A Novel Efficient Method for Extraction and Purification of Recombinant Phenylalanine Dehydrogenase

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Abstract

Growing applications of phenylalanine dehydrogenase (PheDH) enzyme in the medical and pharmaceutical industries encourages researchers to seek simple, fast and economical alternative purification methods. With goal of finding a new technique, the extraction and purification of recombinant *Bacillus badius* PheDH in polyethylene glycol 6000 (PEG-6000) and ammonium sulfate aqueous two-phase systems (ATPS) was studied. Investigation on the effects of PEG molecular weight (MW) and concentration, $(NH_4)_2SO_4$ concentration, pH, temperature and NaCl addition on partition behavior was also performed. The optimized system was 8.5% (w/w) PEG-6000, 17.5% (w/w) $(NH_4)_2SO_4$ and 13% (w/w) NaCl at pH 8.0. The specific activity, yield, purification factor, recovery and partition coefficient were obtained 10424.97 U/mg, 95.85%, 474.3, 141% and 92.57, respectively. Briefly, we described the application of ATPS as an interesting and powerful technique for purifying and recovery of recombinant PheDH.

Keywords: Aqueous two-phase systems (ATPS); Ammonium sulfate; Phenylalanine dehydrogenase (PheDH); Partition; Purification; PEG-6000

Introduction

Amino acid dehydrogenases (AADH, EC 1.4.1.X) are one of the most important enzymes of coenzyme-dependent oxidoreductase superfamily that catalyze the reversible oxidative deamination of an amino acid to its keto acid and ammonia with the concomitant reduction of either NAD^+, $NADP^+$ or FAD. Phenylalanine dehydrogenase (PheDH, L-phenylalanine: NAD^+ oxidoreductase, deaminating; EC 1.4.1.20) is a member of this family that catalyzes the reversible NAD^+-dependent oxidative deamination of L-phenylalanine to

phenylpyruvate. This enzyme serves in the first catabolic step of phenylalanine in bacteria. It was originally discovered by Hummel *et al.* in a strain of *Brevibacterium* species isolated from soil [1]. Latter, the enzyme was found in several bacterial strains including *Bacillus* [2, 3], *Sporosarcina* [3], *Nocardia* [4], *Microbacterium* [5], *Thermoactinomyces* [6] and *Rhodococcus* [7]. Since its discovery, it has received much attention as a biocatalyst in synthesis of phenylalanine and related L-amino acids as basic building blocks for inclusion in foods [8] and production of pharmaceutical peptides [9-11]. This

enzyme has also been used in biosensors and diagnostic kits for phenylketonuria (PKU) screening [12,13].

However, the wide medical and biotechnological applications of this enzyme are often hampered by the requirement for large quantities of highly purified enzyme with appropriate properties. On the other hand, the conventional purification procedures such as precipitation and column chromatography are often tedious and expensive process with low yields. Therefore in the light of above basic demands, aqueous two-phase systems (ATPS) seem to be a good and economical alternative where clarification, concentration and partial purification can be integrated in one step [14-16]. Moreover, the most characteristic feature of ATPS is high water content, which complemented with suitable buffers and salts results in providing a gentle nontoxic environment for bio-molecules. Some important advantages of ATPS are easy to scale up, less energy consumption, less process time, low material cost and high yield. The basis of partitioning in ATPS depends upon properties of the target protein, system components, polymers molecular weight and concentration, salts and pH. The causative mechanisms of phase formation and the solute partitioning is a complex phenomenon that is poorly understood. Therefore, experimentation is necessary to design an optimal system for partitioning of a particular protein. This enzyme has already been purified by means of multistage chromatography columns [3,5,7] and affinity purification method [17]. In this study, we report the partitioning and purification of recombinant PheDH by in ATPS composed of PEG-6000/ $(NH_4)_2SO_4$. The influences of PEG molecular weight (MW) and concentration, pH, $(NH_4)_2SO_4$ concentration, temperature and NaCl salt addition on enzyme partitioning were also studied.

Materials and Methods

Materials

Recombinant *Bacillus badius* PheDH was provided by Professor Yasuhisa Asano (Toyama Prefectural University, Japan). Various PEGs *e.g.* 2000, 4000, 6000, 8000, 10000, 20000 and $(NH_4)_2SO_4$ were purchased from Merck (Darmstadt, Germany). L-phenylalanine and NAD^+ were from Sigma-Aldrich (St. Louis, Mo., USA) and used as substrates for the enzyme assay. The salts and all other chemicals were of analytical grade and Millipore water was used in all experiments. The cultures were grown and cell free extracts were obtained as described previously [2,17].

