Expression, Purification and Characterization of the Proline Dehydrogenase Domain of PutA from *Pseudomonas putida* POS-F84

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Abstract The objective of the present work was to express a truncated form of *Pseudomonas putida* PutA that shows proline dehydrogenase (ProDH) activity. The *putA* gene encoding ProDH enzyme was cloned into pET23a vector and expressed in *Escherichia coli* strain BL-21 (DE3) plysS. The recombinant *P. putida* enzyme was biochemically characterized and its three dimensional structure was also predicted. ProDH encoding sequence showed an open reading frame of 1,035-bp encoding a 345 amino acid residues polypeptide chain. Purified His-tagged enzyme gave a single band with a molecular mass of 40 kDa on SDS-PAGE. The molecular mass of the isolated enzyme was found to be about 40 kDa by gel filtration. This suggested that the enzyme of interest consists of one subunit. The K_m and V_{max} values of recombinant *P. putida* ProDH were estimated to be 31 mM and 132 $\mu\text{mol/min}$, respectively. The optimum pH and temperature for the catalytic activity of the enzyme was about pH 8.5 and 30 °C. The modeling analysis of the three dimensional structure elucidated that Ser-165, Lys-195 and Ala-252 were key residues for the ProDH activity. This study provides data on the cloning, sequencing and recombinant expression of PutA ProDH domain from *P. putida* POS-F84.

Keywords Characterization · Expression · Proline dehydrogenase (ProDH) · *Pseudomonas putida*

Introduction

In the first enzymatic step for proline catabolism, proline is converted to Δ^1 -pyrroline-5-carboxylate by proline dehydrogenase (ProDH; L-proline: FAD oxidoreductase 1.5.99.8) in a FAD-dependent reaction. Electrons from the reduced FAD are subsequently transferred to an acceptor in the electron transport chain. Δ^1 -pyrroline-5-carboxylate is in spontaneous equilibrium with the γ -glutamic acid semialdehyde (GSA) by a non-enzymatic intramolecular Schiff's base reaction. GSA is then oxidized in the second enzymatic step to glutamate by Δ^1 -pyrroline-5-carboxylate dehydrogenase (P5CDH; P5C: NAD⁺ oxidoreductase, 1.5.1.12) in a NAD⁺-dependent reaction. The enzymes of proline catabolism differ widely among the organisms [1]. ProDH and P5CDH appear separate enzymes in eukaryotes and fused bi-functional enzymes known as proline utilization A (PutA) in most bacteria [2]. Multifunctional PutA flavoenzyme contains ProDH and P5CDH domains [3]. In addition to these enzymatic roles, PutA polypeptide has also DNA-binding activity and participates in the transcriptional control of *put* genes [4]. In the absence of proline, PutA accumulates in the cytoplasm and represses transcription of the *put* regulon by binding to the control intergenic region between *putP* and *putA* genes. The *putP* gene encodes the PutP Na⁺-proline transporter. In the presence of proline, PutA associates with the membrane and performs its enzymatic functions. The presence of PutA protein has been reported in different bacteria such as *Escherichia coli* [5], *Pseudomonas aeruginosa* [6], *Salmonella typhimurium* [7], *Bradyrhizobium japonicum* [8] and *P. fluorescence* [3]. In the current paper, we

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report the cloning, expression and purification of ProDH domain of the bi-functional PutA from a newly isolated *P. putida* POS-F84. To best of our knowledge, there has been no report on the ProDH from *P. putida*. The bacterial ProDH enzyme is functionality similar to the human version, so its results can help us to gain more information about the structure and function of human enzyme. The enzyme ProDH has recently received much attention in cancer researches because it plays a role in apoptosis by creating the superoxide. According to these facts, studying the bacterial enzymes involved in proline metabolism can provide valuable information for understanding the human ProDH.

Materials and Methods

Bacterial Strains and Plasmids

The *P. putida* POS-F84 used in this study was obtained during screening program [9]. The *E. coli* strains DHF α and BL-21 (DE3) plysS were obtained from Stratagene (LaJolla, CA, USA). The expression vector of pET-23a was prepared from the National Recombinant Gene Bank (NRGB) of Pasteur Institute of Iran.

