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Rapid One-Step Separation and Purification of Recombinant Phenylalanine Dehydrogenase in Aqueous Two-Phase Systems

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ABSTRACT

Background: Phenylalanine dehydrogenase (PheDH; EC 1.4.1.20) is a NAD^+-dependent enzyme that performs the reversible oxidative deamination of L-phenylalanine to phenylpyruvate. It plays an important role in detection and screening of phenylketonuria (PKU) diseases and production of chiral intermediates as well. The main goal of this study was to find a simple and rapid alternative method for purifying PheDH. **Methods:** The purification of recombinant *Bacillus sphaericus* PheDH was investigated in polyethylene glycol (PEG) and ammonium sulfate aqueous two-phase systems (ATPS). The influences of system parameters including PEG molecular weight and concentration, pH and $(NH_4)_2SO_4$ concentration on enzyme partitioning were also studied. The purity of enzyme was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. **Results:** A single extraction process was developed for separation and purification of recombinant PheDH from *E. coli* BL21 (DE3). The optimized conditions for partitioning and purification of PheDH were 9% (w/w) PEG-6,000 and 16% (w/w) $(NH_4)_2SO_4$ at pH 8.0. The partition coefficient, recovery, yield, purification factor and specific activity values were achieved 58.7, 135%, 94.42%, 491.93 and 9828.88 U/mg, respectively. Also, the K_m values for L-phenylalanine and NAD^+ in oxidative deamination were 0.21 and 0.13 mM, respectively. **Conclusion**: The data presented in this paper demonstrated the potential of ATPS as a versatile and scaleable process for downstream processing of recombinant PheDH. *Iran. Biomed. J. 12 (2): 115-122, 2008*

Keywords: Aqueous two-phase systems (ATPS), Ammonium sulfate, Phenylalanine dehydrogenase (PheDH), Purification, Polyethylene glycol 6,000 (PEG-6,000)

INTRODUCTION

Phenylalanine dehydrogenase (PheDH, L-phenylalanine: NAD^+ oxidoreductase, deaminating; EC 1.4.1.20) is a member of amino acid dehydrogenase family that catalyzes the reversible NAD^+-dependent oxidative deamination of L-phenylalanine to phenylpyruvate. This enzyme plays an important role in carbon and nitrogen metabolism in bacteria and is a key factor in assimilation of L-phenylalanine as an energy source through the tricarboxylic acid cycle during sporulation [1]. It has received much attention as a valuable biocatalyst in synthesis of phenylalanine and related L-amino acids as basic building blocks for inclusion in foods [2] and production of pharmaceutical peptides [3]. PheDH has also been used in biosensors and diagnostic kits for phenylketonuria (PKU) newborn screening [4, 5].

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 [6-10]. ATPS partitioning is generally obtained by the incompatibility between aqueous solutions of two polymers (PEG, dextran, etc.) or a polymer and a salt (phosphate, sulfate, citrate, etc.) at high ionic strength. It is necessary to mention that the polymer-salt systems have the

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advantages of higher selectivity, lower cost and lower viscosity in comparison with polymer-polymer systems [6, 7, 10]. It has been found the desirable biomaterials are usually concentrated into the polymer-rich top phase and the contaminants are remained in salt-rich bottom phase.

Recently, some novel systems such as micro emulsion and affinity phases have been developed [7, 11]. Partitioning in ATPS mainly depends on the physiochemical traits of biomolecules such as charge, shape, size, molecular weight, hydrophobicity and specific binding sites. Moreover, the partition profile is also influenced by van der waals, hydrogen and hydrophobic bonds, static effects and electrostatic interactions between the biomaterial and the phase forming components. As a result, the partition may be affected by altering the system components, the molecular weight and concentration of polymer, the type and concentration of salt, the ionic strength, the system pH and temperature [12- 14]. The causative mechanisms of ATPS partitioning are largely unknown. Although, the mathematical models such as response surface methodology provide some information about phase behavior and partitioning of target bimolecules, no comprehensive theory exists to guide the design of optimal systems. Thus, the experimental data is necessary to obtain an adequate partitioning [6, 7]. In recent years, ATPS has attracted considerable interest in industrial applications due to the multiple advantages such as high water content in both phases (80-90% w/w), low interfacial tension, high yield, low labor cost, low energy consumption and easy to scale up. The polymers themselves also have a stabilizing effect on proteins [6-11]. Recombinant PheDH has been purified by conventional purification methods including ammonium sulfate precipitation followed by chromatography using anion exchange, gel filtration and affinity chromatography or a combination of these usually processing time and expensive [15-17]. In this paper, we report the purification of recombinant PheDH by partitioning in ATPS composed of PEG-6000 and $(NH_4)_2SO_4$.