Enzyme Production

E. coli BL21 (DE3) cells with recombinant *Bacillus badius* PheDH activity were grown in LB broth medium containing ampicillin with 0.1 mg /ml. A 10 ml culture (8 h old) was diluted 100-fold into 1 L of medium in baffled culture flasks and shaken at 37 °C until an OD_{600}=1.0 was reached. The culture was then cooled to approximately 23 °C by stirring the flasks in an ice-water bath for 4 min. The T_7 promoter was induced by addition of 0.005 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG) and shaking at 23°C for 8 h. After cultivation, cells were collected by centrifugation at 3500 rpm for 15 min and kept at −20°C for further uses in purify experiments. The cell pellets were suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 2-mercaptoethanol and then sonicated (20 min total) with a 9-KHz ultrasonic oscillator. This suspension was centrifuged at 1000 rpm at 4°C for 20 min and dialyzed against the same buffer.

Aqueous Two-Phase Extraction

Phase systems were prepared in 15 ml graduated centrifugal tubes by dissolving appropriate amounts of solid PEG-6000 and $(NH_4)_2SO_4$ (Table 1) in 0.1 M potassium phosphate buffer (pH 8.0) at room temperature. Two mL of enzyme solution was added to make a final system of 10 g. Systems were thoroughly mixed by gentle agitation for 1 h and then centrifuged at 3000 rpm at 25°C for 40 min to speed up the phase separation. The volumes of the top and bottom phase were measured and then assayed for enzyme activities and total protein concentrations [14,15].

Enzyme Activity Assay

PheDH activity in the oxidative deamination reaction was assayed spectrophotometrically (Shimadzu UV-visible-1601 PC, Japan) by monitoring the reduction of NAD^+ at 340 nm. Mixture assay contained 10 mM L-phenylalanine, 100 mM glycine-KCl-KOH buffer (pH 10.4), 2.5 mM NAD^+ and enzyme solution in a total volume of 1 ml. One unit of PheDH activity (U) was defined as the amount which produced the formation of 1 μmol NADH per min [18].

Protein Determination

The total protein concentration was determined by a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard [19]. Samples were carefully

Table 1. Partitioning of recombinant PheDH in various ATPS combinations comprising PEG-6000/$(NH_4)_2SO_4$ (pH 8.0)

System	Phase compositions (%, w/w)	K_{enzyme}	R (%)	Y (%)	PF
1	12% PEG-15% $(NH_4)_2SO_4$	0	0	0	0
2	10% PEG-15% $(NH_4)_2SO_4$	0.27	16.16	10.37	27.88
3	8% PEG-15% $(NH_4)_2SO_4$	0.54	32.47	15.25	63.49
4	8% PEG-12% $(NH_4)_2SO_4$	0.49	19.55	17.35	51.20
5	8% PEG-10% $(NH_4)_2SO_4$	0.62	28.75	24.20	74.80
6	8% PEG-17% $(NH_4)_2SO_4$	0.67	12.82	22.30	33.57
7	8% PEG-20% $(NH_4)_2SO_4$	1.34	43.51	42.97	83.95
8	8% PEG-16% $(NH_4)_2SO_4$	1.16	133	22.48	470.66
9	8% PEG-18% $(NH_4)_2SO_4$	10.83	20.3	73.02	85.18
10	7% PEG-17% $(NH_4)_2SO_4$	41.65	72.62	91.23	304.61
11	9% PEG-17% $(NH_4)_2SO_4$	22.90	140	85.13	420.05
12	8.5% PEG-16.5% $(NH_4)_2SO_4$	47.82	79.98	96.71	401.35
13	8% PEG-16.5% $(NH_4)_2SO_4$	53.47	57.09	93.03	295.78
14	9% PEG-16.5% $(NH_4)_2SO_4$	62.41	66.63	93.97	345.20
15	9.5% PEG-17% $(NH_4)_2SO_4$	68.77	66.63	91.13	300.20
16	8.5% PEG-17% $(NH_4)_2SO_4$	18.29	39.91	82.05	206.78
17	8.5% PEG-16% $(NH_4)_2SO_4$	9.02	36.26	69.27	187.88
18	9.5% PEG-16% $(NH_4)_2SO_4$	37.38	39.91	90.33	199.79
19	9.5% PEG-16.5% $(NH_4)_2SO_4$	81.10	49.93	93.29	258.71
20	8% PEG-17.5% $(NH_4)_2SO_4$	33.18	100	89.34	295.40
21	8.5% PEG- 17.5% $(NH_4)_2SO_4$	58.7	135	94.42	491.93
22	9% PEG-17.5% $(NH_4)_2SO_4$	14.80	70.32	78.72	207.74
23	9.5% PEG-17.5% $(NH_4)_2SO_4$	15.73	47.40	79.72	140.04
24	9% PEG-16% $(NH_4)_2SO_4$	53.95	91.61	93.09	430.73

