Development and Application of Aqueous Two-Phase Partition for the Recovery and Separation of Recombinant Phenylalanine Dehydrogenase

Shahbaz Mohammadi, Hamid
Young Research Club, Department of Biochemistry, Science & Research Campus, Islamic Azad University, Tehran, I.R. IRAN

Omidinia, Eskander*[+]
Department of Biochemistry, Pasteur Institute of Iran, Tehran, I.R. IRAN

ABSTRACT: *Aqueous two-phase systems (ATPS) have emerged as a powerful extraction method for the downstream processing of bio-molecules. The aim of this work was to investigate the possibility of utilizing ATPS for the separation of recombinant Bacillus sphaericus phenylalanine dehydrogenase (PheDH). Polyethylene glycol (PEG) and ammonium sulfate systems were selected for our experiment. The effect of different elements such as; type and concentration of PEG, concentration of $(NH_4)_2SO_4$, pH, phase volume ratio (V_R) and tie-line length (TLL) on the extraction behavior and selective separation was also studied. Desirable conditions for differential partitioning was obtained in 8.5 % (w/w) PEG-6000, 17.5 % (w/w) $(NH_4)_2SO_4$ and V_R 0.25 at pH 8.0. PheDH was mainly concentrated into the upper PEG-rich phase in all tested systems. The partition coefficient (K), recovery (R %), yield (Y %), TLL and selectivity were found to be 58.7, 135 %, 94.42 %, 39.89 % (w/w) and 2174, respectively. From the experimental results, it was revealed that the PEG molecular weight, $(NH_4)_2SO_4$ concentration, TLL and pH of system had strong impacts on partition features. The extraction efficiency was increased with elevation of pH and TLL values. In this paper, we described the partitioning behavior in PEG/$(NH_4)_2SO_4$ ATPS in order to evaluate the applicability of ATPS for partitioning and recovery of PheDH.*

KEY WORDS: *Aqueous two-phase systems (ATPS), Ammonium sulfate, Phenylalanine dehydrogenase (PheDH), Polyethylene glycol (PEG), Separation.*

INTRODUCTION

Today, industries desire purification methods which are rapid, simple, efficient and economic. As a matter of fact, the traditional procedures including salt or organic precipitation, dialysis, filtration and chromatographic methods or a combination of these are expensive and time consuming and pose enormous difficulties in large scale

* *To whom correspondence should be addressed.*
+ *E-mail: skandar@pasteur.ac.ir*
1021-9986/08/2/119 9/$/2.90

applications. With regard to these problems, aqueous two-phase systems (ATPS) appear to be a powerful and attractive candidate for separating and purifying a variety of biological products [1-4].

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 advantages of higher selectivity, lower cost and lower viscosity in protein partitioning in comparison with polymer-polymer systems [3, 5].

Also it has been found that the desirable biomaterials are usually concentrated into one phase and contaminants remain in the other. Partitioning in ATPS is mainly dependent upon the physiochemical traits of bio-molecules 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 [1, 3]. 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 [6, 7]. The causative mechanisms of ATPS partitioning are largely unknown. Although, the mathematical models such as response surface methodology (RSM) provide some information about phase behavior and partitioning of target bio-molecules, no comprehensive theory currently exists to guide the design of optimal systems. Thus, the experimental data is necessary to obtain an adequate partitioning [2, 3]. 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 influence on proteins. This will be interesting and suitable from the biotechnology viewpoint [3, 8].

Phenylalanine dehydrogenase (PheDH; EC 1.4.1.20) is a member of amino acid dehydrogenase family that performs the reversible NAD^+-dependent oxidative deamination of L-phenylalanine to phenylpyruvate. This enzyme is present in different species of bacteria and responsible for the first catabolic step of phenylalanine [9]. It has gained considerable interest for use in biosensors or diagnostic kits in phenylketonuria (PKU) newborn screening [10, 11]. Additionally, it can be used in synthesis of phenylalanine and related L-amino acids as basic building blocks for production of foods and pharmaceuticals [12, 13]. The purpose of this reseach was to investigate the possibility of utilizing ATPS for partitioning and recovery of PheDH. The influences of PEG molecular weight (MW) and concentration, pH, $(NH_4)_2SO_4$ concentration and TLL on enzyme partitioning were also studied.

