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Production of Recombinant Proline Dehydrogenase Enzyme from *Pseudomonas fluorescens* pf-5 in *E. coli* System

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Abstract

Proline dehydrogenase (ProDH; 1.5.99.8) belongs to superfamily of amino acid dehydrogenase, which plays a significant role in the metabolic pathway from proline to glutamate. The goal of this research was gene cloning and characterization of ProDH enzyme from *Pseudomonas fluorescens* pf-5 strain. The gene encoding ProDH was isolated by means of PCR amplification and cloned in an IPTG inducible T7-based expression system. The Histidine-tagged recombinant enzyme was purified and its kinetic properties were studied. According to SDS-PAGE analysis ProDH revealed a MW of 40 kDa. The K_m and V_{max} values of *P. fluorescens* ProDH were estimated to be 20 mM and 160 µmol/min, respectively. ProDH activity was stable at alkaline pH and the highest activity was observed at pH 8.5 and 30°C. This study is the first data on the isolation and production of *P. fluorescens* ProDH enzyme in *E. coli* expression system.

Keywords: Cloning; Characterization; Proline dehydrogenase (ProDH); *Pseudomonas fluorescence*

Introduction

The amino acid L-proline is metabolized to glutamic acid in a two-step oxidation reaction. In most bacteria, both enzymatic steps for proline utilization are catalyzed by a multifunctional flavoprotein encoded by the *putA* gene [1]. Multifunctional proline utilization A (PutA) flavoprotein, contains proline dehydrogenase (ProDH; L-proline: FAD oxidoreductase 1.5.99.8) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH; P5C: NAD^+ oxidoreductase, 1.5.1.12) domains. ProDH is an important flavoenzyme in the first step of proline metabolism and catalyzes the conversion of proline to Δ^1-pyrroline-5-carboxylate (P5C) in the presence of FAD as a cofactor. In the second step of proline degradation, P5C is hydrolyzed to glutamate-γ-semialdehyde (GSA), which is then oxidized to glutamate by P5CDH in a reaction requiring NAD^+ cofactor (Fig. 1) [2]. In addition to these enzymatic roles, PutA polypeptide has also DNA-binding activity and participates in the transcriptional control of *put* genes. In the absence of proline, PutA accumulates in

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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 absence of proline, PutA associates with the membrane and performs its enzymatic functions [3, 4]. The presence of PutA protein has been reported in different bacteria such as *E. coli* [4], *Pseudomonas aeruginosa* [5], *P. putida* [6], *s. typhimurium* [7] and *Bradyhizobium japonicum* [8]. In the current paper, we report the gene cloning, and characterization of ProDH domain from *P. fluorescence*. To best of our knowledge, there has been no report on the ProDH from *P. fluorescence*. This enzyme is functionally similar to the human version, so its results can help us to gain more information about the structure and function of human enzyme. ProDH has recently received much attention in cancer researches because it plays a role in apoptosis by creating the superoxide [4]. According to these facts, studying the bacterial enzymes involved in proline metabolism could provide valuable information for understanding the human PRODH. Moreover, ProDH exhibit a high potential for application in biosensors.

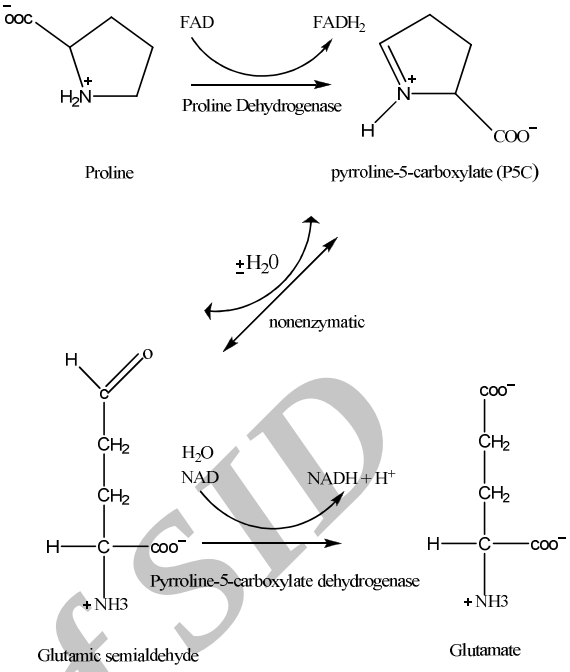

Figure 1. Chemical reactions catalyzed by the bi-functional PutA flavoenzyme in metabolism of proline to glutamate [1].

