Molecular cloning of the aspartate 4-decarboxylase gene from *Pseudomonas* sp. ATCC 19121 and characterization of the bifunctional recombinant enzyme

Nai-Chen Wang · Chia-Yin Lee

Received: 27 February 2006 / Revised: 18 April 2006 / Accepted: 19 April 2006 / Published online: 8 June 2006
© Springer-Verlag 2006

Abstract L-Aspartate 4-decarboxylase (Asd) is a major enzyme used in the industrial production of L-alanine. Its gene was cloned from *Pseudomonas* sp. ATCC 19121 and characterized in the present study. The 1,593-bp *asd* encodes a protein with a molecular mass of 59,243 Da. The Asd from this *Pseudomonas* strain was considerably homologous to other Asds and aminotransferases, and has evolved independently of these enzymes from gram-positive microbes. Productivity rate of the C-terminal His-tagged fusion Asd was at 33 mg/l of *Escherichia coli* transformant culture. The kinetic parameters K_m and V_{max} of the fusion protein were 11.50 mM and 0.11 mM/min, respectively. Gel filtration analysis demonstrated that Asd is a dodecamer at pH 5.0 while 4.4 % of the recombinant protein dissociated into dimer when the pH was increased to 7.0. Asd exhibited its maximum activity at pH 5.0 and specific activity of 280 U/mg, and remained stable over a broad range of pH. The optimum temperature for Asd reaction was 45 °C, and 92 % of the activity remained when the enzyme was incubated at 40 °C for 40 min. This enzyme did not have any preferred divalent cation for catalysis. The recombinant Asd also exhibited aminotransferase activity when D,L-Asp, L-Glu, L-Gln, and L-Ala were utilized as substrates. However, the decarboxylation activity of L-aspartate was 2,477 times higher than its aminotransferase activity. The present study is the first investigation on the important biochemical properties of the purified recombinant Asd.

Introduction

L-Aspartate 4-decarboxylase (Asd; EC 4.1.1.12), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, catalyzes the β -decarboxylation of L-aspartate to L-alanine and CO₂. This enzyme is markedly activated by α -keto acids (Nishimura et al. 1962) and considered as bifunctional because a low-level nonspecific L-amino acid transamination activity was demonstrated in Asd from *Alcaligenes faecalis* (Novogrodsky et al. 1963). Both transamination and β -decarboxylation involve the same quinonoid intermediate which, later on, diverge in the subsequent steps of the individual reactions (Dunathan 1966). To find amino acid residues of Asd directing the process of catalysis would be interesting. The decarboxylases occur in mammals and various microorganisms such as bacteria, actinomycetes, and fungi (Crawford 1958; Wilson and Kornberg 1963; Chibata et al. 1965; Bowers et al. 1970; Rathod and Fellman 1985; El-Rahmany 1994). But up to now, only three *asd* genes have been cloned from microorganisms (Rozzell 1991; Chen et al. 2000; Abe et al. 2002). In addition, more than 300 bacterial genomes were already completely sequenced and *asd* was only found in probably 6 of them. This prompted us to clone another *asd* from strains with a decarboxylase activity to identify the important amino acid residues in the β -decarboxylation reaction.

As an amino acid infusion and food additive component, L-alanine is usually produced by an enzymatic method. Chibata et al. (1965) initially applied Asd for the production of L-alanine using immobilized *Pseudomonas dacunhae* cells. Recently, we constructed an expressing strain to more efficiently obtain a large amount of His-tagged Asd (Chen et al. 2000). Even though the general biochemical investigations of the bacterial Asd were already

N.-C. Wang · C.-Y. Lee (✉)
Graduate Institute of Agricultural Chemistry,
National Taiwan University,
No. 1, Sec. 4, Roosevelt Rd.,
Taipei 10617 Taiwan, Republic of China
e-mail: m477@ntu.edu.tw

undertaken since the 1950s, no detailed characterization has ever been done on His-tagged Asd that was designed for rapid protein purification. In the present study, an *asd* gene with exceptionally high GC content was cloned from *Pseudomonas* sp. ATCC 19121, which had been identified as an alanine-producing strain. The corresponding protein was highly produced as the His-tagged recombinant Asd in *Escherichia coli*. A number of biochemical and biophysical properties of the purified recombinant enzyme were investigated for the first time in the present work and, thus, facilitate further molecular studies and its biotechnological applications.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Pseudomonas sp. ATCC 19121 was purchased from the American Type Culture Collection (Rockville, USA). *Escherichia coli* XL1-Blue and *E. coli* BL21(DE3)pLysS (Novagen) grown at 37 °C overnight in Luria–Bertani (LB) medium were used as hosts for cloning and expression of the gene. When necessary, ampicillin and chloramphenicol were added to a final concentration of 100 and 34 µg/ml, respectively. Plasmids pGEM-T Easy (Promega) and pBluescript II KS (+) (Stratagene) were used as gene cloning vectors, and the pET-21b (Novagen) was used to express the gene.

