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Comparison between Polyvinyl Pyrrolidone/Na_2SO_4 Aqueous Two-Phase Systems and Chromatographic Methods for Purification of Recombinant Phenylalanine Dehydrogenase

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Abstract

Phenylalanine dehydrogenase (PheDH; EC 1.4.1.20) is an important enzyme of amino acid dehydrogenases family that increasingly used as a valuable biocatalyst in neonatal screening kits and synthesis of L-phenylalanine. The goal of this literature was to find a suitable purification method for recombinant *Bacillus badius* PheDH by practical comparison between chromatographic and polyvinyl pyrrolidone (PVP)/Na_2SO_4 aqueous two-phase systems (ATPS) techniques. The partitioning behavior of target enzyme in PVP/Na_2SO_4 ATPS was examined and compared with the obtained results from a chromatographic protocol. Direct comparison of chromatography and ATPS procedures clearly revealed that the ATPS consisting of 8.0% (w/w) PVP, 17.0% (w/w) Na_2SO_4 with pH of 8.0, V_R=0.25 and temperature of 25 °C was the most desirable process for PheDH purification. A specific activity of 1231.42 U/mg, a purification factor of 36.61, a yield of 95.5% and a recovery of 138.9% were achieved. Altogether, we presented a two-phase methodology as a scalable and economically alternative for the production of PheDH enzyme.

Keywords: Comparison; Na_2SO_4; Purification; Phenylalanine dehydrogenase (PheDH); Polyvinyl pyrrolidone (PVP)

Introduction

Phenylketonuria (PKU) is an inborn metabolic disorder that without strict dietary control results in mental retardation, microcephaly and seizures [1]. Newborn PKU screening using phenylalanine dehydrogenase (PheDH; EC 1.4.1.20) is a simple method which has proved sensitive, reliable, rapid and cheap compared to other methodologies. PheDH is being one of the most important amino acid dehydrogenases that catalysis the reversible NAD^+-dependent oxidative deamination of L-phenylalanine to phenylpyruvate [2]. This enzyme occurs in various bacteria and it can be used as a diagnostic reagent in biosensors [3], enzyme chips [4] and neonatal screening kits [2]. Among this family, *Bacillus badius* PheDH has great potential for use in monitoring PKU because it shows higher substrate specificity for L-phenylalanine

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with minimal interference from tyrosine. Also according to Asano's comments, PheDH from *B. badius* is more applicable to the microdetermination of phenylalanine and phenylpyruvate [2]. In addition to its clinical applications, it is being increasingly used as a valuable biocatalyst in synthesis of L-phenylalanine for food and pharmaceutical industries [5]. Thus, developing the extensive biotechnological applications of this enzyme is the mean reason for investigating purification methods. The conventional procedures involve a series of low-efficiency purification steps which increase the cost of enzyme preparation and reduce the yield [6]. We have reported the purification of recombinant *Bacillus badius* PheDH by partitioning in a PEG/ammonium sulfate ATPS [7]. But we realized that this proposed system will not be a big breakthrough in purification of recombinant PheDH without studying the other ATPS and chromatographic procedures. Our objective in this research was to find a more suitable method by practical comparison between chromatographic technique and PVP/Na_2SO_4 ATPS.

Materials and Methods

Materials

Escherichia coli BL21 (DE3) cells with recombinant *B. badius* PheDH activity were provided by Professor Yasuhisa Asano (Toyama Prefectural University, Japan). PVP was purchased from Merck (Darmstadt, Germany). The salts used to prepare ATPS were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA). TSK gel DEAE-Toyopearl 650M was purchased from TOSOH (Tokyo, Japan). DEAE-cellulose and NAD^+ were from Sigma-Aldrich (St. Louis, MO, USA) and L-phenylalanine was obtained from Merck (Darmstadt, Germany). All other chemicals and biochemical reagents were of laboratory grade and double-distilled water was used throughout the experiments.

