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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