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PROLINE DEHYDROGENASE FROM *Pseudomonas fluorescence*: GENE CLONING, PURIFICATION, CHARACTERIZATION AND HOMOLOGY MODELING

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The gene encoding proline dehydrogenase (ProDH) from *Pseudomonas fluorescence* was isolated using PCR amplification and cloned into pET23a expression vector. The expression of the recombinant target enzyme was induced by addition of IPTG. The produced His-fusion enzyme was purified and its kinetic properties were studied. The 3D structure modeling was also performed to identify key amino acids involved in FAD-binding and catalysis. The PCR product contained a 1033 bp open reading frame encoding 345 amino acid residue polypeptide chain. SDS-PAGE analysis revealed a MW of 40 kDa, whereas the native enzyme exhibited a MW of 40 kDa suggesting a monomeric protein. The K_m and V_{max} values of the *P. fluorescence* ProDH were estimated to be 35 mM and 116 μmol/min, respectively. ProDH activity was stable at alkaline pH and the highest activity was observed at 30°C and pH 8.5. The modeling analysis of the three dimensional structure elucidated that Lys-173 and Asp-202, which were oriented near the hydroxyl group of the substrate, were essential residues for the ProDH activity. This study, to our knowledge, is the first data on the cloning and biochemical and structural properties of *P. fluorescence* ProDH.

The amino acid L-proline is metabolized to glutamic acid in a two-step oxidation reaction. In the most bacteria, both enzymatic steps for proline utilization are catalyzed by a multifunctional encoded by the *putA* gene [1]. Multifunctional proline utilizating A flavoprotein (PutA) contains proline dehydrogenase (ProDH; L-proline: FAD oxidoreductase; EC 1.5.99.8) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH; P5C: NAD^+ oxidoreductase, EC 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 this enzymatic role, PutA polypeptide has also DNA-binding activity and participates in the transcriptional control of *put* genes. 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 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 *Escherichia coli* [4], *Pseudomonas aeruginosa* [5], *P. putida* [6], *Salmonella 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 functionality 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, this enzyme exhibits a high potential for application in biosensors.

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 (Germany).

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

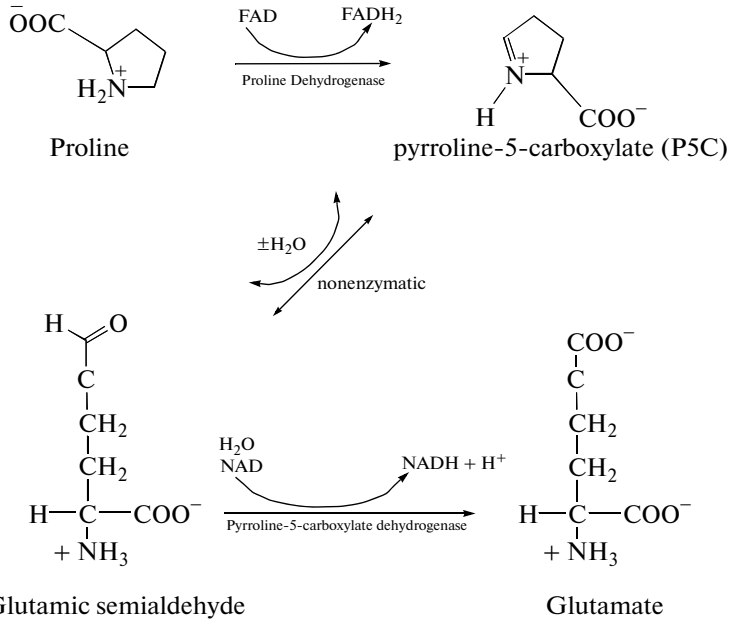

Fig. 1. Chemical reactions catalyzed by the bi-functional PutA flavoenzyme in metabolism of proline to glutamate.

vector of pET-23a was obtained 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 [9]. Sequencing was performed by the commercial services of MacroGen Co. ltd. (Seoul, Korea) with the appropriate sequencing primers.

PCR amplification and construction of expression plasmid for ProDH domain gene. 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., 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*CTGACCTCCTCCCTG-3') and PDHPF5Rev (5'-AGGATCCATGTCGGCGATACG-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, 1*x* 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: preincubation at 95°C for 1 min and then 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 2 min. 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 [9].