MATERIALS AND METHODS

Materials. PEG with several molecular masses of 2,000, 4,000, 6,000, 8,000, 10,000, 20,000 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. Recombinant *Bacillus sphaericus* PheDH was provided by Professor Yasuhisa Asano (Toyama Prefectural University, Toyama, Japan).

Enzyme production. For enzyme production, *E. coli* BL21 (DE3) cells with recombinant *Bacillus sphaericus* PheDH activity were grown in LB (Luria-Bertani) 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 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 3,500 ×g for 15 min and kept at -20°C for further uses. 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 1,000 ×g at 4°C for 20 min to clarity and dialyzed against the same buffer [15, 17].

Aqueous two-phase systems. 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 at room temperature. Enzyme solution (2 ml) was added to make a final system of 10 g. Systems were thoroughly mixed by gentle agitation for 1 h and then centrifuged at 3,000 ×g at 25°C for 40 min to speed up the phase separation [6, 7]. The volumes of the top and bottom phase were measured and then assayed for enzyme activities and total protein concentrations (Fig. 1).

PheDH activity determination. PheDH activity in the oxidative deamination reaction was measured spectrophotometrically (Shimadzu UV-visible-1601 PC, Japan) by following the increase of absorbance at 340 nm. Assay was performed in a reaction mixture containing 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. One unit of PheDH activity (U) was defined as

Table1. Extraction and purification of recombinant PheDH in PEG-6,000 and $(NH_4)_2SO_4$ ATPS at pH 8.0.

Assay	ATPS compositions (%, w/w)	K_{enzyme}	R (%)	Y (%)	PF
1	8% PEG-17% $(NH_4)_2SO_4$	0.67	12.82	22.30	33.57
2	8% PEG-16% $(NH_4)_2SO_4$	1.16	133.00	22.48	470.66
3	8% PEG-18% $(NH_4)_2SO_4$	10.83	20.30	73.02	85.18
4	7% PEG-17% $(NH_4)_2SO_4$	41.65	72.62	91.23	304.61
5	9% PEG-17% $(NH_4)_2SO_4$	22.90	140.00	85.13	420.05
6	8.5% PEG-16.5% $(NH_4)_2SO_4$	47.82	79.98	96.71	401.35
7	8% PEG-16.5% $(NH_4)_2SO_4$	53.47	57.09	93.03	295.78
8	9% PEG-16.5% $(NH_4)_2SO_4$	62.41	66.63	93.97	345.20
9	9.5% PEG-17% $(NH_4)_2SO_4$	68.77	66.63	91.13	300.20
10	8.5% PEG-17% $(NH_4)_2SO_4$	18.29	39.91	82.05	206.78
11	8.5% PEG-16% $(NH_4)_2SO_4$	9.02	36.26	69.27	187.88
12	9.5% PEG-16% $(NH_4)_2SO_4$	37.38	39.91	90.33	199.79
13	9.5% PEG-16.5% $(NH_4)_2SO_4$	81.10	49.93	93.29	258.71
14	8% PEG-17.5% $(NH_4)_2SO_4$	33.18	100.00	89.34	295.40
15	9% PEG-16% $(NH_4)_2SO_4$	58.70	135.00	94.42	491.93
16	9% PEG-17.5% $(NH_4)_2SO_4$	14.80	70.32	78.72	207.74
17	9.5% PEG-17.5% $(NH_4)_2SO_4$	15.73	47.40	79.72	140.04

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

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 BSA as a standard protein [19].

