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Partitioning and purification of menadione induced NAD(P)H oxidase from *Phanerochaete chrysosporium* in aqueous two-phase systems

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ABSTRACT

The partitioning behavior of menadione-induced NAD(P)H oxidase from the white rot fungus *Phanerochaete chrysosporium* has been studied in various poly(ethylene glycol) (PEG)-salt aqueous two-phase systems (ATPSs). The effects of the molecular weight of the PEG, system composition, and pH of the system on NAD(P)H oxidase partitioning were investigated at 25 °C. The best partition parameters were observed in the PEG 2000 18%–K_2HPO_4 13%–NaCl 0.5% (w/w, pH 7.0) system; the partition coefficient of total NAD(P)H oxidase activity (K_e) of the system was 0.232. While the recovery in the bottom phase (R_b) was 87.644%, this value was 20.350% in the top phase of the optimized system. The purification fold was found as 8.331 in the bottom phase. NAD(P)H oxidase purified from *P. chrysosporium* by the PEG–K_2HPO_4 two-phase system was determined to be a homodimer in its native state with a molecular weight of 116 kDa.

1. Introduction

NAD(P)H oxidases are membrane-bound enzyme complexes that catalyze the production of superoxide, a reactive free radical, through the transfer of one electron from NADH or NADPH to oxygen as the electron acceptor, simultaneously forming H_2O_2 or H_2O. It is well-known that these enzymes are the enzymatic sources of reactive oxygen species (ROS) and are important in vascular disease and hypertension together with xanthine oxidase (XO) and uncoupled nitric oxide synthase (NOS). In addition, as previously stated, NAD(P)H oxidases have a high potential for industrial applications due to their excellent cofactor regeneration properties, especially in processes catalyzed by oxidoreductases that require high-cost nicotinamide cofactors (NAD(P)H, $NAD(P)^+$) to complete the reactions [1–4].

The enormous biotechnological potentials of NAD(P)H oxidases have encouraged biotechnologists to approach different downstream strategies to fulfill the industry-imposed stability and productivity requirements for enzyme catalysts. During the last few years, there has been renewed interest in separation methods for biological molecules as alternatives to precipitation, solid phase extraction, and column chromatographies. These techniques are not only expensive but also result in lower yields and technical difficulties such as matrix fouling and viscous slurries. Aqueous two-phase systems (ATPS) formed by mixing a polymer (usually polyethylene glycol, PEG) and a salt (e.g., phosphate, sulfate, or citrate) or two polymers are generally mild systems for enzyme purification. In these systems, denaturation or loss of biological activity are not usually observed, most likely due to the high water content, the stabilizing effect of the polymers, and the low interfacial tension, which protect the proteins [5]. In addition to their mild conditions, these systems attract attention due the advantages including low process time and energy consumption. The success of ATPS in different processes with potential commercial application has been demonstrated by the existence of numerous reports dealing with the recovery of a large number of biological products. Pyruvate kinase, catalase, glucoamylase, c-phycocyanin, glyceraldehyde 3-phosphate dehydrogenase, alkaline xylanase, and penicillin acylase are some of the biomolecules that have been isolated from various sources using ATPS with more than 70% product recovery [6–12].

This study aimed to induce NAD(P)H oxidase activity in *Phanerochaete chrysosporium* with the treatment of the exogenous factor menadione and purify the enzyme using PEG–K_2HPO_4 systems from *P. chrysosporium* crude extract. To the best of our knowledge, this study is the first to test the effectiveness of ATPS for the purification of NAD(P)H oxidase.

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2. Materials and methods

2.1. Material

Polyethylene glycol (PEG) with average molecular masses of 1000, 2000, 3350, 6000, and 8000 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. All other used chemical reagents were of analytical grade. Distilled and deionized water was used.

2.2. Methods

2.2.1. Media and growth conditions of P. chrysosporium

Cultures of *P. chrysosporium* (German Collection of Microorganisms and Cell Cultures, DSMZ-1547) were maintained in potato dextrose agar (PDA) medium (pH 5.6), as described by Beever and Bollard [13]. Sterilization of the medium was carried out by autoclaving at 121 °C for 20 min. Inoculation was performed at 28 °C for seven days in Petri dishes.

P. chrysosporium was cultured in modified Tien and Kirk liquid medium [14] containing (g/L) KH_2PO_4 (2.0), $CaCl_2$ (0.114), $MgSO_4$ (0.7), NH_4Cl (0.12), D-glucose (2.0), thiamin-HCl (1.10–3), Tween 80 (0.05), $FeSO_4{\cdot}7H_2O$ (70 μg), $ZnSO_4{\cdot}7H_2O$ (46 μg), $MnSO_4{\cdot}2H_2O$ (35 μg), and $CoCl_2{\cdot}6H_2O$ (7 μg). The liquid medium was sterilized by autoclaving at 121 °C for 20 min. Incubation was carried out at 28 °C for 10 days with agitation at 150 rpm in a 250-mL Erlenmeyer flask containing 90 mL liquid medium and 10 mL spore suspension (OD_{650}: 0.800).

