Characterization of NADP-dependent L-arginine dehydrogenase as a novel amino acid dehydrogenase and its application to an L-arginine assay

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Abstract

L-Arginine dehydrogenase (L-ArgDH, EC 1.4.1.25) is an amino acid dehydrogenase which catalyzes the reversible oxidative deamination of L-arginine to the oxo analog in the presence of NADP. Although the enzyme activity is detected in the cell extract of *Pseudomonas aruginosa*, the purification and characterization of the enzyme have not been achieved to date. We here found the gene homolog of L-ArgDH in genome data of *Pseudomonas veronii* and succeeded in expression of *P. veronii* JCM11942 gene in *E. coli.* The gene product exhibited strong NADP-dependent L-ArgDH activity. The crude enzyme was unstable under neutral pH conditions, but was markedly stabilized by the addition of 10% glycerol. The enzyme was purified to homogeneity through a single Ni-chelate affinity chromatography step and consisted of a homodimeric protein with a molecular mass of about 65 kDa. The enzyme selectively catalyzed L-arginine oxidation in the presence of NADP with maximal activity at pH 9.5. The apparent K_m values for L-arginine and NADP were 2.5 and 0.21 mM, respectively. The nucleotide sequence coding the enzyme gene (was determined and the amino acid sequence was deduced from the nucleotide sequence. As an application of the enzyme, simple colorimetric microassay for L-arginine using the enzyme was achieved.

Key words L-arginine dehydrogenase, *Pseudomonas veronii,* molecular cloning, NADP-dependent amino acid dehydrogenase, L-arginine determination

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Introduction

More than twenty different amino acid dehydrogenases (EC 1.4.1.-), catalyzing the reversible deamination of amino acids to their corresponding oxo analogs in the presence of NAD(P), have been found so far. Among them, glutamate dehydrogenase, leucine dehydrogenase, alanine dehydrogenase and phenylalanine dehydrogenase have all been well characterized and are widely used for industrial asymmetric production of L-amino acids and their analogs [1-4], which then serve

as important nutrients and pharmaceutical compounds. In addition, amino acid dehydrogenases have been used for sensing amino acids in foods and blood and for enzyme assays [1, 5]. For those reasons, novel amino acid dehydrogenases have the potential to be highly useful [2]. Among these enzymes, NAD(P)-dependent L-arginine dehydrogenase (EC 1.4.1.25, L-ArgDH) is one of the most recently discovered and is known to physiologically function in concert with FAD-dependent D-arginine dehydrogenase for the conversion (epimerization) of D-arginine to L-arginine via its oxo-analog, 5-guanidino-2-oxopentanoate, in *Psuedomonas aeruginosa* POA1 [6]. The gene *DauB* in *P. aeruginosa* has been expressed in *Escherichia coli*, and the product reportedly catalyzes the reversible deamination of L-arginine to 5-guanidino-2-oxopentanoate in the presence of NAD(P). However, the molecular and catalytic properties of L-ArgDH have not yet been reported.

We previously identified a putative L-ArgDH gene, PverR02_12350, within the genome of the nonpathogenic bacterium *P. veronii* (KEGG Organisms: Complete Genomes: www.genome.jp/kegg/catalog/org_list. Html). This gene possesses high amino acid sequence homology (67.8 %) with a gene encoding L-ArgDH in the pathogenic bacterium *P. aeruginosa* POA1. Here, we succeeded in the expressing the *P. veroni*i JCM 11942 gene in *E. coli*, using artificial primers synthesized from the N- and C-terminal sequences inferred from the putative L-ArgDH gene. The expressed product was purified, characterized, and applied to a simple colorimetric assay for L-arginine.

