sequence and the internal sequences of the purified enzyme. Chromosomal DNA was prepared and purified from strain TNJL143-2 cells as described previously (24). The following degenerate oligonucleotide primers were synthesized: 1F, 5'-GTNGTNGAYCCNAARACNGG-3'; 1R, 5'-ACNACNACRTCCNGCRTCNGC-3'; 2F, 5'-ACNACNACRTCCNGCRTCNGC-3'; 2R, 5'-GCNCCRTANGGRTANGCYTC-3'; where N, R, and Y indicate degenerate sites (N, A/C/G/T; R, A/G; Y, C/T). The first PCR amplification was performed with the chromosomal DNA as a template by using primers 1F and 1R, and the PCR product was used as a template for the second (nested) amplification using primers 2F and 2R. These amplifications were completed using a FailSafe™ PCR kit (Epicentre, Madison, WI, USA) and LA Taq DNA polymerase (Takara Bio). The PCR product, which was 1.2 kbp in length, was sized by 0.8% agarose gel electrophoresis and was recovered using a GenElute™ agarose spin column (Sigma-Aldrich Japan, Tokyo, Japan). The recovered DNA fragments were ligated with T-vector using a TOPO-TA cloning kit (Life Technologies Japan, Tokyo, Japan). *Escherichia coli*

TOP10 cells (Life Technologies Japan) were transformed with the ligation mixture. Nucleotide sequences of the plasmid extracted from the transformant cells were determined using a CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA, USA). The GENETYX program (ver. 9.0, Software Development, Tokyo, Japan) was used for the analysis of nucleotide and deduced amino acid sequences. Because the nucleotide sequence thus obtained was found to lack start and stop codons, we further performed PCR amplification using a genomic DNA library of strain TNJL143-2 as a template to obtain the full-length open reading frame sequence of *AcyM*, as follows.

The chromosomal DNA of strain TNJL143-2 was partially digested with the restriction enzyme *Sau3A1*, and the partial digests were separated by electrophoresis on 0.8% agarose gels. DNA fragments, 2–6 kbp in size, were recovered from the agarose gels using a GenElute agarose spin column and were ligated with *Bam*HI-digested and dephosphorylated pUC18 (Takara Bio). This ligation mixture was used as a template for the following PCR amplifications of the 5'- and 3'-terminal ends of the *AcyM* gene. The 5'-terminal end of the *AcyM* gene was amplified with a forward primer designed on the basis of the nucleotide sequence of pUC18, and the reverse primers were designed on the basis of the determined nucleotide sequence of *AcyM* [primer 3R (5'-ATGCCGAGAAGCCGTAGTTCAG-3'); the complementary sequence of positions 313–334 of *AcyM*] for the first PCR and primer 4R (5'-TGGCTGTGCAGGTGCACGAAG-3'); the complementary sequence of positions 168–188 of *AcyM*] for the nested PCR]. Likewise, the 3'-terminal region of the *AcyM* gene was amplified with the forward primers designed on the basis of the determined nucleotide sequence of *AcyM* [5F (5'-AACGGCGAGTGGCAGCGATC-3'); corresponding to positions 415–434 of *AcyM*] for the first PCR and 6F (5'-ATATCGACCGGTGCTCAGC-3'); corresponding to positions 728–748 of *AcyM*] for the nested PCR] and a reverse primer designed on the basis of the nucleotide sequence of pUC18. The PCR products were sized and recovered as described above. The recovered DNA fragments were ligated with T-vector, and the nucleotide sequences were determined as described above. Finally, the 5'-terminal, internal, and 3'-terminal sequences were combined to produce the full-length *AcyM* gene. The nucleotide sequence of the *AcyM* gene has been submitted to DDBJ under accession number AB710379.