DNA manipulations and sequence analysis

Genomic DNA of *Pseudomonas* sp. ATCC 19121 was extracted using the Blood & Tissue Genomic kit (Viogene, Taiwan). Restriction enzymes (New England Biolabs) and T4 DNA ligase (Invitrogen) were used according to the instructions of the suppliers. General DNA manipulation techniques used in this study were applied as described by Sambrook et al. (1989). Both strands of the insert of pBK-*asdP*-A9 were sequenced to confirm its identity. The nucleotide sequences were determined using an ABI Prism 3770 DNA sequencer (Perkin Elmer). The BLAST (Altschul et al. 1997) was used for protein homology search, and the EMBOSS-Align (needle) program on the EMBL-EBI website was used to determine nucleotide identity. ClustalX (Thompson et al. 1997) was used for multiple sequence alignment. A phylogenetic tree was constructed by the neighbor-joining (NJ) method (Saitou and Nei 1987) using the PHYLIP 3.5 program.

Cloning of the *Pseudomonas* sp. ATCC 19121 *asd* gene

A degenerate sense primer, 5'-GGBGTBCGNTTCCTB GGBAAGWC-3' (N, any base; B, C or G or T; W, A

or T), and a degenerate antisense primer, 5'-CCVAGNCGC CAVCCSGT-3' (V, A or C or G; S, C or G), were designed based on the Asd consensus sequences from *P. dacunhae* and *A. faecalis* (Chen et al. 2000). The DNA fragment, amplified by polymerase chain reaction (PCR), was cloned into pGEM-T Easy vector to maintain and analyze the nucleotide sequence. When used as a probe, the *Ava*I–*Bgl*II fragment (252 bp) was labeled with DIG using a DNA labeling kit (Roche), following the protocol of the supplier. After Southern blot analysis, the 8- to 10-kb fragments of the genomic DNA from *Pseudomonas* sp. ATCC 19121 were collected and inserted into the multiple cloning site of pBluescript II KS(+). The ligation mixture was transformed into *E. coli* XL1-Blue, and white colonies were screened by hybridization using the DIG-labeled probe. The plasmid isolated from a positive clone was called pBK-*asdP*-A9.

Construction of Asd expression vector

Pseudomonas sp. ATCC 19121 Asd was expressed in *E. coli* using a T7 expression system according to the manufacturer's instructions (Novagen). The *asd* gene was amplified by PCR using an N-terminal primer, 5'-ATCA CAAGGAGCTCCGCAAT-3', containing a *Sac*I site and a C-terminal primer, 5'-AGAAGCTTCTCCTTGCC CAGCGC-3', including a *Hind*III site and the pBK-*asdP*-A9 as the template. The amplified DNA fragment was digested by *Sac*I and *Hind*III, and then cloned into an expression vector pET-21b to produce hexahistidine-tagged Asd. The resultant expression vector was named pES1, in which the *asd* sequence was verified.

Production and purification of recombinant Asd

After pES1 was transformed into *E. coli* BL21(DE3)pLysS cells, a single colony was inoculated into 5 ml LB media containing ampicillin and chloramphenicol. The overnight culture (2 ml) was transferred to 200 ml of the same media and cultured at 37 °C with shaking. The expression of the *asd* gene was induced by adding β-isopropyl-D-thiogalactoside (IPTG) at a final concentration of 0.4 mM to the cultures that had reached a cell density with an A₆₀₀ of 0.5–1. The temperature was then reduced to 30 °C. Cells were harvested after 5 h, rinsed with 50 mM Na-phosphate buffer (pH 7.4), resuspended in 20 mM Na-phosphate buffer (pH 7.4) with 0.5 M NaCl, and lysed by sonication. The His-tagged Asd was purified to homogeneity using a prepacked fast liquid chromatography (Amersham Pharmacia ÄKTA system) HiTrap chelating column, according to manufacturer's instructions. The recombinant His-tagged Asd was finally eluted with a buffer that contained 360–435 mM imidazole, and was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE), which was performed in 0.375 M Tris–HCl buffer (pH 8.8) with 0.1 % SDS on a 10 % gel, following the method of Laemmli (1970).

