Isolation, purification and characterization of proline dehydrogenase from a *Pseudomonas putida* POS-F84 isolate

Hamid Shahbaz Mohammadi, Eskandar Omidinia*

Department of Biochemistry, Pasteur Institute of Iran, P.O. Box 1316943551, Tehran, I.R. Iran

Abstract
The purpose of this study was to isolate and characterize Proline Dehydrogenase (ProDH) enzyme from microorganisms isolated from soil in Iran. Isolation and screening of L-proline degradative enzymes from soil samples was carried out. The isolate was characterized by biochemical markers and 16S rRNA gene analysis. The target ProDH was purified and the effects of pH and temperature on the activity and stability were tested. Among the 150 isolates recovered from 30 soil samples, only one was identified as *Pseudomonas putida* displayed the highest enzyme activity toward L-proline (2200U/l). The enzyme was identified as a ProDH and had K_m value of 35 mM for L-proline. The molecular mass of the purified ProDH was about 40 kDa, and determined to be a monomeric protein. The N-terminal amino acid sequences of the subunit of *P. putida* enzyme were determined to be MLTSSLTRIIGKSGE. ProDH exhibited high activity at temperature range of 25 to 35ºC and the highest activity was achieved at 30ºC. It was almost stable at temperatures between 25-30ºC for 2 h. The optimum pH of ProDH activity was determined in pH=8.5 and it was stable in pH range of 8.0-9.0 up to 24 h. The enzyme was purified with a yield of 8.5% and a purification factor of 37.7. Briefly, a ProDH flavonzyme was purified and characterized from a *P. putida* bacterium. The specificity of *P. putida* enzyme toward L-proline is advantageous for the application to the L-proline analysis.

Keywords: Characterization; purification; proline dehydrogenase (ProDH); *Pseudomonas putida*

Correspondence to:* **Eskandar Omidinia, Ph.D.
Tel/Fax: +98 21 66402770
E-mail: skandar@pasteur.ac.ir

INTRODUCTION

Microorganisms are preferred to plants and animals as enzyme sources because of their rich diversity in nature, stability in different environments, cheaper production costs, higher enzyme contents and their potential use in biotechnology industry. Isolation and screening of microorganisms from naturally occurring habitats to provide enzymes with novel properties can be very useful (Acehle, 2004). One of the interesting microbial enzymes is Proline Dehydrogenase (ProDH; 1.5.99.8). The human homolog of the ProDH plays critical roles in cancer prevention and schizophrenia (Lee *et al*., 2003). This enzyme is applied for specific determination of plasma proline level in biosensor and diagnostic kits (Zheng *et al*., 2010).

The metabolic pathway of proline includes conversion of proline to Δ^1-pyrroline-5-carboxylate (P5C), followed by conversion to ornithine, metabolism in the urea cycle or to glutamate and introduction into the Tricarboxylic Acid Cycle (TCA) cycle. The degradation of proline to the glutamate proceeds in two oxidative step by the actions of ProDH and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH; P5C: NAD^+ oxidoreductase, 1.5.1.12) enzymes (Fig. 1). At first, ProDH catalyzes the transfer of two electrons from proline to a flavin adenine dinucleotide (FAD) cofactor to generate P5C and $FADH_2$. Electrons from the reduced of the noncovalently associated FAD are subsequently transmitted to an acceptor in the respiratory chain (Menzel and Roth, 1981a). In the second step of proline oxidation, P5C is non-enzymatically hydrolyzed to glutamate-γ-semialdehyde (GSA), which is then oxidized into glutamate by P5CDH in a

