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Short communication

Application of dual-enzyme nanoflower in the epoxidation of alkenes

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ABSTRACT

In this work, a novel organic-inorganic hybrid nanoflower was synthesized by the incorporation of glucose oxidase and lipase and used as the coimmobilized enzyme in the epoxidation of alkenes. Imaging and catalytic performance results suggest that the dual-enzyme nanoflower greatly reduces the diffusion and decomposition of H_2O_2 and facilitates the mass transfer of glucose oxidase/lipase. Furthermore, the controllable generation of H_2O_2 and peracid contributes to the good stability of the dual-enzyme nanoflower, which exhibits a higher catalytic performance than the free enzymes in a dual-enzymatic cascade epoxidation. The yield of epoxidation was up to 82% even after ten cycles, further indicating the good reusability of the dual-enzyme nanoflower and its great potential for application in other enzymatic oxidations.

1. Introduction

Over the past decade, multi-enzymatic cascade reactions have been become an important and popular topic in biochemical research [1–3]. Such reactions offer considerable advantages, including the regeneration of cofactors, shifting reaction equilibria, and overcoming inhibition effects as well as reducing the reaction time, energy, and material demands of a given process [4–6]. Generally, multi-enzymatic cascade systems must be robust and recyclable for practical application [7]. Coimmobilization is a key technique to increase the operational performance of enzymes and, more importantly, to enhance the catalytic activity of enzymes in multi-enzymatic reactions [8–12]. In addition, the stability and reusability of coimmobilized multi-enzymes have attracted interest from an environmental and economic point of view. In recent decades, the application of glucose oxidase to generate hydrogen peroxide *in situ* and thereby decrease problems due to inactivation of enzyme by high hydrogen peroxide concentration has been studied in many multi-enzymatic reactions [13,14]. For example, Sheldon's group has been reported that chloroperoxidase-glucose oxidase system could be applied in the sulfoxidation of thioanisole [15]. This chloroperoxidase-glucose oxidase system has also been used in enantioselective epoxidation of alkenes [16]. Okrasa et al. reported that chiral sulfoxides were synthesized from sulfides using a plant peroxidase from *Coprinus cinereus*-glucose oxidase bienzymatic system [17]. Recently, a novel dual-enzyme (glucose oxidase and lipase) cascade system for the *in situ* generation of peracid has been applied to the oxidation of various amines and alcohols [18,19]. This system combines the catalytic specificity of glucose oxidase and catalytic promiscuity of lipase to construct a dual-enzyme mediated oxidative process. It also has great potential in some other oxidations, such as Baeyer-Villiger oxidation, Dakin oxidation, sulfonation, and epoxidation [20–25]. Considering the low stability and high cost of free enzymes in biocatalysis, it is highly pertinent to establish a facile and efficient coimmobilization method for this useful dual-enzyme system.

In recent years, a simple and versatile immobilization technology to prepare hybrid organic-inorganic nanoflowers composed of various proteins and cupric phosphate has been reported [26]. When an enzyme is encapsulated as the bioactive molecule in the hybrid nanoflower *via* biomimetic mineralization within the protective exteriors, the enzyme activity and stability is enhanced. Similarly, Ge et al. prepared novel protein-inorganic hybrid nanoflowers for the first time and found that the specific activity of carbonic anhydrase or laccase in the nanoflower was increased dramatically [27]. Wu and his coworkers fabricated lipase-incorporated nanoflower and successfully applied the nanoflower to the enantioselective transesterification of (*R,S*)-2-pentanol [28]. Jiang et al. reported a facile, economic and green method based on biomimetic mineralization to acquire lipase-inorganic hybrid nanoflower, which was then employed as an economically viable biocatalyst for biodiesel production [29]. Sun et al. reported that the use of the multi-enzyme (glucose oxidase and horseradish peroxidase) co-

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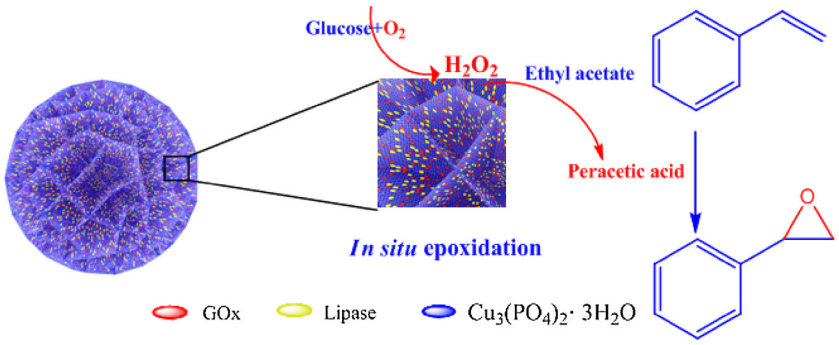

Scheme 1. The process of epoxidation of alkenes mediated by dual-enzyme nanoflower.

embedded hybrid nanoflower as a colorimetric sensor greatly enhanced the sensitivity of glucose detection [30]. These findings encouraged us to continue trying to coimmobilize glucose oxidase (GOx) and lipase *via* this facile technique and subsequently apply the dual-enzyme nanoflower in organic oxidations.