RESULTS

Purification, molecular weight, and stability of AcyM DAA was purified to near homogeneity (Fig. 1) from the crude extract of the *M. natoriense* strain TNJL143-2 in 5 chromatographic steps including anion exchange, hydrophobic interaction, and size

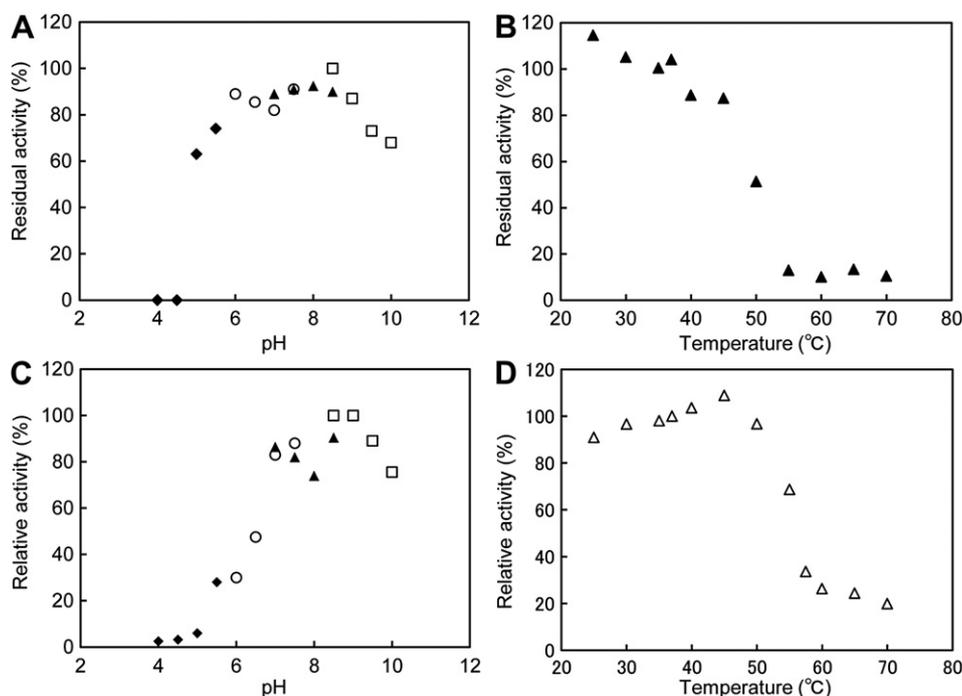


FIG. 2. pH (A) and temperature stabilities (B) of AcyM and effects of pH (C) and temperature (D) on the DAA activity of AcyM. Buffers used for pH studies (A, C) were as follows: pH 4.0–5.6, sodium acetate (closed diamonds); pH 6.0–7.4, potassium phosphate (open circles); pH 7.0–8.6, Tris-HCl (closed triangles); and pH 8.6–10.0, Gly-NaOH (open squares). For temperature stability (B), catalytic activity of the enzyme without the heat treatment was taken to be 100%, and for temperature-activity profiles (D), catalytic activity of the enzyme at 37°C was taken to be 100%. For details, see Materials and methods.

exclusion chromatographies (Table 1). During the present purification, total DAA activity diminished upon the second DEAE-Toyopearl 650 M chromatography and was somewhat restored after hydrophobic interaction chromatography. Some inhibitory substances might have been co-purified and subsequently removed

during these purification procedures. SDS-PAGE of the purified enzyme, AcyM, indicated a protein band corresponding to a molecular weight of 56,000 (Fig. 1). The purified enzyme was eluted at an elution volume corresponding to an approximate molecular weight of 56,000 during gel filtration on a Superdex