Enzyme Production

For the production of recombinant enzyme, *E. coli* BL21 (DE3) cells with recombinant *B. badius* PheDH activity were cultivated in shake flasks containing Luria-Bertani (LB) medium supplemented with 0.1 mg /ml ampicillin at 37 °C and 150 rpm. After 8 h of cultivation, a 10 ml culture broth was transferred into 1 L of LB medium and incubated 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 0.2 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG) and shaking at 23 °C for 5 h. After cultivation, cells were collected by centrifugation (Eppendorf centrifuge 5810 R, Germany) at 3500 rpm for 15 min and kept at -20 °C for further uses. The resulting pellets were dissolved in buffer A (0.1 M potassium phosphate buffer pH 7.0, 0.1 mM EDTA, and 2-mercaptoethanol) and then disrupted (20 min with a 9-KHz ultrasonic oscillator). This suspension was centrifuged at 1000 rpm at 4 °C for 20 min to clarity and dialyzed against the same buffer [6, 7].

Purification of Recombinant PheDH by ATPS

ATPS was carried out by adding defined amounts of solid components (8.0% (w/w) PVP and 17.0% (w/w) Na_2SO_4) and enzyme crude extract to make up the final mass of 10 g. Systems were gently shaken for 1 h and then centrifuged at 3500 rpm for 40 min at 25 °C to complete phase separation. After this treatment, the two-phases became clear and transparent and the interface was well defined. Samples of the top and bottom phases were carefully extracted and analyzed for enzyme activity and total protein [7, 8].

Purification of Recombinant PheDH by Chromatographic Method

In the first step, the crude enzymatic extract was heated at 50 °C for 15 min. After cooling on ice, the enzyme solution was centrifuged at 3500 rpm for 60 min at 4 °C and then precipitated with ammonium sulfate at 30-60% saturation under slow stirring. The precipitate was collected by centrifugation as before, dissolved in buffer A and dialyzed overnight at 4 °C against the same buffer. The dialyzed solution was subjected to anion-exchange chromatography in a DEAE-Toyoperal 650 M column (2.6×2 cm) pre-equilibrated with Buffer A. The column was equipped with a chromatography system (Bio-Rad, BioLogic DuoFlow model) and the elution was carried out using the buffer A containing 0.1 M NaCl at a flow rate of 2 ml/min. The active fractions were pooled, concentrated and applied to a DEAE-cellulose column (2.5×25 cm) equilibrated with the same buffer. Elution was also performed as before. Afterwards, the active enzyme fractions were combined, concentrated by ultrafiltration and loaded on a sephadex G-200 column that had been equilibrated with buffer A. Finally, the fractions containing PheDH activity were analyzed for protein purity [6,9].

Enzyme Activity Assay

Enzyme activity was determined spectrophoto-

metrically (double-beam UV-visible spectrophotometer, Shimadzu 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. One unit of PheDH activity (U) is expressed as the amount of enzyme catalyzing the formation of 1 μmol NADH per minute under the assay conditions [9].

Protein Determination

The concentration of total protein was estimated by a Bio-Rad protein assay kit using bovine serum albumin (BSA) as a standard [10]. The appropriate blank system phase was employed to correct for minor interference from polymer and salt.

Purity Analysis

Finally, the purified samples from ATPS and chromatography protocols were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were bodied for 5 min with the presence of 1% SDS, 80 mM 2-mercatoethonal, 100 mM Tris-HCl buffer (pH 6.8) and 15 % glycerol and loaded onto a 12% SDS ployacrylamide gel. After electrophoresis, the gels were stained with silver staining protocol [11].