Materials and Methods

Chemicals and Enzymes

All chemicals and buffers were obtained from Sigma-Aldrich (St. Louis, USA) and Merck (Germany). Restriction endonucleases, DNA modifying enzymes and molecular mass markers for electrophoresis were purchased from Fermentas.

Bacterial Strains and Plasmids

The *pseudomonas fluorescence* pf-5 wild-type strain (ATCC BAA-477) was used for this research. *E. coli* strains DH5α and BL-21 plysS (DE3) were kindly provided from the National Stratagene (LaJolla, CA, USA). The expression vector of pET-23a was bought from the National Recombinant Gene Bank of Pasteur Institute of Iran.

General Molecular Biology Techniques

Isolation of genomic DNA and plasmid purification was performed as described by Sambrook and Russell [9]. DNA digestions with restriction enzymes, ligations, and transformations were performed by standard procedures. Sequencing was performed by the commercial services of MacroGen Co. ltd. (Seoul, Korea) with the appropriate sequencing primers.

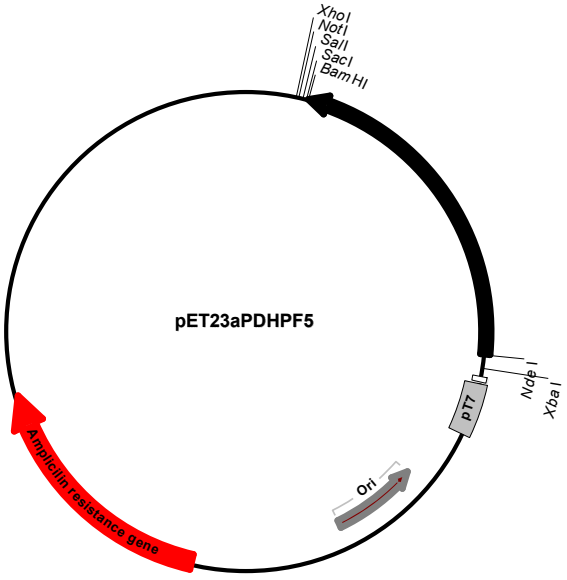

Figure 2. Construction of PDHPF5 expression plasmid pET23aPDHPF5. The PCR fragment corresponding to pdh gene digested with *Nde*I nad *Bam*HI and ligated with the vector pET23a previously digested with *Nde*I and *Bam*HI.

PCR Amplification and Construction of Expression Plasmid

PCR primers were designed based on the available nucleotide sequence of PutA of the *p. fluorescence* genome using DNASIS MAX software (DNASIS version 2.9, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). A 1035-kb DNA fragment containing the truncated ProDH domain was amplified by PCR from the genomic DNA of *p. fluorescence* with specific primers PDHPF5Fw (5'-TAT*CATATG*CTGACCTCC TCCCTG-'3) and PDHPF5Rev (5'-A*GGATCC*ATGTC GGCGATACG-'3), which contained the restriction sites for *Nde*I and *Bam*HI, respectively. PCR amplification was performed in a 50 µl reaction mixture containing 20 pmol of each primer, 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 mg template DNA and 2.5 units of *pfu* DNA polymerase under amplification condition: initial denaturation at 95°C for 2 min followed by 30 cycles, 45 sec of denaturation at 95°C, 45 sec of annealing at 60°C, 1 min of extension at 72°C and a final extension step of 5 min at 72°C. The product of the PCR reaction was cut with *Nde*I and *Bam*HI, gel purified and then ligated into the pET23a (+) expression vector carrying a C-terminal His_6-taq previously digested with the same restriction enzymes. The resulting construct bearing the ProDH gene was named pET23aPDHPF5 (Fig. 2) and transformed into the *E. coli* BL-21 (DE3) plysS. The correctness of the cloned gene was confirmed by nucleotide sequencing and no mutation was revealed.