In this work, we developed a simple method for the preparation of a GOx/lipase nanoflower for the efficient epoxidation of alkenes (Scheme 1). To the best of our knowledge, this is the first time that the incorporation of GOx and lipase in the nanoflower for the epoxidation of alkenes has been reported. In this nanoflower, cupric phosphate was used as the inorganic component, while glucose oxidase from *A. niger* and lipase B from *Candida antarctica* (CalB) were adapted as the organic components to construct the dual-enzyme nanoflower. As shown in Scheme 1, when glucose was added into the system, it reacted with O_2 and GOx on the dual-enzyme nanoflower to produce H_2O_2, which was immediately adopted by lipase on the same nanoflower. This was followed by the perhydrolysis of carboxylic ester and resulted in the *in situ* generation of peracid, which was used in the epoxidation of alkenes. Coimmobilization of this dual-enzyme system can reduce the diffusion resistance of H_2O_2 to lipase and dramatically accelerate the generation of peracid due to the proximity of the two enzymes that creates a microenvironment for H_2O_2-rich lipase. More importantly, the controllable generation of H_2O_2 and peracid by the "feed-on-demand" method in the dual-enzyme nanoflower can avoid enzyme inactivation and improve the stability of the enzyme. The dual-enzyme nanoflower was characterized by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) and then used in the epoxidation of alkenes, for which reaction conditions were optimized.

2. Materials and methods

2.1. Materials

Glucose oxidase from *A. Niger* (GOx) and *Candida antarctica* lipase B (CalB) were purchased from Sigma (Beijing, China). These enzymes were used after lyophilization for the immobilization without further purification. Glucose and alkenes used in this study were purchased from J&K Scientific (Beijing, China). All the other chemical reagents were purchased from Shanghai Chemical Reagent Company (Shanghai, China). All the commercially available reagents and solvents were used without further purification. NMR spectra were recorded on an Inova 500 (500 MHz) spectrometer.

2.2. Preparation of the dual-enzyme nanoflower

$CuSO_4$ solution (120 mM) was added to the phosphate buffer (pH 7.4, 100 mL) containing GOx (glucose oxidase from *A. niger*, 100 mg) and lipase (lipase B from *Candida antarctica*, 100 mg). The system was incubated at 25 °C for 72 h. Then a precipitate with porous, flower-like structures appeared. The prepared dual-enzyme nanoflowers were separated by centrifugation and dried overnight under vacuum. The protein content of the residual solution was measured by the Lowry method with bovine serium albumin (BSA) as a standard for protein concentration. It's noteworthy that no protein could be detected in the residual solution after the co-immobilization process which was in accordance with the previous report [28]. Therefore, the enzyme loading efficiency (%) was about 100%. The SEMs of the dual-enzyme nanoflower were obtained from a JSM-6700 F electron microscope (JEOL, Japan). The FTIR spectrums were recorded by Nicolet 5700 FTIR spectrometer (Thermo, USA).

2.3. General Procedure for the epoxidation of alkenes mediated by the dual-enzyme nanoflower

The reaction was performed in a round bottom flask contained alkene (1 mmol), glucose (1.2 mmol), dual-enzyme nanoflower (protein content: 18 mg, lipase/GOx = 1/1) and a mixed solution (5 mL, PB/EA = 1/9, PB: phosphate buffer (pH 7.0), EA: ethyl acetate). The reaction mixture was stirred at room temperature for 30 h when the oxygen (1 mL/min) was bubbled into the reaction mixture. Then, the mixture was filtered and extracted with dichloromethane. The combined organic phases were dried over Na_2SO_4 (anhydrous) and concentrated under vacuum, and the resulting residue was purified by flash column chromatography on silica gel with EA/hexane (1/4). The experiments were performed triplicate, and all data were obtained based on the average values. All the isolated products were well characterized by their ^{1}H-NMR spectral analysis.

3. Results and discussion

The dual-enzyme nanoflower was prepared by adding $CuSO_4$ solution to phosphate buffer (pH 7.4) containing GOx and lipase. The system was incubated at 25 °C for three days until a precipitate with porous, flower-like structures appeared. Fig. 1 shows the general morphologies of the dual-enzyme nanoflower imaged by SEM. Fig. 1a reveals that the samples consist of large quantities of flower-like particles with diameters in the range of 20–30 μm. As shown in Fig. 1b, the nanoflower has hierarchical structure with high surface-to-volume ratio.

$Cu_3(PO_4)_2{\cdot}3H_2O$, lipase, GOx, and GOx/lipase nanoflower were characterized by FTIR spectra in the region of 400-4000 cm^{-1} to certify the enzyme's presence in the nanoflower (Fig. 2a–d). The vibration bands of PO_4^{3-} can be seen at 1047 cm^{-1}, 989 cm^{-1}, 628 cm^{-1} and 559 cm^{-1} (Fig. 2a). The amide I and II bands of lipase or GOx can be observed at 1646 cm^{-1} and 1533 cm^{-1} (Fig. 2b and c), respectively. The spectra of GOx/lipase nanoflower (Fig. 2d) indicates that the enzymes were incorporated in the nanoflower successfully.

Subsequently, the loading efficiency of the dual-enzyme nanoflower was investigated using 100 mg lipase and 100 mg GOx. After the immobilization process was performed, the protein content in the pooled suspension and washing solution was measured. Since no protein was detected in the above-mentioned solution, the loading efficiency (%)