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Purification of recombinant phenylalanine dehydrogenase by partitioning in aqueous two-phase systems

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Abstract

This study presents the partitioning and purification of recombinant *Bacillus badius* phenylalanine dehydrogenase (PheDH) in aqueous two-phase systems (ATPS) composed of polyethylene glycol 6000 (PEG-6000) and ammonium sulfate. A single-step operation of ATPS was developed for extraction and purification of recombinant PheDH from *E. coli* BL21 (DE3). The influence of system parameters including; PEG molecular weight and concentration, pH, $(NH_4)_2SO_4$ concentration and NaCl salt addition on enzyme partitioning were investigated. The best optimal system for the partitioning and purification of PheDH 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 partition coefficient, recovery, yield, purification factor and specific activity values were of 92.57, 141%, 95.85%, 474.3 and 10424.97 U/mg, respectively. Also the K_m values for L-phenylalanine and NAD^+ in oxidative deamination were 0.020 and 0.13 mM, respectively. Our data suggested that this ATPS could be an economical and attractive technology for large-scale purification of recombinant PheDH.

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

1. Introduction

The amino acid dehydrogenases (EC 1.4.1.X) are a widely class of enzymes 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 as the first catabolism 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 to screen blood serum of neonates for phenylketonuria (PKU) [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, ATPS seems 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 the ATPS is high water content, which complemented with suitable buffers and salts results in providing a gentle nontoxic environment for biomolecules. 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

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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 purification of recombinant PheDH by partitioning in ATPS composed of PEG–6000/$(NH_4)_2SO_4$. The effects of PEG molecular weight (MW) and concentration, pH, $(NH_4)_2SO_4$ concentration and NaCl concentration on enzyme partitioning were also studied. To the best of our knowledge, this is the first report describing the use of ATPS to purify PheDH. These results will be important for further development of an ATPS method as a cheap, economical and powerful technology for PheDH recovery.

2. Experimental

2.1. Materials

Recombinant *Bacillus badius* PheDH was provided by Professor Yasuhisa Asano (Toyama Prefectural University, Japan). Vector pET16b (Novagen Inc. Madison, USA) was used for expression. Various PEGs e.g. 2000, 4000, 6000, 8000, 10000, 20000 and $(NH_4)_2SO_4$ were purchased from Merck (Germany). NAD^+ and NADH were from Sigma–Aldrich (St. Louis, USA) and used as coenzymes 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.

2.2. Bacterial strain, medium and enzyme production

E. coli BL21 (DE3) cells with recombinant *Bacillus badius* PheDH activity were grown in LB broth medium containing 0.1 mg/mL ampicillin. 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 harvested by centrifugation at 3500 rpm for 15 min and kept at −20 °C for further uses in purification 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 dialysed against the same buffer and centrifuged at 1000 rpm at 4 °C for 20 min to clarity. The supernatant was used as a crude enzyme solution in the experiments.

2.3. Aqueous two-phase systems preparation

Phase systems were prepared in 15 mL graduated centrifugal tubes by dissolving appropriate amounts of solid PEG and salts in 0.1 M potassium phosphate buffer (pH 8.0) at room temperature. Two milliliter 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.

2.4. Enzyme assay

PheDH activity in the oxidative deamination reaction was measured 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 the enzyme solution in a total volume of 1 mL. The enzyme activity for the reductive amination was assayed by the oxidation of NADH in a reaction mixture (1.0 mL) 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. One unit of PheDH activity (U) was defined as the amount which produced the formation of 1 (mol NADH per min [18].

