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Gene cloning, nucleotide sequencing, and purification and characterization of the D-stereospecific amino-acid amidase from *Ochrobactrum anthropi* SV3

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The gene encoding the D-stereospecific amino-acid amidase from *Ochrobactrum anthropi* SV3 was cloned and sequenced. Analysis of 7.3 kb of genomic DNA revealed the presence of six ORFs, one of which (*daaA*) encodes the D-amino-acid amidase. This enzyme, DaaA, is composed of 363 amino-acid residues (molecular mass 40 082 Da), and the deduced amino-acid sequence exhibits homology to alkaline D-peptidase from *Bacillus cereus* DF4-B (32% identity), DD-peptidase from *Streptomyces* R61 (29% identity), and other penicillin-recognizing proteins. The DaaA protein contains the typical SXXK, YXN, and H(K)XG active-site motifs identified in the penicillin-binding proteins and β-lactamases. The *daaA* gene modified in the nucleotide sequence upstream from its start codon was overexpressed in *Escherichia coli*. The activity of the recombinant DaaA enzyme in cell-free extracts of *E. coli* was 33.6 $U \cdot mg^{-1}$ with D-phenylalaninamide as substrate, which is about 350-fold higher than in extracts of *O. anthropi* SV3. This enzyme was purified to electrophoretic homogeneity by ammonium sulfate fractionation and three column chromatography steps. On gel-filtration chromatography, DaaA appeared to be a monomer with a molecular mass of 40 kDa. It had maximal activity at 45 °C and pH 9.0, and was completely inactivated in the presence of phenylmethanesulfonyl fluoride or Zn^{2+}. DaaA had hydrolyzing activity toward D-amino-acid amides with aromatic or hydrophobic side chains, but did not act on the substrates for the DD-peptidase and β-lactamase, despite their sequence similarity to DaaA. The characteristics of the recombinant DaaA are similar to those found for the native enzyme partially purified from *O. anthropi* SV3.

Keywords: amidase; amide; D-amino-acid; *Ochrobactrum anthropi*.

D-Amino acids are important chiral building blocks for a number of fine chemicals such as pharmaceuticals, agrochemicals, and food additives, and several attempts have been made to synthesize D-amino acids enzymatically, including the exploitation of enzymes specific for the D-configuration of amino-acid derivatives [1,2]. We have previously isolated and characterized D-stereospecific hydrolases, D-aminopeptidase [3] and alkaline D-peptidase [4] from *Ochrobactrum anthropi* C1–38 and *Bacillus cereus* DF4-B, respectively, to use as catalysts for the production of D-amino acids and their derivatives. D-Aminopeptidase is a D-stereospecific aminopeptidase acting on substrates that include low-molecular-mass D-amino-acid amides, such as D-alaninamide and D-α-aminobutyric acid amide, and peptides with a D-alanine at the N-terminus, such as D-alanylglycine, D-alanyl-L-alanyl-L-alanine and D-alanine oligomers. On the other hand, alkaline D-peptidase is a D-stereospecific endopeptidase that acts on oligopeptides composed of D-phenylalanine such as $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$. D-Aminopeptidase has been used as a catalyst for the D-stereospecific hydrolysis of several racemic amino-acid amides [5] and synthesis of D-amino-acid *N*-alkylamides [6] and D-alanine oligopeptides [7]. Alkaline D-peptidase has also been used for oligomerization of D-phenylalanine with D-phenylalanine methyl ester as a substrate [8]. The amino-acid sequences presumed from genes encoding D-aminopeptidase and alkaline D-peptidase are similar to each other and to penicillin-binding proteins (DD-peptidases) and β-lactamases from some micro-organisms [4,9]. We proposed that the D-aminopeptidase and alkaline D-peptidase are penicillin-recognizing enzymes [10].

D-Amino-acid amidases, which catalyze the stereospecific hydrolysis of D-amino-acid amide to yield D-amino acid and ammonia, have also attracted increasing attention as catalysts for stereospecific production of D-amino acids. D-Alaninamide amidase from *Arthrobacter* species was purified, characterized, and used for the production of D-alanine [11]. (*R*)-Enantioselective amidase from *Comamonas acidovorans* hydrolyzes D-leucinamide and D-phenylalaninamide, despite low stereospecificity [12]. We have also reported the partial purification and characterization of a D-stereospecific amino-acid amidase acting preferentially on aromatic amino-acid amides from bacterial isolates from soil, *O. anthropi* SV3 [13]. In this study, we cloned and sequenced the D-stereospecific amino-acid amidase gene, *daaA*, from *O. anthropi* SV3. The recombinant protein, DaaA, was also produced in *Escherichia coli*, purified and characterized.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

O. anthropi SV3 previously isolated from soil by an acclimation culture technique [13] was used as the source of chromosomal DNA. *E. 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 pBluescriptII SK(–) (Toyobo, Osaka, Japan) and pUC19 (Takara Shuzo, Kyoto, Japan) were used as cloning vectors. *O. anthropi* SV3 was grown in a Medium II containing 2 g K_2HPO_4, 1 g NaCl, 0.2 g $MgSO_4,7H_2O$, 0.5 g yeast extract (Oriental Yeast, Osaka, Japan), 10 mL vitamin mixture solution (see below for details), 5 g sucrose, and 2 g D-valinamide hydrochloride in 1 litre distilled water, pH 7.0. Vitamin mixture solution contained 2 μg biotin, 400 μg calcium pantothenate, 2000 μg inositol, 400 μg thiamine hydrochloride, 400 μg pyridoxine hydroxychloride, 400 μg nicotinic acid, 200 μg *p*-aminobenzoic acid, 200 μg riboflavin, and 10 μg folic acid in 10 mL distilled water. Recombinant *E. coli* JM109 was cultured at 37 °C on Luria–Bertani medium [14] 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.