K_{enzyme}: partition coefficient; Y: yield; PF: purification factor; R: recovery.

withdrawn from each phase and diluted at least 1/10 with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 2-mercaptoethanol before the addition of Bradford solution. This dilution procedure can remove the interference of phase components on the protein assay. Equally diluted solutions from corresponding phase systems without protein extract were used as blanks, which were prepared in the same manner.

Electrophoresis

Samples from crude extract, top and bottom phase in ATPS were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out in 10% homogenous gel [20] consisting of a resolving gel (pH 8.8, 10%, w/v, acrylamide) and a stacking gel of 5% acrylamide (pH 6.8). Electrophoresis was run at 50 v and 12 mA for 5 h.

The gel was stained with Coomassie Blue staining method [20].

Steady-State Kinetics

The initial velocity studies for oxidative deamination reaction were performed by varying the concentration of one substrate in the presence of different fixed concentrations of the other substrate [18]. The kinetic parameters for the best purification system were calculated from the secondary plots of intercepts versus reciprocal concentrations of the other substrate.

Results and Discussion

Effects of Phase Concentrations on the Partitioning and Purification of Recombinant PheDH

In order to find the best extraction conditions for the

partitioning and the purification of PheDH, 24 different systems were evaluated (Table 1). Among these different combinations studied, optimal values for the partition coefficient, top phase yield, purification factor and recovery were observed in PEG-6000 8.5% (w/w) and $(NH_4)_2SO_4$ 17.5% (w/w).
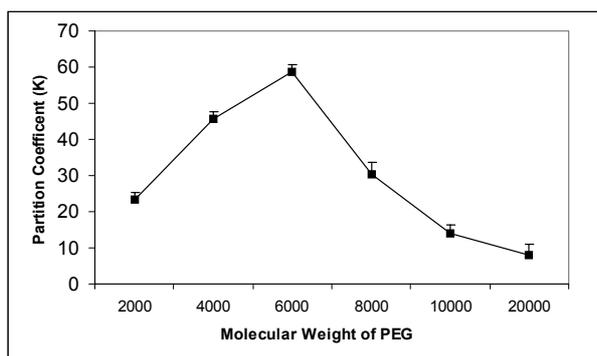
Effects of PEG Molecular Weight

Using on the above results, we select the 8.5% PEG-6000 and 17.5% $(NH_4)_2SO_4$ for the study of the PEG molecular weight effect on PheDH partitioning. Figures 1 and 2 illustrate the effects of different PEGs on the partition coefficient (K) and yield (Y %) of PheDH, respectively. As anticipated, low molecular weight of PEG (2000) and the much higher PEG MW (20000) were not suitable for adequate partitioning. The highest partition coefficient and the highest yield were obtained by the PEG-6000. As seen in Figures 2 and 3, when the MW increased from 2000 to 6000, the K_{enzyme} and the

yield increased. But as the PEG MW increased from 6000 to 20,000, the K_{enzyme} and yield decreased. These complexities in partition parameters can be explained by hydrophobic interactions which play an important role in the mechanism of partitioning [14,21,22]. Therefore the PEG molecular weight should be kept at 6000. In the present work, the partition experiments were carried out in triplicate to estimate experimental errors.
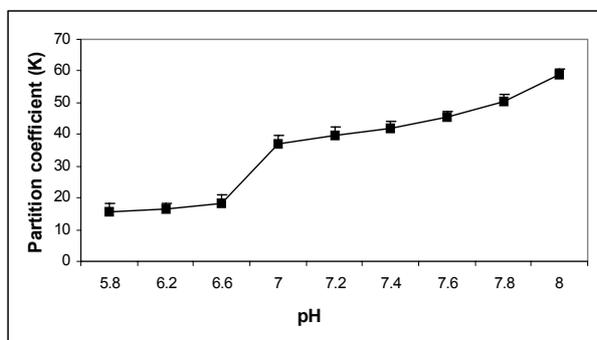
Effects of pH