2.5. Protein assay

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

2.6. Calculations

The purification process in this study was evaluated by parameters being defined: specific activity (SA), partition coefficient (K), purification factor (PF), recovery (R) and yield (Y). These parameters are defined as follows [14]:

Specific activity (SA): is defined as the enzyme activity (U/mL) in the phase sample divided by the total protein concentration (mg/mL) and is expressed in U/mg of protein.

$$\mathrm{SA} = \frac{\text{enzyme activity}}{\text{protein concentration}}$$

Partition coefficient (K): is determined by the PheDH activity in the top phase (A_t) to that in the bottom phase (A_b). It should be mentioned that identical volumes of top and bottom phases were used.

$$K = \frac{A_t}{A_b}$$

Purification factor (PF): is calculated by the ratio between the specific activity in the top phase and the specific activity in the

initial extract (before partition).

$$\mathrm{PF} = \frac{\text{SA in the collected phase}}{\text{initial SA}}$$

Recovery (R): is defined by the ratio of the PheDH activity in the top phase to initial activity in original sample.

$$R\,(\%) = \frac{\text{enzyme activity of the top phase}}{\text{total enzyme activity added to the system}}$$

Yield (Y%): yield in the top phase is determined as

$$Y\,(\%) = \frac{100\,V_t K}{V_t K + V_b}$$

where V_t and V_b are the volumes of the top and bottom phase, respectively.

2.7. 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 [19] 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 silver staining method [19].

2.8. 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.

3. Results and discussion

3.1. Effects of phase compositions on the partitioning and purification of recombinant PheDH

The selection of phase forming components in the ATPS is one of the important points for this technique [14,15]. Nevertheless, the behavior of partitioning and distribution of compounds in these systems is very complex phenomenon due to the several factors such as hydrophobic interactions, hydrogen bonds, charge, steric effects and biomolecule properties [14]. As a matter of fact, no comprehensive and mechanistic theory exists to guide the choice and design of adequate systems. Therefore, application of this method requires experimentation to obtain a suitable and good system for optimal partitioning of biomaterials [14,15]. In this study, PEG-salt ATPS were selected to find the best purification system. The polymer–salt systems have the advantages of higher selectivity, low cost and low viscosity in protein partitioning in comparison with other systems [14–16].

Table 1
Influence of phase composition on partition behavior of PheDH in PEG-salt ATPS at pH 8.0

Phase compositions (%, w/w)	K_{enzyme}	Y (%)	PF
9.5% PEG 6000–17% Mg SO_4	0.00	0.00	0.00
8.5% PEG 6000–17% Mg SO_4	0.00	0.00	0.00
8.5% PEG 6000–16% Mg SO_4	18.4	58.01	1.02
9.5% PEG 6000–17% $(NH_4)_2$ SO_4	68.77	91.13	300.20
8.5% PEG 6000–17% $(NH_4)_2$ SO_4	18.29	82.05	206.78
8.5% PEG 6000–16% $(NH_4)_2$ SO_4	9.02	69.27	187.88
9.5% PEG 6000–17% K_2HPO_4	22.89	40.0	3.40
8.5% PEG 6000–17% K_2HPO_4	2.5	0.04	1.78
8.5% PEG 6000–16% K_2HPO_4	0.00	0.00	0.00
9.5% PEG 6000–17% Na_2HPO_4	0.00	0.00	0.00
8.5% PEG 6000–17% Na_2HPO_4	2.13	58.3	2.26
8.5% PEG 6000–16% Na_2HPO_4	0.43	0.24	16.70

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

The main criteria for selecting PEG as the most suitable polymer were: low cost and fast approach to equilibrium. Another important component present in this system was salt. For selecting the salt type, four different salts were evaluated. The results are summarized in Table 1. According to the preliminary results obtained in this work, PEG/ammonium sulfate ATPS were considered for further study. Afterwards, in order to define the best extraction conditions for the partitioning and the purification of PheDH 24 different systems were evaluated (Table 2). Among these different combinations studied, optimal values for the partition coefficient, top phase yield, purification factor and recovery were observed in PEG 8.5% (w/w) and $(NH_4)_2SO_4$ 17.5% (w/w).