Polymerase Chain Reaction of the *ProDH* Domain

To amplify the *ProDH* domain, forward and reverse primers were designed using DNASIS MAX software (DNASIS version 3.0, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The primers used were PDHPOSFw (5'-TATCATATGCTGACCTCCTCGCTCACC-3') and PDHPOSRev (5'-AGGATCCAATCGGCGATGCGG-3'), which contained the restriction sites for *Nde*I and *Bam*HI, respectively (underlined). Polymerase chain reaction (PCR) conditions were as follows; hot start cycle at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 7 min.

Cloning of ProDH Coding Region of PutA

The resultant PCR product was double digested with *Nde*I and *Bam*HI, gel purified, and then ligated into the pET23a (+) expression vector carrying a C-terminal His₆-tag. The construct bearing the ProDH gene was named pET23aPDHPOS and transformed into the *E. coli* strain DH5 α competent cells for screening purposes. The correctness of the cloned gene was also confirmed by nucleotide sequencing and no mutation was revealed. The isolated pET23aPDHPOS plasmid was then transformed into *E. coli* strain BL-21 (DE3) plysS competent cells for expression purposes.

Expression and Purification of ProDH Domain

Escherichia coli strain BL21 (DE3) plysS cells bearing pET23aPDHPOS construct were cultivated overnight in Luria–Bertani (LB) medium containing 100 mg/ml of ampicillin at 37 °C and 150 rpm. 100 ml preculture broth was transferred into 1 L of LB medium in culture flasks and incubated at 37 °C and 150 rpm. When cell density reached an OD₆₀₀ of 0.6–0.8, ProDH enzyme was expressed by the addition of 0.5 mM sterile isopropyl- β -D-thiogalactopyranoside (IPTG). After 6 h induction at 23 °C, cells were harvested, washed twice with 0.9 % NaCl solution and stored at –20 °C for further uses. Pelleted *E. coli* cell were suspended in lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 10 mM EDTA, pH 8.0), mechanically broken by sonication using a pulse sequence of 15 s on and 10 s off and clarified by centrifugation at 4,000 rpm for 1 h. The precipitate (inclusion bodies) containing recombinant ProDH enzyme was washed twice with wash buffer (50 mM Tris–HCl, 50 mM NaCl, 10 mM EDTA, pH 8.0, 1 % Triton X-100). The washed pellet was resuspended in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 10 % glycerol and 0.1 mM DTT (buffer A) containing 8 M urea and incubated in 4 °C with continuous stirring for 24 h to solubilize the inclusion bodies. Any insoluble material was removed by centrifugation at 4,000 rpm at 4 °C for 1 h. Refolding was performed by stepwise dialysis against descending concentrations of urea. The unfolded ProDH was first dialyzed against buffer A supplemented with 4, 2 M and then without urea. The buffer was changed every 24 h. For reconstitution, the renatured enzyme was dialyzed overnight at 4 °C in buffer A containing 0.15 mM FAD and applied to a Ni²⁺ NTA affinity column (Novagen). Next, the column was washed with 3 column volumes of the lysis buffer containing 50 mM imidazole, and then, ProDH eluted with an elution buffer (50 mM Tris–HCl, 50 mM NaCl, 10 mM EDTA, pH 8.0, 500 mM imidazole) [10].

Enzyme and Protein Assays

ProDH activity was spectrophotometrically assayed using proline as substrate, INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) oxidoreductase assay which was performed by INT as a terminal electron acceptor and Phenazine methosulfate (PMS) as a mediator electron carrier [11]. Assay reaction mixture (final volume, 1.0 ml) contained 100 mM Tris–HCl, 10 mM MgCl₂, 10 % glycerol, pH 8.5, 200 mM L-proline, 0.2 mM FAD, 0.45 mM INT, 0.08 mM PMS and the enzyme. The increase in absorbance at 490 nm was read and corrected for blank values lacking proline. One unit (U) of enzyme is defined as the amount of enzyme that transfers electrons from 1 μ mol of proline to INT per minute at 25 °C. All

assay experiments were done in triplicate and the average results were used for data analysis. The protein content was measured by the method of Bradford using bovin serum albumin (BSA) as a standard [12].