Materials, enzymes, and chemicals

Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo. Alkaline phosphatase from shrimp and *Pwo* DNA polymerase were 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. Substrates for the D-amino-acid amidase were synthesized or purchased from commercial sources as described previously [3,4]. DEAE-Toyopearl 650 M, Butyl-Toyopearl 650 M, and HPLC G-3000 SW and ODS-80Ts columns were purchased from Tosoh Corp. (Tokyo, Japan). Superdex 200 HR 10/30 was from Pharmacia (Uppsala, Sweden).

Cloning of the *O. anthropi* SV3 D-amino acid amidase gene (*daaA*)

For routine work with recombinant DNA, established protocols were used [14]. Total DNA was prepared from *O. anthropi* SV3 by the method of Misawa *et al*. [15], and partially digested with *Sau*3AI. The resulting fragments of 2–9 kbp in size were isolated from the agarose gel by use of Easytrap^{TM} version 2 (Takara Shuzo). The size-fractionated DNAs were ligated into *Bam*HI-digested and alkaline phosphatase-treated pBluescriptII SK(–) using T4 ligase. *E. coli* JM109 was transformed with recombinant plasmid DNA by the method of Inoue *et al*. [16], and ampicillin-resistant transformants were selected. Visualization of the D-amino-acid amidase activity expressed in the transformants was carried out essentially as described by Ikenaka *et al*. [17], with modifications. Recombinant *E. coli* colonies on the plate were transferred to a nitrocellulose filter, and then the filter was floated on the surface of 1 mL of a 10 mg·mL^{-1} lysozyme solution containing 10 μmol EDTA (pH 6.0) until it was wetted from below, and incubated at 30 °C for 30 min. The filter was transferred on to a filter paper to remove the residual lysozyme solution and then put on a dry plastic tray. The filter on the tray was frozen at −20 °C and thawed at room temperature. This procedure was repeated three times to destroy the spheroplasts. The filter was soaked in 2 mL of a reaction mixture [60 μmol potassium phosphate buffer (pH 7.4), 6 mg D-phenylalaninamide, 5 mg phenol, 1.6 units D-amino-acid oxidase, 1.4 units peroxidase, and 0.2 mg 4-aminoantipyrine] and stood at room temperature. A colony corresponding to developed red color was picked up as a positive clone. The *E. coli* transformant carried a 10.3-kb plasmid, designated pDA1.

DNA sequence analysis

An automatic plasmid isolation system (Kurabo, Osaka, Japan) was used to prepare the double-stranded DNAs for sequencing. pDA1 was used as a sequencing template. Nested unidirectional deletions were generated with the Kilo Sequence deletion kit (Takara Shuzo). Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method [18] with M13 forward and reverse oligonucleotides as primers. Sequencing reactions were carried out with a Thermo Sequenase^{TM} cycle sequencing kit and dNTP mixture with 7-deaza-dGTP from Amersham Pharmacia Biotech (Uppsala, Sweden), and the reaction mixtures were run on a DNA sequencer 4000L (Li-cor, Lincoln, NE, USA). Both strands of DNA were sequenced. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB026907. Amino-acid sequences were compared with the BLAST program [19].

Expression of the *daaA* gene in *E. coli*

A modified DNA fragment coding for the D-amino-acid amidase was obtained by PCR. The reaction mixture for the PCR contained in 50 μL 10 mM Tris/HCl, pH 8.85, 25 mM KCl, 2 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, each dNTP at a concentration of 0.2 mM, a sense and an antisense primer each at 1 μM concentration, 2.5 U *Pwo* DNA polymerase and 500 ng plasmid pDA1 as template DNA. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 30 s (first cycle 2 min 30 s), an anealing step at 55 °C for 30 s, and an elongation step at 72 °C for 2 min. The sense primer contained a *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 19 nucleotides (nucleotides 5492–5510 in the sequence of GenBank accession number AB026907) of the *daaA* gene starting with the ATG start codon (bold letters). The antisense primer contained 16 nucleotides, the sequence of which is complementary to nucleotides 6719–6734 in the sequence, and an *Xba*I site (underlined sequence). The two primers were as follows: sense primer, 5′-GAAATTAAGCTT-TAAGGAGGAATAGCCG**ATG**AGTGATTTGAACAACG-3′; antisense primer, 5′-CTACACGtctagaCGC-3′. The amplified PCR product was digested with *Hin*dIII and *Xba*I, separated by agarose-gel electrophoresis, and then purified with EASY-TRAP^{TM} version 2 kit. The amplified DNA was inserted downstream of the *lac* promoter in pUC19, yielding pDAA, and then used to transform *E. coli* JM109 cells.

