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Affinity Purification and Characterization of Recombinant *Bacillus sphaericus* Phenylalanine Dehydrogenase Produced by pET Expression Vector System

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ABSTRACT

Cloning and expression of the L-phenylalanine dehydrogenase gene, from *B. sphaericus* in *E. coli* were done. The gene was cloned in the vector pET16b and transformed into *E. coli* BL21 (DE3). The functional form of the L-phenylalanine dehydrogenase enzyme was purified by affinity purification techniques, taking advantage of the ability of this enzyme to bind to the nucleotide site affinity dye, Reactive Blue 4. Approximately 3 mg of highly purified recombinant enzyme was obtained from 950 mg cell pellet (wet weight). The Relative molecular mass of the L-phenylalanine subunits was about 41 kDa by 10% SDS-PAGE. Using this method, the enzyme was obtained with a yield of 28%, and had a specific activity of 577.3 U/mg protein, which is purified 88 times. This method was provided a facile and effective way for preparing the enzyme with a good yield that suitable for analytical purposes. *Iran. Biomed. J. 6 (1): 31-36, 2002*

Keywords: Bacillus sphaericus, L-phenylalanine dehydrogenase (PheDH), affinity purification, expression,

INTRODUCTION

L-Phenylalanine dehydrogenase (PheDH) [NAD^+ Oxidoreductase, deaminating; EC 1.4.1.20] was discovered in 1984 [1], and since then, has been identified from several bacteria sources, including *Bacillus*, *Sporosarcina* [2, 3], *Nocardia* [4], *Microbacterium* [5] and *Thermoactinomyces* [6].

PheDH has limited substrate activity with phenylpyruvate analogues, although phenylketobutyrate is a reasonably good substrate for the enzyme. Enzyme-catalyzed reductive amination of phenylketobutyrate is potentially useful for the production of optically pure L-homophenyl-alanine, a component of an angiotensin converting enzyme (ACE) inhibitor used in the treatment of hypertension and heart failure [7]. The enzyme is also being developed as a biosensor to screen for phenylketonuria [8, 9] and has industrial uses for the production of optically pure L-phenylalanine [10], a component of the artificial sweetener aspartame [11].

The low yield of enzyme in wild strains triggered researchers to use recombinant DNA techniques in order to obtain sufficient amounts of the PheDH. *E. coli*, which naturally lacks the PheDH, is commonly used as a host for production of heterologous proteins, including PheDH. This enzyme has been already purified by means of multistage chromatography columns [3, 5, 12]. These different purification methods are a little bit tedious and time consuming for laboratory aims. These situations have prompted us to search for a straightforward and simple method for purification of enzyme. This paper describes the heterologous expression of *Bacillus sphaericus* PheDH in catalytically active form in *E. coli* and its purification by chromatography using Reactive Blue 4 dye.

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MATERIALS AND METHODS

Strains, plasmids and chemicals. The *E. coli* JM109 (ATCC 53323) was used as a host strain for subcloning and *E. coli* BL21(DE3) (Novagen, Inc. Madison, USA) was used for protein expression. Transformants were grown at 37°C in Luria and Bertani (LB) medium with or without 1.2% agar containing 0.1 mg/ml ampicillin [13]. Vectors pUC19 and pET16b (Novagen, Inc. Madison, USA) were used for subcloning, sequencing and expression. Restriction enzymes and shrimp alkaline phosphatase were from Boehringer Mannheim (FRG). Reagents for ligation were supplied in a kit by Takara Corp. (Shuzo, Japan). DNA sequencing was performed using the 7-deza-dGTP kit and Sequenase product number US78500 (United States Biochemicals, USA). Long ranger agarose solution was from FMC Corp. (USA). Other chemicals for sequencing were obtained from Wako Crop. (Osaka, Japan). Plasmid purification was done using the QIAGEN plasmid purification kit (FRG). Reactive Blue 4 was purchased from Sigma Corp. (USA).