Expression, solublization, refolding and reconstitution 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 the same conditions. When cell density reached an OD_{600} of 0.6–0.8, ProDH enzyme was expressed by the addition of 0.5 mM of 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 the lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA, pH 8.0), mechanically disrupted by sonication in pulse sequence of 15 s on and 10 s off and clarified by centrifugation at 5000 g for 1 h. The precipitate (inclusion bodies) containing recombinant ProDH enzyme was washed twice with the 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) contaning 100 mM NaCl, 10 mM EDTA, 10% glycerol and 0.1 mM DTT (buffer A) supplemented 8.0 M urea and incubated at 4°C with continuous stirring for 24 h to solubilize the inclusion bodies. Any insoluble material

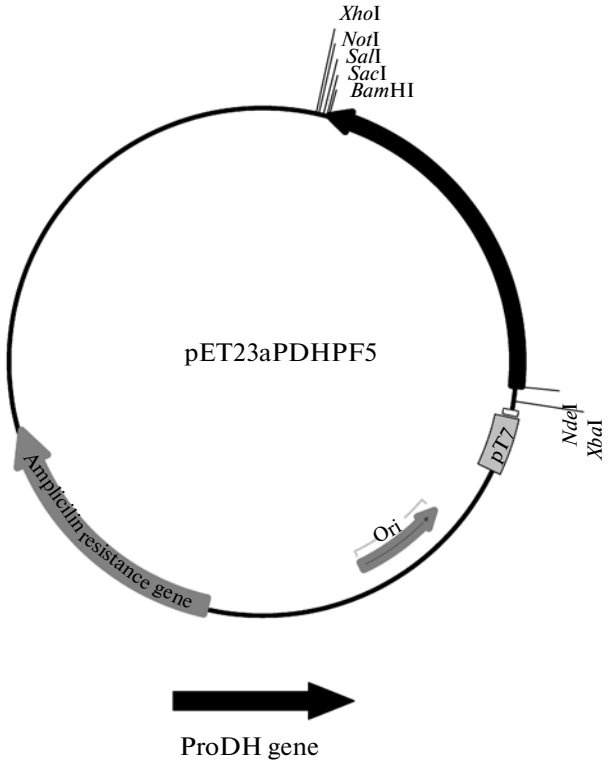

Fig. 2. Construction of PDHPF5 expression plasmid pET23aPDHPF5. The PCR fragment corresponding to *pdh* gene digested with *Nde*I nad *Bam*HI and ligated with the vector pET23a previously digested with *Nde*I and *Bam*HI.

was removed by centrifugation at 5000 g at 4°C for 1 h. Refolding was performed by stepwise dialysis against descending concentrations of urea. The unfolded recombinant ProDH was first dialyzed against buffer A supplemented with 4.0, 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 5000 g at 4°C for 1 h. The supernatant solution containing renaturated proteins was used for further purification [9].

Enzyme activity assay. ProDH activity was measured using the proline: 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) oxidoreductase assay which was performed by INT as a terminal electron acceptor and phenazine methosulfate (PMS) as a mediator electron carrier [10]. The standard reaction mixture was composed of 100 mM Tris-HCl (pH 8.5) containing 10 mM $MgCl_2$, 10% glycerol, 200 mM L-proline, 0.2 mM FAD, 0.45 mM INT, 0.08 mM PMS and the enzyme in a total volume of 1 ml. 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 [11]. 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 bovine serum albumin as a standard [12].

Purity analysis. The ProDH purification was analyzed by SDS-PAGE [9]. This procedure was performed using discontinuous gels (10 × 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 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. Apoferritin (443 kDa), myosine (200 kDa), β-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 deter-

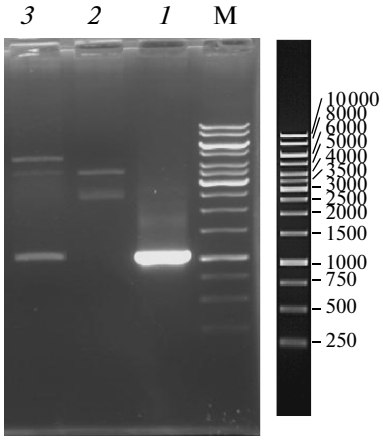

Fig. 3. Analysis of the PCR-amplified ORF of ProDH and confirmation of the cloning of the ProDH gene specific fragment (1035 bp) from *P. fluorescence* in the pET23a. M – 1-kb ladder; *1* – PCR-amplified sample; *2* – isolated plasmid; *3* – *Nde*I and *Bam*HI-digested clones (the presence of the 1035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

mined from Lineweaver-Burk plots of the data obtained form initial rates using UV probe software.