Purification of the D-amino-acid amidase from *E. coli* transformant

E. coli JM109 harboring pDAA was subcultured at 37 °C for 12 h in a test tube containing 5 mL Luria–Bertani medium supplemented with ampicillin. The subculture (5 mL) was then inoculated into a 2-L conical flask containing 500 mL Luria–Bertani medium supplemented with ampicillin and isopropyl thio-β-D-galactoside. After an 8-h incubation at 37 °C with reciprocal shaking, the cells were harvested by centrifugation at 8000 ***g*** for 10 min at 4 °C and washed with 0.9% (w/v) NaCl. All the purification procedures were performed at a temperature lower than 5 °C, unless otherwise noted. The buffer used

throughout this study was Tris/HCl, pH 8.0, containing 0.1 mM dithiothreitol and 5 mM 2-mercaptoethanol. Washed cells from 2.5 L culture were suspended in 100 mM buffer and disrupted by sonication for 10 min (19 kHz; Insonator model 201M; Kubota, Tokyo, Japan). For the removal of intact cells and cell debris, the lysate was centrifuged at 15 000 ***g*** for 20 min at 4 °C. After centrifugation, the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 50–70% saturation was collected by centrifugation and dissolved in 10 mM buffer. The resulting enzyme solution was dialyzed against 10 L of the same buffer for 24 h. The dialyzed solution was applied to a DEAE-Toyopearl 650M column (1.4 × 12.5 cm) which had been equilibrated with 10 mM buffer. After the column had been washed thoroughly with 10 mM buffer, followed by the same buffer containing 50 mM NaCl and 100 mM NaCl, the enzyme was eluted with 100 mL 10 mM buffer containing 150 mM NaCl. The active fractions were then brought to 30% ammonium sulfate saturation and added to a Butyl-Toyopearl 650M column (1 × 12 cm) equilibrated with 10 mM buffer 30% saturated with ammonium sulfate. After the column had been washed with the same buffer, followed by 10 mM buffer 20% saturated with ammonium sulfate, the active fractions were eluted with 10 mM buffer 15% saturated with ammonium sulfate. The active fractions were combined, concentrated by Centricon 10 (Amicon), and applied to a column of Superdex 200 HR 10/30 equilibrated with 50 mM buffer containing 150 mM NaCl. The column was eluted by FLPC (Pharmacia) at 0.5 mL·min^{-1}, and the active fractions were collected and concentrated by Centricon 10.

Enzyme assay

The standard reaction mixture (1 mL) for assaying the D-amino-acid amidase activity contained 0.1 mmol Tris/HCl buffer (pH 8.0), 20 μmol D-phenylalaninamide hydrochloride and an appropriate amount of the enzyme. The reaction was performed at 30 °C for 5 min and stopped by the addition of 0.2 mL 2 M $HClO_4$. The amount of D-phenylalanine formed in the reaction mixture was determined with a Waters 600E HPLC apparatus equipped with an ODS-80Ts column (4.6 × 150 mm) at a flow rate of 1.0 mL·min^{-1}, using the solvent system methanol/5 mM H_3PO_4 (1 : 4, v/v). Absorbance of the eluate was monitored at 254 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol D-phenylalanine·min^{-1} from D-phenylalaninamide under the above conditions. Protein was determined by the method of Bradford [20] using BSA as standard.

Analytical measurements

To estimate the molecular mass of the enzyme, the sample (10 μg) was subjected to HPLC (Waters 600E system) on a TSK G-3000 SW column (0.75 × 60 cm; Tosoh), at a flow rate of 0.5 mL·min^{-1}, with 0.1 M potassium phosphate, pH 7.0, containing 0.1 M NaCl at room temperture. The A_{280} of the eluate was recorded. The 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.). SDS/PAGE analysis was performed by the method of Laemmli [21]. Proteins were stained with Brilliant blue G and destained in ethanol/acetic acid/water (3 : 1 : 6, by vol.).

Substrate specificity

Enzyme activity toward amino-acid amides and oligopeptides was determined by measuring the production of amino acids. The amount of tyrosine and tryptophan formed was quantitatively assayed by HPLC on an ODS-80Ts reverse-phase column (4.6 × 150 mm) at a flow rate of 1.0 mL·min^{-1} using the solvent system methanol/5 mM H_3PO_4 (1 : 9, v/v) and (1 : 4, v/v), respectively. Quantitative analysis of the other amino acids was performed with an amino-acid analyzer (model L-8500; Hitachi, Tokyo, Japan). Enzyme activity toward aliphatic and aromatic amides was determined by measuring the formation of ammonia. The amount of ammonia produced was colorimetrically determined by the phenol/hypochlorite method [22] using Conway microdiffusion apparatus [23].

RESULTS

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

An *O. anthropi* SV3 total DNA library was constructed in *E. coli* JM109. Transformants in *E. coli* JM109 were screened for D-amino-acid amidase activity by the development of a red color as described in Materials and methods. One clone (pDA1) among ≈ 4000 tested developed a faint red color on the replica nitrocellulose filter, which indicated the functional expression of D-amino-acid amidase gene. The plasmid pDA1 containing a 7.3-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 7330-bp long, and six ORFs were present in this region. The (G + C) content of the sequenced DNA amounted to 59%, in good agreement

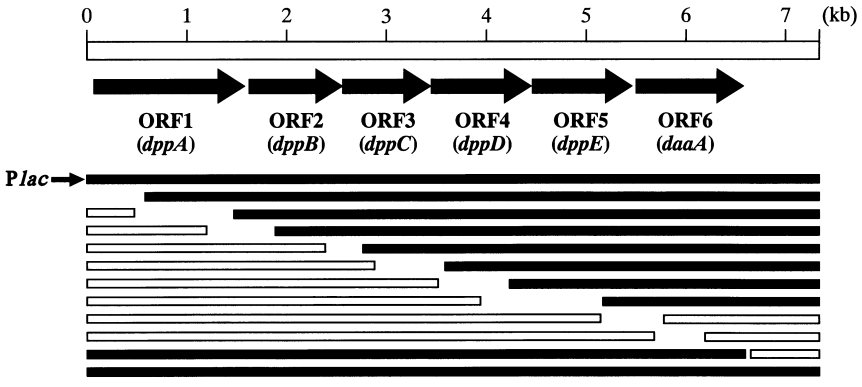

Fig. 1. Physical map of the *O. anthropi* SV3 *daaA* and adjacent regions. The location of *daaA* and *dpp* ORFs and the direction of transcription (arrows) are indicated. Inserted fragments of the nested deletion plasmids were used for the determination of *daaA* location. The orientation of *lacZ* of the vector (small arrow) and the transcription of the six ORFs is the same. Solid and open bars indicate the presence and absence of D-amino-acid amidase activity in the corresponding clone, respectively.