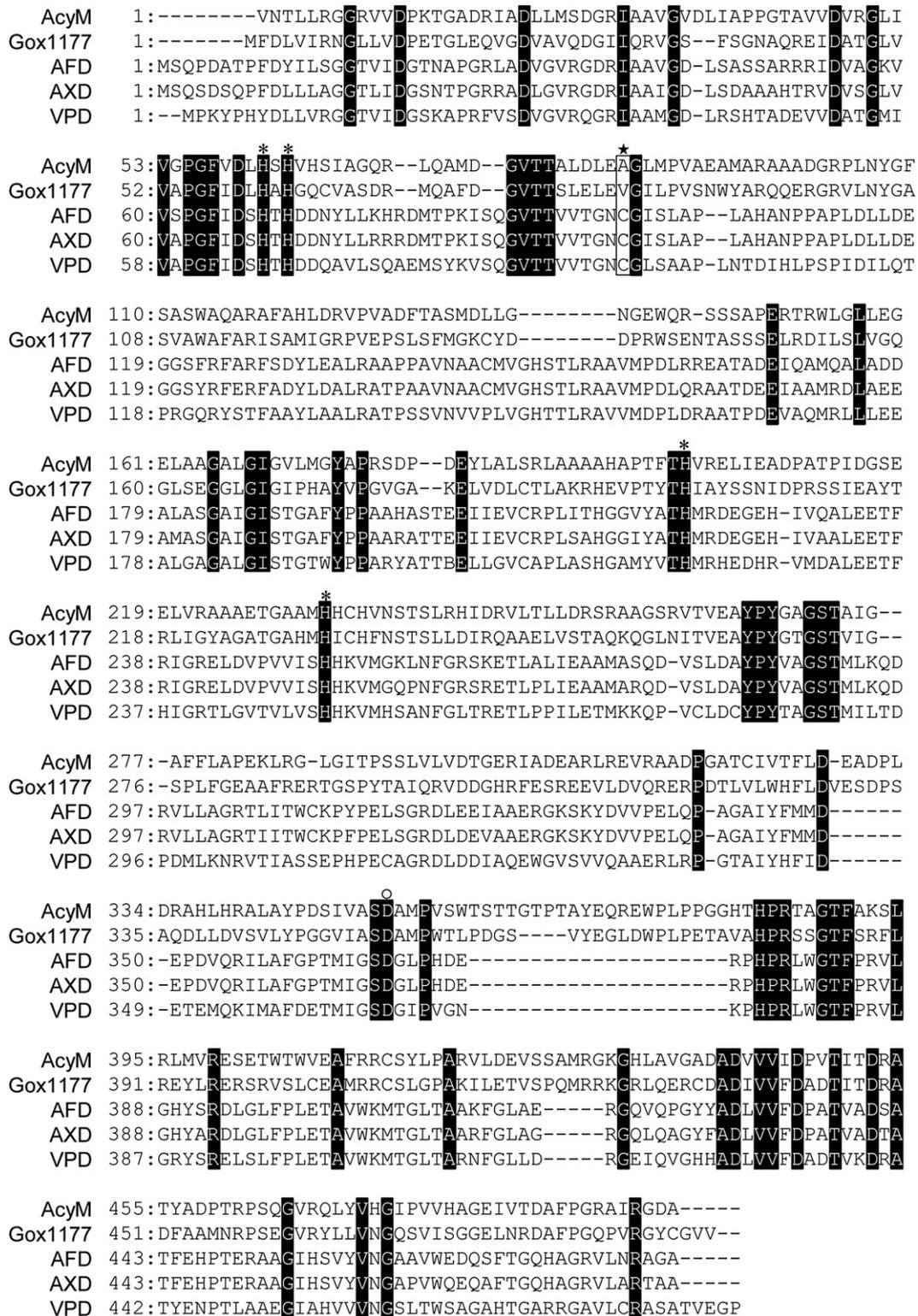


FIG. 3. Alignment of the amino acid sequences of AcyM (this study), Gox1177 (Genbank accession number, AAW60938), AFD (1M7J), AXD (P72349), and VPD (AAM96826). Identical amino acid residues among all of the DAAs are shown in white type on a black background. Putative zinc-binding residues are indicated by asterisks (histidine residues) and closed stars (cysteine residues), respectively, while AcyM does not have cysteine residue in the corresponding position (boxed). Putative catalytic aspartic acid residues are indicated by open circles above the AcyM sequence.

200HR 10/30 column (data not shown). These results suggest that AcyM exists as a monomeric form in solution.

Stability studies showed that the enzyme was stable in the pH range of 6.0–8.5 (at 37°C for 30 min) and retained more than 80% of its original activity after incubation at 45°C (at pH 7.2 for 30 min) (Fig. 2A and B).