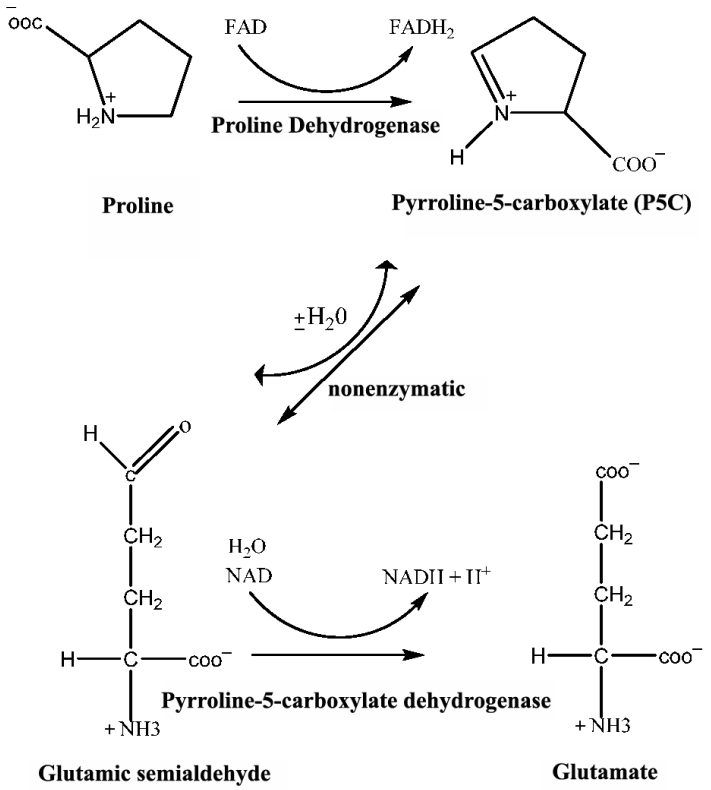

Figure1. Chemical reaction catalyzed by ProDH enzyme in metabolism of proline to glutamate (Baban *et al*., 2004).

NAD^+-dependent reaction. Inherited disorders in proline metabolism cause hyperprolinema diseases in humans. Deficiency of the ProDH and P5CDH activity results in type I and type II hyperprolinemia, respectively (Baban *et al*., 2004). Studying the proline metabolizing enzymes from bacteria could provide valuable information on the structure and function of human version (Cecchini *et al*., 2011; White *et al*., 2007). Furthermore, the bacterial ProDHs are potentially attractive for specific determination of plasma proline in diagnostic kits and proline biosensors (Lee *et al*., 2003; Muro-Pastor and Maloy, 1995). Therefore, in the current research we attempted to isolate these target biocatalysts from soil-inhabiting organisms. Previously screening of proline catabolic enzymes from different microbial sources such as *Escherichia coli* (Baban *et al*., 2004), *Pseudomonas aeruginosa* (Meile and Leisinger, 1982), *Salmonella typhimurium* (Menzel and Roth, 1981a) and *Bradyhizobium japonicum* (Straub *et al*., 1996) has been reported. In this study, we report the purification and characterization of ProDH from a *P. putida* POS-F84 isolate.

MATERIALS AND METHODS

Collection of soil samples: Soil samples were collected from different locations in Tehran: Darekeh, Darband and Farahzad during fall and winter seasons in 2006. The samples were placed in sterile polyethylene bags and maintained at 4ºC until processed.

Isolation and screening of proline degradating enzymes: The preliminary screening of bacteria that produce enzymes of poline degradation was carried out by enrichment culture technique in medium containing proline as a sole source of carbon and nitrogen. One gram of each sample was suspended in Proline Yesat Polypeptone (PYP) selective liquid medium containing; 0.5% L-proline, 1 g NaCl, 2 g K_2HPO_4, 0.5 g $MgSO_4$.$7H_2O$, 5 g yeast extract and 5 g polypeptone in 1 liter of tap water (pH 7.0), then incubated with shaking at 140 rpm for 48 h. Serial dilutions up to 10^{-4} were prepared. From each dilution, 0.05 ml was taken and spread on agar plates and incubated at 37ºC for 24-36 h until the isolates formed colonies. The single uniform colonies were streaked at least three times

to ensure purity (Shahbaz Mohammadia *et al*., 2007).

Biochemical and morphological characterization: Identification of bacterium was performed by morphological and, biochemical characteristics, and specific PCR amplification. Classification as Gram negative or Gram positive was done by Gram staining and KOH test. Morphological characteristics of the isolated bacterium were also performed according to the standard method (Baron and Finegold, 1990). Preliminary identification of isolate was conducted using well-established biochemical tests described in Bergey's manual of Determinative Bacteriology (Krieg and Holt, 1984).

16s rRNA isolation, sequencing and data analysis: Chromosomal DNA from bacterial cell was purified according to the Doi protocol (Asano and Tanetani, 1998). The DNA sample was suspended in 10 mM Tris-HCl buffer (pH 7.6), containing 1 mM EDTA and 10 mM NaCl and stored at -20ºC for further use. Complete 16S rRNA gene sequence was amplified from the chromosomal DNA of the isolated strain with primers 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5'-CTACGGCTACCTTGTTACGA-3') (Scarpellini *et al*., 2004). PCR amplification was performed in total volume of 25 µl containing 20 pmol of each primer, 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 µg template DNA and 2.5 units of *Taq* DNA polymerase. Nucleases free water was used to bring the reaction volume to 25 µl. The PCR product was then analyzed in a 1.5% (w/v) horizontal agarose gel, excised from the gel and purified. The amplified 16S rDNA was cloned into pJET1.2 Vector (MBI Fermentas, St. Leon-Rot, Germany) and transformed into *E. coli* JM 107. Plasmid DNA vector was isolated from the positive clones using plasmid extraction protocol (Sambrook *et al*., 1994). Sequencing was performed by the commercial services of MacroGen Co. ltd. (Seoul, Korea) with the appropriate sequencing primers. The 16S rDNA sequence of the isolate was aligned with the reference 16S rDNA sequences using the Basic Local Alignment Search Tool (BLAST) algorithm available in NCBI (National Center for Biotechnology Information) database. Multiple alignment of sequences and calculations of levels of sequence similarity were performed by using ClustalW. Reference sequences used for phylogentic analysis were recovered from GenBank database. The 16S rRNA genes of the isolated strain and closely related strains from *Pseudomonas* species were aligned separately using the Molecular Evolution Genetic Analysis (MEGA) program, version 4.0 (Tamura *et al.*, 2007). Phylogenic trees were constructed via the Neighbor-Joining (NJ) algorithm using the MEGA version 4.0 (Tamura *et al*., 2007). Bootstrap test was done 1000 times to confirm the reliability and validity of inferred trees.