Expression, and Refolding of Recombinant Enzyme

E. coli BL21 (DE3) plysS cells bearing pET23aPDHPF5 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 OD_{600} 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. Bacterial pellet were suspended in lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA, pH 8.0), mechanically disrupted by sonication in pulse sequence of 15s on and 10 s off and clarified by centrifugation at 4000 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 4000 rpm at 4°C for 1 h. Refolding was performed by stepwise dialysis against descending concentrations of urea [9]. The unfolded recombinant ProDH was first dialyzed against buffer A supplemented with 4 M, 2 M and then without urea. The buffer was changed every 24 h. For reconstitution, the renaturated enzyme was dialyzed overnight at 4°C in buffer A containing 0.15 mM FAD. The dialysate was centrifuged at 10000 rpm at 4°C for 1 h. The supernatant solution containing renaturated proteins were used for further purification.

Enzyme Activity Assay

ProDH activity was measured using the proline: 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. The standard reaction mixture was composed of 100 mM Tris-HCl, 10 mM $MgCl_2$, 10% glycerol, pH 8.5, 200 mM L-proline, 0.2 mM FAD, 0.45 mM INT, 0.08 mM PMS and the enzyme in a total volume of 1ml. The increase in absorbance at 490 nm was estimated and corrected for blank values lacking proline. Also, all values were corrected for the low rate of enzyme-independent proline oxidation observed in assay mixtures containing all components except enzyme. One unit (U) of ProDH activity was defined as the quantity of enzyme, which transfers electrons from 1 µmol of proline to INT per minute at 25°C [10]. All assay experiments were done in triplicate and the average results were used for data analysis.

Protein Determination

Protein concentrations were measured by the method of Bradford using bovin serum albumin (BSA) as a standard [11].

Purity Analysis

The ProDH purification was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [9]. SDS-PAGE was performed using discontinuous gels (10 cm × 10 cm) with a 12% separating gel and a 6% stacking 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 30v and 10mA for 5h. 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), ß-galactosidase (175 kDa), lactate dehydrogenase (142 kDa), fructose-6-phosphate (88 kDa), bovin serum albumin (66 kDa) and ovalalbumin (45 kDa) were used as molecular markers.

Kinetic Analysis

Initial reaction rates of the ProDH were measured with various concentrations of proline. The Michalis-Menten parameter (K_m) was determined from Linweaver-burk plots of the data obtained from initial rates using UVprobe software.

Sequence Alignment

BLAST through NCBI was used to identify homologous structures of ProDH, with default settings against the database of protein sequences in the protein data bank (PDB). Multiple sequence alignment was performed with Clustal W program. Alignments were checked for deletions and insertions in structurally conserved regions and finally fine-tuned manually modified.

Results and Discussion

Cloning and Sequencing of ProDH Gene

Utilizing PCR amplification, a 1035-bp DNA fragment containing ProDH gene domain was obtained (Fig. 3), which was gel purified and cloned into pET-23a in the frame with 6x-His tag. The corresponding plasmid was designated pET23PDHPF5, and transformed in *E. coli* strain BL21 (DE3) pLysS. Among 40 transformants of *E. coli* strain, 20 colonies were selected for plasmid isolation. All the clones exhibited an insert of 1035-bp along with a 3666-bp (pET23a) vector band after digestion with *Nde*I and *Bam*HI (Fig. 3). The restriction pattern confirmed the cloning of ProDH gene (Fig. 2). The nucleotide sequence of the insert DNA of pET23PDHPF5 was determined by the dideoxynucleotide chain termination method using M13 forward and M13 reverse primers. The 1107-bp open reading frame (ORF) of the ProDH gene had a coding capacity of 325 amino acids (Fig. 4). This suggested that the ProDH would be synthesized as 40 kDa enzyme.