Results

In order to achieve an efficient separation condition in ATPS studies, a series of experiments were conducted. Table 1 depicts the compositions and the results of examined systems. Among the all studied systems, a phase system comprised of 8.0% (w/w) PVP+ 17.0% (w/w) Na_2SO_4 was recognized as the best candidate for further experiments. This system gave better partition parameters in terms of recovery (138.9%), partition coefficient (52.25) and yield (95.5%). To determine the effect of salt type upon the enzyme partitioning, 16 kinds of ATPS including 8.0% (w/w) PVP and 17.0% (w/w) different salts were evaluated separately (Table 2). Considering the partition coefficient (K_E=52.25) and recovery (R=138.9%), Na_2SO_4 was selected as the salt phase. To achieve efficient partitioning of recombinant PheDH, it was essential to optimize the partition behavior by selecting a proper pH. Table 3 lists the partition parameters of PheDH in six kinds of ATPS at different pH values. When pH increased up from 7.0 to 8.0, the partition parameters of PheDH raised. From all these analysis, it was decided to carry out further experiments at pH 8.0. Under identical condition of phase composition and optimized experimental conditions, the influence of temperature on the extraction efficiency of recombinant PheDH was studied. As shown in Figure 1, as the temperature increased from 15 to 25 °C, the extraction efficiency raised. However, with increasing temperature from 25 to 55 °C, the partitioning of PheDH towards the PVP-rich phase decreased. In this research, all extraction experiments were done in 25 °C.

The chromatography process involved ammonium sulfate precipitation and two steps of anion-exchange chromatography after cell disruption (see diagram B in Fig. 2). Typical results of this multi-step protocol are shown in Table 3. The enzyme was purified with a yield of 25.12% and a specific activity of 553.57 U/mg from the cell-free extract of *E. coli* BL21 (DE3). To better understanding the efficiency of each presented method, we directly compared the chromatography and ATPS from the technical and economical viewpoints. As found from the Tables 3 and 4, the obtained purification parameters for recombinant PheDH was higher in ATPS strategy. The optimal PVP/Na_2SO_4 partition system consisting five unit operations resulted in a higher *SA* of 1231.42 U/mg and a higher *Y* of 95.5% at the PVP-rich top phase. In contrast, using the chromatographic purification method that involved 10 unit operations, *SA* and *Y* were obtained 553.57 U/mg and 25.12 %, respectively. A schematic representation of ATPS and chromatography processes are shown in Figure 3.

Discussion

Today's biotechnology industry has been demanding efficient and economical downstream processing for the

Table 1. Partition parameters of recombinant PheDH in PVP and Na_2SO_4 ATPS

No.	Total composition (%, w/w)		K_E	R (%)	Y (%)	PF
	PVP	Na_2SO_4				
1	7.44	12.37	49.45	180	84.6	16.45
2	7.83	13.23	54.37	173.4	85.8	12.66
3	8.21	14.2	45.9	142.15	72.08	18.3
4	8.0	17.0	52.25	138.9	95.5	36.61
5	9.0	16.0	62.44	120.55	84.62	20.27
6	11.81	16.9	22.9	138.85	80.16	14.38
7	13.92	17.33	16.18	94.85	92.5	16.75
8	15.12	17.84	14.16	76.8	41.86	4.35
9	19.4	18.81	6.15	42.2	33.33	9.6

Table 2. Influence of salt type on the partitioning behavior of recombinant PheDH in PVP/salt ATPS

No.	salt (w/w, %)	V_R	K_E	K_P	R (%)	Y (%)	PF	S
1	$(NH_4)_2HPO_4$	0.25	21.78	0.57	150.6	85.5	6.7	38.21
2	Na_2CO_3	0.25	29.94	0.47	109.17	87.85	5.46	61.57
3	Na_3PO_4	0.25	0.85	0.021	3.3	15.88	0.86	40.47
4	Na_2SO_4	0.25	52.25	0.58	138.9	95.5	36.61	28.27
5	$C_6H_5O_7Na_3$	0.25	14.5	16.0	118.9	55.5	16.6	90.62
6	$C_4H_{47}Na_2O_4$	0.17	1.01	0.018	1.94	15.15	0.57	56.11
7	K_2CO_3	0.25	4.24	0.142	8.12	51.45	0.3	29.86
8	$(NH_4)H_2PO_4$	0.25	0.21	0.58	0.63	4.99	0.031	0.36
9	$NaClO_3$	0.54	0.13	0.016	11.8	6.54	10.65	8.12
10	$KHCO_3$	0.33	43.5	0.3	129.0	93.54	15.0	145
11	K_2HPO_4-KH_2PO_4	0.25	54.7	0.05	114.8	94.91	11.23	1094
12	CH_3COOK	0.43	25.1	0.48	133.5	91.5	23.45	52.3
13	$(NH_4)C_2O_4$	0.43	0.008	0.1	0.48	0.34	0.06	0.08
14	Na2HPO4-NaH2PO4	0.17	0.36	0.17	24.85	5.97	6.28	2.11
15	NaSCN	0.43	0.09	0.05	1.6	3.71	1.06	1.8
16	NH_4NH_2COO	0.25	0.62	0.23	1.56	7.34	6.04	2.7

