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Self-assembled enzyme–inorganic hybrid nanoflowers and their application to enzyme purification

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ABSTRACT

We report a novel method to synthesize organic–inorganic nanoflowers for crude soybean peroxidase (SBP) purification. A hierarchical flower-like spherical structure with hundreds of nanopetals was self-assembled by using crude SBP as the organic component and $Cu_3(PO_4)_2{\cdot}3H_2O$ as the inorganic component. The structure of the hybrid nanoflowers was confirmed by Fourier-transform infrared spectroscopy, X-ray diffraction, and energy-dispersive X-ray spectroscopy, and the enzymatic activity of SBP embedded in the hybrid nanoflowers was evaluated using guaiacol as substrate. Compared with free crude SBP in solution, SBP embedded in hybrid nanoflowers exhibited enhanced enzymatic activity (∼446%). The hybrid nanoflowers also exhibited excellent reusability and reproducibility during cycle analysis. These results demonstrate that synthesis of hybrid nanoflowers is an effective enzyme purification strategy.

1. Introduction

Given rapid developments in enzyme catalysis technology and nanoscience, enzyme-embedded nanomaterials have attracted increased attention because of their special properties, particularly their functional and structural availability; these properties offer new opportunities with which to improve the biological functions of enzymes and expand their applications in areas such as industrial biocatalysis, biosensors, and bioanalytical devices [1–6].

Nanomaterials with a larger surface-to-volume ratio than that of bulk materials have been proposed as substrates in enzyme immobilization [7,8]. Several types of nanomaterials, such as nanoporous silica, magnetic nanoparticles, electrospun nanofibers, carbon nanotubes, and polymer beads, have been developed [9–13]. However, while most of these enzyme-embedded nanomaterials exhibit enhanced stability in comparison with the free enzymes in solution, the activities of the former are often reduced after immobilization by the use of organic solvents, significant mass transfer of the substrate in the immobilized enzymes, and chemical reactions between proteins and the nanomaterials [14–16]. Activity enhancement seldom occurs after immobilization [17–19]. Therefore, a facile synthetic method for preparing controlled and well-defined hierarchical enzyme-embedded nanomaterials is highly desirable.

Zare et al. reported a method for preparing immobilized enzymes with greatly enhanced activities [20]. Hybrid nanoflowers with $Cu_3(PO_4)_2{\cdot}3H_2O$ and various enzymes were synthesized via a facile method, and the same principle has been used by many other researchers. Wang et al. [21] for example, prepared $CaHPO_4$–α-amylase hybrid nanoflowers for a nanobiocatalytic system, while Lin et al. [22] prepared horseradish peroxidase (HRP)-inorganic hybrid nanoflowers to detect hydrogen peroxide and phenol. Sun et al. [23] synthesized multi-enzyme co-embedded organic–inorganic hybrid nanoflowers for a colorimetric sensor. Considering their many advantages, enzyme–inorganic hybrid nanoflowers have not been fully investigated, especially during application with crude enzymes. Further development is thus necessary to explore the complete benefits of this type of hybrid nanoflowers.

Soybean peroxidase (SBP), which is obtained from soybean hull coats, belongs to class III of the plant peroxidase superfamily, similar to HRP. Given the abundance of its source, wide range of substrates, high thermostability, and stability within a relative wide range of pH values, SBP may be developed as a substitute for HRP [24–26]. SBP has recently generated research attention because of its high specificity, which may be of great use in a wide range of

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applications, such as biocatalysis [27], bioremediation and wastewater treatment [28], diagnostic tests and therapeutics [29], and biosensors [30]. However, the traditional process of purifying SBP limits its wider applicability. An effective means to decrease the price of an enzyme is reduction of its purification cost, which can be achieved by either reducing the number of purification steps and/or increasing recovery rates.

In this study, we extract crude SBP from soybean hull coats and synthesize SBP-inorganic hybrid nanoflowers by using crude SBP, aqueous $CuSO_4$ solution, and phosphate buffered saline (PBS). Compared with free crude SBP, SBP embedded in hybrid nanoflowers shows enhanced enzymatic activity.

2. Experimental

2.1. Materials

Dry soybean hull coats were purchased from the market of agricultural products. Copper sulfate pentahydrate, guaiacol, ammonium sulfate, acetone, sulfuric acid, H_2O_2, phosphoric acid (H_3PO_4, >85%), and Coomassie brilliant blue G-250, bovine serum albumin (BSA) and other chemicals were analytical reagent grade quality, and used without further purification. All aqueous solutions were prepared using pure water.