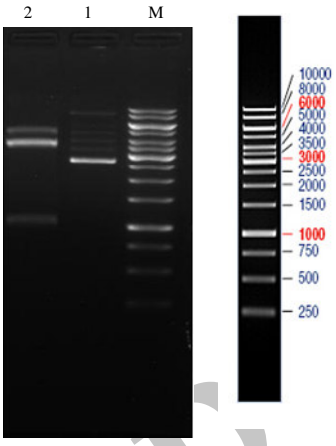

Figure 3. Analysis of the PCR-amplified ORF of ProDH and confirmation of the cloning of the ProDH gene specific fragment (1035 bp) from *P. fluorescence* in the pET23a. lane M, 1-kb ladder; lane 1, isolated plasmid; lane 2, *Nde*I and *Bam*HI-digested clones (the presence of the 1035 bp fragment is shown).

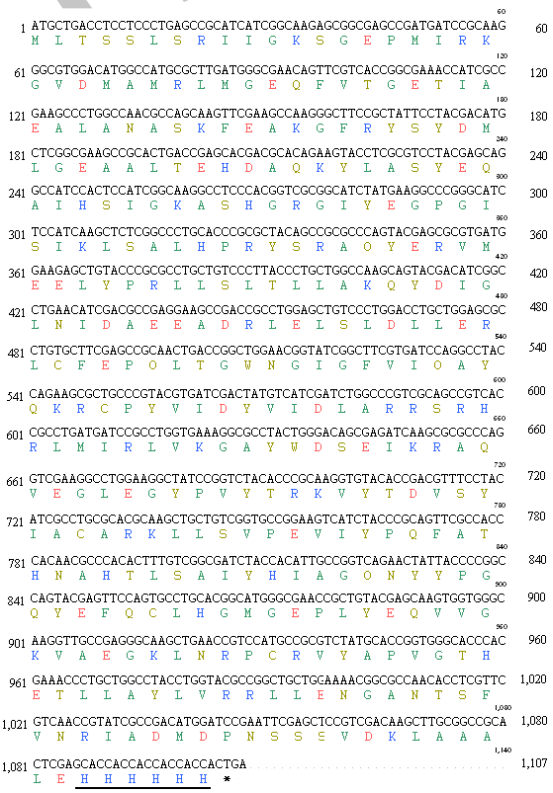

Figure 4. Nucleotide sequence of the *Nde*I and *Bam*HI fragment subcloned from PDH in pET23a. The predicted amino acid sequence is in the single-letter code. The underline sequence represents the His-tag region. The numbers on the left are nucleotide accounts.

Expression and Purification

ProDH was purified to homogeneity by affinity chromatography from the recombinant *E. coli* strain BL21 (DE3) pLysS carrying pET23PDHPF5 with an overall yield of 72% and a purification factor of 11. The purified enzyme gave a single band with a molecular mass of 40 kDa on SDS-PAGE (Fig. 5). The molecular mass of the isolated enzyme was found to be about 40 kDa by gel filtration. This result indicated that the target enzyme consists of one subunit. The observed band matched with the expected molecular weight for recombinant ProDH.

Kinetic Parameters, Substrate Specificity and Effect of Temperature and pH

Initial velocity experiments were done by varying the concentration of L-proline. The K_m and V_{max} values of *P. fluorescence* ProDH were calculated to be 20 mM and 160 µmol/min, respectively. The K_m value is lower than that reported for other ProDH enzymes. For example, K_m value of proline for the PutA enzymes in *P. aeruginosa* [5] and *S. typhimurium* [7] has been reported 45 mM and 43 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 [10, 13]. Therefore, the higher affinity of *P. fluorescence* ProDH toward proline made this enzyme very attractive for use in biosensors and protein engineering studies. The ability of ProDH to catalyze the dehydrogenation of various amino acids was examined. L-proline (100%) was the most preferred substrate for the ProDH reaction (Table 1). The enzyme also showed weak activities towards L-Threonine and L-Alanine. The following amino acids were inert for the ProDH reaction: D-proline, L-Hydroxyproline, L-Arginine, Aspartate, and Glycine. Moreover, chelating agents such as EDTA did not inhibit the enzyme. Similar results have been observed for *P. aeruginosa* [5] and *S. typhimurium* [7] ProDHs. The ProDH reaction exhibited its maximal activity at temperature range of 25 to 30°C, and its highest activity was achieved at 30°C (Fig. 6A). As can be seen (Fig. 6A), a sharp decrease in enzyme activity was observed above 30°C and was completely inactivated at 70°C. From this feature, it was concluded that like many other ProDHs [5,7], the *P. fluorescence* ProDH was a form of mesophilic enzymes. Similar results have been reported for ProDHs isolated from *P. aeruginosa* [5] and *P. putida* [6]. The effect of various pH values on the enzymatic reaction of ProDH were evaluated in the pH range from 3.0 to 12.0 at 30°C. ProDH had a good activity in the range of pH 7.0-9.0 with optimal pH at 8.5 (Fig. 6B). Similar results have been reported for other bacteria ProDHs [14].