Table 3. Effect of pH on partitioning behavior of recombinant PheDH in 8.0% (w/w) PVP and 17.0% (w/w) Na_2SO_4 ATPS

Assay	pH	SA	R (%)	Y (%)	PF
1	7.0	560.20	85.56	81.92	10.86
2	7.2	925.15	97.08	83.08	13.29
3	7.4	945.08	112.55	86.15	15.44
4	7.6	1187.54	125.18	89.11	16.73
5	7.8	1204.53	135.37	92.03	18.56
6	8.0	1231.42	138.9	95.5	31.42

Table 4. Purification of recombinant *B. badius* PheDH by chromatographic purification method

Purification steps	Activity (U/l)	Total protein (mg/l)	SA (U/mg)	Y (%)	PF
Crude extract	20360	1490	13.66	100	1
Heat treatment	20360	1050	22.17	100	1.62
Ammonium sulfate (30-60%)	17700	454	38.98	86.93	2.85
DEAE-Toyopearl	14927	61.68	242	73.31	17.71
DEAE-cellulose	10550	36.36	290.1	51.82	21.23
Sephadex G-200	5115	9.24	553.57	25.12	40.52

partitioning and purification of many diverse bio-molecules that are produced and studied in modern biology [12,13]. Generally, the downstream costs are higher for enzymes used for therapy and diagnostics than for industrial enzymes. Hence in order to reduce these costs, it is important to improve the downstream processing [14]. Taking this hypothesis into account in our experiments, for success in the commercial production of recombinant PheDH that is recognized as an industrially important enzyme, we decided to compare the ATPS and chromatographic procedures for

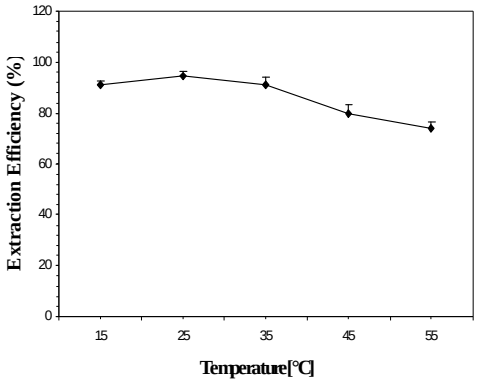

Figure 1. Temperature dependence of the extraction efficiency of recombinant PheDH in 8.0% (w/w) PVP and 17.0% (w/w) Na_2SO_4 (pH 8.0) ATPS.

large downstream processing in an industrial scale. The use of polymer-salt systems in bio-processing researches has been well documented [15,16]. In this study, polymer-salt ATPS were selected to design the purification process. In our pervious paper, we have reported the purification of recombinant *Bacillus badius* PheDH in a two-phase system composed of 8.5% (w/w) PEG-6000, 17.5% (w/w) $(NH_4)_2SO_4$ and 13% (w/w) NaCl [7]. As part of a continuing project, we here decided to obtain a better extraction condition from the other polymer/salt systems. For this purpose, we selected the PVP/Na_2SO_4 ATPS. As observed in Table 2, parameters in terms of recovery and yield (R > 100% and Y% > 90%). Two different explanations could be considered for this result. The first reason was related to the higher salting-out power of Na_2SO_4 that led to effective partition of recombinant target enzyme to the top phase. Generally, the stabilizing effect of salts depends on the nature of anion and cation. The anions with a higher valence such as phosphate are better salting-out agents than anions with a lower valence because the higher valence hydrates more water and thus decreases the amount of water available to hydrate polymer [17]. The second possible reason was that compared with other salts, Na_2SO_4 resulted in an appropriate medium pH for the target enzyme. Therefore, Na_2SO_4 was chosen as the salt phase for further experiment.