The partition behaviors of PheDH with different pH values of 0.1 M potassium phosphate buffer were investigated. Figures 3 and 4 show the effects of pH on the partition coefficient (K) and top phase yield (Y %) over the pH range 5.8~8.0, respectively. The optimal values for the partition coefficient and top phase yield were obtained at pH 8.0. Based on the above results, pH 8.0 was chosen for PheDH partitioning in this study. The amounts of K_{enzyme} and yield reported in this study were an average value of triplicate experiments.
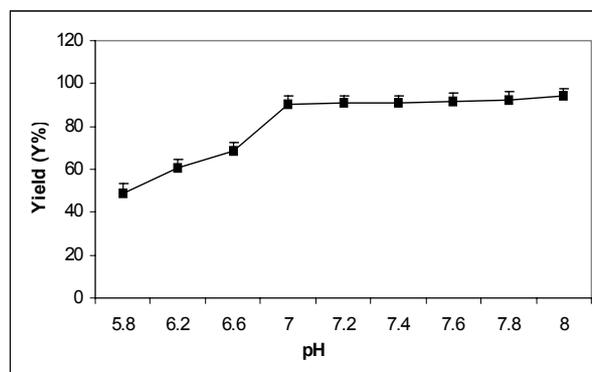

Figure 1. Effects of PEG molecular weight on PheDH partition coefficient (K) in systems containing PEG 8.5%-$(NH_4)_2SO_4$17.5% (pH 8.0).
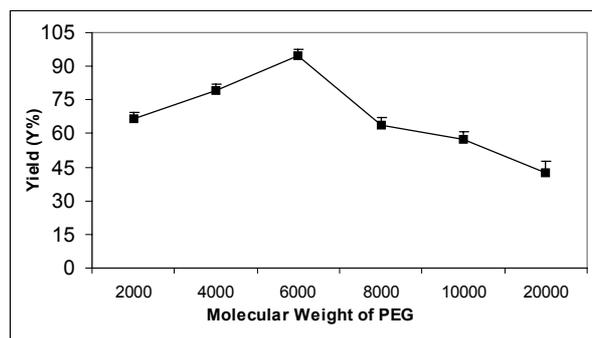
Figure 2. Effects of PEG molecular weight on the PheDH yield (Y) in systems containing PEG 8.5%-$(NH_4)_2SO_4$17.5% (pH 8.0).

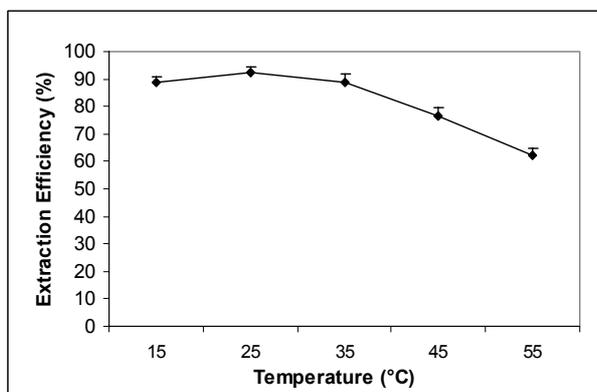**Figure 3.** Effects of pH on PheDH partition coefficient (K) in PEG-6000 8.5%- $(NH_4)_2SO_4$17.5% systems.

Figure 4. Effects of pH on the PheDH yield(Y) in PEG-6000 8.5%-$(NH_4)_2SO_4$17.5% systems.
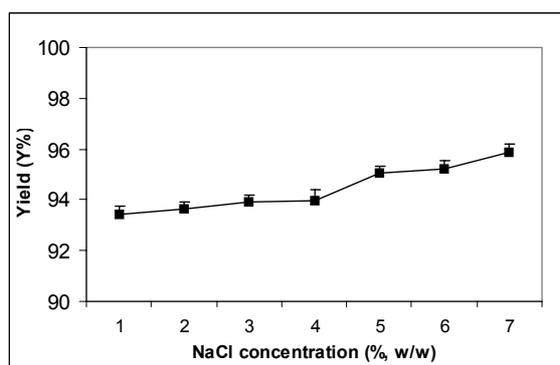
Effects of Temperature

The influence of temperature on the extraction efficiency of PheDH was also investigated. Within temperature range of 15-55°C, the extraction efficiency varied with the value of 62-92%. As shown in Figure 5, the best experimental result for the enzyme extraction was obtained at 25°C. In this research, all extraction experiments were done within 20-30°C.