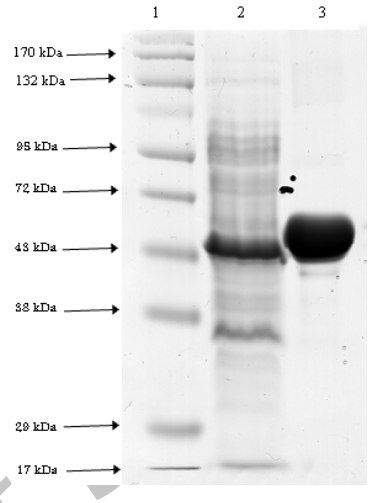

Figure 5. SDS-PAGE electrophoresis of the purified ProDH. Protein samples of various stages of the purification process. Lane 1: molecular weight markers; lane 2: pellet of the cell lysate; lane 3: purified enzyme.

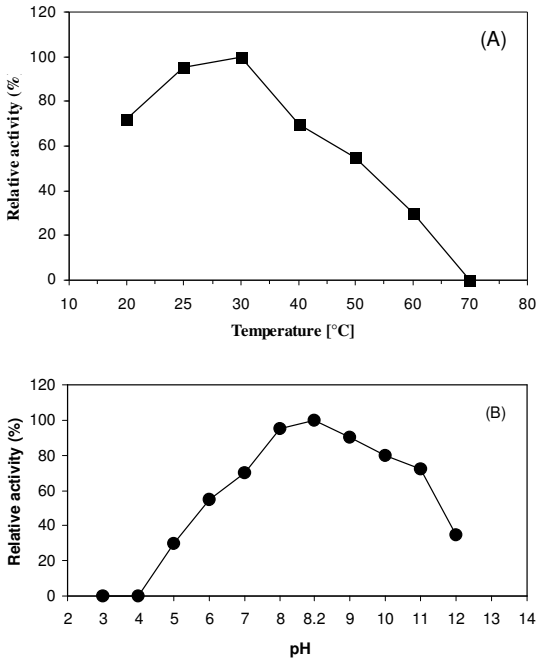

Figure 6. Influence of temperature (A) and pH (B) on the activity of ProDH from *P. fluorescence*.

Figure 7. Sequence alignment of *P. fluorescence* ProDH sequence with *E. coli* using DNASIS MAX software. Identical residues are highlighted in yellow.

Table 1. Substrate specificity for the ProDH reaction of *P. fluorescence* ProDH

Amino acid	Concentration (mM)	Relative activity (%)
L-Proline	200	100
D-Proline	200	0
L-Hydroxyproline	200	100
L-Tryptophan	10	0
L-Arginine	10	62
L-Serine	10	55
L-Glutamate	10	0
L-Histidine	10	72
L-Threonine	10	66
L-valine	10	33
L-Leucine	10	42
L-Alanine	10	48
Glycine	10	52
Aspartate	10	0

Each value represents the average of three experiments.

In summary, we isolated the gene encoding of ProDH enzyme from *P. fluorescence*, expressed it in *E. coli* BL-21 (DE3) plysS with a C-terminal His-tag, and examined the biochemical characteristics of recombinant enzyme. The target enzyme is a good candidate for specific determination of proline amino acid in biosensors. Modeling studies also provided valuable information about the active site of *P. fluorescence* ProDH.

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