Evaluation of Enzyme Purity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using discontinuous gels (10 × 10 cm) with a 12 % separating gel and a 6 % stacking gel [10]. Protein bands were visualized by staining with 0.025 Coomassie brilliant Blue R-250 in the mixture of 50 % methanol and 10 % acetate. Apoferritin (443 kDa), myosine (200 kDa), β -galactosidase (175 kDa), lactate dehydrogenase (142 kDa), fructose-6-phosphate (88 kDa), BSA (66 kDa) and ovalalbumin (45 kDa) were used as molecular markers.

Effect of pH and Temperature on Enzyme Activity

The effect of temperature on the enzymatic reactions of ProDH was analyzed by performing the enzyme assay at various temperatures (30–70 °C). The effect of pH on the enzymatic reactions of ProDH was evaluated by measuring the activity in 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). All experiments were done triplicate and repeated at least twice [13].

Prediction of 3D Structure of *P. putida* ProDH

The BLAST through NCBI was used to identify homologous structures by searching the structural database of protein sequences in the protein data bank (PDB). The crystal structure of PutA from *E. coli* K12 with bound FAD (PDB code: 1k87) was selected as a template for homology modeling of *P. putida* POS-F84 ProDH. The amino acid sequence of *P. putida* POS-F84 ProDH was aligned with sequence of PutA from *E. coli* K12 extracted from its crystal structure using the DNASIS MAX program. Homology model of ProDH was constructed using the homology modeling program MODELER version 9v9 (default parameters). Two scoring functions, DOPE (discrete optimized protein energy) score and RAPDF (residue-specific all-atoms conditional probability density function) score were used to select the final model. The geometry of loop regions was corrected using MODELER/Refine Loop command. The stereochemical, volume and surface properties of the model were evaluated by PROCHECK server [14]. Visual analysis and manipulation of the model were

done with PyMOL version 0.99, which was also used for illustrations.

Results and Discussion

Isolation and DNA Sequence Analysis of ProDH Domain of PutA From *P. putida* POS-F84

To characterize PutA ProDH domain from *P. putida* POS-F84, ProDH domain was isolated, aligned with other ProDH coding sequences in data Bank and compared with of various *Pseudomonas* strains. The genomic DNA of *P. putida* POS-F84 strain was isolated. Accordingly, a DNA fragment containing ProDH domain (1,035 bp) was obtained by PCR with primers designed on the basis of conservative sequences of ProDHs from *Pseudomonas* strains. The target domain was sequenced and compared with those of other ProDHs. The nucleotide sequence showed remarkable similarity to the ProDH domains of others *Pseudomonas* strains in Genbank sequence database. Sequence comparison result indicated that it was closely similar to ProDH from *P. putida* (identity, 70 %).

Expression and Purification of Recombinant ProDH

Expression plasmid of *P. putida* POS-F84 ProDH, pET23PDHPOS, carrying a fragment coding for the ProDH domain was constructed and transformed in *E. coli* strain DH5 α . Amongst approximately 60 transformants of *E. coli* recombinant cells, seven colonies were selected for plasmid isolation. All the clones exhibited an insert of 1,035 bp along with a 3.6 kb vector band after digestion with *Nde*I and *Bam*HI (Fig. 1). The restriction pattern confirmed the cloning of the ProDH domain. DNA sequencing of the insert was performed using M13 forward and M13 reverse primers. The pET23PDHPOS construct was transformed into *E. coli* strain BL21 (DE3) pLysS for expression of recombinant target enzyme. The open reading frame (ORF) coding for the ProDH gene comprised 1,104 base pairs encoding 368 amino acids. The expressed enzyme was purified to homogeneity by Ni²⁺ NTA affinity chromatography from the recombinant *E. coli* strain BL21 (DE3) pLysS carrying pET23PDHPOS with an overall yield of 72 % and a purification factor of 11. Purified His-tagged enzyme gave a single band with a molecular mass of 40 kDa on SDS-PAGE (Fig. 2). The molecular mass of the isolated enzyme was found to be about 40 kDa by gel filtration. This suggested that the enzyme of interest probably consists of one subunit. The observed band matched with the expected molecular weight for recombinant *P. putida* POS-F84 ProDH.