EXPERIMENTAL

Materials

Polyethylene glycol (PEG) with average molecular weight (MW) of 2000, 4000, 6000, 8000, 10000, 20000 and $(NH_4)_2SO_4$ were purchased from Merck (Darmstadt, Germany). L-Phenylalanine and NAD^+ were obtained from Sigma-Aldrich (St. Louis, Mo, USA) and used for enzyme activity assay. The salts and all other chemicals were of analytical grade and double-distilled water for all experiments was obtained from a Milli-Q system (Millipore Inc., MA, USA). Recombinant *Bacillus sphaericus* PheDH was kindly provided by Professor Yasuhisa Asano (Toyama Prefectural University, Japan).

Cell cultivation and enzyme production

E. coli BL21 (DE3) cells harboring plasmid pET16b with recombinant *Bacillus sphaericus* PheDH activity were cultivated as described in *Omidinia et al*. [14]. Briefly, a recombinant strain of *B. sphaericus* was grown in Luria-Bertani (LB) medium supplemented with 0.1 mg /ml of ampicillin at 37 °C and 140 rpm. After 8 h, 10 ml culture was transferred as a seed into 1 L of LB medium in culture flasks and shaken at 37 °C until an OD_{600}=1.0 was reached, 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. Cells were harvested by centrifugation at 3500 rpm for 15 min, washed with 0.9 % NaCl solution, centrifuged and suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 2-mercaptoethanol. The cells were disrupted for 20 min total (15 s run and 10 s pause) by a 9-KHz sonic oscillator.

Table 1: Partitioning of recombinant PheDH in various ATPS combinations comprising PEG-6000/$(NH_4)_2SO_4$. In the present research, the partition experiments were done in triplicate to estimate experimental errors.

No.	PEG (w/w, %)	$(NH_4)_2SO_4$ (w/w, %)	K_E	K_P	Recovery (%)	Yield (%)	Selectivity
1	8.5	16.5	47.82	0.035	79.98	96.71	336.3
2	8	16.5	53.47	0.027	57.09	93.03	1980
3 4	9	16.5	62.41	0.023	66.63	93.97	2713
4	9.5	17	68.77	0.034	66.63	91.13	3993
5	8.5	17	18.29	0.032	39.91	82.05	571
6	8.5	16	9.02	0.016	36.26	69.27	563
7	9.5	16	37.38	0.026	39.91	90.33	1433
8	9.5	16.5	81.10	0.031	49.93	93.29	2616
9	8	17.5	33.18	0.04	100	89.34	829
10	8.5	17.5	58.7	0.027	135	94.42	2174
11	9.5	17.5	15.73	0.046	47.40	79.72	342
12	9	16	53.95	0.026	91.61	93.09	2075

The resulting homogenate was centrifuged at 3500 rpm at 4 °C for 60 min to clarity and dialyzed against the same buffer.

Phase diagram determination

Phase diagram for the PEG-$(NH_4)_2SO_4$ systems was determined by cloud-point method according to Albertsson [1]. A salt solution of known concentration was added drop-wise to a known amount of a concentrated stock of PEG. At a critical point (the could-point) the mixture became turbid and a two-phase system was formed. The composition of this mixture was noted and provided a point on the binodal curve. Then, 0.1 M potassium phosphate buffer (pH 8.0) was added drop-wise to the tube to get a clear one-phase system and more salt solution was added again to afford a two-phase system. The composition of this mixture was noted and so on.

Preparation of ATPS

Aqueous two-phase partitioning experiments were preformed at room temperature by mixing required amounts of solid PEG and $(NH_4)_2SO_4$ (table 1.), 0.1 M potassium phosphate buffer (pH 8.0) and 2 ml cell culture supernatant, in 15 ml graduated tubes with conical tips [3]. Millipore water was added to obtain 10 g of the final weight. Systems were thoroughly agitated for 1 h and then centrifuged at 3000 rpm at 25 °C for 40 min to speed up the phase separation and to reach the equilibrium state. After this attitude, a well defined interface and transparent bottom and top phases were visualized (see Fig. 1) The volumes of the top and bottom phase were carefully measured and then analyzed for enzyme activities and total protein concentrations (see diagram in Fig. 2).