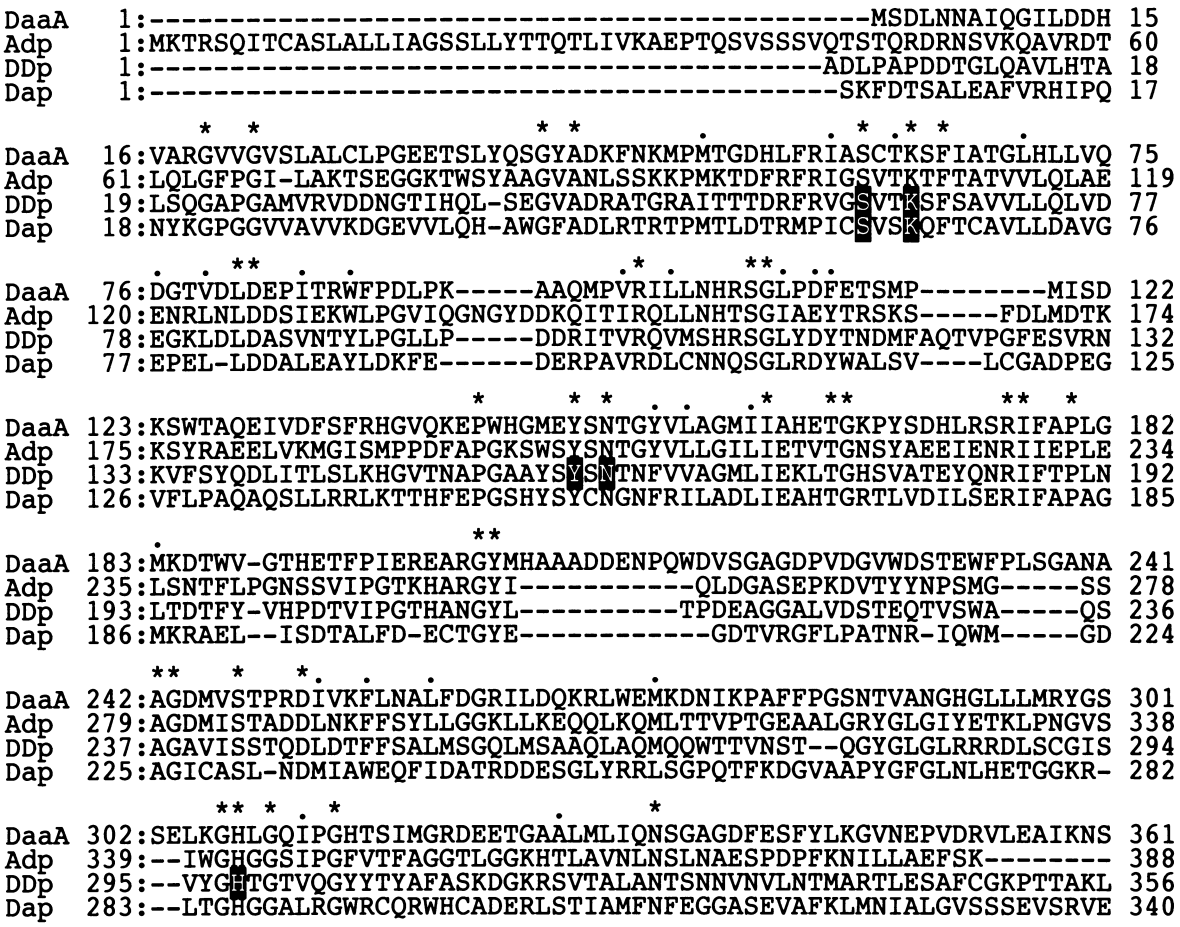

Fig. 2. Comparison of the amino-acid sequences of D-amino-acid amidase (DaaA) from *O. anthropi* SV3 and other homologous proteins. Identical and conserved amino acids among the sequences are marked by asterisks and dots, respectively. Dashed lines indicate gaps introduced for better alignment. Adp, alkaline D-peptidase from *B. cereus* DF4-B; DDp, DD-peptidase from *Streptomyces* R61; Dap, D-aminopeptidase from *O. anthropi* C1-38. The Adp sequence contains predicted signal peptide composed of 39 amino-acid residues [4]. Proposed active-site residues Ser62, Lys65, Tyr159, Asn161, and His298 in DDp and Ser61 and Lys64 in Dap are in black boxes.

