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The influence of synthesis conditions on enzymatic activity of enzyme-inorganic hybrid nanoflowers

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ABSTRACT

In this work, we synthesized hierarchical flower-like structures by using lipase and papain as organic components and $Cu_3(PO_4)_2{\cdot}3H_2O$ as the inorganic component. These hybrid-nanoflower structures were confirmed by Fourier transform infrared spectroscopy, X-ray diffraction, and energy-dispersive X-ray spectroscopy. By changing the synthesis conditions, including enzyme concentration, pH, and temperature for the nanoflowers, we can control the morphology and enzymatic activity of nanoflower. Enzyme concentration and synthesis temperature affect nanoflower size and petal density, whereas pH influences petal density only. Furthermore, we found the optimal conditions to improve the enzymatic activity of lipase-$Cu_3(PO_4)_2{\cdot}3H_2O$ and papain-$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers. The appropriate enzyme content, flower size, and flower density were the critical factors for high enzymatic activity.

1. Introduction

Enzymes are environmentally friendly and highly efficient catalysts important for the chemical industry [1]. Enzyme immobilization is advantageous for commercial application because of its convenience in handling, ease of separation of enzymes from the reaction mixture and reuse, low product cost, and possible increase in thermal and pH stabilities [2]. Furthermore, immobilization may improve some other enzymatic properties, such as activity or selectivity [3,4].

Lipase and papain are the most widely used enzymes [5–9]. Lipases are obtained from animals, plants, and natural or recombinant microorganisms; moreover, lipases have been found to hold a plethora of applications as significant biocatalysts in food and pharmaceutical industries and technologies [10,11]. Lipases can catalyze esterification, interesterification, and transesterification reactions in non-aqueous media (organic solvents and supercritical fluids), particularly for biofuel production [12–17]. Lipases undoubtedly play an important role and are widely used in synthetic organic chemistry because of their high activity, wide variety of sources, and broad range of substrates. Meanwhile, papain exists in papaya roots, stems, and fruits and belongs to the cysteine protease family [7]. This enzyme has been widely used in biocatalysis, biomedicine, leather production, cosmetic and textile industries [7–9], and scar therapeutics [18]. Several methods have been developed to immobilize lipase and papain on different supports [19,20]. However, mass-transfer limitation, stability, activity loss, and limited reusability were the main problems for such approach's further applicability. The use of immobilized enzymes offers advantages in easy separation and recycling. Hence, developing an efficient support to improve the enzymatic properties of immobilized enzymes is an important endeavor [21]. Shortly before, some scholars researched on hybrid complexes in depth to synthesized microcapsules, nanoparticles or nanocrystal by use of proteins [22,23–26].

Recently, Richard N. Zare et al. [27] have reported an encouraging breakthrough in enzyme immobilization with the synthesis of hybrid organic–inorganic nanoflowers showing significantly enhanced activities and stabilities. The method is simple and green and consumes low amounts of energy; hence, this approach has attracted substantial research attention. Some enzymes for aqueous-phase catalysis, including laccase [28], lipase [29], horseradish peroxidase [30–33], soybean peroxidase [34], lactoperoxidase [35], glucose oxidase [31], his-tagged proteins [36], urease [37], trypsin [38], chymotrypsin [39], papain [40], lactoglobulin [41], and apo-α-lactalbumin [42] are selected as organic components of hybrid nanoflowers. As demonstrated by Richard N. Zare et al., nanoflowers were accidentally discovered when $CuSO_4$ was

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added to PBS containing bovine serum albumin (BSA) at pH 7.4 and 25 °C. After showing the generalizability of this method for the preparation of nanoflowers, the group used α-lactalbumin, laccase, carbonic anhydrase, and lipase in place of BSA to form nanoflowers. The enzyme–inorganic nanoflowers usually exhibited much higher catalytic activities and stabilities than those of free enzymes and conventionally immobilized enzymes. However, only the lipase nanoflower showed almost the same activity with free lipase [27]. Scholars claim that metal-free enzymes, such as lipase-based nanoflowers, do not show higher activity than that of the free enzymes. Nevertheless, the factors affecting the enzymatic activity of the nanoflowers remain to be ascertained.

In the current study, we synthesized hierarchical flower-like lipase–inorganic and papain–inorganic hybrid materials by using lipase or papain, copper chloride aqueous solution, and PBS under certain conditions. By changing the incubation conditions, including enzyme concentration, incubation temperature, and pH of the nanoflowers, we studied each condition that might affect the nanoflower morphology. The enzymatic activities of lipase and papain embedded in hybrid nanoflowers were further evaluated using *p*-nitrophenyl palmitate [43,44] and Nα-benzoyl-L-arginine ethyl ester [40] as substrates. In addition, we studied the conditions affecting both nanoflower enzymatic activity and nanoflower morphology. Under optimal conditions, the lipase–inorganic and papain–inorganic hybrid materials showed higher enzymatic activity than that of free enzymes because of the former's suitable enzyme content and hierarchical structure.