Sequence alignment and homology modeling. 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). The crystal structure of PutA from *E. coli* K12 with bound FAD (PDB code: 1k87) was selected as a template for homology modeling. The quality of the 1k87 hit was indicated by a score of 82 bits, an E-value of 7e-14 and 88% identity. 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 before 3D modeling. The three-dimensional model of ProDH protein was constructed using the homology modeling program Modeler version 9v4 (default parameters), based upon the crystal structure of *E. coli* K12 ProDH. Furthermore, FAD was docked into the protein model. The geometry of loop regions was corrected using MODELER/Refine Loop command. The minimized model was then analyzed further and validated using Ramachandran plots obtained from the PROCHECK server [13]. Visual analysis and manipulation of the model were done with PyMOL program, which was also used for illustrations.

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

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.

RESULTS AND DISCUSSION

Cloning and sequencing of ProDH gene from *P. fluorescence*. After 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 the *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 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 [9] 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.

Expression and purification of recombinant enzyme. 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.

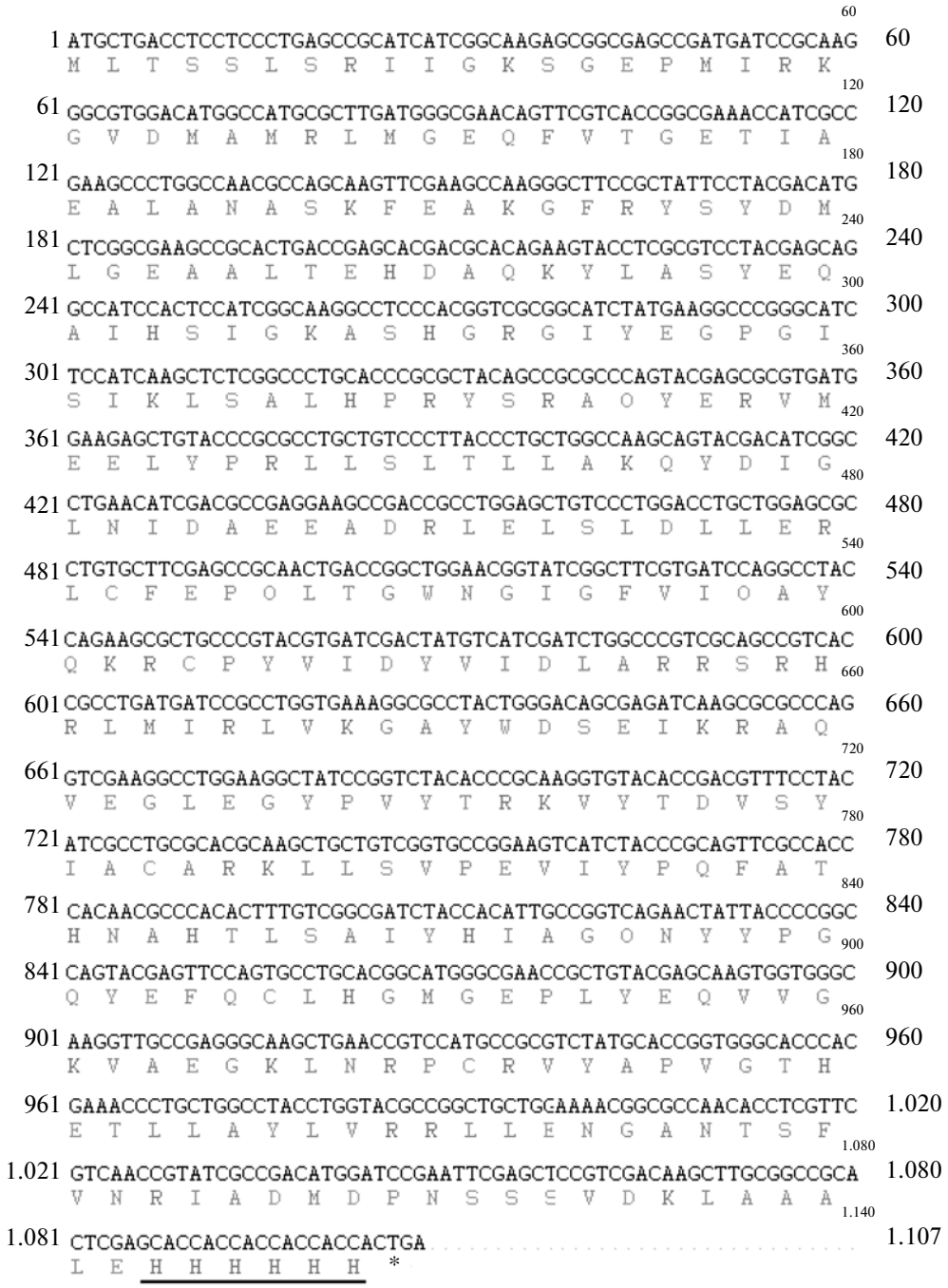

Fig. 4. Nucleotide sequence of the *Nde*I and *Bam*HI fragment subcloned from PDH in pET23a. The predicted amino acid sequence is in the single-letter code. The underline sequence represents the His-tag region. The numbers on the left are nucleotide accounts.