Determination of specific activity, partition coefficient, purification factor, recovery and yield. The purification process in this study was evaluated by parameters including: specific activity, partition coefficient, purification factor, recovery and yield. These parameters defined as follows [6, 7]:

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.

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

Partition coefficient (K_E): is determined by the PheDH activity in the top phase (A_t) to that in the bottom phase (A_b).

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

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).

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

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

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

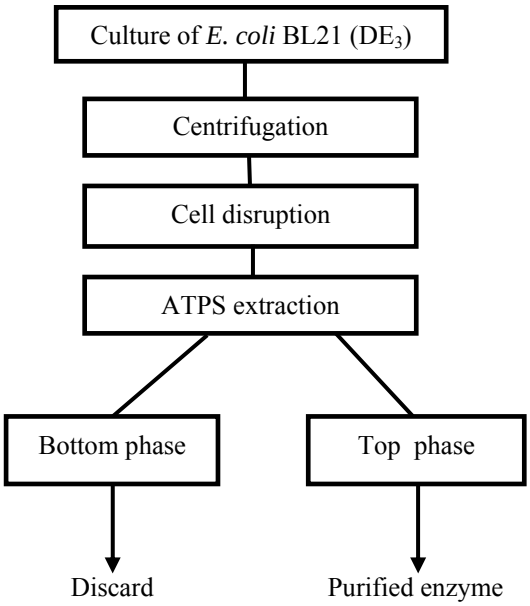

Fig. 1. Flow chart of recombinant PheDH purification in ATPS.

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.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis was carried out in 10% homogenous gel [20]. The gels were stained by Coomassie Brilliant Blue R-250. The molecular mass markers were phosphorylase b (94 kDa), BSA (66.5 kDa), carbonic anhydrase (30 kDa) and α-lactalbumin (14.4 kDa).

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. The kinetic parameters for the best purification system were calculated from the secondary plots of intercepts versus reciprocal concentrations of the other substrate [18].

RESULTS AND DISCUSSUION

Effects of PEG and $(NH_4)_2SO_4$ concentrations. The choice of optimal system for selective separation of PheDH activity was preformed as described by Hatti-Kaul [6]. In order to find the best extraction condition for partitioning and the purification of PheDH, 17 different systems were evaluated (Table 1). As shown in Table 1, there was no regular relation between the partition parameters and phase concentrations. Therefore, the optimized condition was verified experimentally. Also, the reproducibility of the extraction in these systems was confirmed by repeating the process several times. Among these different combinations studied, optimal values for partition coefficient, the K_E, top phase yield, purification factor and recovery were observed in PEG-6,000 9% (w/w) and $(NH_4)_2SO_4$ 16% (w/w).

Effects of PEG molecular weight. Based on the pervious findings, ATPS composed of 9% PEG-6,000 and 16% $(NH_4)_2SO_4$ was selected to investigate the effect of different molecular masses of PEG. The partitioning and extraction of biomaterials are strongly dependent on the PEG molecular mass [21, 22]. This behavior is usually attributed to hydrophobic interactions between the PEG chains and the hydrophobic area of biomolecule. In general, with increase of PEG molecular mass, the extraction efficiency decreases. At high molecular weight (MW), the preferential interaction between the PEG and the protein domain decreases. This results to high viscosity and bad reproducibility. Low molecular mass is also unsuitable because that the exclusion effect decreases and as a result the polymer can attract all proteins (contaminant and desired proteins) to the upper phase. Therefore, it can be said that the intermediate molecular mass of PEG is the best choice for ATPS experiments. However, there is no general rule about the mechanism governing partition and even in some studies, these parameters show opposite results [23]. As shown in Figure 2, increase in PEG molecular weight from 2,000 to 6,000 resulted in increase of partition efficiency. Conversely, when the PEG molecular weight increased from 6,000 to 20,000, the partition efficiency decreased. The highest partition parameters were obtained by PEG-6,000. These data suggested that PheDH has great hydrophobic surface which enhances enzyme-polymer interactions. Our findings in this research were supported by other literatures [24]. Briefly, the PEG molecular weight should be kept at 6,000 for the next experiments.