Materials and methods

Materials

L-Arginine, NADP and NAD were purchased from Wako Pure Chemical Corporation (Tokyo, Japan). Methoxy-phenasine methosulfate (m-PMS), water soluble tetrazolium 1 (WST-1) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) were from Chemical Dojin Co., Ltd. (Kumamoto, Japan). Yeast extract and Luria-Bertani (LB) medium were from Becton, Dickinson and Company (Sparks, MD, USA), and hipolypepton S was from Nihon Pharmaceutical Co., Ltd. (Tokyo). *P. veronii* JCM 11942 was obtained from the Japan Collection of Microorganisms (Wako, Saitama, Japan). Ni Sepharose 6 First Flow resin was purchased from Cytiva (Tokyo, Japan). Infusion vector was obtained from Takara Bio Inc. (Kusatsu, Shiga, Japan). All other chemicals were of reagent grade.

Cloning a putative ArgDH gene from *P. veronii* JCM 11942 and determination of the gene sequence

P. veronii JCM 11942 was aerobically grown in the medium（150 mL in a 0.5 L flask）containing 0.5% hipolypepton, 0.3% beef extract, and 0.5% NaCl for 24 h at 30℃, after which the cells 1.3 g (wet weight) were harvested by centrifugation. The cells were homogenized using a Multi-beads

Shocker (Yasui Kikai, Osaka, Japan), and the crude DNA extract was prepared using the phenol-chloroform-isoamyl alcohol method. To remove RNA, the DNA extract was treated with RNase A, after which the genomic DNA was purified using phenol-chloroform-isoamyl alcohol. The L-ArgDH gene was amplified by PCR using the genomic DNA as the template with the primers 5'-GGAGATATACATATGTCCAGCACGCCCCATGTCATC-3' (forward) and 5'-GCTCGAATTCGGATCGCGCTGCAGCTGGTACAGCG-3' (reverse). The forward and reverse primers contained the sequence of pET21a (underlined) and the putative ArgDH gene, *PverR02_12350*, queried from the Genome Net database. The expression vector pET21a was linearized using inverse PCR with the primers 5'-CATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTT-CTAGAGGGG-3' (forward) and 5'-GATCCGAATTCGAGCTCCGTCGACAAG-3' (reverse). The amplified target gene fragments were ligated into the linearized pET21a vector using an In-Fusion HD cloning kit (Takara Bio Inc.). The sequence of the L-ArgDH gene inserted into pET21a was determined by Hokkaido System Science (Hokkaido, Japan).

Growth conditions for *E. coli* BL21(DE3) transformed with pET21a-L-ArgDH and overexpression of L-ArgDH

E. coli BL21(DE3) was transformed with the prepared expression vector (pET21a-L-ArgDH) and cultured to logarithmic phase (OD_{660} = 0.5) at 37ºC in 150 mL of LB medium containing 0.1

mg/mL ampicillin. IPTG (final 0.1 mM) was then added to the culture medium, and the incubation was continued for an additional 3 h at 37ºC. The cells were then collected by centrifugation and stored at −80ºC until use.

Activity and protein assays

The standard assay for L-ArgDH activity was performed at 30°C and entailed measuring the initial rate of NADPH formation from NADP and L-arginine. The reaction mixture consisted of 0.2 M glycine-KOH buffer (pH 9.5), 10 mM L-arginine (pH 9.5), 1.25 mM NADP and the enzyme in a final volume of 1.00 mL. The mixture solution (0.96-0.99 mL) without enzyme was incubated at 30°C for about 3 min in a cuvette with 10-mm light path. The reaction was then started by addition of enzyme (0.01-0.04 mL). The initial increase in absorbance at 340 nm was measured under temperature-controlled cell conditions at 30°C. One unit of enzyme was defined as the amount of enzyme producing 1 μmol of NADPH per minute at 30°C. The absorption coefficient (6.2 mM^{-1} cm^{-1}) of NADPH at 340 nm was used. Protein concentrations were calculated from the absorbance at 280 nm (1.0 mg/mL=0.80 obtained from the amino acid composition of L-ArgDH). Replicability was ensured by making the measurements triplicate.