Determination of PheDH activity

Dehydrogenase activity of recombinant PheDH in the oxidative deamination reaction was assayed spectrophotometrically (Shimadzu UV-visible-1601 PC, Japan) using phenylalanine as substrate by monitoring the reduction of NAD^+ at 340 nm. The reaction mixture is consisted of 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. 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. In all enzyme assays, control systems without L-phenylalanine or sodium phenylpyruvate were

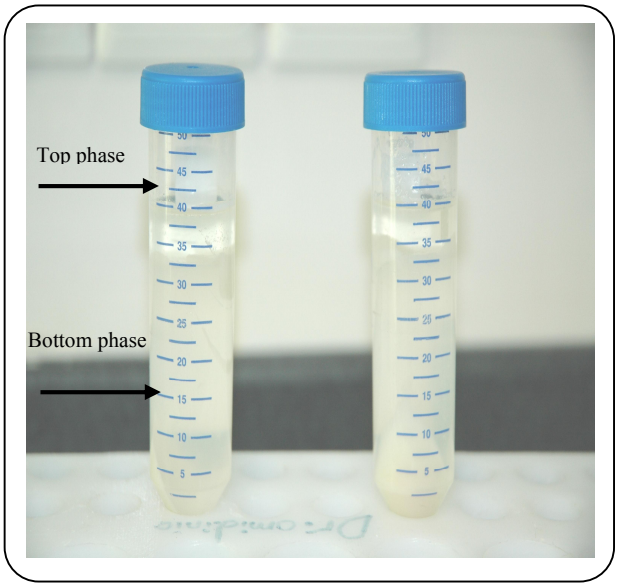


Fig. 1: A picture of ATPS in this research.

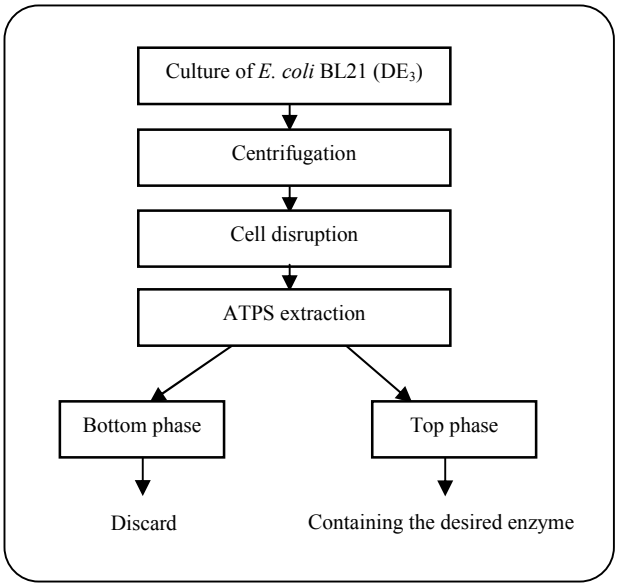


Fig. 2: Flow chart for the differential partitioning of PheDH.

used as reference and no interference from phase components was observed. One unit of PheDH activity (U) was defined as the releasing of 1 μmol NADH per min [9].

Determination of protein content

The total protein content in both phases was determined by a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard protein [15]. 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 Bradford solution. It 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.

Determination of partition parameters

In order to achieve an effective separation of target bio-molecule, the distribution behaviors between the phases were evaluated by the following parameters [1, 3]:

Partition coefficient (K_E): is calculated by the given equation:

$$K_E = \frac{A_t}{A_b} \quad (1)$$

Where A_t and A_b denote the PheDH activity in the top and bottom phase, respectively. Similar partitioning coefficient for enzyme concentration (K_P) can be given as:

$$K_p = \frac{P_t}{P_b} \quad (2)$$

Where P_t and P_b corresponded to PheDH concentrations in the top and bottom phases, respectively.

$$R(\%) = \frac{\text{enzyme activity of the top phase}}{\text{total enzyme activity added to the system}} \quad (3)$$

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

$$Y(\%) = \frac{100 V_t K}{V_t K + V_b} \quad (4)$$

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

Phase volume ratio (V_R): is defined as the ratio of volume in the upper phase (V_t) to that in the bottom phase (V_b).

$$V_R = \frac{V_t}{V_b} \quad (5)$$

Tie-line length (TLL) is described as the straight lines that connect the composition values of the top and bottom phase in equilibrium. They will be on different sides of the binodial curve and never cross each other. It was calculated according to following formula:

$$TLL(\%, w/w) = \sqrt{(P_T - P_B)^2} + \sqrt{(S_T - S_B)^2} \quad (6)$$

Where P_T and P_B represent the PEG concentrations in the top and in the bottom phase, respectively and S_T and S_B are the ammonium sulfate concentrations in the top and in the bottom phase, respectively.