Catalytic properties and substrate specificity The optimum pH and temperature of NADF hydrolysis catalyzed by the purified DAA were found to be pH 8.5 and 45°C, respectively (Fig. 2C and D). Among *N*-acetyl-D-amino acids examined, the enzyme displayed the highest activity with NADF. Relative activities of the following *N*-acetyl-D-amino acids to that of NADF (taken to be 100%) were *N*-acetyl-D-leucine, 59.9%; *N*-acetyl-D-methionine, 25.1%; *N*-acetyl-D-tryptophan, 14.4%; and *N*-acetyl-D-valine, 6.0%. The corresponding *N*-acetyl-L-amino acids were inert as substrates. The AcyM-catalyzed hydrolysis of NADF followed Michaelis–Menten kinetics with apparent K_m and k_{cat} values of 2.5 mM and 41 s⁻¹, respectively. However, The DAA activity of AcyM was inhibited by high concentrations of a substrate (NADF),

as previously reported for known DAAs (29), with an estimated IC₅₀ value of 0.31 M.

Effect of various reagents on enzyme activity Known DAAs are zinc-containing enzymes (4,18,22,23) and some of them are known to be strongly inhibited by ZnCl₂ and EDTA (8,14,15,30). Thus, effects of metal ions and chelators on catalytic activity of AcyM were examined. The enzyme was inhibited by the following compounds (final concentration, 1 mM): CuCl₂ (residual activity, 7%), HgCl₂ (1%), and ZnCl₂ (4%), but not by CaCl₂, CoCl₂, FeCl₂, MnCl₂, or CdCl₂. AcyM displayed full activity in the presence of 1 mM EDTA and showed 65% of its full activity even in the presence of 20 mM EDTA. In this regard, the catalytic activity of a DAA from *Deffluviobacter* sp. A131-3 was also shown to be insensitive to 5 mM EDTA (19).

Molecular cloning, sequencing, and sequence similarity We determined the *N*-terminal and the 7 internal amino acid sequences of the AcyM that was purified from the cells of strain TNJL143-2 (Supplemental Fig. S1). Degenerate PCR primers were designed based on the internal sequences #1 and #7