Nucleotide sequence accession number: The full 16S rDNA and 16S-23S rDNA intergenic spacer (ITS) sequences of the isolated strain have been deposited in the GenBank database under the following accession numbers; GU208206 (16S rDNA) and GU289189 (ITS).

Enzyme activity assay: The standard reaction mixture comprized of 200 mM Tris-HCl (pH=8.5), 200 mM L-proline, 0.2 mM FAD, 0.4 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), 0.08 mM Phenazine methosulfate (PMS) and the enzyme (22 U) in a total volume of 1 ml. The increase in absorbance at 490 nm was estimated and corrected for blank values lacking proline. One unit (U) of ProDH activity was defined as the quantity of enzyme, which transfers electrons from 1 µmol of proline to INT per min at 25ºC (Becker and Thomas, 2001).

Purification of ProDH from *P. putida*: The ProDH purification was performed by the modifications of methods previously described (Satomura *et al*., 2002). The purification procedure was as follows.

Step 1. *Detergent extraction and solubilization*: ProDH production was obtained in the PYP selective liquid medium. The cells were harvested in the late exponential phase by centrifugation at 5000 ×g for 30 min at 4ºC and washed twice with 0.9% NaCl solution. The washed cells were suspended in 50 ml of buffer A (100 mM Tris-HCl, pH 8.0) and disrupted by ultrasonic oscillator for 20 min. Cells and insoluble materials were removed by centrifuged at 4000 × g for 1 h at 4ºC. The supernatant was used as the crude extract centrifuged at 110,000 ×g for 15 min at 4ºC in a Beckman Ti 90 rotor (Beckman Ultracentrifuge). In order to wash the membranes, the supernatant was discarded and the pellet was resuspended in 50 ml of buffer A and then centrifuged at 110,000 ×g for 15 min at 4ºC. The supernatant was then removed and the pellet was resuspended in 30 ml of buffer B (100 mM Tris-HCl, pH 8.2 containing 0.1% (w/v) Tween-20) to solubilize membrane bound enzyme. Finally, the resuspended pellet was allowed to equilibrate at 25ºC

for 20 min and then centrifuged at 110,000 ×g for 20 min at 4ºC. A small aliquot of the supernatant containing the solubilized ProDH was used for enzyme assay and its remainder was precipitated with ammonium sulfate as described below.

Step 2. *Ammonium sulfate precipitation*: The enzyme solution from step 2 was brought to 50% saturation by adding of solid ammonium sulfate solution under gentle stirring at 4ºC. The supernatant was then centrifuged for 30 min at 5000 rpm to collect the precipitate. The pellet was redissolved in 20 ml of buffer C 70 mM Tris-HCl, pH 8.2 containing 0.5% Tween-20 (w/v) and 10% glycerol (v/v) and dialyzed overnight at 4ºC against the same buffer solution.

Step 3. *DEAE-Toyopearl chromatography*: The dialyzed fraction was concentrated by ultrafiltration with an Amicon PM10 membrane, and subjected to a DEAE-Toyopearl column (diameter, 3 cm; length, 15 cm) equilibrated with buffer C using a fast performance liquid chromatography (FPLC) system (Sykam). The column was washed first with buffer C and subsequently washed with buffer C containing 50 mM KCl. ProDH was eluted with a stepwise linear salt gradient of KCl concentration (50-150 mM) in buffer C at a flow rate of 3 ml/min. Fractions of 3 ml per tube were collected. The active enzyme fractions were pooled, concentrated, and stored at 4ºC for various experiments.

Step 4. *Sephadex G-200 chromatography*: The concentrated enzyme was loaded on a Sephadex G-200 gel filtration (2.5 cm × 120 cm), which had been equilibrated with buffer A. The flow rate was maintained at 1 ml/min. Fractions of 3 ml each were collected and absorbance at 280 nm was recorded.

Protein determination: Total protein was determined by the method of Bradford using bovine serum albumin (BSA) as standard (Bradford, 1976).