Estimation of Asd activity and protein content

Asd activity was determined by measuring the amounts of L-alanine appearance. One unit of decarboxylase activity was defined as the amount of enzyme that produced 1 $\mu\text{mol/min}$ of L-alanine at 37 °C. The standard Asd activity assay was performed by adding 10–50 nM Asd to the reaction mixture that contained 0.5 mM PLP, 1 mM α -ketoglutarate, and 0.1 M K-phosphate buffer (pH 6.8). After preincubation at 37 °C for 40 min, 40 mM L-aspartate (neutralized) was added to trigger the reaction, which was subsequently terminated by boiling. The resulting L-alanine was quantified by three independent methods: (a) A coupled L-amino acid oxidase assay was used as described by Rozzell (1991). (b) An OPA-derivatized high performance liquid chromatography (HPLC) analysis: Reaction products (50 μl reaction mixture) were derivatized by 200 μl *o*-phthaldialdehyde (OPA) reagent solution (Sigma) for 2 min. Analysis using a HPLC Hydrocell RP 10S 150 $\times$ 4.6 mm column (BioChrom Labs, Terre Haute, US), with a linear 14 min gradient of acetonitrile/water mixture (95:5), from 0 to 80 %, with 0.1 % TFA (trifluoroacetic acid), was monitored at 333 nm by a Photodiode Array Detector (Waters). (c) An AccQ-derivatized HPLC analysis: One quarter volume of 10 % 5-sulfosalicylic acid was added to precipitate the protein, and the cool-treated and centrifuged mixture was then filtered through a 0.22- μm filter. Amino acids were derivatized by AccQ (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; Waters) reagent, and HPLC separations were performed on a Symmetry C18 (5 μm , 250 $\times$ 4.6 mm; Waters) column with fluorescence detection. The protein concentration was estimated as described by Bradford (1976) using the Bio-Rad protein assay with bovine serum albumin as the standard.

Biochemical investigations

The kinetics of the recombinant Asd were analyzed in 10 mM K-phosphate buffer (pH 6.8), containing 0.2 mM PLP and 1 mM α -ketoglutarate at 37 °C for 60 min; then, a coupled L-amino acid oxidase reaction was performed with color development for 10 min. The amounts of L-alanine were estimated by OD₅₂₀ with 0–14 mM L-alanine solution as standard (method a).

Enzyme assays were conducted in the standard reaction mixture for 10 min at various temperatures to determine the effect of temperature on Asd activity. A thermostability experiment on Asd was performed by incubating 2.5 μg enzyme samples in 0.1 M phosphate buffer (pH 6.8) at

temperatures between 30 and 60 °C for 10 to 60 min. After incubation, the residual activity was determined using the standard activity assay, and L-alanine was quantified by method a.

The effect of pH on Asd activity was investigated at 37 °C in 75 mM citrate buffer (pH 3.0–5.0), acetate buffer (4.0–5.5), phosphate buffer (6.0–7.5) and Tris buffer (7.5–9.0). The purified enzyme was maintained at 4 °C for 1 day in various buffers to measure its pH stability. The residual activity was analyzed using an HPLC Hydrocell column (method b).

In the examination of the effect of metal ions on Asd, purified active fractions were pooled and dialyzed against 50 mM acetate buffer (pH 5.0) with 10 mM EDTA for 18 h with one buffer change, and then dialyzed against metal-ion-free 50 mM acetate buffer (pH 5.0) for 24 h with three buffer changes to elucidate the effect of chemical reagents on the enzyme activity. The dialyzed enzyme was incubated for 40 min with various metal ions at 37 °C in 100 mM acetate buffer (pH 5.0). The decarboxylation reaction was performed for 20 min by adding 25 mM L-aspartate, and alanine was subsequently quantified by method b.

Aminotransferase assays

Variable amino acids (D-alanine, D-aspartate, D-cysteine, and 20 L-amino acids present in proteins except glycine) and 3 α -keto acids (α -ketoglutarate, oxaloacetate, and pyruvate) were used to investigate the transamination activity of Asd. The purified enzyme was added to 35 nM in 0.3 M acetate buffer (pH 5.0) that contained 0.5 mM PLP and 40 mM α -keto acid. After preincubation at 37 °C for 40 min, 40 mM L-amino acid was added to start the reaction, then followed by boiling to stop it. Products were analyzed using detection method c. One unit of aminotransferase was defined as the amount of enzyme that catalyzes the formation of 1 μmol of the product (glutamate, aspartate, or alanine) per minute.

Gel filtration analysis

The purified recombinant Asd (35 μg) was eluted at 1 ml/min with 50 mM acetate buffer (pH 5.0) or 50 mM phosphate buffer (pH 6.0 or 7.0) on a TSK G3000SW column (7.5 mm ID $\times$ 30.0 cm; Tosoh) and recorded by an online UV detector. The column was calibrated using the Gel Filtration Standard (Bio-Rad) over a molecular mass range of 1.35–670 kDa. Asd was also eluted by fast protein liquid chromatography (FPLC) using a HiPrep 16/60 Sephacryl S-300 HR column (Amersham Pharmacia) to confirm the elution profile and the molecular weight of proteins in major peaks with the same buffer containing 0.15 M NaCl. The protein standards (MW-GF-1000),

Table 1 Comparison of *Pseudomonas* sp. ATCC 19121 Asd with Asd-like sequences from other species