3.2. Effects of PEG molecular weight on PheDH partitioning in PEG 6000–$(NH_4)_2SO_4$ systems

From the previous results, we select the 8.5% PEG and 17.5% $(NH_4)_2SO_4$ for the study of the PEG molecular weight effect on PheDH partitioning. The partition behavior of total protein and enzymes depended on the PEG MW. The PEGs with different degree of polymerization influenced the extraction efficiency by changing the phase diagram i.e. the compositions of phases and the number of polymer–enzyme interactions [20,21]. Usually, as the PEG molecular weight increases, the partitioning of enzyme into the top phase decreases. But in some studies, these parameters show opposite results [22]. Unfortunately there is not much information of such behavior. Figs. 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 PEG (2000) and the much higher PEG MW (20,000) were not suitable for adequate partitioning. The highest partition coefficient and the highest yield were obtained with the PEG-6000. As seen in Figs. 1 and 2, 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 [14,20,21]. Therefore, the PEG molecular weight should be kept at 6000. In the present

Table 2
Partition parameters obtained for recombinant PheDH in PEG-6000/$(NH_4)_2SO_4$ ATPS at pH 8.0

System	Compositions (%, w/w)	K_{enzyme}	R (%)	Y (%)	PF
2	10% PEG–15% $(NH_4)_2$ SO_4	0.27	16.16	10.37	27.88
3	8% PEG–15% $(NH_4)_2$ SO_4	0.54	32.47	15.25	63.49
4	8% PEG–12% $(NH_4)_2$ SO_4	0.49	19.55	17.35	51.20
5	8% PEG–10% $(NH_4)_2$ SO_4	0.62	28.75	24.20	74.80
6	8% PEG–17% $(NH_4)_2$ SO_4	0.67	12.82	22.30	33.57
7	8% PEG–20% $(NH_4)_2$ SO_4	1.34	43.51	42.97	83.95
8	8% PEG–16% $(NH_4)_2$ SO_4	1.16	133	22.48	470.66
9	8% PEG–18% $(NH_4)_2$ SO_4	10.83	20.3	73.02	85.18
10	7% PEG–17% $(NH_4)_2$ SO_4	41.65	72.62	91.23	304.61
11	9% PEG–17% $(NH_4)_2$ SO_4	22.90	140	85.13	420.05
12	8.5% PEG–16.5% $(NH_4)_2$ SO_4	47.82	79.98	96.71	401.35
13	8% PEG–16.5% $(NH_4)_2$ SO_4	53.47	57.09	93.03	295.78
14	9% PEG–16.5% $(NH_4)_2$ SO_4	62.41	66.63	93.97	345.20
15	9.5% PEG–17% $(NH_4)_2$ SO_4	68.77	66.63	91.13	300.20
16	8.5% PEG–17% $(NH_4)_2$ SO_4	18.29	39.91	82.05	206.78
17	8.5% PEG–16% $(NH_4)_2$ SO_4	9.02	36.26	69.27	187.88
18	9.5% PEG–16% $(NH_4)_2$ SO_4	37.38	39.91	90.33	199.79
19	9.5% PEG–16.5% $(NH_4)_2$ SO_4	81.10	49.93	93.29	258.71
20	8% PEG–17.5% $(NH_4)_2$ SO_4	33.18	100	89.34	295.40
21	8.5% PEG–17.5% $(NH_4)_2$ SO_4	58.7	135	94.42	491.93
22	9% PEG–17.5% $(NH_4)_2$ SO_4	14.80	70.32	78.72	207.74
23	9.5% PEG–17.5% $(NH_4)_2$ SO_4	15.73	47.40	79.72	140.04
24	9% PEG–16% $(NH_4)_2$ SO_4	53.95	91.61	93.09	430.73

K_{enzyme}: partition coefficient; Y: yield; PF: purification factor; R: recovery. (−) No phase separation.

work, the partition experiments were carried out in triplicate to estimate experimental errors.

3.3. Effects of system pH on PheDH partitioning in PEG 6000–$(NH_4)_2SO_4$ systems

The partition behaviors of PheDH at different pH values were also investigated. Figs. 3 and 4 show the effects of system pH on the partition coefficient (K) and top phase yield (Y%) over the pH range 5.8–8.0, respectively. In ATPS, partitioning of biomolecules is obviously influenced by system pH. The partition is determined by the kind of ions present and the ratio between the different ions and not really the ionic strength. The