Selectivity (S) is determined as the ratio of K_E to the K_P.

$$S = \frac{K_E}{K_P} \quad (7)$$

It is necessary to mention that all the partition experiments were done in triplicate and the average results were reported.

RESULTS AND DISCUSSION

Influence of PEG and $(NH_4)_2SO_4$ concentrations on enzyme partitioning

Partitioning between the two phases of an ATPS is a complex function guided by several factors such as interaction of the partitioned substance and the phase components (e.g. hydrogen bonds, van der waals, hydrophobic and electrostatic interactions), steric and conformational effects and also the phase composition. Generally, in order to define the adequate system, the phase composition is often arbitrarily chosen and partitioning of the interested molecule between the two phases and the interface is investigated. This is followed by further systematic changes in the phase composition and the different parameters to obtain an efficient separation [3].

In this study, the choice of desired system to achieve selective separation for PheDH activity was preformed as described above. For this purpose, the partitioning of recombinant PheDH was studied in 12 different ATPS of PEG/$(NH_4)_2SO_4$ (table 1). As can be found from table 1, there was no regular relation between the partition parameters and phase concentrations. Therefore the optimal condition was verified experimentally. Also the reproducibility of the extraction in these systems was confirmed by repeating the process several times. Results from the laboratory studies suggested that the ATPS composition of 8.5 % (w/w) PEG-6000 and 17.5 % (w/w) $(NH_4)_2SO_4$ could be an excellent system for PheDH separation. Therefore, this ATPS was chosen as the optimal system for the following investigations.

Influence of PEG molecular mass on enzyme partitioning

Using the pervious findings, the ATPS composed of 8.5 % PEG and 17.5 % $(NH_4)_2SO_4$ was selected to investigate the effect of different types of PEG on enzyme partitioning. In order to compare the effect of the different molecular masses of polymer, the PEG and ammonium sulfate concentrations were chosen at the same distance from the critical point of binodial diagrams. According to the Albertsson's comments, the selection of the best molecular weight of polymer is generally the first step in the ATPS experiments [16]. It has been demonstrated that the molecular weight of PEG affects the composition of phases and the number of polymer-protein interactions [17]. This influence is usually attributed to hydrophobic interactions between the chains of PEG and the hydrophobic area of bio-molecule.

Therefore, it can be said that besides PEG exclusion effect, the partitioning is also governed by the chemical structure of protein. A high hydrophobical surface of a protein exposed to solvent is a factor that favors the partition equilibrium displacement to the PEG-rich phase. Hydrophobicity is also changed by the type and molecular weight of polymer. Meanwhile, the PEG-salt interactions must be considered, for example in the PEG/phosphate systems due to the repulsion between the PEG and the phosphate molecules, the PEG phase has higher self energy than the phosphate phase [18]. In general, with increase of PEG molecular mass the extraction efficiency decreases. At high molecular weight (MW), the preferential interactions between the PEG and the protein domain decrease and this leads to a reduction in K_E. One reason could be that the increase in polymer MW increases the exclusion effect, thus the polymer acquires a more compact conformation with intra-molecular hydrophobic bonds and hinders the partition of biomaterial into the top phase. This would resulted in significant transfer of target protein to the salt rich phase, high viscosity and bad reproducibility. Low molecular mass is also unsuitable for adequate partitioning because 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 considering all these descriptions, there is no general rule about the mechanism

governing partition and in some studies, these parameters show opposite results [19, 20]. As can be seen in Fig. 3, PEG molecular weight did not show any regular partitioning behavior. Increase in PEG molecular weight from 2000 to 6000 resulted to increase of the partition efficiency. Conversely, when the PEG molecular weight increased from 6000 to 20000, the partition efficiency decreased. This can possibly be attributed to the differential spatial conformation that PEG with various molecular mass adopts in solution and leads to a differential polymer-enzyme interaction according to the relative composition of the PEG mixture [3]. As anticipated, the low (2000, 4000) and high (8000, 10000, 20000) MW of PEG were not helpful for obtaining suitable separation and the highest partition parameters (R, Y, K_E) were obtained by PEG-6000. Hence, these results were in agreement with the above descriptions in the sense that the intermediate MW is favorable for ATPS extraction. The obtained extraction parameters also suggested that the PheDH had great hydrophobic surfaces which enhanced the enzyme-polymer interactions. On the other hand, the high presence of hydrophobic regions at the molecular surface of enzyme tended to increase its preference for separation into the polymer phase. Similar behaviors were observed for lysozyme in PEG-sodium citrate system [21] and lipase in PEG-phodphate as well [22]. In conclusion, the PEG molecular weight should be kept at 6000 for the next experiments.