Electrophoresis: The ProDH purification was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using discontinuous gels (10 cm × 10 cm) with a 6% stacking and a 12% separating gel. The protein samples were boiled for 5 min in 10 mM Tris-HCl buffer (pH 7.0) containing 1% SDS, 80 mM 2-mercatoethonal and 15% glycerol. Electrophoresis was run at 30 v and 10 mA for 5 h. Protein bands were visualized by staining with 0.025 Coomassie brilliant Blue R-250 in the mixture of 50% methanol and 10% acetate (Sambrook *et al*., 1994).

Determination of N-terminal amino acids: The N-terminal amino acid sequence of the enzyme was analyzed with an automated Edman degradation protein sequencer. The phenylthiohydantoin derivatives (PTH-Xaa) were separated and identified using the protein sequencer ppsq-10 (Shimadzu).

Effects of temperature and pH on the enzyme activity and stability: Reaction mixture was pre-incubated at the desired temperatures for 4 min. The thermal stability of ProDH was examined by incubating at different temperatures ranging from 30 to 70ºC for 60 min and then cooling on ice-cold water. Residual activity was measured at every 10 min interval under standard assay conditions. The non-heated enzyme was used as a control. The effect of pH on the enzymatic reaction of ProDH was evaluated by measuring the activity using the following buffer systems: 0.1 M sodium acetate (pH 3.0-5.0), 0.1 M Potassium phosphate (pH 6.0-7.5), 0.1 M Tris-HCl (pH 8.0-9.0), 0.1 M glycine-NaOH (pH 9.0-11.0) and 0.1 M Sodium carbonate (pH 11.5-12.0). To study the influence of pH on the stability of ProDH, crude enzyme was mixed with the above mentioned buffers at a ratio of 1:1 and then incubated at 4ºC for 24 h. Aliquots was taken at time intervals of 4 h and the residual activity was calculated under standard assay conditions. All experiments were done in triplicate and repeated at least twice (Zhu *et al*., 2007).

Determination of kinetic parameters: The kinetic parameters for the purified enzyme were calculated from the secondary plots of intercepts versus reciprocal concentrations of the L-proline substrate. (Shahbaz Mohammadia *et al*., 2007).

RESULTS

Screening for L-proline metabolizing enzymes: During the primary screening in medium containing L-proline as the sole carbon and nitrogen source, 150 bacteria were isolated. To confirm these selections, bacterial isolates, which were capable of utilizing L-proline were cultivated in the production medium, and the enzyme activity was measured. Among the isolates, only POS-F84 which was isolated from the river clay, was capable of metabolizing proline (activity,

2200U/l) was used for experiments.

Characterization and phylogenetic analysis of POS-F84 isolate: The isolated bacterium was Gram negative, rod shape and motile. The results of the biochemical tests are shown in Table 1. Biochemical characterization revealed that isolate POS-F84 might be similar to *P. putida*. Further identification was conducted by comparative sequence analysis of the 16S rDNA of isolate and other bacteria in the Genbank database. The 16S rDNA sequence of POS-F84 had 99% similarity with the corresponding sequences of *P. putida*. Multiple alignment and phylogenetic analysis revealed the isolate was more closely related to *P. putida* (Fig. 2).

Purification and molecular mass determination: The ProDH was purified to homogeneity from the *P. putida* POS-F84 with a yield of 14.0% and a purification factor of 2.8 (Table 2). The molecular mass of the enzyme was determined to be about 40 kDa by native PAGE (Fig. 3). SDS-PAGE gel analysis of purified target enzyme showed a single band indicating that the enzyme consists of one subunit (Fig. 4).

Substrate specificity and kinetic parameters: The specificity of the ProDH reaction with different substrates was examined. L-proline (100%) and L-Hydroxyproline (100%) were the most preferred substrates for ProDH reaction (Table 3). The enzyme also showed weak activities towards L-Threonine, L-Alanine and Acetaldehyde. The following amino acids were inert for the ProDH reaction: D-proline, L-Arginine, Aspartate, and Glycine. Moreover, chelating agents such as EDTA did not inhibit the enzyme. The K_m value of ProDH reaction for proline was also calculated to be 35 mM.

Table 1. Biochemical properties of the POS-F84 isolate.

Characteristic	POS-F84
Triple Sugar Iron Agar (TSIA)	Alkaline/Alkaline
Methyl red (MR)	-
Citrate	+
Voges-Proskauer (VP)	+
Indole production	-
Urea hydrolysis	-
Oxidase reaction	+
Catalaze	+
H_2S Production	-
Inositol fermentation	-
Mannitol fermentation	-
Sorbitol fermentation	-
Arginine dihydrolase	+
L-Rhamnose fermentation	-
Saccharate	-
α-Amylamine	-
Lysine Decarboxylase	-
L-Arabinose fermentation	+
O-Nitrophenyl-β-D-Galactopyranoside	-
Glucose	+
Gelatin hydrolysis	-
Lecithinase (egg yolk reaction)	-
Levan formation from sucrose	-
Ornithine Decarboxylase	-

Table 2. Purification of *P. putida* ProDH.