2.2. Measurements

IR spectra were taken on a Spectrum 10 infrared spectrophotometer (Perkin-Elmer, USA). The UV–Vis absorption spectra were recorded on a Perkin-Elmer LAMBDA35 (USA). Scanning electron microscopy (SEM) was performed on a JEOL JSM 6460 electron microscope with primary electron energy of 15 kV and transmission electron microscope (TEM) was performed on a JEM2100 with electron energy of 200 kV. The X-ray diffraction (XRD) was measured on a Riguku D/MAX2550 diffractometer with Cu Kα radiation (50 kV, 200 mA, λ = 0.154 nm) and a scanning step of 0.02°. Energy dispersive X-ray spectroscopy (EDS) was recorded with Oxford INCA.

2.3. Extraction and preliminary purification of SBP

2.3.1. Crude extract of SBP

Soybean hull powders (20 g) were mixed with 150 mL of PBS (0.02 M, pH 6.0). The mixed solutions were shaken at 36 °C for 24 h. After filtration, the mixture was centrifuged to obtain the original enzyme solution.

Solid ammonium sulfate (194 g/L) was added to the original enzyme solution to achieve approximately 30% saturation. After centrifugation, solid ammonium sulfate (362 g/L) was added to the supernatant to achieve 80% saturation of the original enzyme solution.

After ammonium sulfate precipitation, the enzyme solution was collected, and a 0.4-fold volume of acetone (at −20 °C) (original enzyme solution) was mixed into it. Acetone was mixed with the supernatant to form a final volume of 1.8-fold volumes of acetone after centrifugation. Precipitates in the mixture were dissolved by distilled water and freeze-dried after dialysis.

2.3.2. SBP activity and purity assays

The activity and RZ value of the original enzyme solution were assayed through the following methods.

Guaiacol was used as the substrate and PBS (10 mM, pH 7.4) was used as the solvent. Previous studies have reported that oxidation of guaiacol may be utilized to assay enzymatic activity. In the presence of SBP and H_2O_2, guaiacol can be oxidized into a water-soluble, red product (3,3-dimethoxy-4,4-biphenoquinone) with an absorption maximum at approximately 470 nm [31]. The test procedure was as follows. Guaiacol (0.028 mL) and 30% hydrogen peroxide (0.019 mL) were mixed with 50 mL of PBS to produce the reaction solution. SBP solution (1 mL) that had been diluted to a proper concentration was then added to 3 mL of the reaction solution. The solution was mixed rapidly, and its absorbance was determined at 470 nm by using a spectrophotometer; here, the solution without enzyme was used as a blank. The absorbance was recorded at intervals of 1 min for approximately 5 min. One unit of peroxidase activity (U) was defined as the amount of enzyme required to change the absorbance value within 1 min. SBP purity was expressed as an RZ value [32]. The RZ value is defined as the ratio of the absorbances obtained at wavelengths of 403 and 275 nm. SBP purity can thus be estimated from the UV absorption data.

2.4. Synthesis and characterization of enzyme–inorganic hybrid nanoflowers

2.4.1. Synthesis of hybrid nanoflowers

To synthesize the nanoflowers, 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (0.1 M, pH 7.4) containing SBPs of different concentrations, followed by incubation at 25 °C for 3 d. Blue precipitates were collected after centrifugation, washed thrice with pure water, and then dried under vacuum at room temperature.

2.4.2. Enzymatic activity test

SBP hybrid nanoflowers (2 mg) were transferred to 3 mL of the reaction solution described in the previous section. The solution was mixed rapidly, allowed to react for 5 min, and then immediately centrifuged to isolate the nanoflowers. The supernatant was subjected to UV–Vis absorption spectroscopy, and its absorbance was measured at 470 nm. For comparison, the activities of free SBP and Cu^{2+} in solution were also determined using the same procedures.

3. Results and discussion

3.1. Extraction and preliminary purification of SBP

The enzymatic activities, RZ values, and purification times at each enzyme synthesis step are described as follows. The activity of the original enzyme solution was 85 U/mg and its RZ value was 0.05. After ammonium sulfate precipitation, enzyme activity increased to 181 U/mg, the RZ value increased to 0.11, and the purification multiple was 2.13. Acetone was used to purify the enzyme, and an enzyme activity of 541 U/mg, RZ value of 0.42, and purification multiple of 6.34 were obtained. Extraction and preliminary purification yielded crude SBP with an enzymatic activity of 541 U/mg.

3.2. Synthesis and characterization of enzyme–inorganic hybrid nanoflowers

To synthesize the enzyme–inorganic hybrid nanoflowers, aqueous $CuSO_4$ solution was added to PBS solution containing crude SBP of different concentrations at pH 7.4 and 25 °C. After 3 d, blue porous nanoflowers were obtained. The formation mechanism of the embedded organic–inorganic hybrid nanoflowers is illustrated in Fig. 1A. The mechanism comprised three steps: (1) nucleation and formation of primary crystals, (2) complex formation of SBP molecules with Cu^{2+} through coordination of amide groups in the SBP (protein) backbone, and (3) complete formation of nanoflowers [20].

The general morphologies of the SBP-$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers were determined by scanning electron microscopy (SEM) and are shown in Fig. 1B; this figure illustrates that the hybrid