Recombinant DNA techniques and subcloning. DNA manipulations including cloning, ligation, amplification, and transformation followed the methods described by Sambrook *et al.* [13]. Restriction endonucleases and other enzymes were used as recommended by the suppliers. The plasmid pBDHH1DBL (PheDH gene of *B. sphaericus*, referred to as *pdh*, inserted in the *Bam*H I site of pUCC) was kindly provided by the Biotechnology Research Center, (Toyama Prefectural University, Japan). In order to isolate the insert from the construct, pBDH1DBL was amplified in *E. coli* JM109. This recent recombinant vector purified from *E. coli* cells and restricted with *Bam*H I to yield a 1.5-kb fragment which was subsequently subcloned into *Bam*H I linearized pET16b. The resulting construct was designated as pETDH. It was used to transform *E. coli* BL21 (DE3). The recombinant clones containing the *pdh* gene on the 1.5-kb *Bam*H I fragment were screened on LB plate containing ampicillin (0.1 mg/ml), and some colonies were selected for digestion with *Hind* III, *Eco*R I, and *Bam*H I. After confirming the presence of the *pdh* gene and determining its orientation by restriction analysis, a representative clone was digested with *Bam*H I and the 1.5 kb *Bam*H I fragment was subcloned into pUC19. The recombinant construct (referred to as pUCDH) was sequenced using the dye terminator sequencing kit (USB Corporation, Ohio, USA). Once the integrity of the sequence was confirmed, the recombinant vector (pETDH) was selected for over expression of the PheDH.

Transformation and expression. Competent cells of *E. coli* JM109 and *E. coli* BL21 (DE3) were prepared and transformed with pUCDH and pETDH according to the Hanahan protocol [14]. The PheDH producer clone was named *E. coli* BL21pETDH and was used to express the *pdh* gene in *E. coli* BL21(DE3) under control of the T7 promoter.

Cell culture and protein overexpression. *E. coli* BL21pETDH cells were cultured in LB broth supplemented with ampicillin at 0.1 mg/ml. A 10 ml culture (8 h old) was diluted 100-fold into 1 L of medium in baffled culture flasks (200 ml/L) and shaken at 37ºC until an OD_{600} = 1.0 was reached. The culture was then chilled to approximately 23ºC by swirling the flasks in an ice-water bath for 4 min. Sterile IPTG was added to a final concentration of 0.005 mM and shaking resumed at 23ºC for 8 h. The cells were harvested by centrifugation and the cell pellets were rapidly frozen in liquid nitrogen and stored at –20ºC.

Purification and PheDH assay. All purification procedures were performed at 4ºC. About 8 g of wet cell mass was suspended in 20 ml of buffer A (50 mM Tris-HCl pH 8.3, 0.1 mM EDTA, 5 mM 2-mercaptoethanol) containing 1 mg/ml lysozyme. This solution was left at room temperature for 20 min and sonicated (50 min total) with a ultrasonic oscillator. The slurry was centrifuged at 12,000 × g at 4ºC for 1 h to clarify. Cell-free homogenate was placed at 50ºC for 10 min. After cooling on ice, the mixture was recentrifuged as before. The clear supernatant was brought to 0.3 M $(NH_4)_2SO_4$ salt saturation: the salt was added very slowly and under constant stirring, while kept on ice. After the salt was completely dissolved, the suspension was incubated at 4ºC for 2 h with gentle stirring, and then centrifuged at 12,000 × g at 4ºC for 1 h. The supernatant was recovered and more $(NH_4)_2SO_4$ was added to bring the solution to 0.6 M saturation. After incubating at 4ºC for 2 h with slow stirring, the suspension was recentrifuged (12,000 × g, for 1 h, at 4ºC). The precipitate was collected and dissolved in 2 ml of a buffer B (consisting of 25 mM Tris-HCl, pH 8.3, 2.5 mM 2-mercaptoethanol, 0.05 mM EDTA), at 4ºC. The resulting solution

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was dialyzed against buffer B in order to remove remaining salts.