Effects of NaCl Concentration

To achieve a more proper system, the influence of NaCl concentration on the partitioning behavior of PheDH was tested. As illustrated in Figures 6 and 7, inclusion of NaCl concentration increased significantly the partition coefficient and yield of PheDH. Therefore, NaCl salt addition in ATPS containing 8.5% (w/w) PEG-6000/17.5% (w/w) $(NH_4)_2SO_4$ (pH 8.0) can be used to efficiently separate PheDH. Finally, the best ATPS for PheDH partitioning was 8.5% (w/w) PEG-6000, 17.5% (w/w) $(NH_4)_2SO_4$ and 13% (w/w) NaCl at pH 8.0. All experiments were run in triplicate.

Conclusion

The extraction and purification of recombinant PheDH was carried out in single-step operation where the enzyme was strongly partitioned to the top PEG-rich phase (Fig. 8). From the experimental results, it was found that molecular weight of PEG, pH and

Figure 5. Effects of temperature on the extraction efficiency of PheDH in systems containing PEG-6000 8.5%-$(NH_4)_2SO_4$17.5% (pH 8.0).
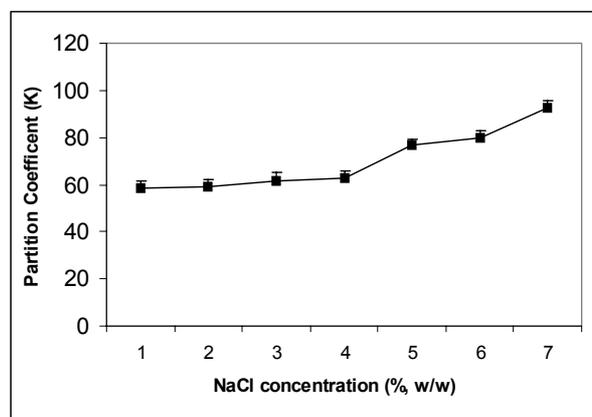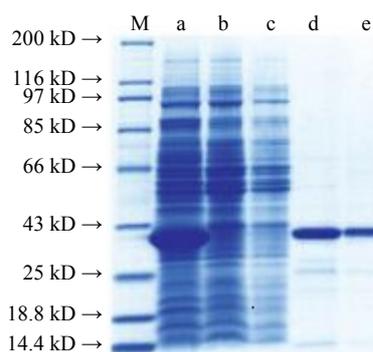
Figure 6. Effects of NaCl concentration on PheDH partition coefficient (K) in systems containing PEG-6000 8.5%-$(NH_4)_2SO_4$17.5% (pH 8.0).

Figure 7. Effects of NaCl concentration on the PheDH yield (Y) in systems containing PEG-6000 8.5%-$(NH_4)_2SO_4$17.5% (pH 8.0).

Figure 8. SDS-PAGE gel electrophoresis of recombinant *Bacillus badius* PheDH. M: molecular mass markers. Lane a and b: crude extract of *E. coli* BL21. Lane c: bottom phase obtained from ATPS consisting of 8.5% PEG-6000, 17.5% (w/w) $(NH_4)_2SO$ and 13% (w/w) $NaCl_4$ (pH 8.0). Lane d and e: top phase of the same ATPS.

temperature had significant effects on enzyme partitioning and purification. The best suitable system was 8.5% (w/w) PEG-6000, 17.5% (w/w) $(NH_4)_2SO_4$ and 13% (w/w) NaCl under pH 8.0. The specific activity of PheDH enzyme was 10424.97 U/mg that was not comparable to the values reported for PheDH from *B. badius* (67.8 U/mg) [2], *Microbactrium sp.* (37.1 U/mg) [5], *Thermoactinomyces* (86.2 U/mg) [6], *R. maris* (65.2 U/mg) [7] and *B. sphaericus* (577.3 U/mg) [17]. The molecular weight of PheDH was estimated to be about 41 kD by SDS-PAGE, which was similar to the previously reported value [2]. The Michaelis constants in the oxidative deamination were 0.021 mM for L-phenylalanine and 0.13 mM for NAD^+. Also the catalytic efficiency (K_{cat}/K_m) for PheDH was 108.9 ($s^{-1}.mM^{-1}$). The main purpose of this study was to purify PheDH with appropriate properties for the design of a PKU diagnostic kit.

Acknowledgements

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