with the value found for the *O. anthropi* DNA, which is within 56–59% [24]. The six putative ORFs (ORF1 to ORF6) exhibited a bias to G or C in the third position of the codon, fulfilling the rules for coding regions [25]. Potential Shine/Dalgarno sequences were found upstream of the putative ATG start codons, except for ORF4 and ORF5. A physical map of this gene cluster is shown in Fig. 1. To determine which ORF encodes the D-amino-acid amidase, the nested deletions generated as described above were checked for D-amino-acid amidase activity. Deletion clones, which harbored the complete sequence of ORF6, expressed D-amino-acid amidase activity (Fig. 1). ORF6 has therefore been designated *daaA*. This gene consists of 1089 bp and codes for a protein of 363 amino acids (molecular mass 40 082 Da). A potential Shine/Dalgarno (GGAG) sequence lies just 7 nucleotides upstream from the start codon ATG. Alignment by the SwissProt and NBRF-PIR databases using the BLAST program showed that the deduced primary structure of DaaA is similar to those of alkaline D-peptidase from *Bacillus cereus* DF4-B (31.7% identical over 281 amino acids [4]; GenBank accession number D86380), DD-peptidase from *Streptomyces* R61(29.0% identical over 279 amino acids [26]; GenBank accession number P15555), penicillin-binding protein from *Bacillus subtilis* (28.2% identical over 316 amino acids [27]; GenBank accession number P32959), β-lactamases from *Serratia marcescens* (25.2% identical over 202 amino acids; GenBank accession number AB008454) and *Enterobacter cloacae* (23.5% identical over 234 amino acids [28]; GenBank accession number S00405), D-aminopeptidase from *O. anthropi* C1-38 (26.7% identical over 191 amino acids [9] GenBank accession number A42209), esterase from *Pseudomonas* sp. (24.8% identical over 218 amino acids [29]; GenBank accession number A44832), and butane-1,4-diol diacrylate esterase from *Brevibacterium linens* IFO 12171 (33.0% identical over 91 amino acids [30]; GenBank accession number AB020733). Figure 2 shows the alignment of the primary structures of DaaA from *O. anthropi* SV3, alkaline D-peptidase from *B. cereus* DF4-B, DD-peptidase from *Streptomyces* R61, and D-aminopeptidase from *O. anthropi* C1-38. The motifs (SXXK, YXN, and HXG) found in the active site of DD-peptidase [31] were conserved in DaaA as well as in alkaline D-peptidase and D-aminopeptidase. No significant homology was observed with other amidases such as (*S*)-enantioselective amidase from *Rhodococcus rhodochrous* [32] (*R*)-enantioselective amidase from *C. acidovorans* [12], pyrazinamidase/nicotinamidase (PncA) from *Mycobacterium tuberculosis* [33], and wide-spectrum aliphatic amidase from *Brevibacterium* sp. [34].

When the other ORFs (ORF1–5) contained in plasmid pDA1 flanking the *daaA* ORF were compared with other sequences in the databases, it was observed that their deduced amino-acid

sequences showed homologies to those of the dipeptide/oligopeptide transporter family. They were therefore designated *dppABCDE*. The first of the five genes (*dppA*) codes for a protein of 508 amino acids with a molecular mass of 55 661 Da. DppA has 26.9% identical amino acids with the dipeptide transporter protein (DppA) of *Bacillus firmus* (GenBank accession number U64514) and 26.6% identical amino acids with the periplasmic dipeptide-binding protein of *Helicobacter pylori* (GenBank accession number B64557). The next gene is *dppB* which codes for a protein of 313 amino acids with a molecular mass of 34 749 Da. DppB has 38.0% identical amino acids with the permease protein (YliC) of ABC transporter of *E. coli* (GenBank accession number P75798) and 34.2% identical amino acids with the oligopeptide ABC transporter, permease protein of *Thermotoga maritima* (GenBank accession number AE001772). The next ORF *dppC* encodes a protein of 291 amino acids with a molecular mass of 32 182 Da. DppC has 41.7% identical amino acids with the permease protein DppC of dipeptide transport system from *E. coli* (GenBank accession number P37315) and 37.3% identical amino acids with the permease protein of dipeptide ABC transporter from *H. pylori* (GenBank accession number D64557). The protein encoded by the *dppD* gene consists of 334 amino-acid residues with a molecular mass of 35 504 Da. DppD has 51.1% identical amino acids with the ATP-binding protein of oligopeptide ABC transporter from *T. maritima* (GenBank accession number AE001726) and 46.0% identical amino acids with the ATP-binding protein of the dipeptide-transport system from *B. subtilis* (GenBank accession number P26905). The gene product of *dppE* is a protein of 335 residues with a molecular mass of 36 538 Da. DppE has 51.6% identical amino acids with the ATP-binding protein of oligopeptide ABC transporter from *T. maritima* (GenBank accession number AE001726) and 50.2% identical amino acids with the ATP-binding protein (OppF) of oligopeptide ABC transporter from *Treponea denticola* (GenBank accession number AF042861).

In the region of DNA upstream of the *daaA* translational start codon, sequences related to the −35 and −10 consensus promoter regions were not identified, and the intergenic region separating the 3′ end of *dppE* from the 5′ end of *daaA* is only 36 bp, suggesting transcriptional coupling between *dpp* genes and *daaA*, although its physiological role is not clear.