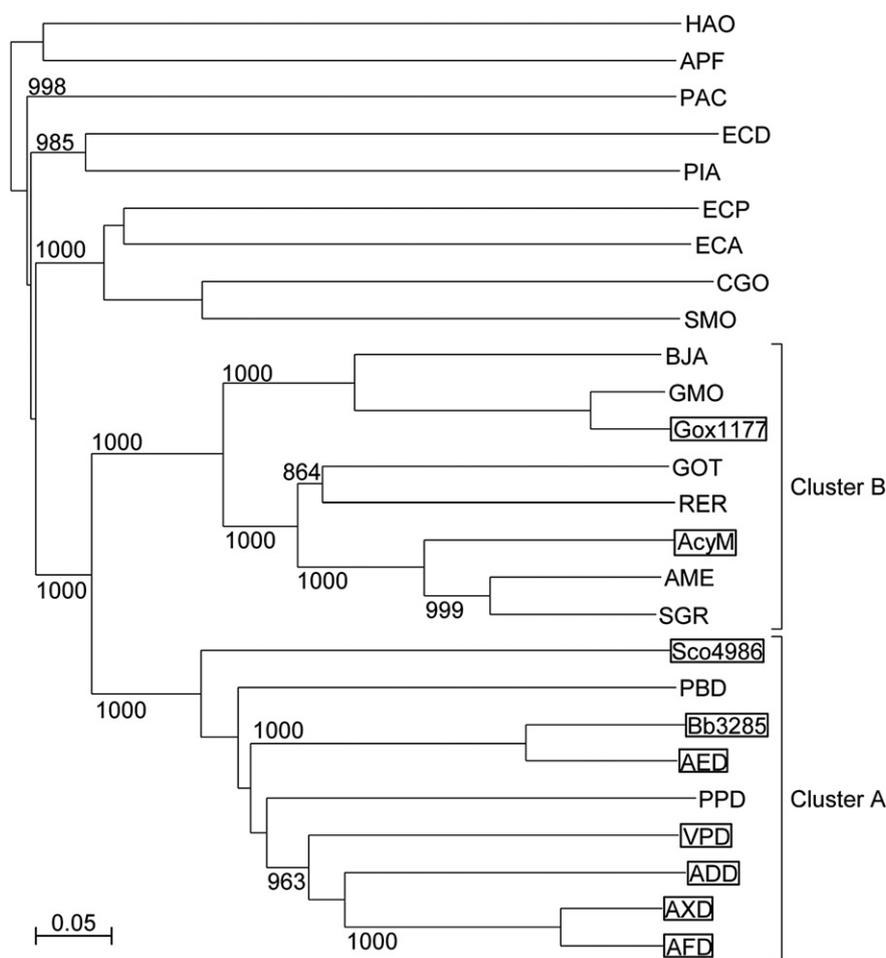


FIG. 4. Molecular phylogenetic tree of DAAs and related enzymes from α/β -barrel amidohydrolase superfamily. Names of biochemically characterized DAAs (Gox1177, AcyM, Sco4986, Bb3285, AED, VPD, ADD, AXD, and AFD) are boxed. The tree was constructed from a CLUSTALW multiple alignment (35) using the neighbor-joining method (36). Numbers indicate bootstrap values greater than 80. The lengths of lines indicate the relative distances between nodes. Phylogenetic Cluster A corresponds to Cluster 3 described by Cummings et al. (20) and Gox1177 belongs to Cluster 4 [Cummings et al. (20)]. The protein sequences used (from top to bottom) are as follows: HAO, *Haloarcula marismortui* dihydroorotase (DDBJ/GenBank/EMBL accession code, AAV48091); APF, *Arthrobacter pascens* *N*-substituted formamide deformylase (Q68AP4); PAC, *Pseudomonas* sp. atrazine chlorohydrolase (NP_862474); ECD, *Escherichia coli* cytosine deaminase (NP_414871); PIA, *Pseudomonas* sp. *N*-isopropylammelide isopropyl amidohydrolase (NP_862508); ECP, *E. coli* phenylhydantoinase (ACB18339); ECA, *E. coli* allantoinase (EG142395); CGO, *Corynebacterium glutamicum* putative dihydroorotase (NP_600825); SMO, *Streptococcus mutans* putative dihydroorotase (NP_721593); BJA, *Bradyrhizobium japonicum* putative amidohydrolase (BAL07365); GMO, *Gluconobacter morbifer* putative DAA (EHH69608); Gox1177, *Gluconobacter oxydans* DAA (AAW60938); GOT, *Gordonia otitidis* putative amidohydrolase (GAB33800); RER, *Rhodococcus erythropolis* putative DAA (EEN84530); AcyM; *M. natoriense* TNJL143-2 DAA (this study, AB710379); AME, *Amycolatopsis mediterranei* putative DAA (ADJ45242); SGR, *Streptomyces griseoaurantiacus* putative DAA (EGG49866); Sco4986, *Streptomyces coelicolor* DAA (NP_629138); PBD, *Pyrococcus abyssi* putative DAA (NP_125384); Bb3285, *Bordetella bronchiseptica* DAA (NP_889821); AED, *A. xylosoxydans* subsp. *xylosoxydans* A-6 α -glutamate aminoacylase (P94211); PPD, *Photobacterium profundum* putative DAA (CAG19683); VPD, *Variovorax paradoxus* Iso1 DAA (AAC38151); ADD, *A. xylosoxydans* subsp. *xylosoxydans* A-6 α -aspartate aminoacylase (P94212); AXD, *A. xylosoxydans* A-6 DAA (P72349); and AFD, *A. faecalis* DAA (PDB ID, 1M7J).

(Supplemental Fig. S1), which had a sequence similarity to the conserved regions of the DAAs reported thus far. A DNA fragment encoding a partial *AcyM* gene was amplified from the chromosomal DNA of strain TNJL143-2 by PCR. After obtaining the 5'- and 3'-ends of the *AcyM* gene, the full-length nucleotide sequence of the *AcyM* gene, 1485 bp in length, was obtained. The deduced amino acid sequence of *AcyM* contained all of the partial amino acid sequences that we determined with the purified enzyme (Supplemental Fig. S1). The translation initiation codon of *AcyM* was found to be GTG, as has often been identified in bacterial genes (31).