The dialysate was added to Reactive Blue 4 agarose slurry (30 ml) pre-equilibrated with buffer B. The mixture was incubated on ice for 30 min (gently shaking periodically to facilitate enzyme adsorption to the beads). The whole slurry was washed with three bed volumes of Buffer B and centrifuged at 5,000 × g at 4º C for 20 min). The PheDH enzyme was eluted with 20 ml of buffer C (buffer B containing 1 M KCl) and precipitated with $(NH_4)_2SO_4$ (0.6 M saturation). The PheDH protein was recovered by centrifugation as before, and solubilized in buffer B. The protein solution was then dialyzed and applied to a column (20 × 1 cm; 15 ml bed volume) of Reactive Blue 4 agarose, pre-equilibrated as before with buffer B. The column was flushed with 3 bed volumes using buffer B (flow rate of 15 ml/h). The enzyme was eluted with 15 ml of buffer C (flow rate 5 ml/h). Fractions containing high enzyme activity, as determined by the reduction of NAD^+ with L-phenylalanine [10], were pooled and analyzed by SDS-PAGE.

Enzyme activity. The enzyme activity was measured by the reduction of NAD^+ at 25°C using L-phenylalanine as a substrate in a 1 ml reaction mixture containing 100 mM glycine-KCl-KOH buffer (pH 10.5), 2.5 mM NAD^+, 10 mM L-phenylalanine and the enzyme sample. To determine the K_m and V_{max} values of PheDH, various concentrations of L-Phe and NAD^+ were employed [10]. Reductive amination was carried out at 25°C in a reaction mixture containing 100 mM glycine-KCl-KOH buffer (pH 9.0), 0.1 mM NADH, 200 mM NH_4Cl, 10 mM sodium phenylpyruvate, and enzyme solution (total 1.0 ml). All reactions were monitored at 340 nm. One unit of the enzyme activity in oxidative deamination reaction was defined as the amount of enzyme used to catalyze the formation of 1 μmol NADH/min [10]. The protein concentration was determined spectrophotometrically (absorbance at 280 nm using the absorption coefficient $A_{1\%/1cm}$= 6.3) [15] or with a Bio-Rad protein assay kit.

RESULTS AND DISCUSSION

Construction and transformation. *Pdh* genes have been successfully cloned from different sources [10] and by us with conventional methods. In order to study the *pdh* gene, we cloned it into the pUC19 and pET16b plasmids. The construct pETDH coding for the PheDH was made as described in the experimental procedure (Figs. 1 and 2). *E. coli* BL21 (DE3) and JM109 cells were transformed with pETDH and pUCDH, respectively, as described in the materials and methods.

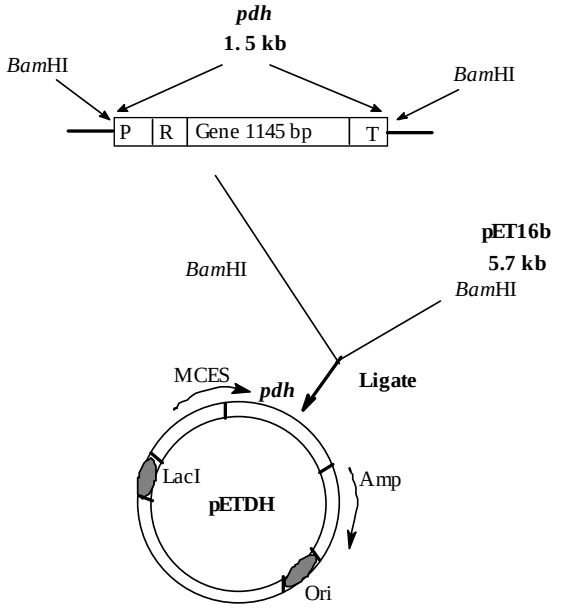

Fig. 1. Schematic of *B. sphaericus* phenylalanine dehydrogenase (*pdh*) gene and making expression construction. P, promoter; R, ribosome binding site; T, terminator; TC, tetracycline; MCES, multiple cloning expression site.