Enzyme purification

E. coli cells expressing the recombinant enzyme (wet weight: about 1.3 g wet weight obtained from a 150 mL culture medium) were suspended in 20 mM NaH_2PO_4-Na_2PO_4 buffer (pH 7.9) solution (6 mL) containing 10% glycerol, 0.5 M NaCl, 0.01% 2-mercaptethanol and 5 mM imidazole, and disrupted by sonication. The crude extract (about 7 mL) was prepared as the supernatant clarified by centrifugation (12,000 g x10 min) and applied on a Ni Sepharose High Performance column (φ10 x 66 mm). The column was washed first with the same buffer solution (about 36 mL with a 3-mL fraction) and then with the same buffer solution (30 mL) but with 60 mM imidazole. The active enzyme was eluted with 20 mM KH_2PO_4-K_2HPO_4 buffer solution (pH 7.2, about 40 mL) containing 10% glycerol, 0.5 M NaCl, 0.01% 2-mercaptethanol and 400 mM imidazole. The active enzyme solution was dialyzed against 20 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.2) containing 10% glycerol and 0.01% 2-mercaptethanol and then stored in refrigerator (4℃ or -20℃) before use.

Effects of glycerol and pH on enzyme stability

The recombinant *E. coli* cells were suspended in 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.2) supplemented with 0.01% 2-mercaptoethnol and disrupted by sonication with or without addition of 10% glycerol. After centrifugation (12,000 g for 10 min), the supernatant was stored at 4℃ and the time-dependent changes in L-ArgDH activity were monitored.

To assess the effect of pH on enzyme stability, the enzyme was incubated for 30 min in different

pH buffer solutions (50 mM, pH 6-9.5) containing 10% glycerol and 0.01% 2-MET, after which the activity of an aliquot (10 μL) was measured under the standard assay conditions for NADP-dependent L-ArgDH formation.

Effect of pH and temperature on enzyme activity

Enzyme activity was measured in different pH buffers under the standard assay conditions. The buffers used were 0.2 M K_2HPO_4-KH_2PO_4 (pH 6.8-8.5) and glycine-KOH (pH 9-10.5). To evaluate the effect of temperature on its stability, the enzyme (in 20 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.5, containing 10% glycerol and 0. 01% 2-MET) was incubated for 30 min at different temperatures, after which the remaining activity was measured under the standard assay conditions.

Polyacrylamide electrophoresis (PAGE)

Native-PAGE (disc gel) was carried out at 4°C on a 7.5% polyacrylamide gel, as previously described [7]. The protein was then stained using 0.025% Coomassie brilliant blue R-250 in 50% methanol and 10% acetate. In addition, active staining was performed at 30°C using a reaction mixture (5.0 mL) containing 0.20 M K_2HPO_4-KH_2PO_4 buffer (pH 8.0), 1.0 mM NADP, 0.2 mM m-PMS, 1 mM INT, and 10 mM L-arginine (pH 8.0) or other amino acids (pH 8.0) until a red band of sufficient intensity was visible after performing the native-PAGE. Sodium dodecyl sulfate (SDS)-

PAGE was carried out on 12.5% polyacrylamide gel using the method of Laemmli [8]. Precision Plus protein standards (Bio-Rad Laboratories, CA, USA) were used as the molecular mass standards. The protein sample was boiled for 5 min in 10 mM Tris-HCl buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol. Protein bands were visualized by staining with 0.025% Coomassie brilliant blue R-250 in 50% methanol and 10% acetate.

Substrate specificity and kinetic parameters

The ability of amino acids to serve as electron donors was assessed based on activity staining on native disc PAGE. Activity staining was performed as described above with various amino acids (10 mM, pH 8) other than L-arginine in the assay mixture. To determine the Michaelis constants (K_m) for L-arginine and NAD(P), initial velocity analyses were carried out under the standard assay conditions, after which the K_m values for the substrates were determined using double reciprocal plots of initial velocity and substrate concentration. The data were fitted to the Michaelis-Menten equation.