Purification steps	Activity (U/l)	Total protein (mg/l)	SA (U/mg)	Y (%)	PF
Detergent extraction and solubilization	2200	120	18.3	100	1.0
Ammonium sulfate (50%)	1460	52.0	28	66.36	1.5
DEAE-Toyopearl	630	18.0	35	28.63	1.91
Sephadex G-200	310	6.0	51.6	14.0	2.8

SA, Y and PF represent the specific activity, yield and purification factor, respectively.

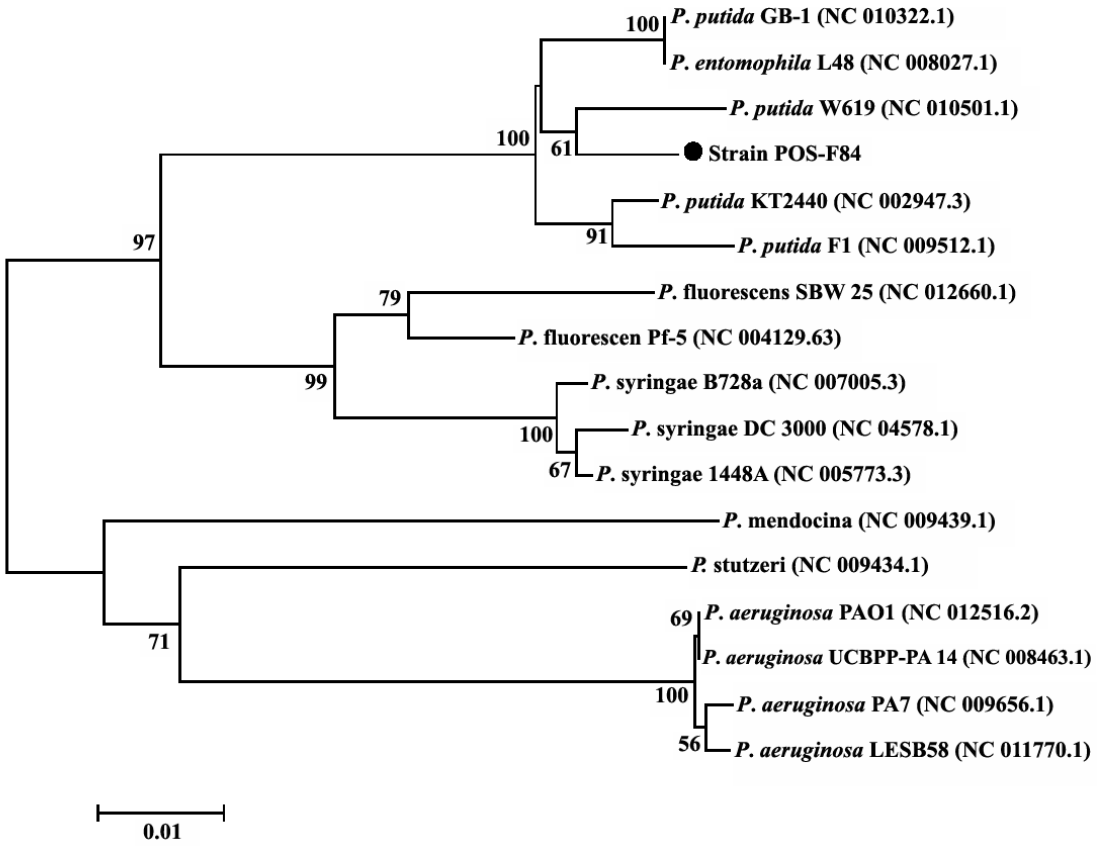

Figure 2. Phylogenetic analysis of the 16S rDNA sequences of POS-F84, and closely related members in the genus *Pseudomonas*. Numbers at nodes are levels of bootstrap support (%) based on Neighbor-Joining (NJ) method of 1,000 resampled datasets. The bootstrap value below 50% is not shown. The scale bar indicates 0.01 nucleotide substitution per position. ●Indicates the isolate.