2. Experimental

2.1. Materials

Type II Pocine Pancreas Lipase was purchased from Sigma-Aldrich-Fluka Chemical Co (St. Louis, MO, USA). Papain was purchased from Shanghai Yuanye Bio Technology Co., Ltd. Coomassie brilliant blue G-250 was purchased from Tianjin Kemiou Chemical Regent Co., Ltd. $CuCl_2$ was purchased from Shenyang Renagent Industry. P-Nitrophenyl palmitate (p-NPP) and Nα-benzoyl-L-arginine ethyl ester (BAEE) was purchase from Aladdin Reagent Co., Ltd. Triton X-100 (TX-100) and p-NP (*p*-Nitrophenol) was purchase from Tianjin Guangfu Fine Chemical Co., Ltd. D-Gum acacia powder was purchase from Sinopharm Chemical Reagent Co., Ltd. DTT was purchase from Beijing Solarbio Science and Technology Co., Ltd. NaCl, KCl, Na_2HPO_4, KH_2PO_4 and Na_2CO_3 was purchase from Tianjin Damao Chemical Reagent company. All of these chemicals were analytical reagent grade quality and used without further purification. All aqueous solutions were prepared using pure water.

2.2. Measurement

The UV–vis absorption spectrum was 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 9 kV and energy dispersive X-ray spectroscopy (EDS) was recorded with Oxford INCA. The FT-IR spectrum was taken on a Perkin-Elmer Spectrum two infrared spectrophotometer. 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°.

2.3. Synthesis method

Enzyme-inorganic hybrid nanoflower were synthesized as follow: 20 μL of aqueous $CuCl_2$ solution (120 mM) was added to 3 mL of PBS (0.01 M, pH 6.0, 7.4, 8.0, 9.0) containing different concentrations of enzyme, then the mixture was incubated at 25 °C for 3 days. Then the blue precipitates were settled, washed for three times by deionized water and collected after centrifugation (3500 rpm for 5 min). The blue precipitates were dried into the powder by using the technology of the vacuum freeze-drying. While the supernatant need to be measured by Coomassie Brilliant Blue G-250.

2.4. Determination of encapsulation yield and enzyme weight percentage

2.4.1. Protein standard curve

The protein concentration in the supernatant was measured by Bradford protein assay method using BSA as standard to get a protein standard curve [45]. BSA (10 mg) was added to 100 mL NaCl (0.15 mol/mL) to obtain 0.1 mg/mL protein standard solution. Then the BSA standard solution was diluted to be 10, 20, 30, 40, 50, 60 and 70 μg/mL with NaCl (0.15 mol/mL). Then 4 mL Commassie Brilliant Blue G-250 solution was added to 1.0 mL diluted protein solution. The absorptions of different concentrations protein solutions were examined by UV/Vis absorption spectrophotometer at 595 nm (The standard curve of BSA was given in Supplementary Fig. S1).

2.4.2. Encapsulation yield of enzyme–inorganic hybrid nanoflowers

The encapsulation yield of enzyme was defined as the ratio of the reduced enzyme amount to the total amount of enzyme added [46]. The Branford protein assay was employed to examine the protein concentration in the supernatant. Commassie Brilliant Blue G-250 (4 mL) was added to 1 mL supernatant. Then, the absorptions of supernatant solution were detected at 595 nm by UV/Vis absorption spectrophotometer. Through the protein standard curve, the enzyme concentration in the supernatant could be obtained. The calculation of the encapsulation yield was shown in Supplementary Formula S1.

2.4.3. Weight percentage of enzyme in hybrid nanoflowers

The weight percentage of enzyme was defined as the weight ratio of the enzyme, which is actually embedded in nanoflowers to the total nanoflowers weight. For enzyme content measured, the dried hybrid nanoflowers were calcined at 700 °C for 2 h by muffle furnace. After removing the organic enzyme in hybrid nanoflowers, the remaining part was inorganic metal salt $Cu_3(PO_4)_2$. The calculation of weight percentage of enzyme in hybrid nanoflower was given as the following equation:

$$W = \frac{G_N - G_0}{G_N} \times 100\% \quad (1)$$

where W is the weight percentage, G_N is the weight of nanoflowers, and G_0 is the weight of $Cu_3(PO_4)_2{\cdot}3H_2O$.

2.5. Determination of enzymatic activity

2.5.1. Enzymatic activity of lipase

The enzymatic activity of lipase-inorgainc hybrid materials and lipase were determined by using *p*-nitrophenyl palmitate (*p*-NPP) as substrate. Lipase could catalyze p-NPP hydrolyzed into *p*-nitrophenol(p-NP) which can be detected at 402 nm. (the standard curve of p-NP was shown in Supplementary Fig. S2). 1 vol of a 16.5 mM solution of p-NPP in 2-propanol mixed with 9 vol PBS (0.01 M, pH 7.4) containing 0.4% (w/v) Triton X-l00 and 0.1% (w/v) arabic gum as the substrate solution. After preheating for 5min(150 rpm, 37 °C), 4 mL of this substrate solution and 1 mL PBS (0.01 M, pH 7.4) containing lipase-inorgainc hybrid nanoflower (the amount of lipase embedded in nanoflowers was equivalent to 1 mg lipase) were mixed. Then the mixture was incubated for 5 min