Molecular mass determination

The molecular mass of the native enzyme was determined by gel filtration column chromatography using a Superdex 200 pg column (2.6 × 60 cm). Ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa) and α-chymotrypsinogen (25 kDa) served as molecular standards (GE

Healthcare). Subunit molecular mass was determined by SDS-PAGE (12.5% acrylamide slab gel, 1 mm thick) using the same marker proteins (Takara Bio).

Sequence alignments

The amino acid sequences of L-ArgDHs and the putative enzyme were collected from the KEGG database (https://www.genome.jp/kegg/) and aligned using Clustal W multiple sequence alignment software (ver. 2.1) [9] via the DNA Data Bank of Japan (DDBJ) website (https://www.ddbj.nig.ac.jp/index.html).

Determination of L-arginine using L-ArgDH

The enzymatic method for determining L-arginine levels using L-ArgDH consists of two reaction steps. The first step is the quantitative conversion of L-arginine to its oxo analog and ammonia with conversion of NADP to NADPH by L-ArgDH. The reaction mixture (0.40 mL) consisted of 100 mM glycine-KOH buffer (pH 9.5), 2.5 mM NADP, the enzyme (0.42 U) and L-arginine (≦100 μM). The second step is the chemical conversion of NADPH to reduced WST-1 (formazan) via a redox mediator (m-PMS). The reaction mixture was incubated at 30°C for 10 min and then 400 mM K_2HPO_4-KH_2PO_4 buffer solution (pH 6.8, 0.40 mL) containing 2 mM m-PMS (0.10 mL) and 5 mM WST-1 (0.10 mL)

were added giving a final volume of 1.00 mL. The formazan formed from WST-1 was determined by measurement of absorbance at 438 nm.

Results and Discussion

Expression, stabilization, and purification of NADP-dependent L-ArgDH

A putative gene encoding L-ArgDH in the nonpathogenic bacterium *P. veronii* JCM 11942 was successively expressed in *E. coli* cells, after which the cell extract exhibited strong NADP-dependent L-arginine dehydrogenase activity. However, the enzyme in the crude extract containing 20 mM phosphate buffer, pH 7.9 or 7.2, supplemented with 0.01% 2-MET was somewhat unstable, and nearly all activity was lost within 2 days (Fig. 1). We therefore evaluated the stabilizing effect of adding glycerol to the enzyme solution and found that the enzyme was greatly stabilized by the addition of 10% glycerol to the crude extract (Fig. 1). In the presence of 10% glycerol, no loss of enzyme activity was observed for at least one month at temperatures of 4°C or -20°C. The enzyme was therefore stored in 20 mM phosphate buffer (pH 7.2) supplemented with 10% glycerol and 0.01% 2-MET unless otherwise stated. The enzyme was then effectively purified to homogeneity (Fig. 2) with a high yield using His-tagged affinity chromatography (Table 1).

Enzymological properties

Using gel filtration chromatography, the molecular mass of the native enzyme was determined to be 66 kDa. In addition, SDS-PAGE gave a single band, and the subunit molecular mass of was calculated to be 34 kDa. This indicates the enzyme consists of dimeric structure with two identical subunits. The ability for L-arginine and several other amino acids to serve as electron doners was evaluated based on activity staining on native-PAGE. When L-arginine was used as the electron doner, a distinct activity band was detected, and the mobility was identical to that of the protein staining band (Fig. 2). By contrast, no activity band was observed after activity staining on native-PAGE with D-arginine, L-lysine, L-ornithine, L-citrulline, L-leucine, L-phenylalanine, L-histidine, L-glutamate, glycine or L-alanine. This means that this enzyme is highly selective for L-arginine. As the electron acceptor, NADP was much more preferable than NAD; the reaction rate for NADP was about 4 times higher than that for NAD under the standard assay conditions. This is in contrast to the findings of Lie and Lu [6], who reported that using *P. aeruginosa* L-ArgDH, the reaction rate with NADP was nearly the same as with NAD.