Production of the DaaA in *E. coli*

When *E. coli* harboring pDA1 was cultivated in the presence of isopropyl thio-β-D-galactoside at 37 h, DaaA activity was detected in the supernatant of the sonicated cell-free extracts obtained at 15 000 ***g***. However, the level of DaaA activity in the supernatant was 7.40 U·mg$^{-1.}$ To enhance the DaaA activity in the cell-free extract, we improved the sequence upstream from the ATG start codon by PCR, with plasmid pDA1 as a template as described in Materials and methods. The resultant plasmid, pDAA, in which the *daaA* gene was under the control of the *lac* promoter of pUC19 vector, was introduced into *E. coli* JM109 cells. A protein corresponding to the predicted molecular mass of 40 kDa was produced when the *lac* promoter was induced by isopropyl thio-β-D-galactoside (data not shown). When *E. coli* JM109 harboring pDAA was cultivated in Luria–Bertani medium supplemented with ampicillin and isopropyl thio-β-D-galactoside for 8 h at 37 °C, the level of DaaA activity in the supernatant of the sonicated cell-free extracts of the transformants was 33.6 U·mg^{-1}, which was much higher than that of *E. coli* containing pDA1 and about 350-fold above that of *O. anthropi* SV3 [13].

Table 1. Summary of purification of DaaA.

	Total protein (mg)	Total activity (U)	Specific activity (U·mg^{-1})	Yield (%)
Cell free extract	797	26 800	33.6	100
Ammonium sulfate	218	16 100	73.9	60.1
DEAE-Toyopearl	34.4	9 440	274	35.2
Butyl-Toyopearl	11.5	3 900	339	14.6
FPLC Superdex 200	5.7	2 090	367	7.81

Purification of the DaaA from *E. coli* transformant

Recombinant DaaA was purified from the *E. coli* JM109 harboring pDAA with a recovery of 7.81% by ammonium sulfate fractionation and DEAE-Toyopearl, Butyl-Toyopearl, and FPLC Superdex 200 column chromatography (Table 1). The final preparation gave a single band on SDS/PAGE with a molecular mass of ≈40 kDa (Fig. 3). This value is in good agreement with that estimated from the deduced amino-acid sequence of DaaA. The molecular mass of the native enzyme was about 38 kDa according to gel-filtration chromatography, indicating that the native enzyme was a monomer. The purified enzyme catalyzed the hydrolysis of D-phenylalaninamide to D-phenylalanine at 367 U·mg^{-1} under the standard conditions.

Stability

The purified enzyme could be stored without loss of activity for more than 2 months at 0 °C in the buffer (pH 8.0) or at −20 °C in the buffer containing 50% glycerol. The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 5 min in 130 mM Tris/HCl (pH 8.0) containing 0.1 mM dithiothreitol and 5 mM 2-mercaptoethanol, a sample of the enzyme solution was taken and the DaaA activity was assayed under the standard conditions. It

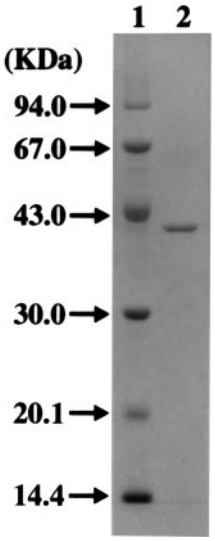

Fig. 3. SDS/polyacrylamide slab gel electrophoresis of DaaA. Lane 1, molecular-mass standards [phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)]; lane 2, purified DaaA (5 μg).

exhibited the following activity: 50 °C, 0%; 45 °C, 1.3%; 40 °C, 49%; 35 °C, 95%; 30 °C, 96%; 25 °C, 100%. The stability of the enzyme was also examined at various pH values. The enzyme was incubated at 30 °C for 5 min in the following buffers (final concentration 100 mM): acetic acid/sodium acetate (pH 4.0–6.0), Mes/NaOH (pH 5.5–6.5), potassium phosphate (pH 6.5–8.5), Tris/HCl (pH 7.5–9.0), ethanolamine/HCl (pH 9.0–11.0), glycine/NaCl/NaOH (pH 10.0–13.0). Then a sample of the enzyme solution was taken, and the DaaA activity was assayed under the standard conditions. The enzyme was most stable in the pH range 6.5–9.5, and 55% of its initial activity was retained even at pH 11.0.

Effects of pH and temperature

The optimal pH for the activity of the enzyme was measured in the buffers described above. The enzyme showed maximum activity at pH 8.5–9.5 (Fig. 4A). The enzyme reaction was carried out at various temperatures for 5 min in 0.1 M Tris/HCl (pH 8.0), and enzyme activity was found to be maximal at 45 °C (Fig. 4B). Above 45 °C, it decreased rapidly, possibly because of instability of the enzyme at the higher temperatures.

Inhibitors

Various compounds were investigated for their inhibitory effects on enzyme activity. Dithiothreitol and 2-mercaptoethanol were removed from the enzyme solution by use of Centricon. Thereafter, we measured the enzyme activity under standard conditions after incubation at 30 °C for 10 min with various compounds at 1 mM. The enzyme was completely inhibited by phenylmethanesulfonyl fluoride, indicating that the enzyme has an active-site serine residue. The enzyme was also completely inhibited by Zn^{2+} ($ZnSO_4$ and $ZnCl_2$), and inhibited 79% by $CdCl_2$, 78% by $PbCl_2$, 61% by $NiCl_2$, 44% by $CoCl_2$, 43% by $HgCl_2$, 41% by $AgNO_3$, and 31% by $CuCl_2$. Other inorganic compounds such as LiBr, H_2BO_3, NaCl, $MgSO_4$, $MgCl_2$, $AlCl_3$, KCl, KI, $CaCl_2$, $CrCl_3$, $MnSO_4$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $Fe(NH_4)_2(SO_4)_2$, RbCl, Na_2MoO_4, $SnCl_2$, and $BaCl_2$ did not influence the activity. Carbonyl reagents such as hydroxylamine, phenylhydrazine, D,L-penicillamine, and D-cycloserine were not inhibitory toward the enzyme. Chelating reagents, e.g. *o*-phenanthroline, 8-hydroxyquinoline, EDTA, and α,α′-dipyridyl, and thiol reagents such as 5,5′-dithiobis-(2-nitorobenzoate), iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide also had no significant effect on the enzyme.