Species	Accession	Nucleotide identity (%)	Amino acid identity (%)	Number of amino acids
Aspartate 4-decarboxylase:				
<i>Pseudomonas</i> sp.	AAQ07948	(100)	(100)	531
<i>Alcaligenes faecalis</i>	AAK58507	77.4	85	533
<i>Pseudomonas dacunhae</i>	BAC65229	78.0	84	533
<i>Zymomonas mobilis</i>	AAV90306	65.3	68	530
<i>Ralstonia metallidurans</i>	ZP_00597251	64.1	61	538
<i>Bacteroides fragilis</i> YCH46	BAD48268	54.5	46	548
<i>Bacteroides fragilis</i> NCTC9343	CAH07159	54.6	46	548
<i>Bacteroides thetaiotaomicron</i>	AAO75842	54.1	45	557
<i>Clostridium perfringens</i>	BAB80047 ^a	41.3	42	528
<i>Enterococcus faecalis</i>	AAO80840	43.3	42	536
<i>Tetragenococcus halophila</i>	BAB92080	48.9	38	532
<i>Lactobacillus sakei</i>	YP_394920	49.9	37	535
Aminotransferase				
<i>Ralstonia eutropha</i>	AAZ62070	68.1	62	541
<i>Ralstonia eutropha</i>	ZP_00168540	67.7	63	536
<i>Burkholderia fungorum</i>	ZP_00282851	62.0	60	554
<i>Burkholderia dolosa</i>	ZP_00982909	64.1	58	546
<i>Vibrio splendidus</i>	ZP_00990937	57.0	50	532
<i>Clostridium perfringens</i>	BAB80707	48.0	44	540
<i>Clostridium beijerinckii</i>	ZP_00910857	49.2	45	545
<i>Lactobacillus casei</i>	ZP_00386474	54.6	44	527
<i>Lactobacillus acidophilus</i>	AAV43507	46.2	39	532

^a Hypothetic protein

ranging from 29 to 2,000 kDa, used in the FPLC analysis were obtained from Simga.

Generation of polyclonal anti-Asd antisera and Western blot analysis

His-tagged recombinant Asd was affinity-purified on a nickel chelate column followed by preparative gel electrophoresis. The gel-purified Asd was then injected into a rabbit to raise antibodies. For Western analysis, eluted fractions were first collected and concentrated by micro-concentrators (Nanosep 10K, Pall Filtron). Proteins quantified as 0.5 µg were denatured by boiling in SDS sample buffer and then resolved by 12 % SDS-PAGE, then transblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore). The membrane was blocked in 12 mM phosphate buffer (pH 7.4) containing 0.8 % (w/v) NaCl, 0.02 % (w/v) KCl (PBS buffer), and 5 % (w/v) skim milk for overnight incubation at 4 °C, and then probed with the rabbit anti-Asd serum for 1 h at room temperature. The membrane was washed three times, 15 min each time, with PBS buffer containing 0.1% (v/v) Tween 20, and then incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma). The immunocomplex was visualized by a colorimetric detection system using the BCIP/NBT substrate from Sigma.

Nucleotide sequence accession number

The complete nucleotide sequence reported in this paper has been deposited in the NCBI nucleotide sequence database under accession number AF506011.

Results

Cloning and sequence analysis of the *Pseudomonas* sp. ATCC 19121 *asd* gene

After screening the *NotI*-digested *Pseudomonas* sp. ATCC 19121 genomic library using the 252 bp probe as described in “Materials and methods” section, one positive clone was identified as having the *asd* gene. Sequencing of the fragment cloned in pBK-*asdP*-A9 revealed a nucleotide sequence of 8.8 kb and includes six open reading frames (ORF) transcribed in the same direction. Functions of the ORFs were predicted for proteins in the database. A putative membrane protein-coding region (ORF2), which encoded a polypeptide with 470 amino acids, was found upstream of the *asd* (ORF3). The Shine-Dalgarno sequence (AGGAGA) was found ahead of both ORF2 and ORF3, and the intergenic noncoding region between them was 55 bp. In the cloned 8.8-kb fragment, two putative

Fig. 1 Unrooted phylogenetic tree based on gene sequences of Asds and homologous proteins using NJ method to show the evolutionary relationships. Sequences were retrieved from GenBank according to the accession number of proteins listed in Table 1. Two branches with only Asds were oval-circled with solid line, and enzymes from gram-positive microorganisms were oval-circled with dotted line

promoters were found upstream of the *asd*. One was about 650 bp upstream of the ORF2, while the other one located in the ORF2 was approximately 1,250 bp upstream of the *asd*. Five ORFs were simultaneously identified for the anti-sense sequence of the 8.8-kb insert. However, no significant similarity was found from among the deduced proteins during the databank search.