Next, the effects of temperature and pH on the enzyme's stability were examined. After incubation for 30 min, the enzyme retained all of its activity at temperatures up to 35℃, but it lost more than 90% of its activity at 45℃ (Fig. 3). In addition, the enzyme activity increased with increasing temperature up to 30℃. At that temperature, the enzyme was stable between pH 7 and 8.5

in the presence of 10% glycerol and 0.01% 2-MET (Fig. 4), but the activity markedly declined below pH 6.5 or above pH 9 (Fig. 4). From the analysis of the pH effect on L-arginine oxidation, the maximum activity was observed at around pH 9.5 (Fig. 5A). Similarly high optimum pHs for activity were previously observed with other amino acid dehydrogenases, including alanine, glutamate, phenylalanine, leucine and tryptophan dehydrogenases [1, 11]

Plots of the initial velocity versus both L-arginine and NADP concentrations gave typical Michaelis-Menten type kinetics. Using double reciprocal plots of the initial velocities and substrate concentrations, the apparent *K*m values for L-arginine and NADP were determined to be 2.5 mM and 0.20 mM, respectively.

After determining the gene sequence of *P. veronii* JCM 11942 L-ArgDH, the deduced amino acid sequence (315 amino acid residues) was aligned with those of *P. aeruginosa* PO1 and the putative *P. veronii* L-ArgDHs (Fig. 6). Of the 315 amino acid residues comprising *P. veronii* JCM 11942 L-ArgDH, only two, A42 and N95, differed from those (P42 and S95, respectively) of the putative *P. veronii* PverR02_12350 enzyme (sequence homology: 99.4%). By contrast, the sequence of *P. veronii* JCM 11942 L-ArgDH had only 67.8% homology with the *P. aeruginosa* PAO1 enzyme, which is rather low for an enzyme from the same *Pseudomonas* family. In addition, a phylogenetic tree prepared using the Clustal W data base with the amino acid sequences of L-ArgDHs in the *Psuedomonas* family showed that *P. veronii* L-ArgDH formed a different cluster from that of the *P.*

aeruginosa enzyme (Fig. 7). This is consistent with *P. veronii* L-ArgDH exhibiting different catalytic properties than the *P. aeruginosa* enzyme. As mentioned, for example, *P. veronii* L-ArgDH is much more selective for NADP over NAD as the electron acceptor, whereas the *P. aruginosa* PAO1 enzyme utilizes NADP and NAD equally [6]. This tree shows that ArgDH widely distributes among *Pseudomonas* species, and we are now investigating the three-dimensional structure of *P. veronii* JCM 11942 L-ArgDH as a new amino acid dehydrogenase.

L-Arginine determination using L-Arg DH

The simple colorimetric assay for L-arginine consisted of two steps because of the different optimum pHs for enzymatic NADPH formation with L-arginine catalyzed by L-ArgDH (pH 9.5) and the reduction of WST1 to formazan with NADPH (pH≦8.0). A linear relationship was obtained between the L-arginine concentration (≦100 μM) and absorbance at 438 nm (Fig. 8). This colorimetric method is simple, rapid (the reaction time ≦ 10 min) and highly specific for L-arginine. The method is applicable to many food and clinical analyses because L-arginine is an intermediate in the human urea-cycle and is an essential amino acid in humans. Although a colorimetric method for assaying L-arginine using five different enzymes has been reported [12], our simple method for assaying L-arginine may be much more attractive for industrial and clinical uses.