Fig. 1 Analysis of the cloning of the ProDH domain specific fragment (1,035 bp) from *P. putida* POS-F84 in the pET23a. lane M, 1-kb ladder; lane 1, isolated plasmid; lane 2, *Nde*I and *Bam*HI-digested clones (the presence of the 1,035 bp fragment is shown)

Fig. 2 SDS-PAGE analysis of the purified ProDH. Protein samples were stained with Coomassie Brilliant Blue. Lane M: molecular mass standards; lane 1: pellet of the cell lysate; lane 2: supernatant of the cell lysate; lane 3: pure recombinant ProDH

Enzymatic Properties of Recombinant *P. putida* POS-F84 ProDH

Kinetic parameters of the purified ProDH were measured, where rates were calculated at six L-proline concentrations and average two assays. The K_m and V_{max} values for *P. putida* ProDH were calculated to be 31 mM and 132 $\mu\text{mol}/\text{min}$, respectively. The K_m value was lower than that reported for other ProDH enzymes. For example, K_m value of proline for the ProDH domains of PutA enzymes

in *P. aeruginosa* [6], *S. typhimurium* [7] and *P. fluorescence* [3] has been reported 45, 43, and 35 mM respectively. 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 [11]. Therefore, the higher substrate affinity of *P. putida* ProDH toward proline made this enzyme very attractive for use in biosensors. The substrate specificity of the enzyme was studied with various amino acids. The highest activity was achieved with L-proline (100 %), although the enzyme exhibited weak activities with L-Threonine and L-Alanine. However, D-proline, L-Hydroxyproline, L-Arginine, Aspartate, and Glycine were inert as substrates. The target enzyme required FAD cofactor as prosthetic group, but NAD^+ and NADP^+ were inert. These results for *P. putida* ProDH were consistent with earlier reports. However, this is the first report of such study with ProDH from *P. putida* POS-F84. Recombinant ProDH exhibited activity at temperature range from 25 to 30 $^{\circ}\text{C}$, and its highest activity was achieved at 30 $^{\circ}\text{C}$. The enzyme of interest lost considerable amount of its original activity at above 30 $^{\circ}\text{C}$ and was nearly inactivated at 70 $^{\circ}\text{C}$. From this feature, it can be inferred that like many other ProDHs [6], the *P. putida* ProDH was a form of mesophilic enzymes. The obtained data were in good agreement with those previously ProDHs from *P. aeruginosa* [6], *P. putida* [15] and *P. fluorescence* [3]. 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 $^{\circ}\text{C}$. ProDH had a good activity in the range of pH 7.0–9.0, and optimum pH was observed to be 8.5. It was in agreement with previous reports for ProDH from other bacteria ProDHs; *Vibrio vulnificus* [16] and *S. typhimurium* [7].

Multiple Amino Acid Sequence Alignment and Enzyme Structural Modeling

The multiple alignment of *P. putida* POS-F84 ProDH amino acid sequence with several other species revealed 96 and 99 % of identity between different ProDH sequences (Fig. 3). In comparison with *E. coli* K12, the percentage of identity failed to 88 %. Sequence analysis also indicated the existence of 301 identical amino acids along the polypeptide chain in comparison of all ProDH sequences. The analysis of these amino acids using the data related to the crystal structure of the ProDH from *E. coli* K12 (the only one so far crystallized and modelised ProDH) indicated that these amino acids were localized next to the active site (not shown) and both structures are almost identical (not shown). Based on this evidence, it was taken as a template for ProDH of *P. putida* POS-F84. We constructed the three dimensional structure of the *P. putida* enzyme expressed from recombinant *E. coli* BL-21 (DE3)