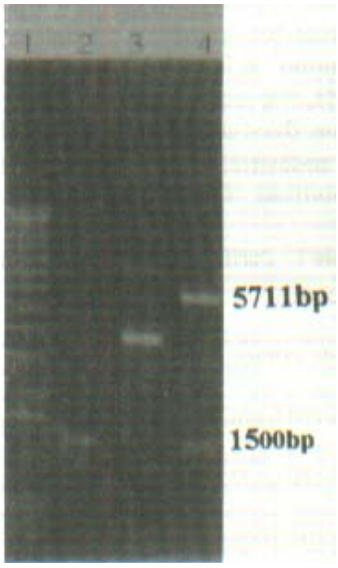

Fig. 2. Agarose gel analysis of construct and vector: Lane 1, one kbp marker; Lane 2, *pdh* insert; Lane 3, pETDH uncut; Lane 4, pETDH *Bam*H I cut.

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Expression and purification. The product of pETDH gene was determined by measuring the oxidative activity of NADH as an indicator [10], and was purified to near homogeneity with a final yield of 28% (Fig. 3). The purification steps and the yields are presented in Table 1.

Fig. 3. Silver stained SDS-PAGE. Lane 1, protein marker; lane 2, purified PheDH; lane 3, batch-wise Reactive Blue 4; lane 4, cell free extract.

Researchers have purified PheDH by means of multistage purification methods [3, 5, 12]. For instance, Misono *et al.* [12] after using 8 steps of purification such as, ammonium sulfate fractionation and column chromatographies, 85-fold enhancement of PheDH specific activity was obtained. These different purification methods are tedious and time consuming; nevertheless, there is no need to purify PheDH in a high grade in order to construct for example a diagnostic kit or apply it for an amino acid production. In order to purify PheDH, we used Reactive Blue 4 dye. Reactive triazine dyes are robust affinity ligands promising for industrial-scale bioprocesses, and their immobilized forms are exploited in downstream processing [16]. Dyes offer clear advantages over biologicals ligands [16,17]. Earlier studies confirmed the effectiveness of biomimetic dye affinity chromatography for the purification of glutamate dehydrogenase [18], formate dehydrogenase [19], alcohol dehydrogenase [20] and lactate dehydrogenase [21]. Reactive Blue 4 has been made by linking commercial dichlorotriazine with agarose beads and exhibit as the biomimetic moiety linked to the chlorotriazine ring, an α-keto acid structure [16]. It should be noted that this is the first report about using Reactive Blue 4 to purify PheDH. The present affinity method provides a simple and effective way for preparing PheDH enzyme with a good yield that is suitable for analytical purposes.

The specific activity of pheDH enzyme was 577.3 U/mg that is comparable to the values reported for PheDH from *B. badius* (68 U/mg) [2] and *Rhodococcus maris* (162 U/mg) [12]. The PheDH enzyme can be stored in 50% glycerol without loss of activity for more than 7 months, in contrast to other PheDH, which have been reported to lose the activity rapidly [4, 12]. Interestingly, *E. coli* BL21pETDH exhibited an PheDH activity (6200 U/L) that was over 140 times greater than the wild type *B. sphaericus* SCRC-R79a, (44 U/L). The expression level of recombinant PheDH of *B. badius* in *E. coli* reported to be 6890 U/L culture [2]. In contrast, Hanson *et al.* [7] (2000), reported a huge amount of production over 19000 U/L culture for the recombinant *T. intermedius* PheDH in *E. coli*. Seemingly, the different results were obtained to the promoters, mRNA construction and its topology, and condition of expression e.g. optimization of IPTG induction, type of cell line, media, and incubation circumstances.