Conclusions

We found the gene homolog of L-ArgDH in genome data of *Pseudomonas veronii.* The *P. veronii* JCM11942 gene was successively expressed in *E. coli*, and the gene product exhibited strong NADP-dependent L-ArgDH activity. The enzyme was unstable but was stabilized by the presence of 10% glycerol under neutral pH conditions. The enzyme was purified to homogeneity through a single Ni-chelate affinity chromatography step and consisted of a homodimeric protein with a molecular mass of about 65 kDa. The enzyme selectively catalyzed L-arginine oxidation in the presence of NADP, with maximal activity at pH 9.5. The apparent K_m values for L-arginine and NADP were 2.5 and 0.21 mM, respectively. Furthermore, A simple colorimetric microassay for L-arginine was achieved using the enzyme.

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Statements and declarations

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Competing interest

The authors declare that they have no competing interests.

Author contribution All authors contributed to the study conception and design, and performed

material preparation, data collection and analysis. Toshihisa Ohshima mainly wrote the manuscript in discussion during the preparation of the manuscript.

Table 1. Summary of L-ArgDH purification

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	95.2	153	1.61	100
Ni-chelate affinity chromatography	19.9	61.4	3.09	40

Legends to Figures

Fig. 1. Effect of glycerol addition on L-ArgDH storage. L-ArgDH was stored at 4°C in 20 mM H_2PO_4-K_2HPO_4 phosphate buffer (pH 7.2) containing 0.01% 2-MET with (●) or without (■) 10% glycerol. Aliquots (10 μL) of the enzyme solution are depicted, and the activity was measured under the standard assay conditions (n=3).

Fig. 2. Native disc-PAGE of purified L-ArgDH. The enzyme (about 125 μg/tube) was applied on a disc gel and the gel was stained with CBB (A) or from L-arginine activity (B) after electrophoresis.

Fig. 3. Effect of temperature on L-ArgDH stability. Solution containing the purified enzyme (20 mM KH_2PO_4-K_2HPO_4 buffer, pH 7.2, containing 10% glycerol and 0.01% 2-ME) was incubated for 30 min at different temperatures. The activity remaining in aliquots (10 μL) of the solution was then measured under the standard assay conditions. (n=3)

Fig. 4. Effect of pH on L-ArgDH stability. The enzyme was incubated in 50 mM buffer solutions (10% glycerol +0.01% 2-ME) with different pHs for 30 min at 30°C. The activity remaining in aliquots (10 μL) of the solution was the measured under the standard assay conditions. The buffers used were KH_2PO_4-K_2HPO_4 (pH 6.0-8.5) and glycine-KOH (pH 9.0-10). (n=3)

Fig. 5. Effects of temperature (A) and pH (B) on L-ArgDH activity. Enzyme activity was measured at different temperatures (A) and in different pH buffers (B) under the standard assay conditions. The buffers used in (B) were 0.2 M KH_2PO_4-K_2HPO_4 (■), Tris-HCl (▲), and Gly-NaOH (◆).

Fig. 6. Amino acid sequence of *P. veronii* JCM 11942 L-ArgDH and the alignments with the sequences of the putative *P. veronii* Pver_12550 and *P. aeruginosa* PA01_PA3862 L-ArgDHs. (●): Amino acid residues in the sequence of *P. veronii* JCM 11942 L-ArgDH that differed from those in the sequence of the putative *P. veronii* Pver_12550 L-ArgDH. (—): Adenine binding motif (GXGXXA(G)) for NADP. The sequence added as a his-tag at the C-terminus of *P. veronii* JCM 11942-L-ArgDH is DPNSSSVDKLAAALEHHHHHH. The sequence alignment was prepared using Clustal W [9] .

Fig. 7. Condensed phylogenetic tree based on the amino acid sequences of *P. veronii* JCM 12942 L-ArgDH and homologous proteins from *Pseudomonas* and *Burkholderia* strains. The phylogenetic tree was prepared using Clustal W [9] 1994) and NJ plot (Perriére and Gouy, 1996). Abbreviations:

P.: *Pseudomonas* and B.: *Burkholderia*. Rectangles show *P. veronii* JCM 11942 and *P. aeromonas* PAO1 L-ArgDHs; the others are putative ornithine cyclodeaminase or L-ArgDH.