Fig. 3 Multiple amino acid sequence alignment of ProDH sequences from different *Pseudomonas* strains using DNASIS MAX software. Highly identical residues are depicted in *black* and the less strongly conserved are in *grey*

plysS through the procedure of homology modeling. The 1,000 models were evaluated and the one with the lowest DOPE score was chosen for further modeling. The low homology loops near the active site regions of modeled enzyme were refined to access proper folding with minimum steric clash. Fig. 4 shows the final modeled structure

from *P. putida* ProDH. The minimized model was then analyzed and validated using Ramachandran plot obtained from the PROCHECK server. In the *P. putida* ProDH model, 94.7 % of residues were in the most favored regions with only 1.3 % in disfavored regions. The achieved data confirmed the reliability of final modeled structure.

Fig. 4 Ribbon diagram of predicted structure of *P. putida* ProDH. The β -strands are colored in *light green*, and the α -helices in *red*. The key catalytic Ser-165, Lys-195 and Ala-252 residues are shown. (Color figure online)

Bioinformatics analysis also revealed that *P. putida* enzyme contained 51 % α -helices, 7 % β -strand, and 41 % coils. Moreover, we used the three dimensional homology modeling to identify key amino acids involved in FAD-binding site and catalysis reaction. As seen in the 3D structure of ProDH of *P. putida* presented in Fig. 4, Ser-165, Lys-195 and Ala-252 residues, which were located near the hydroxyl group of the substrate in the model, were essential for the ProDH activity. The structure presented here provided valuable information on substrate and FAD interactions of the *P. putida* enzyme and, as well as biochemical experiments, such as mutagenesis.

In the present study, we described the production and purification of recombinant form of ProDH from *P. putida* bacterium, which was isolated from a river clay sample. The biochemical characteristics of recombinant enzyme were also investigated. The specificity of *P. putida* ProDH toward L-proline could be advantageous for the L-proline analysis.

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References

1. Menzel R, Roth J (1981) Purification of the *putA* gene product. *J Biol Chem* 256:9755–9761
2. Cecchini MN, Monteoliva IM, Mari'a EA (2011) Proline dehydrogenase contributes to pathogen defense in Arabidopsis. *Am Soc Plant Biol* 155:1947–1959
3. Shahbaz Mohammadia H, Omidinia E (2012) Proline dehydrogenase from *Pseudomonas fluorescence*: gene cloning, purification, characterization and homology modeling. *Appl Biochem Microbiol* 48:167–174
4. 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
5. 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
6. Meile L, Leisinger T (1982) Purification and properties of the bifunctional proline dehydrogenase/1-pyrroline-5-carboxylate dehydrogenase from *Pseudomonas aeruginosa*. *Eur J Biochem* 129:67–75
7. Menzel R, Roth J (1981) Enzymatic properties of the purified PutA protein from *Salmonella typhimurium*. *J Biol Chem* 256:9762–9766
8. Straub PF, Reynolds 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 *Bradyrhizobium japonicum*. *Appl Environ Microbiol* 62:221–229
9. Shahbaz Mohammadia 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
10. Sambrook J, Fritsch EF, Maniatis T (1994) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York
11. Becker DF, Thomas EA (2001) Redox properties of the PutA protein from *Escherichia coli* and the influence of the flavin redox state on PutA-DNA interactions. *Biochemistry* 40:4714–4722
12. Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
13. Zhu W, Cha D, Cheng G, Peng Q, Shen P (2007) Purification and characterization of a thermostable protease from a newly isolated *Geobacillus* sp. YMTC 1049. *Enzyme Microb Technol* 40:1592–1597
14. Laskowski R, Macarthur M, Moss D, Thornton J (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *G Appl Crys* 26:283–291
15. Vilchez S, Molina L, Ramos C, Ramos J (2000) Proline metabolism by *Pseudomonas putida*: cloning, characterization, and expression of the *put* genes in the presence of root exudates. *J Bacteriol* 182:91–99
16. Lee GH, Park NY, Lee MH, Choi SH (2003) Characterization of the *Vibrio vulnificus* putAP operon, encoding proline dehydrogenase and proline permease and its differential expression response to osmotic stress. *J Bacteriol* 185:3842–3852