The deduced amino acid sequence of *AcyM* was 70% identical to the hypothetical protein of *Streptomyces griseoauranticus* M45 with an accession code of EGG49866. Among the biochemically characterized DAAs, the highest sequence identity (41%) was found with Gox1177 DAA from *Gluconobacter oxydans* (18). However, it showed a low similarity (24–27% identity) to the amino acid sequences of AFD as well as to all other characterized DAAs, which showed an identity of at least 50% to one another (4,18) (Fig. 3).

DISCUSSION

Primary structures of AFD and other characterized DAAs share several conserved amino acid residues that are important for metal binding and catalytic functioning of these DAAs. In the crystal structure of AFD (consisting of 484 amino acids), His221 and His251 provide ligands to a tightly bound zinc ion (located at β site) (26,32–34) that is essential for activity, and His68 and His70 provide ligands to another loosely bound, catalytically unimportant zinc ion (located at α site) (Amino acid numbering with the *N*-terminal methionine residue is based on the primary structure shown in Fig. 3). These 4 histidine residues are all conserved among primary structures of biochemically characterized DAAs (4,18,23,32–34). Cys97 of AFD serves as a bridging ligand to the zinc ions at the α and β sites (18,22,23). This metal coordination by a cysteine residue has been uniquely identified in DAAs among crystal structures of enzymes of the amidohydrolase superfamily (22,23). Moreover, the aspartic acid residue (e.g., Asp367 of AFD) that is located near the α site and proposed to play a general acid/base role during DAA catalysis is also conserved among DAAs (4,22,23). In the primary structure of *AcyM*, the amino acid residues corresponding to His68, His70, His221, His251, and Asp367 of AFD were also conserved (i.e., His61, His63, His201, His232, and Asp352, respectively; Fig. 3). However, the cysteine residue that corresponds to Cys97 of AFD was not conserved in *AcyM* and was replaced by alanine (Ala86). This is also the case for Gox1177 (18), where the corresponding position was occupied by valine (Val85). This observation might be related to the observed differential sensitivities of the DAA activities to EDTA between *AcyM* and other DAAs. In future studies it would be of great interest to examine metal binding by *AcyM*.

It has been shown that DAAs are members of the α/β -barrel amidohydrolase superfamily (4,18,21,22) and nearly all DAAs that have been characterized thus far form a single phylogenetic cluster, which is termed here Cluster A (Fig. 4) [corresponding to Cluster 3 according to Cummings et al. (18)]. The phylogenetic analysis consistently showed that *AcyM* also belongs to the α/β -barrel amidohydrolase superfamily (Fig. 4). However, *AcyM* was distant from Cluster A and appeared to form a separate cluster (Cluster B) along with Gox1177 (Fig. 4), which was the only biochemically characterized DAA of the cluster (18). Both of these enzymes, as well as hypothetical proteins with high sequence similarity to *AcyM* (e.g., the hypothetical protein of *S. griseoauranticus* M45 with an accession code of EGG49866; see above), are devoid of the cysteine residue that corresponds to Cys97 of AFD, and this might be a common feature of the Cluster B DAAs.

A variety of DAAs exhibiting different amino-acid specificities have been reported and can be used as optical resolution catalysts for the production of a variety of *D*-amino acids according to their substrate specificity. For example, AXD (7,8) preferentially hydrolyze *N*-acylated derivatives of *D*-leucine and have been used for the industrial production of neutral *D*-amino acids (4). The strain A-6 also produces 2 distinct DAAs with the highest preference for *N*-acyl-*D*-aspartic acid (11,12) and *D*-glutamic acid (9,10), respectively. DAAs with the highest preference for *N*-acylated forms of *D*-methionine (15,30), *D*-phenylalanine (18,20), and *D*-valine (19) have also been identified. Specificity studies showed that the purified *AcyM* displayed the highest preference for NADF and can potentially be used as an optical resolution catalyst to produce *D*-phenylalanine. It is important to note, however, that DAAs are generally inhibited by high concentrations of substrate (29), and this was also the case for *AcyM*. This point must be addressed in the future studies of *AcyM* to make it possible to use this enzyme as an efficient catalyst for the optical resolution of *D,L*-phenylalanine.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiosc.2012.05.015.

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