The *asd* contained 1,593 bp ORF and encoded a 531-amino-acid Asd with a theoretical molecular mass of 59,243 Da for each subunit. The estimated isoelectric point of the translated protein was 5.62. A putative stem-loop structure was found 116 bp downstream of the stop codon (TAG). The GC content of the cloned *asd* was 65.4 %. This is 9.4 % higher than that of the *asd* from *P. dacunhae* CCRC 12623, and is the highest from among the *asds* in the databank.

Table 1 reveals that the *Pseudomonas* sp. cloned *asd* is 77–78 % identical to the *asd* genes from *P. dacunhae* and *A. faecalis*. The deduced Asd protein from *Pseudomonas* sp. ATCC 19121 shares 45–46 % of identity with the Asds from *Bacteroides* and 37–42 % of identity with those from gram-positive bacteria. When compared with the putative aminotransferases, the Asd exhibits 39–63 % of identity with these extraordinarily long aminotransferases.

We further investigated these similarities using the phylogenetic analysis based on DNA sequences of the relative Asds and aminotransferases. The resultant neighbor-joining tree clustered most of the Asds in two

independent branches (Fig. 1). The *asd* sequences of *Pseudomonas* and *Alcaligenes* were apparently separate from those of the gram-positive microorganisms whose branches were interrelated with the putative Asd and aminotransferases. When compared with the Asd protein sequences, all those listed in Fig. 2 include an aminotransferase class-I PLP attachment site since all such sequences were PLP-dependent enzymes. There were 98 fully conserved amino acid residues from the Asds (indicated as stars in Fig. 2). Most of the residues were also conserved when compared with the putative aminotransferases.

Expression and purification of the recombinant Asd

Plasmid pES1, with codons for the His-tag at the 3' end of the *asd* gene, was constructed and expressed in *E. coli* BL21(DE3)pLysS under the control of the T7 promoter. The recombinant Asd was purified from cell lysates by nickel affinity chromatography and yielded of 3.3 mg of enzyme protein for every 100 ml of culture broth. The specific activity of the homogeneously purified Asd was 280 U/mg at pH 5.0 (37 °C).

Asd productivity was examined by SDS-PAGE (Fig. 3). The molecular mass of the major signal of the 63-kDa protein is in agreement with the molecular weight of 62,657 Da deduced from the recombinant *asd*. Under native conditions, gel-filtration chromatography of the purified recombinant Asd was conducted at pH 5.0 in

Fig. 2 Multiple alignment of deduced amino acid sequences of Asds and putative Asds. Strains with accession numbers are illustrated as in Table 1. Identical amino-acid residues and conservative substitutions are marked as stars and dots, respectively. Residues symbolized in black boxes were supposed to be the three points mentioned by Graber

et al. (1999). The *triangular mark* represents the lysine residue forming Schiff base with PLP during catalyzation. The *boxed conserved region* was predicted as the aminotransferase class-I PLP attachment site. The ATP/GTP binding motif is *boxed as dotted line*. Sequences were retrieved from GenBank and aligned with Clustal X (version 1.83)

acetate buffer. A significant peak with a molecular weight of approximately 760 kDa was demonstrated (Fig. 4) and suggested that Asd exists with a dodecameric structure. When Asd was eluted in phosphate buffer (pH 6.0), a small peak was observed and calibrated as Asd dimer. As the pH kept on rising, more Asd was present in the homodimeric form at pH 7.0 than at pH 6.0 even though most of them still existed in the dodecameric form. The same phenomenon was also observed from the FPLC analysis, and dimers were quantified to shift from 2.5 % (at pH 6.0) of the total protein to 4.4 % (at pH 7.0). Peaks of the calculated dimer and dodecamer were both

collected and verified as Asd by the Western blot analysis (Fig. 4).

Effects of temperature and pH on the recombinant Asd

The optimum temperature for Asd activity was at 45 °C (Fig. 5a). The enzyme exhibited at least half of its maximum activity at temperatures between 30 and 50 °C, and less than 20 % at 60 °C. The recombinant enzyme was stable at temperatures below 40 °C. As shown in Fig. 5b, Asd maintained 80 % of its activity after 1 h of incubation at 50 °C, but nearly lost all of it at 60 °C.

Fig. 3 SDS-PAGE (10 % polyacrylamide) of recombinant Asd from *Pseudomonas* sp. ATCC 19121. Asd overproduced by *E. coli* BL21 (DE3)pLysS/pES1 transformant was purified using the HiTrap Chelating column. Lane 1 molecular weight marker, lane 2 crude enzyme extract (40 μ g protein), lane 3 purified Asd (10 μ g protein). Proteins were stained with Coomassie blue R-250

Figure 5c plots the pH-dependence of recombinant Asd at 37 °C. The activity was maximum at pH 5.0 in the acetate buffer. Moreover, to measure its pH stability in various buffers, the purified Asd was maintained at 4 °C for 1 day. Figure 5d shows that the purified Asd was fully stable at pH 4.0–8.5.