Table 1. Purification of PheDH from recombinant *E. coli* BL21/pETDH.

Steps	Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery (%)	Fold purification
Crude extract	6200	950	6.5	100	1.0
30% $(NH_4)_2SO4$	5952	815	7.3	96	1.1
60% $(NH_4)_2SO4$	5580	649	8.6	90	1.3
Reactive Blue 4	2232	37	60.3	36	9.3
60% $(NH_4)_2SO4$	2165	32	67.6	35	10.4
Reactive Blue 4	1732	3	577.3	28	88.8

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Features of PheDH. PheDH exhibits a narrow range of subunit molecular masses between 36 and 42 kDa [11]. This 41-kDa recombinant *B. sphaericus* PheDH subunit calculated from its SDS-PAGE mobility agreed with the previously reported value [3]. There is considerable variation in the quaternary structures of these enzymes. The enzymes from *B. badius*, *B. sphaericus*, *S. ureae* and *Microbacterium* sp. DM 86-1 have been reported to be octamers [10]. On the other hand, the *T. intermedius* PheDH has been reported to be hexamer [6], the *R. maris* K-18, and *Rhodococcus* sp. M4 enzymes have been shown to be a dimmer [11]. The Nocardia sp. 239 enzyme has been pointed as a monomer. The molecular mass of PheDH was estimated to be approximately 340 kDa (not shown) by gel filtration on G-200 Sephadex column (1.5 × 85 cm). This result was supported by previous report wild type of PheDH [3].

The substrate specificity of this recombinant PheDH enzyme is shown in Table 2. The inert amino acids were D-phenylalanine, L-lysine, glycine, L-alanine, L-glutamic acid, L-asparagine, L-proline, L-serine and L-arginine. The K_m value determined from the secondary plots of intercepts against reciprocal concentration of the substrate. K_m values for L-phenylalanine, L-tyrosine and NAD^+ were 0.24 mM, 0.48 mM and 0.19 mM, respectively. The optimum pH for the oxidative deamination of the recombinant enzyme was 11 and 10.2 for the reductive amination. The above-mentioned features of heterologously produced PheDH enzyme were compatible to those of the wild type PheDH enzyme [3, 10].

Table 2. Substrate specificity of recombinant *B. sphaericus* PheDH.

Amino acid	Relative activity (%)
L-phenylalanine	100.0
L-tyrosine	74.0*
L-norleucine	4.5
L- valine	3.0
L-methionine	2.0
L-tryptophan	1.8
L-leucine	1.0

*Measured at 0.3 mM.

Note: The oxidative deamination reaction was carried out under the standard reaction conditions [10]. The concentration of amino acid was 10 mM unless indicated.

In brief, the *pdh* gene of *B. sphaericus* has been successfully cloned in pET system and expressed in *E. coli*. We used a simple and effective affinity method for preparing PheDH enzyme with a good yield that is suitable for analytical purposes. This is the first report about using Reactive Blue 4 to purify PheDH.