Table 3 lists the partition parameters of PheDH in six kinds of ATPS composed at different pH values. When pH increased up from 7.0 to 8.0, the partition parameters of PheDH raised. The reason could be considered for this effect was that with increase of pH, negative electrical charges on the oxygen atom of PVP molecules would decrease and so the complex formation tendency of PVP units with anion species reflected in increase of partition coefficient as per on the Flory-Huggins theory [8]. Moreover, above isoelectric point (the isoelectric point of *Bacillus badius* PheDH is 5.3), PheDH was negatively charged and PVP behaved as a positively charged molecule and thereby polyanions of the target enzyme were attracted by the PVP–rich phase. As result, PheDH gave partitioned to the top phase. As shown in Figure 1, as the temperature increased from 15 to 25 °C, the extraction efficiency raised. This behavior could be explained on the basis of temperature effect on the PVP hydrophobicity. Generally, when the temperature is raised, water molecules are driven from the PVP phase to the salt phase, so the PVP phase becomes more concentrated and the salt density in the bottom phase decreases. This caused hydrophobicity difference would increase the strength hydrophobic interactions between the interested enzyme and the PVP molecules and lead to effective partitioning. This is also known as the salting-out phenomenon which resulted in higher extraction efficiency values as the temperature increases [8, 18]. There were a good agreement between this partition feature of recombinant PheDH and the reported results in other literatures [17, 19]. However, with increasing

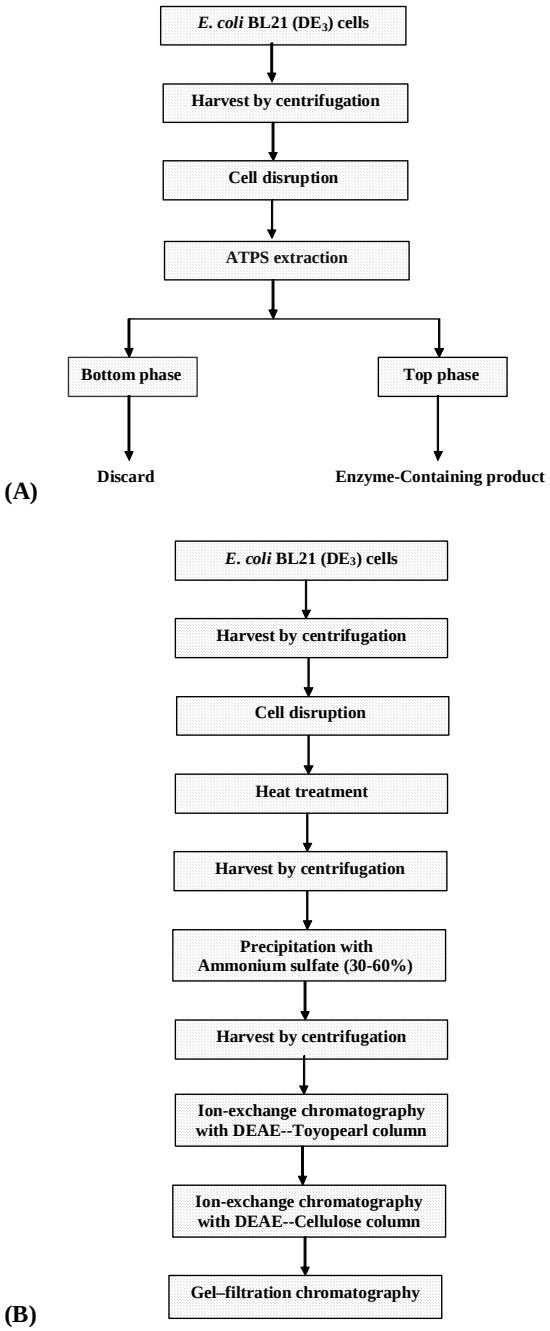

Figure 2. Flow chart of recombinant PheDH purification in PVP/Na_2SO_4 ATPS (A) and chromatography (B) protocols.