Effect of metal ions and reagents

The activity of recombinant Asd was strongly inhibited by 10 mM Co^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} , Ni^{2+} , and Zn^{2+} (Table 2) to below 10 % of the residual value, while the presence of 1 mM Hg^{2+} in the reaction mixture decreased the enzyme activity to an undetectable level. Zinc ions in 0.1 mM reduced the Asd activity by approximately 5% while

10 mM Ca^{2+} reduced it by 14%. Mg^{2+} ions moderately inhibited the Asd activity at 64%. All the added metal ions suppressed the recombinant Asd activity, while 10–20 mM EDTA improved the enzyme activity by 10%. The presence of 1 mM of the reducing agent DTT or 2-mercaptoethanol increased the Asd activity by 7%.

Kinetics and substrate as well as reaction specificity of the recombinant Asd

Using L-aspartate as the substrate for the purified recombinant Asd, the K_m value in β -decarboxylation reaction was estimated at 11.50 mM using detection method a. The maximum velocity was 0.11 mM/min at pH 6.8 in 10 mM phosphate buffer containing 0.2 mM PLP. Although Asd is highly specific to the β -decarboxylation of L-aspartate, we tried to examine its β -elimination ability towards other amino acids. In the present study, various kinds of amino acids such as those described in the “Materials and methods” section of the aminotransferase assay were used to perform enzyme reactions independently in the presence of α -ketoglutarate at pH 5.0. A tiny quantity of the eliminated product was examined by detection method c with a specific activity of 0.128 U/mg when L-asparagine was used as substrate. When the substrate was changed to D-aspartate or L-lysine, one quarter of the product was generated when compared to the reaction of L-asparagine. During the tests with L-glutamate and L-glutamine, a very small amount of the elimination product was detected (Table 3).

The recombinant Asd also displayed aminotransferase activity, as shown in Table 3. The amino group could be transferred from either L-aspartate or D-aspartate to α -ketoglutarate under the Asd catalysis and produced glutamate with specific activities of 0.113 and 0.042 U/mg, respectively. With about 26 and 22% of the former and the latter specific activities, respectively, pyruvate was also a good amino acceptor when the amino acids were L-glutamine and L-glutamate. Under the same conditions, no transamination reaction of the tested amino acids was observed when oxaloacetate served as the amino group acceptor.

Discussion

The present work demonstrated the *asd* cluster from *Pseudomonas* sp. ATCC 19121, and presumably encoded three enzymes involved in the amino acid metabolism and the two membrane proteins. In the present study, *asd* is located 55 bp downstream of the probable aspartate-alanine antiporter gene, and oriented contrary to the *asp* operon in the *Tetragenococcus halophilus* reported by Abe et al.

Fig. 4 HPLC gel-filtration chromatographic analyses of the recombinant Asd. Recombinant Asd incubated in acetate buffer (pH 5.0) was stored at 4 °C for 2 h. Profiles of the enzyme elution at pH of 5.0 and 7.0 are labeled. Each peak depicted as dodecamer and dimer, which collected during elution, was proved to be Asd through Western analysis

Fig. 5 Effect of pH and temperature on Asd activity and stability. **a** Optimal temperature of Asd reaction, **b** thermal stability of the recombinant Asd, **c** optimal pH for Asd reaction, and **d** pH stability of the recombinant Asd. Reaction conditions were as described in “Materials and methods” section

(2002). Its RBS sequence is found 7 bp upstream of the ATG initiation codon. Combining the actions of the Asd and the antiporter was supposed to establish an electrochemical proton gradient and thereby exploited for ATP

Table 2 Effect of divalent cations and various reagents on the recombinant Asd activity

Reagents	Conc. (mM)	Relative activity ^a (%)
None	—	100
Ca ²⁺	10	86.1
Co ²⁺	10	6.9
Cu ²⁺	0.1	20.3
	10	0.5
Fe ²⁺	0.1	72.1
	10	7.4
Hg ²⁺	1	0.0
Mg ²⁺	10	64.0
Mn ²⁺	10	7.8
Ni ²⁺	10	8.4
Zn ²⁺	0.1	94.9
	10	7.7
EDTA ^b	10	110.9
	20	111.3
2-Mercaptoethanol	1	107.1
	2	101.1
DTT ^c	1	107.4
	10	105.2

^a Values shown are the means of duplicate determinations with a standard deviation of less than 20 %

^b EDTA, ethylenedinitrilotetraacetic acid

^c DTT, dithiothreitol

synthesis by H⁺-coupled F₁F₀ATP synthases (Dimroth and Schink 1998). Meanwhile, an ATP/GTP binding motif was observed in the Asd sequence, and the aspartate β-decarboxylation was supposed to contribute to the conservation of energy in the studied *Pseudomonas* strain being studied. The other two genes of potential interest are the *ilvD* (dihydroxy-acid dehydratase) and the *dgdR* (2,2-dialkylglycine decarboxylase repressor protein). The former dehydrates dihydroxy acids in the biosynthesis of L-valine and L-isoleucine from pyruvate and threonine, respectively, while the latter regulates the expression of the *dgd* gene and suppresses the generation of L-alanine and CO₂ from pyruvate and 2,2-dialkylglycine. DgdR influences the enzymatic production of L-alanine. Because it is the same product as the β-decarboxylation of aspartate, Asd was assumed to cooperate with the DgdR control in vivo. All genes in the *asd* cluster from *Pseudomonas* sp. were supposed to be involved in the metabolic pathways of pyruvate and aspartate families.