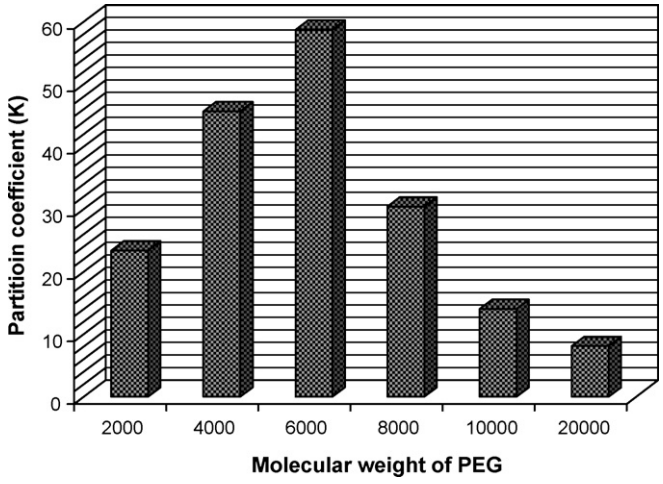

Fig. 1. Effects of PEG molecular weight on PheDH partition coefficient (K) in systems containing PEG 8.5%–$(NH_4)_2SO_4$17.5% (pH 8.0).

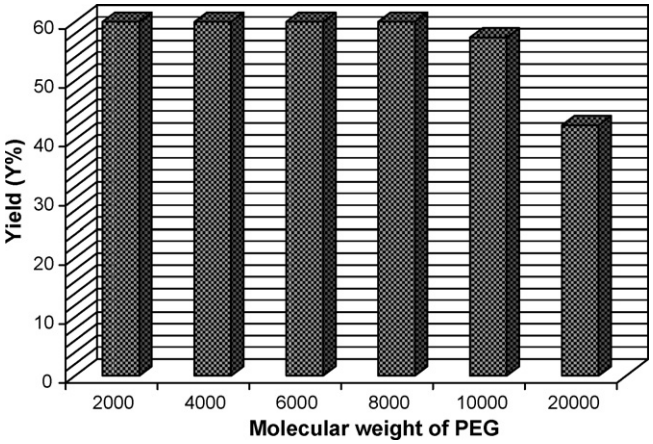

Fig. 2. Effects of PEG molecular weight on the PheDH yield (Y) in systems containing PEG 8.5%–$(NH_4)_2SO_4$17.5% (pH 8.0).

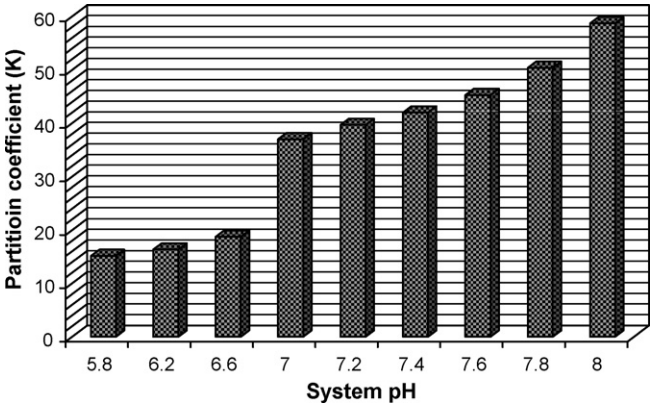

Fig. 3. Effects of system pH on PheDH partition coefficient (K) in PEG-6000 8.5%–$(NH_4)_2SO_4$17.5% systems.

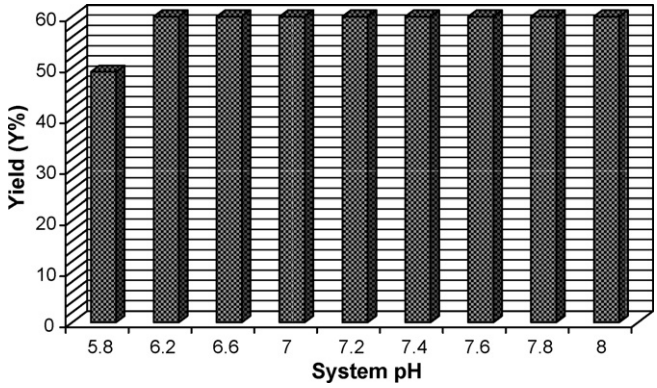

Fig. 4. Effects of system pH on the PheDH yield (*Y*) in PEG-6000 8.5%–$(NH_4)_2SO_4$ 17.5% systems.