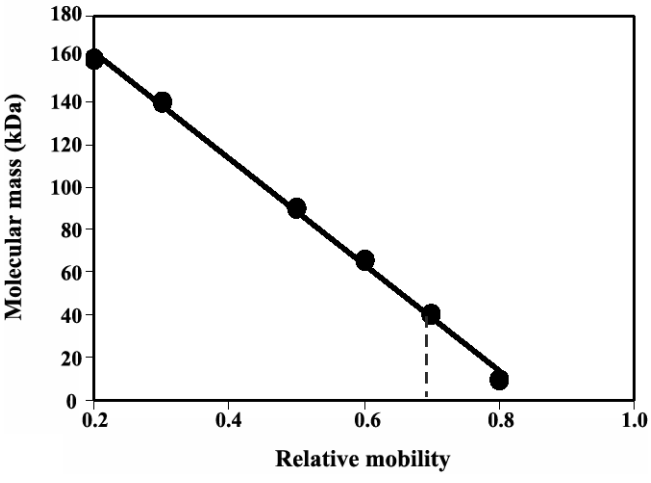

Figure 3. Determination of the molecular mass of native ProDH. The molecular mass was determined on the native PAGE using a 6% gel as described in the text. RNA polymerase (160 kDa), lactate dehydrogenase (142 kDa), glutamate dehydrogenase (90 kDa), bovin serum albumin (66 kDa), and cytochrome *c* (12.4 kDa) were used as the molecular mass standards.

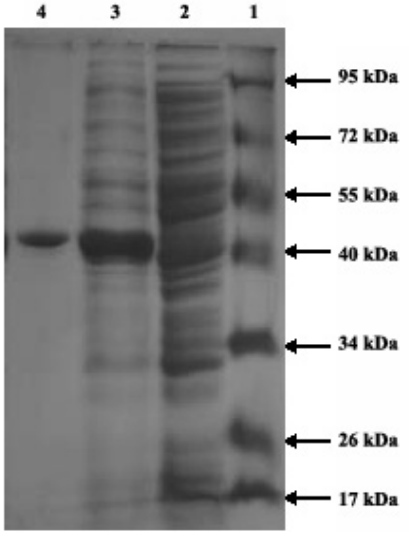

Figure 4. SDS-PAGE of ProDH. Purification steps of ProDH. Lane 1: molecular standard markers lane 2: supernatant of the cell lysate; lane 3: pellet of the cell lysate; lane 4: sample from sephadex G-200 column.

Table 3. Substrate specificity for the ProDH reaction of *P. putida*.

Amino acid	Concentration (mM)	Relative activity (%)
L-Proline	10	100
D-Proline	10	0
L-Hydroxyproline	10	0
L-Tryptophan	10	12
L-Arginine	10	0
L-Serine	10	11
L-Glutamate	10	13
L-Histidine	10	19
L-Threonine	10	32
L-Valine	10	9
L-Leucine	10	17
L-Alanine	10	22
Glycine	10	0
Aspartate	10	0

N-terminal amino acid sequences: The N-terminal amino acid sequence of the subunit (15-amino acid residues) of *P. putida* enzyme were determined to be MLTSSLTRIIGKSGE. The obtained result strongly supported a type of polypeptide catalyzing the ProDH reaction.

Effects of temperature and pH on the enzyme activity and stability: The ProDH reaction exhibited its maximal activity at temperature range of 25 to 30ºC, and its highest activity was achieved at 30ºC (Fig. 5). As can be seen (Fig. 5), a sharp decrease in enzyme activity was observed above 30°C and was completely inactivated at 70°C. For examination of the temperature effect on enzyme stability, the residual activity of ProDH incubated at different temperatures (25-50ºC) for a period of one hours was measured (Fig. 6). The

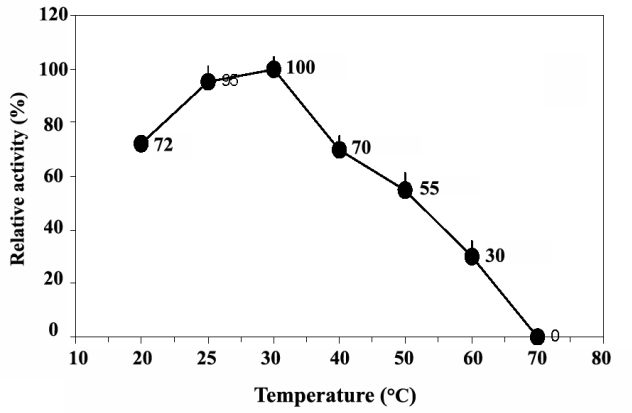

Figure 5. Effect of temperature on the activity of ProDH of *P. putida*.

ProDH was almost stable at temperatures between 25-30ºC for one hour, but lost 27%, 45% and 85% of its initial activity after incubation for one hour at 35, 40 and 45ºC, respectively. At 50ºC, target ProDH was completely inactivated after 50 min. The effect of various pH values on the enzymatic reaction of ProDH was evaluated in the pH range from 3.0 to 12.0 at 30 ºC. ProDH had a good activity in the range of pH 7.0-9.0 with optimal pH at 8.5 (Fig. 7). The effect of pH on enzyme stability was also tested by the measurement of residual activity after incubation at different pH values for 24 h. ProDH was highly stable between pH 8.0-9.0, while at pH 10, only 45% of its original activity was retained (Fig. 8).