Fig. 8. Calibration curve for L-arginine determination in an assay using L-ArgDH. Oxid and Red m-PMS: methoxy-phenagine methosulfate oxidized and reduced forms, respectively. WST-1 water soluble tetrazolium.

Figures

Figure 1

Effect of glycerol addition on L-ArgDH storage. L-ArgDH was stored at 4□ in 20 mM H2PO4-K2HPO4 phosphate buffer (pH 7.2) containing 0.01% 2-MET with (□) or without (□) 10% glycerol. Aliquots (10 µL) of the enzyme solution are depicted, and the activity was measured under the standard assay conditions (n=3).

Figure 2

Native disc-PAGE of purified L-ArgDH. The enzyme (about 125 µg/tube) was applied on a disc gel and the gel was stained with CBB (A) or from L-arginine activity (B) after electrophoresis.

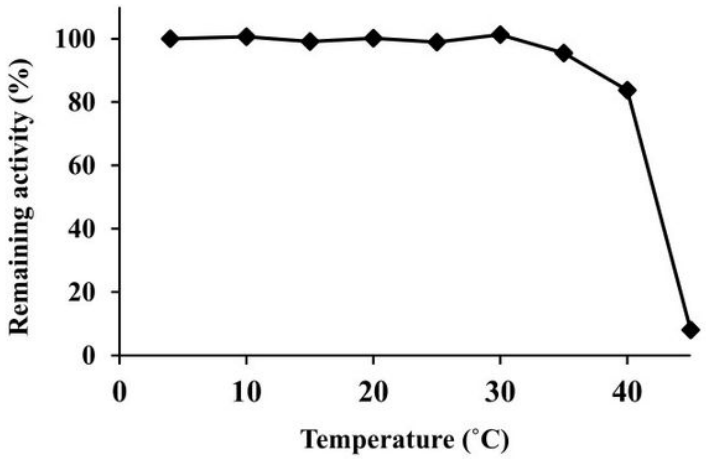

Fig. 3.
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Figure 3

Effect of temperature on L-ArgDH stability. Solution containing the purified enzyme (20 mM KH2PO4-K2HPO4 buffer, pH 7.2, containing 10% glycerol and 0.01% 2-ME) was incubated for 30 min at different

temperatures. The activity remaining in aliquots (10 μL) of the solution was then measured under the standard assay conditions. (n=3)

Figure 4

Effect of pH on L-ArgDH stability. The enzyme was incubated in 50 mM buffer solutions (10% glycerol +0.01% 2-ME) with different pHs for 30 min at 30□. The activity remaining in aliquots (10 μL) of the solution was the measured under the standard assay conditions. The buffers used were KH2PO4-K2HPO4 (pH 6.0-8.5) and glycine-KOH (pH 9.0-10). (n=3)

Figure 5

Effects of temperature (A) and pH (B) on L-ArgDH activity. Enzyme activity was measured at different temperatures (A) and in different pH buffers (B) under the standard assay conditions. The buffers used in (B) were 0.2 M KH2PO4-K2HPO4 (□), Tris-HCl (□), and Gly-NaOH (□).

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P. veronii JCM11942 ArgDH     1 -MSSTPHVIQQAQARELLAQIDVPQILHKLFRDLAAGLAVQPAQQLVAFP 49
P. veronii PverR02_12350      1 -MSSTPHVIQQAQARELLAQIDVPQILHKLFRDLAAGLAVQPPQQLVAFP 49
P. aeruginosa PAO1_PA3862     1 MSAATPLIVQQAEAEQLLARIDVLQAMRQLFLDLAAGQALQPAQQLVEFP 50

P. veronii JCM11942 ArgDH       KGAGDFINYLGVLAEDGVYGVKTSPYIVGEQGPLVTAWTLLMSMHNGQPL 99
P. veronii PverR02_12350        KGAGDFINYLGVLAEDGVYGVKTSPYIVGEQGPLVTAWTLLMSMHSGQPL 99
P. aeruginosa PAO1_PA3862       AGRGDFINYLGVLAQEQVYGVKTSPYIVREQGPLVTAWTLLMSMQTGQPL 100