Effects of pH. The partition behaviors of PheDH with different pH values were also investigated. According to Albertsson's equation, the partition coefficient of a charged biomaterial is influenced by short range (van der waals) and long-range (electrostatic) molecular interactions as follows [6]:

$$1nK_p = 1nK^0{}_p + \left(\frac{Z_P F}{RT}\right)\ \Delta\Psi$$

Where K_p and K^0_p are partition coefficient at a given pH and the isoelectric point (p*I*). The $\Delta\Psi$ is the difference of interfacial potential between the top and bottom phases $(\Psi_{top} - \Psi_{bottom})$ which influences the partitioning behavior of target bio-molecule. The Z_P, F, R and T denote the net protein charge, Faraday constant, universal gas constant and absolute temperature, respectively. Figure 3 shows the effects of pH on the partition parameters. When pH rose from 5.8 to 8.0, the partition coefficient, yield, recovery and purification factor were increased. However, changing pH from 8.0 to 11 caused decrease in extraction efficiency.

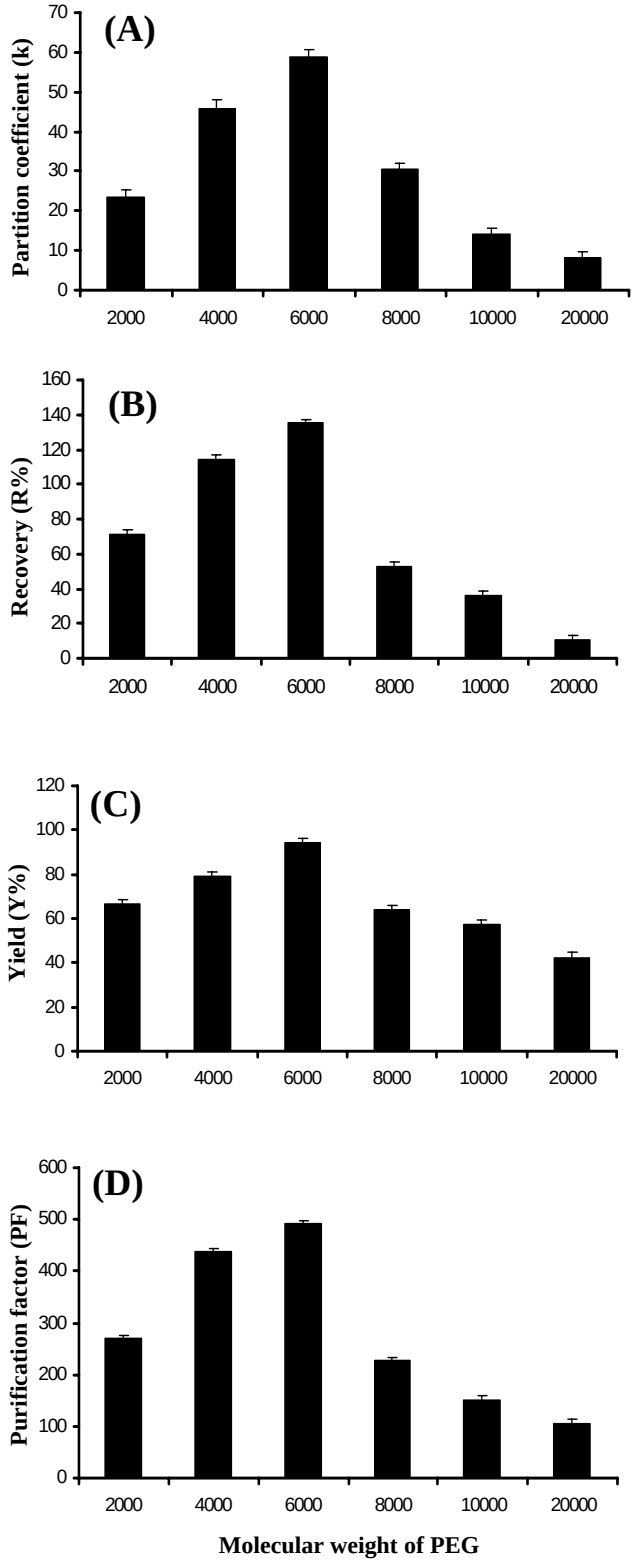

Fig. 2. Influences of PEG molecular weight on partition coefficient **(A)**, recovery **(B)**, yield **(C)** and purification factor **(D)** in systems containing 9% PEG-6000 and 16% (w/w) $(NH_4)_2SO_4$ (pH 8.0). The partition experiments were carried out in triplicate to estimate experimental errors.