Kinetic parameters, substrate specificity and effect of temperature and pH. Initial velocity experiments were done by varying the concentration of L-Pro. The K_m and V_{max} values of *P. fluorescence* ProDH were calculated to be 35 mM and 116 μmol/min, respectively. The K_m value is lower than that reported for other bacterial 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 literature, high K_m value of ProDHs for proline is one of the common features of proline metabolizing enzymes in bacteria [11, 14]. Therefore, the higher affinity of *P. fluorescence* ProDH toward proline made this en-

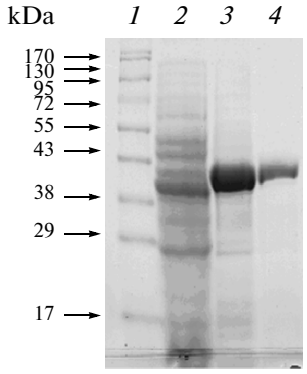

Fig. 5. SDS-PAGE of the purified ProDH. Protein samples of various stages of the purification process. *1* – molecular weight markers; *2* – supernatant of the cell lysate; *3* – pellet of the cell lysate; *4* – purified enzyme.

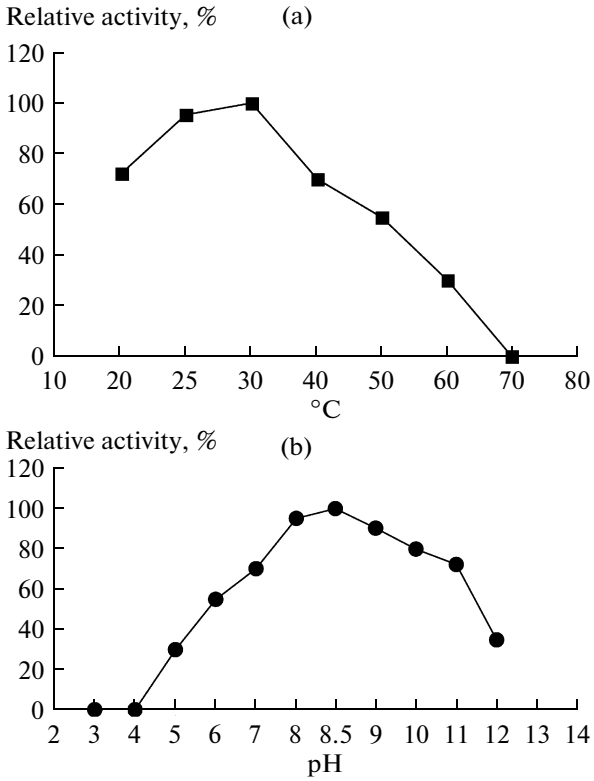

Fig. 6. Influence of temperature (a) and pH (b) on the activity of ProDH from *P. fluorescence*.

zyme 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-Pro (100%) was the most preferred substrate for the ProDH reaction (Table). The enzyme also showed weak activities towards L-Val, L-Leu and L-Ala. The following amino acids were inert for the ProDH reaction: D-Pro, Asp, L-Glu, and L-Trp. Moreover, chelating agents such as EDTA did not inhibit the enzyme. Similar results have been observed for the *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 was observed above 30°C and enzyme activity 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 bacterial ProDHs [14].