Considerable percentage of identities, 84 % for the gene and 93% for the amino acid sequence, were exhibited between Asds from *A. faecalis* and *P. dacunhae*. However, based on the phylogenetic analysis (Fig. 1), a lower percentage of identity of the Asd was observed between *Pseudomonas* sp. and *P. dacunhae* (Table 1) from the same genus—*Pseudomonas*. This suggested that Asd from *P. dacunhae* is more closely related to enzyme evolution of *A. faecalis* than to that of *Pseudomonas* sp. ATCC 19121. Moreover, the GC content of *asd* from *Pseudomonas* sp. ATCC 19121 and *P. dacunhae* were found to be 65.4 and 56%, respectively.

Table 3 Substrate specificity of the recombinant Asd on β -elimination and transamination activity

Substrate ^a	Keto acid	Specific activity (U/mg)	Relative activity ^b (%)
β -Elimination			
L-Asparagine	α -Ketoglutarate	0.128	100.0
D-Aspartate	α -Ketoglutarate	0.032	25.1
L-Lysine	α -Ketoglutarate	0.028	21.7
L-Glutamine	α -Ketoglutarate	0.014	10.7
L-Glutamate	α -Ketoglutarate	0.009	6.8
Transamination			
L-Aspartate	α -Ketoglutarate	0.113	100.0
D-Aspartate	α -Ketoglutarate	0.042	37.6
L-Glutamine	Pyruvate	0.030	26.4
L-Glutamate	Pyruvate	0.025	22.1
L-Alanine	α -Ketoglutarate	0.015	13.2

^a In addition to the data listed above, no reaction product could be detected when using other tested amino acids as the substrate

^b Detection method c is too sensitive to correctly quantify the excess alanine produced in the β -decarboxylation reaction of L-aspartate when α -ketoglutarate exists. Therefore, the reaction of L-asparagine that can be well quantified as that of the highest level was defined to be 100 % in the β -elimination reaction. As for the transamination reaction, the specific activity between L-aspartate and α -ketoglutarate was defined to be 100%

These differences in both the GC content and sequence identities of Asd were probably due to the genetic diversity of *Pseudomonas* (Dogan and Boor 2003).

Like other microbial Asds, our His-tagged recombinant Asd has similar optimal pH values and optimal reaction temperatures. Furthermore, the pH stability results (Fig. 5d) in the present study suggested that the enzyme is stable over a broad pH range. Based on the gel-filtration profiles (Fig. 4), majority (>95 %) of recombinant Asd exhibited the dodecameric form as the pH shifted from 5.0 to 7.0. A similar phenomenon was previously observed in the sedimentation equilibrium test for purified wild-type Asd from *A. faecalis* by Bowers et al. 1970. To identify the electron microscopic appearance of the recombinant Asd, we found the dodecameric holoenzyme to be very similar to the wide-type Asds from *P. dacunhae* (Tate and Meister 1970) and *A. faecalis* (Bowers et al. 1970) at pH 5.0 (data not shown).

The effect of metal ions on pure Asd has never been reported. However, El-Rahmany found that Mn^{2+} , Ni^{2+} , Co^{2+} , and Fe^{2+} (all in 10 mM) enhanced the Asd activity by 20–38 %, while 10 mM EDTA reduced it by 62 % in cell-free extracts of *Cunninghamella elegans* and *Penicillium citrinum* (El-Rahmany 1994). In the present investigation, the purified recombinant Asd was dialyzed as described in the “Materials and methods” section. Because metal ions could inhibit the coupled L-amino acid oxidase reaction and bias the quantification of Asd product, L-alanines were analyzed by HPLC. All metal ions examined herein inhibited the Asd activity, while the four metal ions mentioned earlier did not increase but inhibit the Asd activity by up to 90%.

In the present study, the recombinant Asd β eliminated L-asparagine in detectable amounts. L-Aspartate was still the best substrate for the β -decarboxylation reaction with a

specific activity of about 2,187-fold. While D-aspartate served as the substrate in the presence of α -ketoglutarate in the Asd catalyzed reaction mixture, a little amount of both alanine produced by β -decarboxylation and glutamate produced by transamination, were determined. As compared to the reaction between L-aspartate and α -ketoglutarate, L-aspartate was β -decarboxylated 8,715 times more and transaminated 2.7 times more than the D-aspartate under the catalyzation of the recombinant Asd. It suggested that the Asd recombinant protein is not strongly stereospecific for L-amino acids in the transamination reaction.