Electrostatic molecular interactions between the charged bio-molecules and the phases were responsible for this adverse influence of pH [6, 7]. This phenomenon has been observed by others as well [25, 26]. Finally, ATPS of pH 8.0 was chosen as the optimal pH for enzyme partitioning.

Purification of recombinant PheDH by aqueous two-phase extraction. In the present research, PEG-6,000 and $(NH_4)_2SO_4$ ATPS were investigated for the partitioning and purification of recombinant *Bacillus sphaericus* PheDH. The extraction and purification were carried out in one partition step where the recombinant enzyme was strongly partitioned to the top PEG-rich phase (Fig. 4). Under the most favorable conditions with 9% (w/w) PEG-6,000 and 16% (w/w) $(NH_4)_2SO_4$ at pH 8.0, partition coefficient and yield were achieved 58.7 and 94.42%, respectively. PEG/salt ATPS is widely used to purify different enzymes and proteins [6, 7]. As representative examples of extraction process that exploit ATPS are the processing of β-mannanase (yield = 83%, K_E= 7.06) [11], β-glucanase (yield = 65.3%, K_E = 2.84) [23], lysozyme (yield = 70%) [24], alkaline protease (yield = 62.2%, K_E = 41.2) [26], papain (yield = 88.8%) [27], Ipomoea peroxidase (yield = 93%, K_E = 0.01) [28], proteinase (yield = 69%, K_E= 1.94) [29], polyphenol oxidase (yield = 97%, K_E= 32.3) [30] and β-glucosidase (yield = 92%, K_E = 0.5) [31]. Comparison of partition coefficient and yield values among these results and our study proved that the proposed method here would be useful and desirable for downstream processing of PheDH. Also the specific activity of PheDH enzyme in this study was 9828.88 U/mg that was comparable to the values reported for PheDH from *B. badius* (67.8 U/mg) [17], *Microbacterium sp.* (37.1 U/mg) [16], *Thermoactinomyces* (86.2 U/mg) [15], *R. maris* (65.2 U/mg) [15] and *B. sphaericus* (577.3 U/mg) [15]. The molecular weight of PheDH was estimated to be about 41 kDa by SDS-PAGE, which was similar to the previously reported value [2].The Michaelis constants for L-phenylalanine and NAD^+ in oxidative deamination were obtained 0.21 and 0.13 mM, respectively.

In conclusion, the process described in this work could be used as a benefit, interesting and economical technique for the recovery and purification of recombinant PheDH. This study might open up new possibility in the separation and purification of other amino acid dehydrogenases.