pH affected the charge of targeted protein and ion composition, surface character of contaminating materials and causes variation in their partitioning into the top and bottom phase [14,16]. According to Albertsson's equation, the partition coefficient of a charged biomaterial is influenced by the interfacial potential, ψ as follows [20]:

$$\ln K_p = \ln K_p^0 + \left(\frac{FZ}{RT}\right)\psi.$$

where K_p is the partition coefficient of a charged protein in a two-phase system containing ionic species providing an interfacial potential of ψ and $\ln K_p^0$ denote the protein partition coefficient at the isoelectric point (p*I*). *Z*, *F*, *R* and *T* are the number of charges per protein molecules, Faraday constant, universal gas constant and absolute temperature, respectively. The term (*FZ*/*RT*) is the electrostatic interactions which are responsible of the effect of pH on solute partition behaviors. In general, negatively charged proteins prefer the top phase and positively charged proteins partition to the bottom phase [14]. The optimal values for the partition coefficient and top phase yield were obtained at pH 8.0. Therefore, this was in agreement with this rule since the isoelectric point of *Bacillus badius* PheDH is 5.3 [18] and was more negatively charged at this pH. 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.

3.4. Effects of NaCl concentration on PheDH partitioning in PEG 6000–$(NH_4)_2SO_4$ systems

To achieve a more proper system for enzyme partitioning, the influence of NaCl concentration on the partitioning behavior of PheDH was also tested. It is well known that salt concentration alters the partition behavior of biological materials. Generally, addition of neutral salts such as NaCl affects partitioning and extraction in ATPS by inducing the phase separation or by changing the electrical potential [23,24]. The addition of NaCl has been reported to be beneficial to targeted material [14,25,26]. However, the opposite behavior was also observed [22,27]. As illustrated in Figs. 5 and 6, inclusion of NaCl concentration increased significantly the partition coefficient and yield

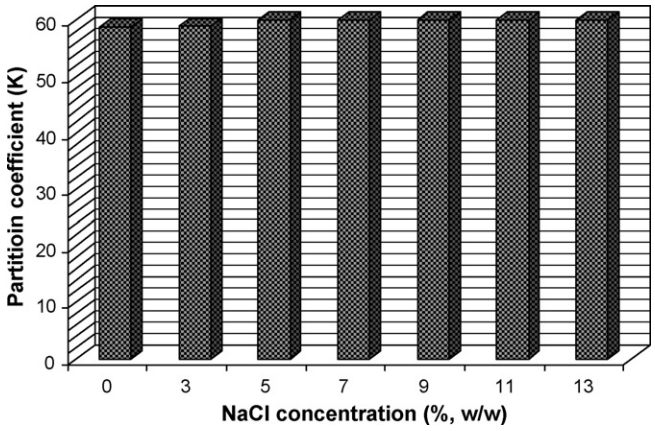

Fig. 5. Effects of NaCl concentration on PheDH partition coefficient (*K*) in systems containing PEG-6000 8.5%–$(NH_4)_2SO_4$17.5% (pH 8.0).

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.

3.5. Presented method efficiency

Conventional purification methods for the recovery and purification of PheDH including ammonium sulfate precipitation followed by chromatography using anion exchange, gel filtration and affinity chromatography or a combination of these are usually need long processing time and are expensive [2,5,17]. Nevertheless, further progress in utilizing PheDH for clinical and pharmaceutical purposes will depend on the efficiency of the purification methods. In the best purification procedure that has been reported by Omidinia et al. [17], recovery, specific activity and purification factor were 28%, 577.3 U/mg and 88.8, respectively, which is not very attractive for industrial-scale preparation. Therefore, new approaches are necessary to minimize time and costs. In the present study, PEG-6000 and $(NH_4)_2SO_4$ ATPS were investigated for the partitioning and