temperature from 25 to 55 °C, the partitioning of PheDH towards the PVP-rich phase decreased. In this respect, it may be concluded that the salting out effects in the bottom phase become less dominant with elevating temperature to 55 °C [17].

As can be found from Figure 2, a number of different unit operations were needed for the purification of target enzyme in chromatography. But, implementation of ATPS after centrifugation resulted in decrease of the number of unit operations from 10 to 5. Furthermore, the shorter process time (4 h versus 48 h) and the lower total cost (4 € versus 20 €) were additionally features that confirmed the suitability of proposed technique. In the case of chromatography, the operating cost was estimated by considering that the resins can be re-used up to 100 times. However, for the ATPS method, the potential savings for the recycling of PVP and phosphate were not considered. The purity of target enzyme in ATPS process was also comparable to that obtained from the chromatographic purification. Figure 3 shows the SDS-PAGE analysis of recombinant PheDH obtained by ATPS and chromatography. The purified enzyme from ATPS and chromatography methods appeared as a single band on silver stained SDS-PAGE, corresponding to a molecular mass of 41 kDa. This result was in agreement with the observations of Asano et al. [8]. Also, the enzyme purified by ATPS could be stored at -20 °C for nine months without losing its activity where the obtained PheDH in chromatographic method was stable three months. Therefore, our reported ATPS was cost-effective, time-saving and important from the commercial viewpoint. Similar results have been reported in the case of using PVP-salt two-phase process for downstream processing of other enzymes such as lipase [20,21], amyloglucosidase [22] and Ipomoea peroxidase [23].

In this communication, we compared the ATPS and chromatographic procedures for large-scale downstream processing of recombinant PheDH. Our optimal system was an ATPS containing 8.0% (w/w) PVP, 17.0% (w/w) Na_2SO_4, pH of 8.0, V_R=0.25 of 25 °C. The best results in terms of *Y* (95.5%) and *SA* (1231.42 U/mg) which are key criteria for industrially compatible procedures were obtained by ATPS. Moreover, ATPS process was performed in shorter time and lower total cost that confirmed the superiority of this technique.

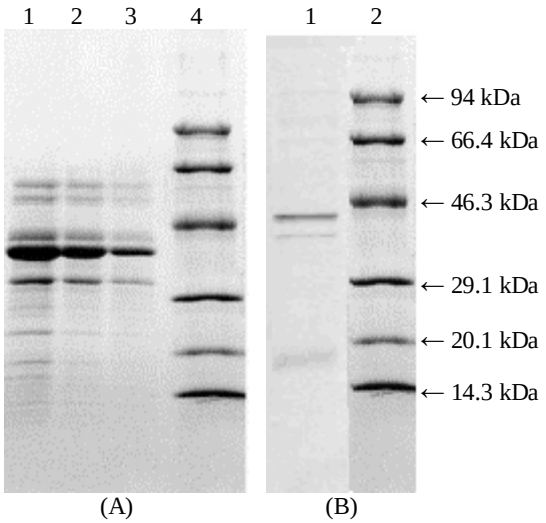

Figure 3. SDS-PAGE electrophoresis of recombinant PheDH. A) Purification steps of recombinant PheDH in chromatography protocol. Lane 1: DEAE-Toyoperal column; lane 2: DEAE-cellulose column; lane 3: sephadex G-200 column; lane 4: molecular standard markers. B) ATPS containing 8.0% (w/w) PVP and 17.0% (w/w) Na_2SO_4. Lane 1: Top phase of system; lane 2: molecular standard markers.

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