P. veronii JCM11942 ArgDH       LLCDAHELTTARTAATTALAVDALAPLAARRLAIIGSGKVAQAHLRYVQN 149
P. veronii PverR02_12350        LLCDAHELTTARTAATTALAVDALAPLAARRLAIIGSGKVAQAHLRYVQN 149
P. aeruginosa PAO1_PA3862       LLCDAARLTTARTAATTAVAVDALAPAEACRLALIGSGPVAHAHLQYVKG 150


P. veronii JCM11942 ArgDH       LRDWQHISLFSPSLASASPATLAQLTGLDPRLSIADSCAAAVADADVIML 199
P. veronii PverR02_12350        LRDWQHISLFSPSLASASPATLAQLTGLDPRLSIADSCAAAVADADVIML 199
P. aeruginosa PAO1_PA3862       LRDWQGVRVHSPCLDERR---LQSLRAIDPRAEAAGSLEEALDEADVILL 197

P. veronii JCM11942 ArgDH       CTSSAGPVLDPAHLSKPALITSISTNAPRAHEVPPHSLNAMQVFCDYRQT 249
P. veronii PverR02_12350        CTSSAGPVLDPAHLSKPALITSISTNAPRAHEVPPHSLNAMQVFCDYRQT 249
P. aeruginosa PAO1_PA3862       CTSSARAVIDPRQLKRPALVTSISTNAPRAHEVPAESLAAMDVYCDYRHT 247

P. veronii JCM11942 ArgDH       TPDAAGEMLIASEQHGWDKRAVMGDLPELLSDMAQRPDYQRPVFFRSIGL 299
P. vronii PverR02_12350J        TPDAAGEMLIASEQHGWDKRAVMGDLPELLSDMAQRPDYQRPVFFRSIGL 299
P. aeruginosa PAO1_PA3862       TPGSAGEMLIAAEQHGWSPEAIRGDLAELLSAQAPRPEYRRPAFFRSIGL 297

P. veronii JCM11942 ArgDH       GLEDIALANALYQLQR-  315 100%
P. veronii PverR02_12350        GLEDIALANALYQLQR-  315  99.4%
P. aeruginosa PAO1_PA3862       GLEDVALANALYRLRQAG 315  67.0%
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Fig. 6

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Figure 6

Amino acid sequence of P. veronii JCM 11942 L-ArgDH and the alignments with the sequences of the putative P. veronii Pver_12550 and P. aeruginosa PA01_PA3862 L-ArgDHs. (□): Amino acid residues in the

sequence of P. veronii JCM 11942 L-ArgDH that differed from those in the sequence of the putative P. veronii Pver_12550 L-ArgDH. (—): Adenine binding motif (GXGXXA(G)) for NADP. The sequence added as a his-tag at the C-terminus of P. veronii JCM 11942-L-ArgDH is DPNSSSVDKLAAALEHHHHHH. The sequence alignment was prepared using Clustal W [9] .

Figure 7

Condensed phylogenetic tree based on the amino acid sequences of P. veronii JCM 12942 L-ArgDH and homologous proteins from Pseudomonas and Burkholderia strains. The phylogenetic tree was prepared using Clustal W [9] 1994) and NJ plot (Perriére and Gouy, 1996). Abbreviations: P.: Pseudomonas and B.: Burkholderia. Rectangles show P. veronii JCM 11942 and P. aeromonas PAO1 L-ArgDHs; the others are putative ornithine cyclodeaminase or L-ArgDH.

Figure 8

Calibration curve for L-arginine determination in an assay using L-ArgDH. Oxid and Red m-PMS: methoxy-phenagine methosulfate oxidized and reduced forms, respectively. WST-1 water soluble tetrazolium.