DISCUSSION

The amino acid L-proline is catabolized into glutamate by the actions of two enzymes, ProDH and P5CDH

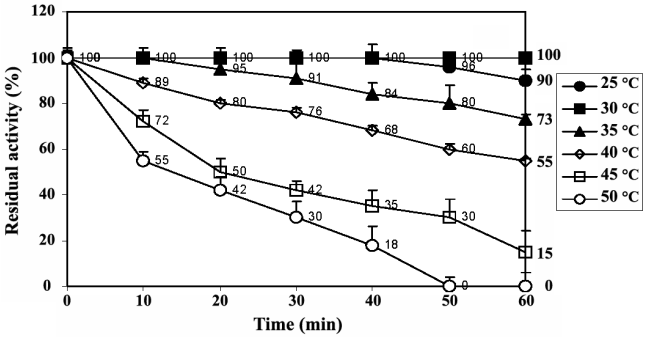

Figure 6. Influence of temperature on the stability of ProDH reaction from *P. putida*.

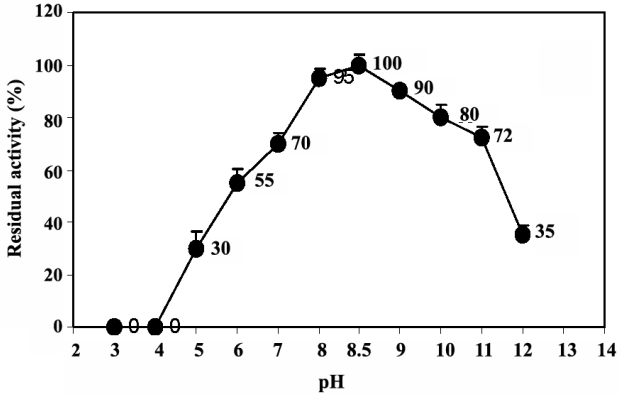

Figure 7. Effect of pH on the ProDH reaction of *P. putida*.

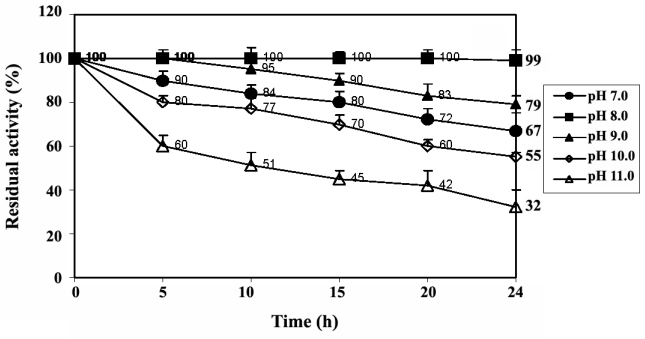

Figure 8. Influence of pH on the stability of ProDH reaction from *P. putida*. Enzyme sample were incubated in different buffers and aliquots were collected at different time intervals for enzyme assay.

(Menzel and Roth, 1981a). The bacterial enzymes participating in proline metabolism are potentially attractive models for studying the structure and function of human version and also specific determination of plasma L-proline in diagnostic kits and biosensors (Lee *et al*., 2003; Muro-Pastor and Maloy, 1995). Considering the medical applications of enzymes catabolizing proline in diagnosis of inborn errors of proline metabolism, we attempted to isolate these target biocatalysts from soil-inhabiting organisms. At theend of screening program, only one bacterium from the river clay, was found to be capable to produce ProDH and selected for further studies. To identify the POS-F84 isolate, biochemical and genetic analysis was performed. Phylogenetic analysis revealed the isolate was a newly isolated *P. putida*. PAGE analysis of the purified enzyme showed a single band with an estimated molecular mass of 40 kDa. Based on the subunit molecular weight of 40 kDa obtained by SDS-PAGE, we interpret that one electrophoretic band observed under native condition represents the monomeric protein. The reported molecular mass for this enzyme varies from 40 kDa to 45 kDa (Satomura *et al.*, 2002).

The molecular weight of the target protein was in agreement with the available observations for ProDH enzyme. Similar results have been observed for *P. aeruginosa* (Cecchini *et al*., 2011) and *S. typhimurium* (White *et al*., 2007) ProDHs. While these observations were in agreement with the existence of a ProDH enzyme, definite evidence for this notion came from the determination of the N-terminal amino acids of the purified enzyme. The *P. putida* ProDH possessed different kinetic characteristics from the other ProDH and had lower K_m value for proline than that reported (Meile and Leisinger, 1982; Menzel and Roth, 1981a). As it has been noted in the literatures, high K_m value of ProDHs for proline is one of the common features of proline metabolizing enzymes in bacteria (Lee *et al*., 2003). For example, K_m value for proline for the ProDH enzymes in *P. aeruginosa* (Meile and Leisinger, 1982), and *S. typhimurium* (Menzel and Roth, 1981a) has been reported 45 mM and 43 mM, respectively. Therefore, the higher affinity of *P. putida* ProDH toward proline made this enzyme very attractive for use in biosensors and protein engineering studies. The ProDH described in this research differed from the ProDH enzymes with respect to the binding of FAD, while the purified enzymes from the *P. aeruginosa* (Meile and Leisinger, 1982), and *S. typhimurium* (Menzel and Roth, 1981a) had retained FAD, *P. putida* ProDH was fully dependent on added FAD for catalyzing the ProDH reaction.