2.2.2. NAD(P)H oxidase activity induction with menadione

As described earlier, NAD(P)H oxidase activity induction was carried out by the treatment of stationary-phase *P. chrysosporium* cells with 0.75 mM menadione for 6 h [15]. The menadione-treated cells were harvested and washed several times with 20 mM potassium phosphate buffer (pH 7.4) at 4 °C, and the crude extracts were then prepared.

2.2.3. Preparation of crude extract from P. chrysosporium

The harvested cells were resuspended in 20 mM potassium phosphate buffer (pH 7.4) in a volume equal to three-times of the wet weight of the cells. The optimized homogenization procedure was performed for 3 min at 9000 rpm with 30-s intervals. The cell debris in the homogenate was removed by centrifugation at 15,000 rpm and for 15 min at 4 °C. The crude extract was not frozen before use.

2.2.4. NAD(P)H oxidase activity assays

NAD(P)H oxidase activity was determined by spectrophotometry. The procedure was based on the disappearance of NADPH at 340 nm [16]. The decreases in A_{340} were recorded in two 5-min intervals. A millimolar extinction coefficient of 6.22 was used to calculate the NADPH disappearance.

2.2.5. Preparation of aqueous two-phase systems

Stock solutions of PEG (40%, w/w) and K_2HPO_4 (40%, w/w) were prepared in deionized water. The appropriate amounts of the stock solutions were mixed with distilled water in a graded 15-mL glass centrifuge tube to obtain the desired system composition with a total weight of 5 g. Enzyme extract was added to the system as the final component to avoid possible precipitation and denaturation risks. The tubes were mixed by vortexing for 60 s to allow redistribution of the components. Complete phase separation was achieved by low-speed batch centrifugation at 2500 rpm for 5 min. The systems were left standing at 25 °C for 1 h after phase separation to reach equilibrium. The equilibrium state was characterized by the absence of turbidity in both the top and bottom phases. After the phases were carefully separated, both phases were appropriately diluted for the determination of NAD(P)H oxidase activities and total protein amounts. To correct for polymer and salt interference in the total protein assays, a blank system was prepared for each set of conditions, and the samples were read against the blanks. All partition experiments were conducted in triplicate, and the averages were used in the calculations.

2.2.6. Total protein assay

Samples withdrawn from each phase were diluted 10-fold with a known amount of distilled water, and total protein contents were measured by Bradford protein assay [17]. BSA was used as a standard. An identically diluted solution of the corresponding phase from a system containing no NAD(P)H oxidase extract was used as a blank.

2.2.7. Native molecular weight determination of NAD(P)H oxidase from P. chrysosporium

To determine the native molecular weight of NAD(P)H oxidase from *P. chrysosporium*, a standard protein mixture instead of a sample was applied to the Sephacryl S-200 gel filtration column (bed volume, 36 mL). The column was eluted (0.2 mL/min) with 5 mM potassium phosphate buffer (pH 7.4). In the column, β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) were used as molecular weight markers with blue dextran (200 kDa) for determining the void volume. The molecular weight for NAD(P)H oxidase was estimated from the calibration curve.

2.2.8. Electrophoretic procedures

SDS–PAGE analyses of the crude extract, the bottom phase of the optimized ATPS, and the NAD(P)H oxidase active fractions of the Sephacryl S-200 gel filtration column were performed according to the method of Laemmli [18]. Protein solutions were mixed at 1:4 (v/v) ratios with the SDS–PAGE sample buffer (0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) and boiled for 5 min. The samples were loaded onto the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 150 V per gel using a Protean II Cell apparatus (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gel was stained overnight with Coomassie Brilliant Blue R-250. Protein patterns were visualized after washing the gel several times with 40% (v/v) methanol and 10% (v/v) acetic acid solution to remove Coomassie particles until a clear background was observed. A spectra multicolor broad range protein ladder supplied the SDS–PAGE molecular weight standards.

2.3. Calculation of the partition parameters

The partition coefficient is described as the ratio of the total enzyme activity or protein concentration in the top phase to that in the bottom phase.

$K_e = A_t/A_b$, and $K_p = C_t/C_b$, where A_t and A_b are the total enzyme activities, and C_t and C_b are the total protein concentrations of the top and bottom phases, respectively.

The specific activity of the enzyme (SA, expressed in U/mg protein), the purification fold (PF), and the recovery (R) in the top and bottom phases were also calculated according to the given equations to evaluation efficiency of the systems:

$$SA_t = A_t/C_t \quad \text{and} \quad SA_b = A_b/C_b$$

$$PF_t = (SA)_t/(SA)_i \quad \text{and} \quad PF_b = (SA)_b/(SA)_i$$

$$R_t = A_t/A_i \quad \text{and} \quad R_b = A_b/A_i$$