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REFERENCES

1. Hummel, W., Weiss, N. and Kula, M.R. (1984) Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity. *Arch. Microbiol. 7: 47-52*.
2. Asano, Y., Nakazawa, M.V., Endo, K., Hibino, Y., Ohmori, M., Numao, N. and Kondo, K. (1987) Phenylalanine dehydrogenase of *Bacillus badius*: Purification, characterization and gene cloning. *Eur. J. Biochem. 168: 153-159*.
3. Asano, Y., Nakazawa, A. and Endo, K. (1987) Novel phenylalanine dehydrogenase from *Sporosarcina ureae* and *Bacillus sphearicus*: Purification and characterization. *J. Biol. Chem. 262: 10346-10354*.
4. Pasquo, A., Britton, K.L., Baker, P.J., Brearley, G., Hinton, R.J., Moir, A.J., Stillman, T.J. and Rice, D.W. (1998) Crystallization of NAD^+-dependent phenylalanine dehydrogenase from Nocardia sp. 239. *Acta. Crystallogr. Biol. Crystallogr. 54: 269-272*.
5. Asano, Y. and Tanetani, M. (1998) Thermostable phenylalanine dehydrogenase from a mesophilic *Microbacterium* Strain DM86-1. *Arch. Microbiol. 169: 220-224*.
6. Takada, H., Yoshimura, T., Ohshima, T., Esaki, N. and Soda, K. (1991) Thermostable phenylalanine dehydrogenase of Thermoactinomyces intermedius: cloning, expression and sequencing of its gene. *J. Biochem. 109 :371-376*.
7. Hanson, R.L., Howell, J.M., Laporte, T.L., Donovan, M.J., Cazzulino, D.L. *et al.* (2000) Synthesis of allysine ethylene acetal using phenylalanine dehydrogenase from *Thermoactinomyces*

[DOR: 20.1001.1.1028852.2002.6.1.4.5]

intermedius. *Enzyme. Microb. Technol. 26: 348-358*.

8. Huang, T., Warsinke, A., Kuwana, T. and Scheller F.W. (1998) Determination of L-phenylalanine based on an NADH-detecting biosensor. *Anal. Chem. 70: 991-997*.
9. Rivero, A., Allue, J.A., Grijalba, A., Palacios, M. and Merlo, S.G. (2000) Comparison of two different methods for measurement of phenylalanine in dried blood spots. *Clin. Chem. Lab. 38: 773-776*.
10. Asano, Y. (1999) Phenylalanine dehydrogenase. In: *Encyclopedia of bioprocess technology: fermentation, biotechnology and bioseparation*. (Flinckinger, M.C. and Drew, S.W. eds.), Vol. 2, John Wiely and Sons, Inc., New York, USA, pp. 1955-1963.
11. Brunhuber, N.M.W., Thodden, J.B., Blanchard, J.S. and Vanhooke, J.L. (2000) Rhodococcus L-Phenylalanine dehydrogenase: kinetics, mechanism, and structural basis for catalytic specificity. *Biochemistry 39: 9174-9187*.
12. Misono, H., Yonezawa, J., Nagata, S. and Nagasaki, S. (1989) Purification and characterization of a dimeric L-phenylalanine dehydrogenase from Rhodococcus maris K18. *J. Bacteriol. 171: 30-36*.
13. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1994) Molecular Cloning: A laboratory manual. 2nd ed., Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York.
14. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol. 166: 557-580*.
15. Whitaker, J.R. and Granum, P.E. (1980) An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal. Biochem. 109: 156-159*.
16. Labrou, N.E. (2000) Dye-ligand affinity chromatography for protein separation and purification. *Methods Mol. Biol. 147: 129-139*.
17. Clonis, Y.D., Labrou, N.E., Kotsira, V.P., Mazitsos, C., Melissis, S. and Gogolas, G. (2000) Biomimetic dyes as affinity chromatography tools in enzyme purification. *J. Chromatogr. A. 891: 33-44*.
18. Yang, B. and LeJohn, B. (1994) $NADP^+$ -activable, NAD^+ -specific glutamate dehydrogenase: purification and immunological analysis. *J. Biol. Chem. 269: 4506-4512*.
19. Labrou, N.E. (2000) Improved purification of Candida boidinii formate dehydrogenase. *Bioseparation 9: 99-104*.
20. Labrou, N.E. (2000) Dye affinity labeling of yeast alcohol dehydrogenase. *J. Enzyme Inhib. 15: 487-496*.
21. Labrou, N.E. and Clonis Y.D. (1995) Biomimetic dye affinity chromatography for purification of bovine heart lactate dehydrogenase. J. *Chromatogr. A. 718: 35-44*.

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