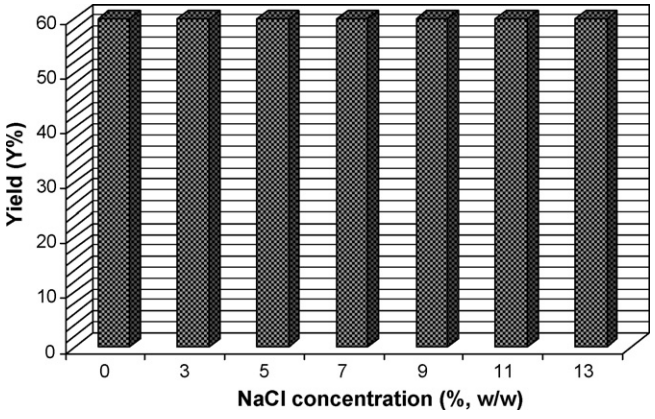

Fig. 6. Effects of NaCl concentration on the PheDH yield (*Y*) in systems containing PEG-6000 8.5%–$(NH_4)_2SO_4$17.5% (pH 8.0).

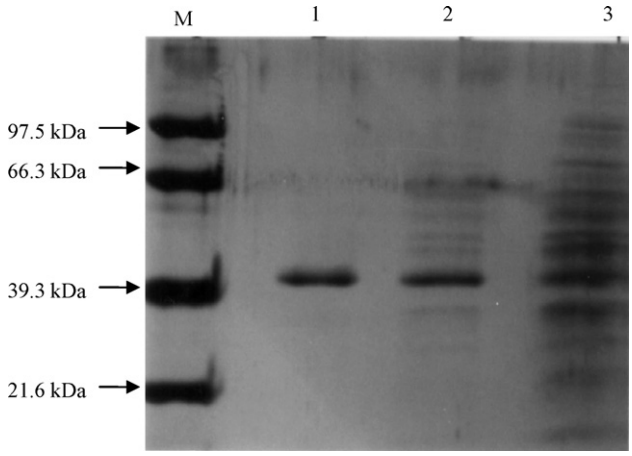

Fig. 7. SDS-PAGE profile of recombinant *Bacillus badius* PheDH purified by partitioning in ATPS. Lane 1: top phase obtained from ATPS consisting of 8.5% PEG-6000, 17.5% (w/w) $(NH_4)_2SO_4$ and 13% (w/w) NaCl (pH 8.0). Lane 2: bottom phase of the same ATPS. Lane 3: crude extract of *E. coli* BL21. M: molecular weight markers. The protein bands were stained with silver staining protocol.

purification of recombinant *Bacillus badius* PheDH. 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. 7). The MW of the subunit of the PheDH was estimated to be about 41 kDa by SDS-PAGE, which was similar to the previously reported value [2]. The specific activity of PheDH enzyme in this study was 10424.97 U/mg that is to compare to the values reported for PheDH from *B. badius* (67.8 U/mg) [2], *Microbactrium sp.* (37.1 U/mg) [5], *Thermoactinomyces sp.* (86.2 U/mg) [6], *R. maris* (65.2 U/mg) [7] and *B. sphaericus* (577.3 U/mg) [17]. Interestingly, the enzyme purified by ATPS could be stored at −20 °C for several months without losing its activity, in contrast to other PheDH, which have been reported to lose the activity rapidly [4,7]. The main purpose of this study was to purify PheDH with appropriate properties for the design of a phenylketonuria diagnostic kit. As a result, we were successful in this aim. It should be noted that this is the first report about using ATPS to purify PheDH. The presented method also opens new opportunities for the separation and purification of other amino acid dehydrogenases.

4. Conclusion

Compared with traditional methods, this one-step extraction method is simpler, faster and more efficient. The best suitable 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 with partition coefficient, 92.57; recovery, 141%; yield, 95.85%; and purification factor, 474.3. The Michaelis constants in the oxidative deamination were 0.020 mM for L-phenylalanine and 0.13 mM for NAD^+. From the experimental results, it was found that molecular weight of PEG, pH system and NaCl concentration had significant effects on enzyme partitioning and purification. In short, we report here the partitioning and purification of recombinant PheDH in aqueous two-phase systems as a cheap, economical and powerful technology for phenylalanine dehydrogenase recovery.

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