Table 2. Substrate specificity of purified DaaA. The activity for D-phenylalaninamide, corresponding to 367 U·mg^{-1}, was taken as 100%. The following compounds were not substrates for the DaaA: L-phenylalaninamide, L-tyrosinamide, L-tryptophanamide, L-leucinamide, L-alaninamide, L-methioninamide, L-norvalinamide, L-prolinamide, L-histidinamide, L-serinamide, L-valinamide, L-isoleucinamide, L-glutaminamide, L-threoninamide, L-*allo*-threoninamide, L-isoasparagine, D-isoglutamine, L-isoglutamine, D-lysinamide, L-lysinamide, D-asparaginamide, L-asparaginamide, glycinamide, propionamide, acetamide, *n*-butyramide, benzamide, nicotinamide, D-phenylalanyl-D-phenylalanine, D-phenylalanyl-D-phenylalanyl-D-phenylalanine, D-phenylalanyl-D-phenylalanyl-D-phenylalanyl-D-phenylalanine, L-alanyl-L-alanine, L-alanyl-D-alanine, L-alanyl-D-alanyl-L-alanine, D-alanyl-L-alanine, D-alanyl-D-alanine, D-alanyl-D-alanyl-D-alanine, D-alanyl-D-alanyl-D-alanyl-D-alanine, D-alanylglycine, L-alanylglycine, D-alanylglycylglycine, glycylglycine, glycylglycylglycine, N^{α},N^{ε}-diacetyllysyl-D-alanyl-D-alanine, L-phenylalanine methylester, penicillin G, and ampicillin.

Substrate	Relative activity (%)
D-Phenylalaninamide	100
D-Tyrosinamide	98
D-Tryptophanamide	79
D-Leucinamide	37
D-Alaninamide	23
D-Methioninamide	17
D-Norleucinamide	16
D-Norvalinamide	12
D-Phenylalanine-*p*-nitroanilide	9.3
D-Prolinamide	6.2
D-Leucine-*p*-nitroanilide	5.1
D-Histidinamide	2.5
D-Isoleucinamide	2.5
D-Serinamide	0.92
D-Valinamide	0.50
D-Threoninamide	0.46
D-Glutaminamide	0.39
D-*allo*-Threoninamide	0.34
D-Isoasparagine	0.16
D-Alanine-*p*-nitroanilide	0.15
D-Phenylalanine methyl ester	130

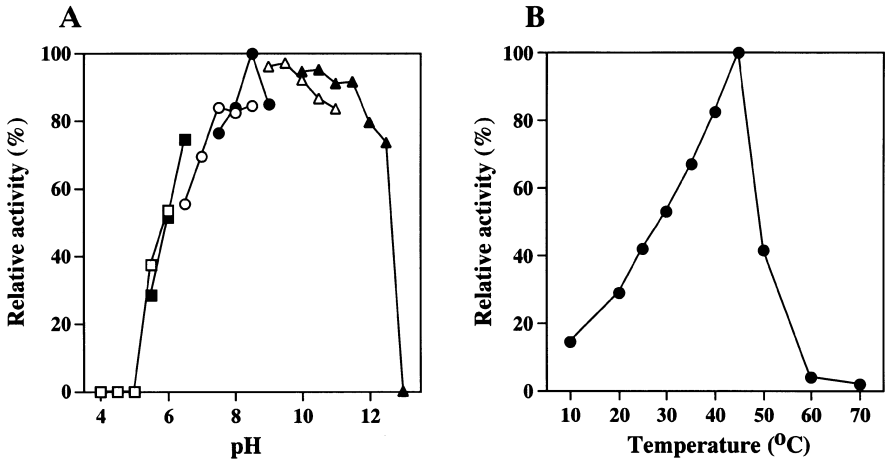

Fig. 4. Effect of pH and temperature on the activity of DaaA. (A) To assess the effect of pH, the reactions were carried out at 30 °C in the following buffers (100 mM): acetic acid/sodium acetate (□), Mes/NaOH (■), potassium phosphate (○), Tris/HCl (●), ethanolamine/HCl (△), and glycine/NaCl/NaOH (▲). (B) Reactions were carried out at various temperatures.

The presence of a stereoisomeric substrate L-phenylalaninamide did not affect the enzyme activity. The activity was not inhibited by β-lactam compounds such as penicillin G and ampicillin which are inhibitors of DD-peptidase.

Substrate specificity

To study the substrate specificity, the enzyme was used to hydrolyze D- or L-amino-acid amides, oligopeptides, aliphatic amides, and aromatic amides, and the activity was assayed (Table 2). Besides D-phenylalaninamide, the enzyme was remarkably active towards D-tyrosinamide, D-tryptophanamide, D-leucinamide, D-alaninamide, D-methioninamide, D-norleucinamide, and D-norvalinamide. D-Prolinamide, D-isoleucinamide, and D-valinamide however, were hydrolyzed, at much lower rates than the above aromatic and hydrophobic D-amino acid amides. L-Amino-acid amides, oligopeptides, aliphatic amides, aromatic amides, and β-lactams were not substrates of the enzyme. D-Phenylalanine methyl ester was active as a substrate. The apparent K_m values for D-phenylalaninamide, D-tyrosinamide, and D-tryptophanamide were 0.44, 0.50, and 0.48 mM, respectively, whereas V_{max} values for the three substrates were 370, 333, and 323 U·mg^{-1}, respectively.