Altering the reaction specificity of PLP-dependent enzyme by point mutations has attracted some interest in recent times. Few *asd* sequences were cloned until recently, and the consequent lack of *asd* sequence information has retarded the progress in elucidating the structural relationship between Asd and aminotransferase. In Fig. 2, residues marked as black squares correspond to the three residues mutated in aspartate aminotransferase, based on the molecular modeling of the partial Asd structure and using tyrosine aminotransferase as template (3tat in Protein Data Bank). Asd residues, which were supposed to interact with α - and β -carboxyl groups of the substrate, L-aspartate, and the O-3' of cofactor PLP were the same as those mutated in aspartate aminotransferase. Therefore, we have proposed that these three residues could not be the only key determinants in enzyme catalysis.

In summary, an *asd* cluster involved in the metabolic pathway of aspartate families was cloned in the present study. Moreover, biochemical characterization and reaction specificity studies of the recombinant Asd led to interest in the protein structure of the holoenzyme. Because no Asd structure has yet been determined, the authors are currently undertaking the analysis of X-ray diffraction data from this

recombinant Asd. From these results, we expect that amino acid residues governing the pathway of the reaction intermediates could be identified in the near future.

Acknowledgements This work was supported by grants 90AS-2.1.1-FD-Z4(1) and 91AS-3.1.1-FD-Z5(3) from the Council of Agriculture, and was partially supported by grant NSC92-2313-B-002-092 and NSC94-2313-B-002-049 from the National Science Council, Taipei, Taiwan.

References

- Abe K, Ohnishi F, Yagi K, Nakajima T, Higuchi T, Sano M, Machida M, Sarker RI, Maloney PC (2002) Plasmid-encoded *asp* operon confers a proton motive metabolic cycle catalyzed by an aspartate-alanine exchange reaction. *J Bacteriol* 184:2906–2913
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Bowers WF, Czubaroff VB, Haschemeyer RH (1970) Subunit structure of L-aspartate β -decarboxylase from *Alcaligenes faecalis*. *Biochemistry* 9:2620–2625
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Chen CC, Chou TL, Lee CY (2000) Cloning, expression and characterization of L-aspartate β -decarboxylase gene from *Alcaligenes faecalis* CCRC 11585. *J Ind Microbiol* 25:132–140
- Chibata I, Kakimoto T, Kato J (1965) Enzymatic production of L-alanine by *Pseudomonas dacunhae*. *Appl Microbiol* 13: 638–645
- Crawford LV (1958) Studies on the aspartic decarboxylase of *Nocardia globetula*. *Biochem J* 68:221–225
- Dimroth P, Schink B (1998) Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. *Arch Microbiol* 170:69–77
- Dogan B, Boor KJ (2003) Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. *Appl Environ Microbiol* 69:130–138
- Dunathan HC (1966) Conformation and reaction specificity in pyridoxal phosphate enzymes. *Proc Natl Acad Sci USA* 55:712–716
- El-Rahmany TA (1994) Comparison of L-aspartate 4-carboxy-lyases of *Cunninghamella elegans* and *Penicillium citrinum*. *Microbiol Res* 149:253–257
- Graber R, Kasper P, Malashkevich VN, Strop P, Gehring H, Jansonius JN, Christen P (1999) Conversion of aspartate aminotransferase into an L-aspartate β -decarboxylase by a triple active-site mutation. *J Biol Chem* 274:31203–31208
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Nishimura JS, Manning JM, Meister A (1962) Studies on the mechanism of activation of aspartic acid β -decarboxylase by α -keto acids and pyridoxal 5'-phosphate. *Biochemistry* 1:442–447
- Novogrodsky A, Nishimura JS, Meister A (1963) Transamination and β -decarboxylation of aspartate catalyzed by the same pyridoxal phosphate-enzyme. *J Biol Chem* 238:PC1903–PC1905
- Rathod PK, Fellman JH (1985) Identification of mammalian aspartate-4-decarboxylase. *Arch Biochem Biophys* 238:435–446
- Rozzell JD (1991) Method and compositions for the production of L-alanine and derivatives. US Patent No. 5,019,509
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425
- Sambrook J, Fritsch EF, Manniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
- Tate ST, Meister A (1970) Regulation and subunit structure of aspartate β -decarboxylase. Studies on the enzymes from *Alcaligenes faecalis* and *Pseudomonas dacunhae*. *Biochemistry* 9:2626–2632
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Wilson EM, Kornberg HL (1963) Properties of crystalline L-aspartate 4-carboxy-lyase from *Achromobacter* sp. *Biochem J* 88:578–587