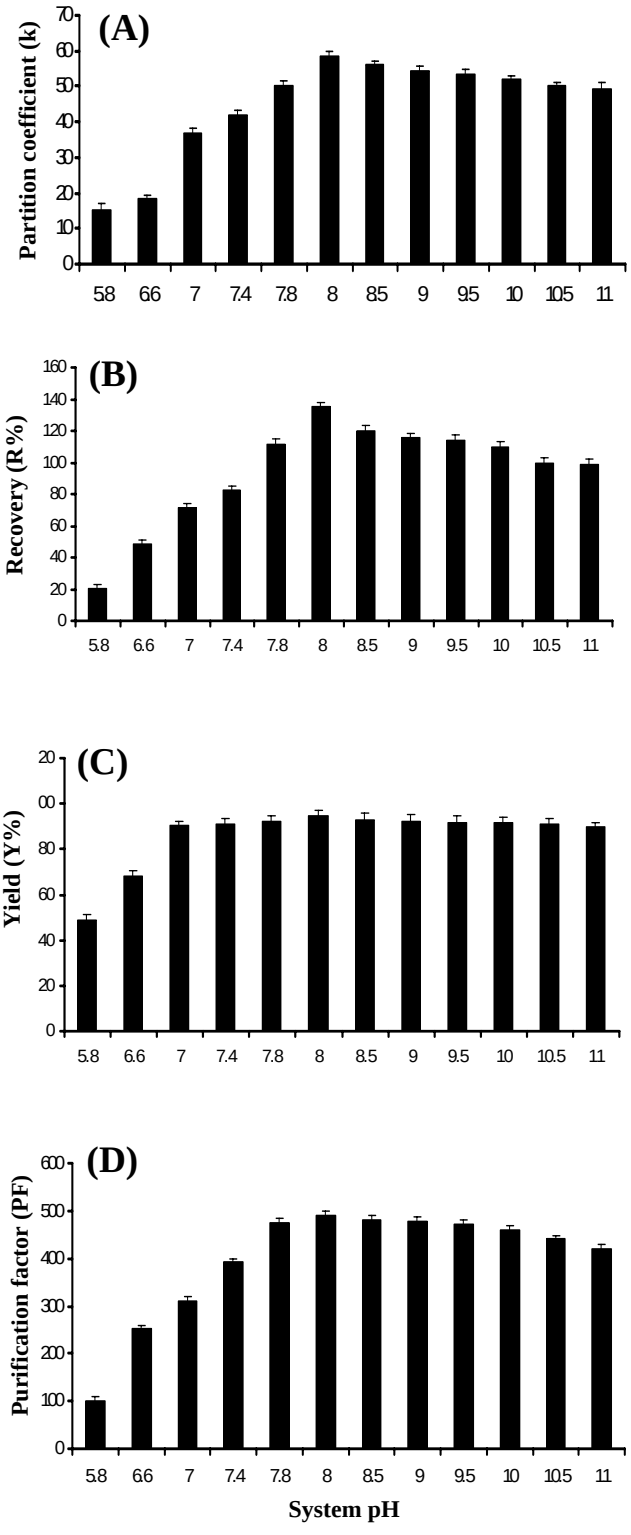

Fig. 3. Influences of pH on partition coefficient **(A)**, recovery **(B)**, yield **(C)** and purification fact **(D)** in systems containing 9% PEG-6000 and 16% (w/w) $(NH_4)_2SO_4$ (pH 8.0). The amounts of $K_{enzyme,}$ recovery, yield and purification factor reported in this study were an average value of triplicate experiments.

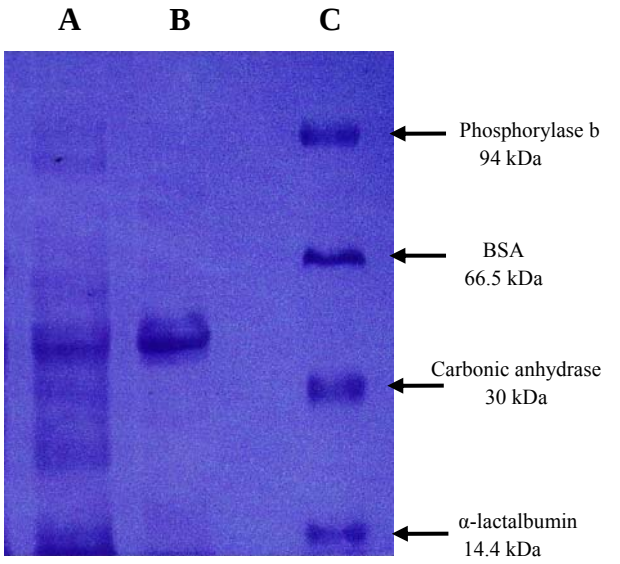

Fig. 4. Electrophoresis SDS-PAGE of recombinant PheDH. Lane A: bottom phase after extraction by ATPS consisting of 9% PEG-6000 and 16% (w/w) $(NH_4)_2SO_4$ (pH 8.0). Lane B: upper phase of the same ATPS. Lane C: molecular markers. The protein bands were stained with Coomassie Brilliant Blue R-250.

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