In summary, a ProDH enzyme was purified and characterized from a *P. putida* POS-F84 isolated from river clay.

Acknowledgements

The authors are grateful to Dr. Esfahani for his helpful discussions during this project the Research Grant Number 374 from the Pasteur Institute of Iran.

References

Acehle W (2004). *Enzymes in industry, Production and Applications*. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim. PP. 244-246.

Asano Y, Tanetani M (1998). Thermostable phenylalanine dehydrogenase from a *mesophilic Microbacterium* strain DM86-1. *Arch Microbiol*. 169: 220-224.

Baban BA, Vinod MP, Tanner JJ, Becker DF (2004). Probing a hydrogen bond pair and the FAD redox properties in the proline dehydrogenase domain of *Escherichia coli* PutA. *Biochim Biophys Acta*. 1701: 49-59.

Baron EJ, Finegold SM (1990). *Bailey and Scotts's diagnostic microbiology*, 8th Ed. The C.V. Mosby Company, St. Louis. Baltimore. Philadelphia. Toronto. PP. 363-407.

Becker DF, Thomas EA (2001). Redox properties of the PutA protein from *Escherichia coli* and the influence of the flavin redox state on PutA-D NA interactions. *Biochemistry*. 40: 4714-4722.

Bradford MM (1976). Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem*. 72: 248-254.

Cecchini MN, Monteoliva IM, Marýa EA (2011). Proline dehydrogenase contributes to pathogen defense in Arabidopsis. *American Society of Plant Biologists*. 155: 1947-1959.

Krieg NR, Holt JG (1984). Bergey's Manual of systematic Bacteriology. Vol.1. Baltimore: Williams and Wilkins Co.

Lee YH, Nadaraia S, Gu D, Becker DF, Tanner JJ (2003). Structure

of the proline dehydrogenase domain of the multifunctional PutA flavoprotein. *Nat Struct Biol*. 10: 109-114.

Menzel R, Roth J (1981). Purification of the *putA* gene product. *J Biol Chem*. 256: 9755-9761.

Meile L, Leisinger T (1982). Purification and properties of the bifunctional ptoline dehydrogenase/1-pyrroline-5-carboxylate dehydrogenase from *Pseudomonas aeruginosa. Eur JBiochem*. 129: 67-75.

Sambrook J, Fritsch E.F, Maniatis T (1984). Molecular Cloning: A laboratory manual 2nded., Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York.

Satomura T, Kawakami R, Sakuraba H, Ohshima T (2002).Dye-linked D-proline dehydrogense from hyperthermophilic archaeon *Pyrobaculum islanddicum* is a novel FAD-dependent amino acid dehydrogenase. *J Biol Chem*. 227: 12861-12867.

Scarpellini M, Franzetti L, Galli A (2004). Development of PCR assay to identify *Pseudomonas fluoresens* and its biotype. *Microbiol Lett.* 236: 257-260.

Shahbaz Mohammadi H, Omidinia E, Sahebghadam Lotfi A, Saghiri R (2007). Preliminary report of NAD^+- dependent amino acid dehydrogenases producing bacteria isolated from soil. *Iranian Biomed J*. 11: 131-135.

Straub PF, Reynolids PHS, Althomsons S, Mett V, Zhu Y, Shearer G, Kohl DH (1996). Isolation, DNA sequence analysis, and mutagenesis of a proline dehydrogenase gene (*putA*) from *Bradyhizobium japonicum*. *Appl Environ Microbiol*. 62: 221-229.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol*. 24: 1596-1599.

White TA, Krishnan N, Becker DF, Tanner JJ (2007). Structure and kinetics of monofunctional proline dehydrogenase from *Thermus thermophilus*. *J Biol Chem*. 282: 14316-14327.

Zheng H, Lin L, Okezaki Y, Kawakami R, Sakuraba H, Ohshima T, Takagi K, Suye S (2010). Electrochemical behavior of dye-linked L-proline dehydrogenase on glassy carbon electrodes modified by multi-walled carbon nanotubes. *Beilstein J Nanotechnol*. 1: 135-141.

Zhu W, Cha D, Cheng G, Peng Q, Shen P (2007). Purification and characterization of a thermotable protease from a newly isolated *Geobacillus* sp. YMTC 1049. *Enzyme Microb Technol*. 40: 1592-1597.