Influence of system pH on enzyme partitioning

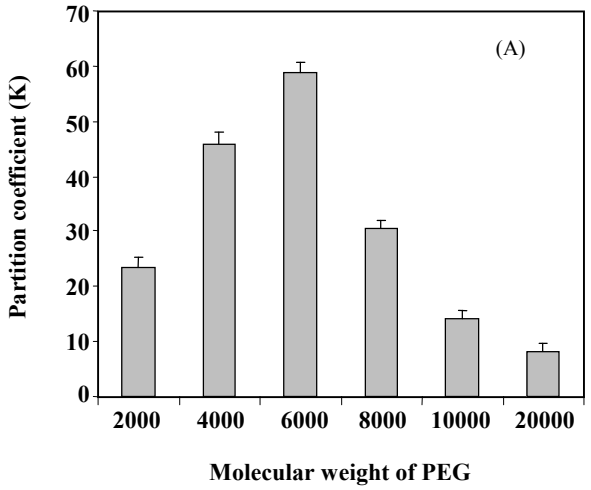
Another important factor which influences the partitioning of bio-molecules is system pH [1, 3]. To evaluate the effect of pH on enzyme partitioning, the partition behavior of PheDH with different pH values was also studied. The partition is determined by the kind of ions present and the ratio between them. The pH affected the charge of bio-molecules their ion composition, and the surface character of contaminating materials and caused variation in their partitioning into the top and bottom phase [3]. Fig. 4 depicts the effect of pH on the partition parameters of enzyme. As can be found, when pH was raised from 5.8 to 8.0, the K_{enzyme}, yield and recovery were increased. The optimal values for the partition parameters were obtained at pH, 8.0. According to the *Forciniti* and Hall's comments, the hydrophobic interactions are stronger at a pH closer to the pI of the protein (the isoelectric point of *Bacillus sphaericus* PheDH is 5.3) [3]. Therefore, there was a general increase of affinity behavior to the PEG rich phase with increasing the system pH. This behavior also could be explained on the basis of Albertsson's equation, which takes into account electrostatic and non- electrostatic (van der waals) molecular interactions [1]:

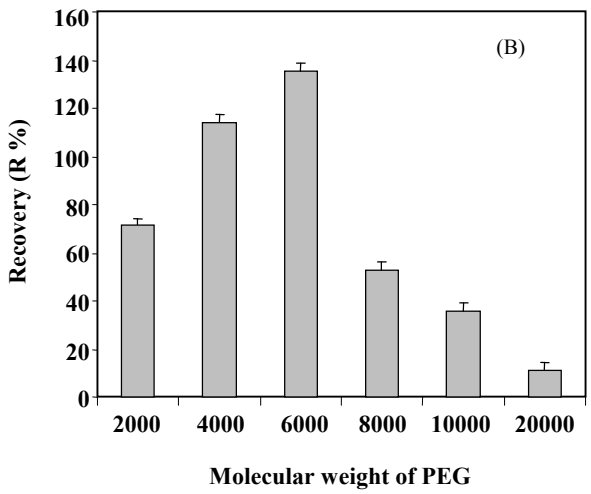
$$\ln K_p = \ln K_p^0 (\frac{Z_p F}{RT}) \Delta\Psi \quad (8)$$

Where K_p and K^0_p are the partition coefficients at a given pH and isoelectric point (pI) and $\Delta\Psi$ is the difference of interfacial potential between the top and the 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. With increase of pH, positive electrical charges on the oxygen atom of PEG will decrease and so will the complex formation tendency of PEG with anion species which is reflected in the increase of partition coefficient as mentioned in the Flory-Huggins theory [3]. This phenomenon was observed by others as well [20, 23]. Finally, ATPS of pH 8.0 was chosen as the optimal pH for PheDH partitioning.