DISCUSSION

In this paper, we cloned the *daaA* gene expressing D-stereospecific amino acid amidase activity from *O. anthropi* SV3, and determined its nucleotide sequence. *daaA* was located immediately downstream of the hypothetical dipeptide/oligopeptide transporter gene (*dpp*) cluster. We also purified and characterized the recombinant D-amino-acid amidase, DaaA, produced in *E. coli* transformant. Characteristics such as molecular mass, subunit structure, optimum pH and temperature, and substrate specificity were mostly the same as for the D-amino-acid amidase partially purified from the parent strain [13], indicating that the *daaA* gene actually codes for the D-amino-acid amidase. As DaaA has become abundantly available by the DNA technique, the recombinant *E. coli* cells producing DaaA or the purified DaaA may be applicable to optical resolution of amino-acid amides to yield D-amino acids.

Ozaki *et al*. [11] reported the purification and characterization of a D-stereospecific amino-acid amidase (D-alaninamide amidase) from *Arthrobacter* sp. NJ-26 and its application to the production of D-alanine from racemic D,L-alaninamide. The D-alaninamide amidase can hydrolyze D-alaninamide and glycinamide but cannot hydrolyze D-leucinamide and D-phenylalaninamide. On the other hand, DaaA reported here acted on D-phenylalaninamide and D-leucinamide as well as D-alaninamide, but it could not act on glycinamide (Table 2). The D-alaninamide amidase is a 67-kDa monomer, whereas DaaA is a 40 082-Da monomer. The D-alaninamide amidase was strongly inhibited by *p*-chloromercuribenzoate, and the presence of cysteine in the reaction mixture reduced the inhibitory effect, suggesting the involvement of a thiol group at the active center of the enzyme. DaaA, on the other hand, was completely inhibited by phenylmethanesulfonyl fluoride, showing that the enzyme probably has a serine residue in its active site. These observations demonstrate that DaaA is distinct from the D-alaninamide amidase.

An (*R*)-enantioselective amidase (*R*-amidase) from *C. acidovorans* KPO-2771-4 isolated as a micro-organism that enantiomerically hydrolyzes racemic 2-(3′-benzoylphenyl)-propionamide (ketoprofen amide) to produce *R*-(−)-ketoprofen, acts preferentially on D-isomers of leucinamide and phenylalaninamide [12]. However, the stereospecificity of the enzyme toward the amino-acid amides is less strict than that of DaaA: the relative activities of the *R*-amidase for D-leucinamide, L-leucinamide, D-phenylalaninamide, and L-phenylalaninamide were 251, 2.3, 137, and 45.0%, respectively, when the enzyme activity for ketoprofen amide was taken as 100%. DaaA does not have significant sequence homology with the *R*-amidase.

We found that DaaA shows homology with the sequences of alkaline D-peptidase from *B. cereus* DF4-B, DD-peptidase from *Streptomyces* R61, penicillin-binding protein from *B. subtilis*, class-C β-lactamases from *S. marcescens* and *E. cloacae*, and D-aminopeptidase from *O. anthropi* C1-38. Of the above enzymes, DD-peptidase, which hydrolyzes the terminal D-alanyl-D-alanine peptide bond of a carbonyl donor peptide in the nascent cell wall peptidoglycan, has been intensively studied with respect to the catalytic mechanism. The Ser62 in the SXXK motif of DD-peptidase is responsible for nucleophilic attack on the carbonyl carbon of the peptide bond to be hydrolyzed in a peptide substrate or the β-lactam carbonyl carbon in an antibiotic molecule [35]. In the crystal structure of the enzyme, Ser62, Lys65, Tyr159, Asn161, and His298 constitute the active site of the enzyme [31]. We previously showed that Ser61 and Lys64 of D-aminopeptidase, which are comparable with Ser62 and Lys65 in DD-peptidase, function as the active-site residues of the enzyme [9]. Perfect conservation of these residues in the D-stereospecific amino-acid amidase DaaA as well as alkaline D-peptidase and D-aminopeptidase (Fig. 3) suggests that DaaA could be categorized as a new member of the family of penicillin-recognizing enzymes, including DD-peptidase, penicillin-binding protein, β-lactamase, D-aminopeptidase, and alkaline D-peptidase, and that the catalytic mechanism of DaaA could be analogous to those of the other members. However, DaaA could act on neither N^{α},N^{ε}-diacetyl-lysyl-D-alanyl-D-alanine which is a substrate for DD-peptidase, nor β-lactam antibiotics such as penicillin G and ampicillin which are substrates for β-lactamase. Furthermore, DaaA was inactive on the peptide substrates containing D-alanine or D-phenylalanine which are good substrates for D-aminopeptidase and alkaline D-peptidase, respectively (Table 2). Therefore, DaaA may differ from the other members of the family with respect to its substrate recognition. The biological role of DaaA is still not known. The parent strain, *O. anthropi* SV3, was isolated as a D-valinamide degrader from a 3-month acclimation culture using D-valinamide as sole nitrogen source [13]. The DaaA may have evolved to be active exclusively toward D-amino-acid amides from a common ancestor of the penicillin-recognizing enzyme family.

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