Amino acid sequence alignment and homology modeling of 3D structure. The search for the closet paralog led to the structure of ProDH from *E. coli* K12. Based on this evidence, *E. coli* K12 ProDH was taken as a template for ProDH of *P. fluorescence* pf-5. The amino acid sequence of *E. coli* K12 ProDH displayed 88% identity when aligned with that of 3D structure (Fig. 7). We constructed the three dimensional structure of the *P. fluorescence* ProDH based on its similarity to the structure of the previously crystallized ProDH from *E. coli* K12. The 100 models were evaluated and the one with the lowest DOPE score was chosen for further analysis. The Ramachandran plot for local backbone conformation of each residue in the final model was produced by PROCHECK. In the *P. fluorescence* ProDH model, φ and Ψ dihedral angles of 100% of residues were located within the allowed regions (94.7% most favored). This result expressed the strong confidence in the homology model. Moreover, we used the three dimensional homology modeling to identify key amino acids involved in FAD-binding and catalysis. The 3D structure of ProDH from *P. fluorescence* is presented at Fig. 8. As seen in the 3D structure of ProDH of *P. fluorescence* presented in Fig. 8, Lys-173 and Asp-202, which were oriented near the hydroxyl group of the substrate in the model were essential for the ProDH activity. The model provided considerable information on substrate and FAD interactions with the active site of the *P. fluorescence* ProDH.

We isolated the gene encoding of the 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 the *P. fluorescence* ProDH.

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                    10        20        30        40        50        60
Pseudomonas fluorescens Pf-5 LTSSLSRIIG KSGEPMIRKG VDMAMRLMGE QFVTGETIAE ALANASKFEA KGFRYSYDML
Escherichia coli K-12        LSRSLNRIIG KSGEPLIRKG VDMAMRLMGE QFVTGETIAE ALANARKLEE KGFRYSYDML
                    70        80        90       100       110       120
Pseudomonas fluorescens Pf-5 GEAALTEHDA QKYLASYEQA IHSIGKASHG RGIYEGPGIS IKLSALHPRY SRAQYERVME
Escherichia coli K-12        GEAALTAADA QAYMVSYQQA IHAIGKASNG RGIYEGPGIS IKLSALHPRY SRAQYDRVME
                   130       140       150       160       170       180
Pseudomonas fluorescens Pf-5 ELYPRLLSLT LLAKQYDIGL NIDAEEADRL ELSLDLLERL CFEPQLTGWN GIGFVIQAYQ
Escherichia coli K-12        ELYPRLKSLT LLARQYDIGI NIDAEESDRL EISLDLLEKL CFEPELAGWN GIGFVIQAYQ
                   190       200       210       220       230       240
Pseudomonas fluorescens Pf-5 KRCPYVIDYV IDLARRSRHR LMIRLVKGAY WDSEIKRAQV EGLEGYPVYT RKVYTDVSYI
Escherichia coli K-12        KRCPLVIDYL IDLATRSRRR LMIRLVKGAY WDSEIKRAQM DGLEGYPVYT RKVYTDVSYL
                   250       260       270       280       290       300
Pseudomonas fluorescens Pf-5 ACARKLLSVP EVIYPQFATH NAHTLSAIYH IAGQNYYPGQ YEFQCLHGMG EPLYEQVVGK
Escherichia coli K-12        ACAKKLLAVP NLIYPQFATH NAHTLAAIYQ LAGQNYYPGQ YEFQCLHGMG EPLYEQVTGK
                   310       320       330       340       350       360
Pseudomonas fluorescens Pf-5 VAEGKLNRPC RVYAPVGTHE TLLAYLVRRL LENGANTSFV NRIAD..... ..........
Escherichia coli K-12        VADGKLNRPC RIYAPVGTHE TLLAYLVRRL LENGANTSFV NRIAD..... ..........
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Fig. 7. Sequence alignment of *P. fluorescence* ProDH sequence with *E. coli* using DNASIS MAX software. Identical residues are highlighted in grey.