Effect of system Tie-line length on enzyme partitioning

The influence of Tie-line length (TLL) on enzyme partitioning was also investigated. The effect of PEG and ammonium sulfate concentrations on partitioning is defined as a function of TLL. Various TLL describe different selectivity or partition pattern for different protein components [3]. As a consequence, the study of different selectivity conditions is critical. In these ATPS (V_R = 0.25 in all experiments), increasing the ammonium sulfate concentration in the bottom phase resulted in the increase of TLL (table 2), whereas the phase volume practically remained constant (0.25). Higher TLL caused a better partitioning of PheDH into the top phase and subsequent increase of the extraction efficiency (table 2). At the highest TLL (39.89 %), a yield about 94.42 % was obtained in the top phase and the partition coefficient was 58.7. This can be attributed to the salting out effect of ammonium sulfate which caused molecular exclusion of PheDH to move to the upper phase. Therefore, it was





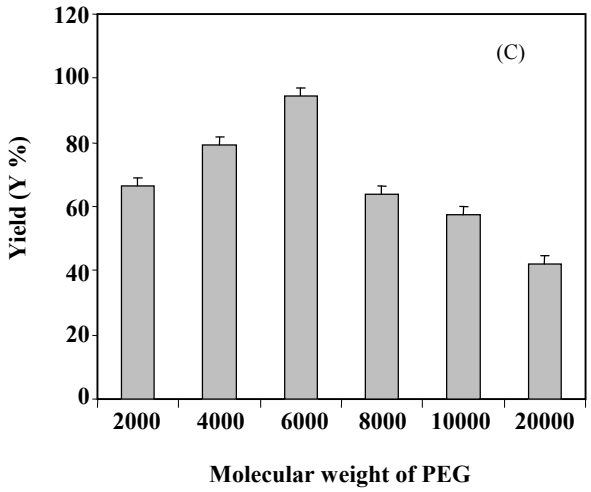
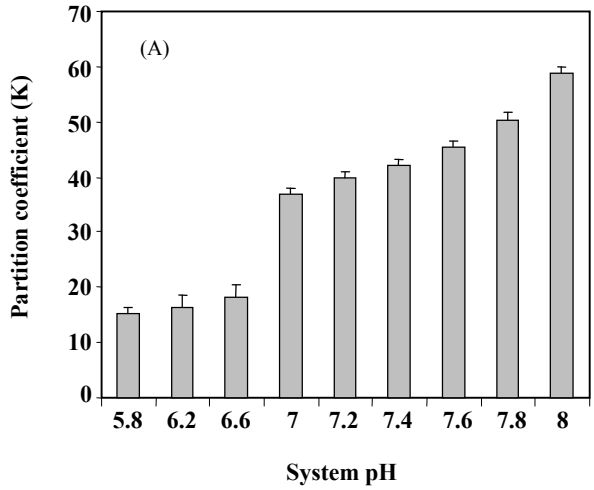
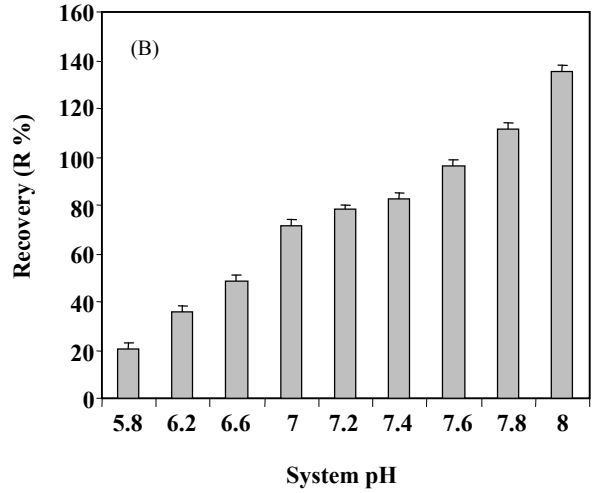


Fig. 3: Influences of PEG molecular weight on partition coefficient (A), recovery (B) and yield (C) in PEG/$(NH_4)_2SO_4$ ATPS. In the present work, the partition experiments were carried out in triplicate to estimate experimental errors.





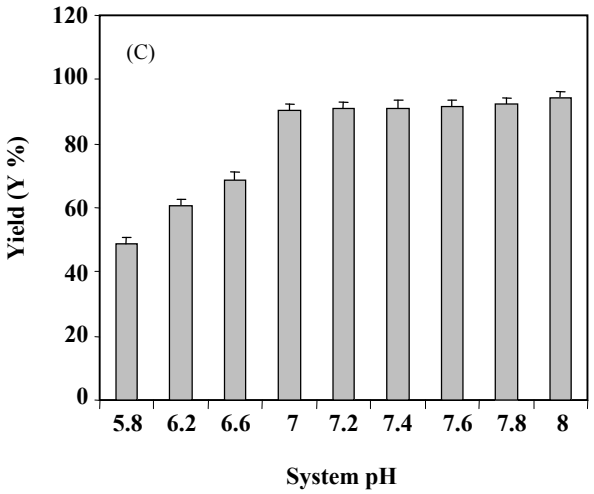


Fig. 4: Influences of system pH on partition coefficient (A), recovery (B) and yield (C) in PEG-6000/$(NH_4)_2SO_4$ ATPS. The amounts of K_{enzyme}, Recovery and yield reported in this study were an average value of triplicate experiments.

Table 2: The effect of TLL on the partition coefficient and phase volume ratio of PheDH in PEG-6000/$(NH_4)_2SO_4$ ATPS.

System	TLL (%,w/w)	K_E	Phase volume ratio (V_R)
1	9.4	47.82	0.25
2	8.3	53.47	0.25
3	6.8	62.41	0.25
4	9.8	68.77	0.25
5	24	18.29	0.25
6	21	9.02	0.25
7	23	37.38	0.25
8	31	81.10	0.25
9	38.8	33.18	0.25
10	39.89	58.7	0.25
11	36	15.73	0.25
12	8.9	53.95	0.25

decided to select the ATPS with a TLL of 39.89 % (w/w) for PheDH partitioning.

CONCLUSIONS

The partitioning of recombinant *Bacillus sphaericus* PheDH produced by *E. coli BL21* (DE_3) in PEG and ammonium sulfate ATPS was successfully performed. The obtained finding in this study suggested that this partition procedure using PEG/$(NH_4)_2SO_4$ could be employed as an efficient and attractive tool for the extraction and recovery of recombinant PheDH. The final optimized system was 8.5 % (w/w) PEG-6000 and 17.5 % (w/w) $(NH_4)_2SO_4$ at pH 8.0 with V_R=0.25, selectivity of 2174, K_P of 0.027 and TLL of 39.89 % (w/w). The experimental results revealed that molecular weight of PEG, pH and TLL had significant effects on enzyme partitioning. Longer TLL and higher pH resulted in better partitioning into the top phase. Finally, we here conclude that the partitioning of recombinant PheDH in ATPS could be used as a beneficial, interesting and economical technique for separating and recovery of PheDH. The next step will be the purification this enzyme in ATPS.

Acknowledgements

This work was performed within the framework of the Ministry of Health, Treatment and Medical Education project, Food and Drug deputy.

Nomenclatures

NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
OD	Optical density

Received : 9th March 2007 ; Accepted : 27th January 2008

REFERENCES

[1] Albertsson, P. A., "Partition of Cell Particles and Macromolecules", 3rd Ed., New York, John Wiley & Sons, (1986).

[2] Ratio-Palomares, M., Practical Application of Aqueous Two-Phase Partition to Process Development for the Recovery of Biological Products, *J. Chromatogr. B,* **807**, 3 (2004).

[3] Hatti-Kaul, R., "Methods in Biotechnology: Aqueous Two-Phase Systems: Methods and Protocols", Humana Press Inc., Totowa, NJ, (1999).

[4] Bensch, M., Selbach, B. and Hubbuch, J., High Throughput Screening Techniques in Downstream Processing: Preparation, Characterization and Optimization of Aqueous Two-Phase Systems, *Chem. Eng. Sci.,* **62**, 2011 (2007).

[5] Rahimpour, F., Mamoa, G., Feyzi, F., Maghsoudi, S. and Hatti-Kaul, R., Optimizing Refolding and Recovery of Active Recombinant *Bacillus Halodurans* Xylanase in Polymer-Salt Aqueous Two-Phase System Using Surface Response Analysis, *J. Chromatogr. A,* **1141**, 32 (2007).

[6] Huddleston, J., Veide, A., Kohler, K., Flanagan, J., Enfors, S. and Lyddiatt, A., The Molecular Basis of Partitioning in Aqueous Two-Phase Systems, *Trends Biotechnol.,* **9**, 381 (1991).

[7] Tub´ıo, G., Nerli, B. and Pic´o, G., Partitioning Features of Bovine Trypsin and Chymotrypsin in Polyethyleneglycol-Sodium Citrate Aqueous Tow-Phase Systems, *J. Chromatogr. B,* **852**, 244 (2007).