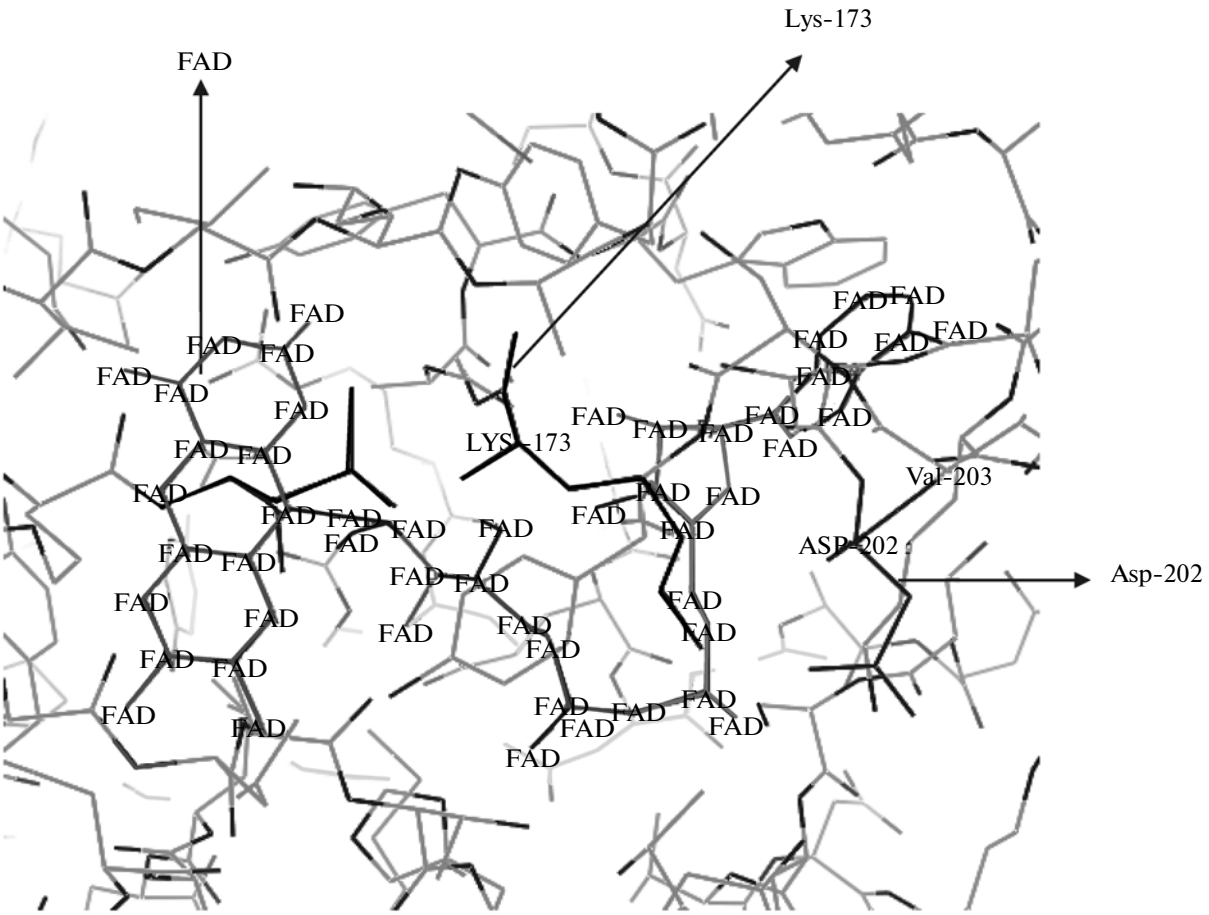

Fig. 8. Modeling of ProDH from *P. fluorescence* based on homology modeling. Stick model showing the conserved residues interacting with FAD. The figure was created with Pymol.

ACKNOWLEDGMENTS

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