[8] Shibusawa, Y., Takeuchi, N., Tsutsumi, K., Nakanoa, S., Yanagida A., Shindo, H. and Ito, Y., One-Step Purification of Histone Deacetylase from *Escherichia Coli* Cell-Lysate by Counter-Current Chromato-

graphy Using Aqueous Two-Phase System, *J. Chromatogr. A,* **1151**, 1583 (2007).

[9] Asano, Y., "Phenylalanine Dehydrogenase In: Encyclopedia of Bioprocess Technology: Fermen-tation, Biotechnology and Bioseparation, (Flinckinger, M. C. and Drew, S. W., Eds.), Vol. 2, John Wiley & Sons, Inc., New York, USA, pp. 1955-63 (1999).

[10] Weiss, D. J., Dorris, M., Loh, M. and Peterson, L., Dehydrogenase Based Reagentless Biosensor for Monitoring Phenylketonuria, *Biosens. Bioelectron,* **22**, 2436 (2006).

[11] Tachibana, S., Suzuki, M. and Asano, Y., Application of an Enzyme Chip to the Microquantification of L-phenylalanine, *Anal. Biochem.,* **359**, 72 (2006).

[12] Busca, P., Paradisi, F., Moynihan, E., Maguire, A. R. and Engel**,** P. C., Enantioselective Synthesis of Non-Natural Amino Acids Using Phenylalanine Dehydrogenases Modified by Site-Directed Mutagenesis, *Org. Biomol. Chem.,* **2**, 2684 (2004).

[13] Mihara, H., Muramatsu, H., Kakutani, R., Yasuda, M., Ueda, M., Kurihara, T. and Esaki, N., N-Methyl-L-amino Acid Dehydrogenase from *Pseudomonas putide*: A Novel Member of an Unusual $NAD(P)^+$-Dependent Oxidoreductase Superfamily, *Eur. J. Biochem.,* **272**, 1117 (2005).

[14] Omidinia, E., Taherkhani, H., Asano, Y., Khatami, S., Omumi, A., Ghadiri, A., Van der Lelie, D., Rashid Pouraie, R., Mirzahoseini, H. and Samadi, A., Affinty Purification and Characterization of Recombinatant *Bacillus sphaericus* Phenylalanine Dehydrognase Produced by PET Expression Vector System, *Iran Biomed. J.,* **6**, 31 (2002).

[15] Bradford, M. M., A Rapid and Sensitive for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem.,* **72**, 248. (1976).

[16] Albertsson, P. A., Cajarville, A., Brooks, D. E. and Tjerneld, F., Partition of Proteins in Aqueous Polymer Two-Phase Systems and the Effect of Molecular Weight of the Polymer, *Biochem. Biophys. Acta.,* **926**, 87 (1987).

[17] Forciniti, D., Hall, C. K. and Kula, M. R., Influence of Polymer Molecular Weight and Temperature on Phase Composition in Aqueous Two-Phase Systems, *Fluid Phase Equil.,* **61**, 243 (1991).

[18] Forciniti, D., Hall, C. K. and Kula, M. R., Protein Partitioning at the Isoelectric Point: Influence of Polymer Molecular Weight and Concentration and Protein Size, *Biotechnol. Bioeng*, **38**, 986 (1991).

[19] Bim, M. A., and Franco, T. T., Extraction in Aqueous Two-Phase Systems of Alkaline Xylanase Produced by *Bacillus pumillus* and its Application in Kraff Pulp Bleaching, *J. Chromatogr. B,* **743**, 394 (2000).

[20] Guo-qing, H., Xiu-yan, Z., Xing-jun, T., Qi-he, C. and Hui, R., Partitioning and Purification of Extracellular β-1, 3-1, 4-Glucanase in Aqueous Two-Phase Systems, *J. Zhejiang Univ. Sci.,* **6B**, 825 (2005).

[21] Su, C. K. and Chiang , B. H., Partitioning and Purification of Lysozyme from Chicken Egg White Using Aqueous Two-Phase System, *Process Biochem.,* **41**, 257 (2006).

[22] Bradoo, S., Saxena, R. K. and Gupta, R., Partitioning and Resolution of Mixture of Two Lipase from *Bacillus stearothermophilus* SB-1 in a Aqueous Two-Phase System, *Process Biochem.,* **35**, 57 (1999).

[23] Wongmongkol, N. and Prichanont, S., Partition of Alkaline Protease in Aqueous Two-Phase Systems of Polyethylene Glycol 1000 and Potassium Phosphate, *Korean J. Chem. Eng.,* **23**, 71 (2006).