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Carbon nanotube-lipase hybrid nanoflowers with enhanced enzyme activity and enantioselectivity

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ABSTRACT

Various nanoflowers are synthesized as supports for different methods of enzyme immobilization; however, the activities of these immobilized enzymes are limited because of their confinement in the nanoflowers. In order to increase the performance of nanoflowers, in this study, different protein-phosphate hybrid nanostructures were successfully synthesized and further enhanced by carbon nanotubes (CNTs) under the same conditions. Only $Cu_3(PO_4)_2$ complex nanostructures exhibited flower-like structures and showed excellent results after enhancement with CNTs in this framework. An esterification reaction between lauric acid and 1-dodecanol was used to test enzyme activity during immobilization, revealing that the $Cu_3(PO_4)_2$/CNT/protein complex exhibited 68-fold higher activity relative to free lipase and 51-fold higher than that of $Cu_3(PO_4)_2$/*Burkholderia cepacia* lipase hybrid nanoflowers in the absence of CNTs. All three hybrid nanostructures showed good performance and exhibited excellent reusability in resolution reactions between 1-phenylethanol and vinyl acetate. Additionally, the substrate enantiomeric excess (ee_s) reached 98% in only 10 min, and the corresponding $Cu_3(PO_4)_2$/CNT/protein complex could be recycled eight times without obvious loss of activity. This approach involving nanoflowers enhanced with CNTs will be highly beneficial for decreasing mass-transfer resistance and providing enhanced enzyme loading along with promising potential for industrial application.

1. Introduction

Immobilization is a good way to be used to protect enzyme conformation and enhance their performance during biocatalytic reactions. Currently, immobilization methods and materials have considerable effects on the performance of the immobilized enzymes. Adsorption, entrapment, covalent bonding, and other methods are usually used for immobilization, with different methods exerting different effects on enzyme conformation and activity (Singh et al., 2013). The immobilization supports can decrease enzyme distortion under extreme reaction conditions and provide a suitable and limited microenvironment. This system also protects enzymes from sudden changes of temperature, reaction media, or mechanical force, which are less susceptible to affect enzyme conformation and activity in the presence of immobilized carriers. Lipase, a lipolytic enzyme which functions at oil-water interface, has advantages in reactions using organic solvents as reaction media. Recently, various materials were used as supports for lipases immobilization, such as silk fibers (Chatterjee et al., 2009), microcapsule (Su et al., 2016), magnetic protein (Gao et al., 2017a,b), mesoporous silica nanospheres (Gao et al., 2017a,b), nanoflowers (Ge et al., 2012a,b), and so on. These materials can be used to enhance and stabilize the activity of the immobilized lipase via the following methods: the biocompatible biomaterials, such as silk fibers, chitosan, agarose, sodium alginate, are used as support materials due to their high affinity to lipase, biodegradability and renewability; the heterofunctional carriers with adsorption coupling sites and covalent binding sites, can connect with lipase molecules via a quick adsorption and then covalent bonding, and the immobilized lipases can be activated by the hydrophobic adsorption groups and be stabilized by the covalent bonding groups on the surface of the carriers (Guajardo et al., 2015); some special proteins like magnetic protein or self-assembling protein can present advantages in self producing by the cells and decreasing in output cost of industry application, some proteins used as supports can help enzymes to keep higher reactive activity by forming polymeric bodies (Heyman et al., 2007), meanwhile, some inactive proteins like bovine serum albumin were added together with lipases in cross-linking immobilization to decrease the enzyme distortion caused by cross-linking reagents (Shah et al., 2006); biosurfactants, protective reagents and hydrophobic interface of supports can be used to activate and protect lipases in immobilization (Chatterjee et al., 2009; Su et al.,

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2014). The structures, properties, and enzyme-loading potential of the immobilization materials strongly influence enzyme performance through the existence of mass-transfer resistance between substrate and enzyme, interactions with enzyme molecules, and between carriers and enzymes (Li et al., 2017a). Some materials have special properties, such as porous materials (Gao et al., 2017a,b), nanoflowers (Ge et al., 2012a,b), nanotrees (Kharissova and Kharisov, 2010), nanoleaves (Kharissova and Kharisov, 2010), nanopalms (Zhu et al., 2006), nanoneedles (Zhang et al., 2008a,b), and nanomushrooms (Ohtani et al., 2009), that can improve the surface area of the carrier and enhance enzyme loading, thereby improving the stability and enzyme activity of the immobilized enzymes (Li et al., 2017a).

Nanoflowers have been widely used in biomedicine (Zhou et al., 2016), biocatalysis (Altinkaynak et al., 2016; Cui and Jia, 2017), photocatalysis (Wu and Qi, 2007), electrocatalysis (Zhu et al., 2011), oxygen reduction (Yang et al., 2010a,b), energy storage (Ge et al., 2012a,b; Zhang et al., 2008a,b), sensors (Sun et al., 2014), nanomaterial creation (Kharissova and Kharisov, 2010), and other areas (Kharissova and Kharisov, 2010). The petal shapes of nanoflowers are flaky (Zhang et al., 2009), filamentous (Yang et al., 2010a) and acicular (Cao et al., 2010), with these shapes beneficial for increasing carrier surface area. Many inorganic (including carbon or metal/metal oxide/metal alloy nanoflowers), organic, and organic-inorganic hybrid materials can be used to synthesize nanoflowers, which can be produced by many different methods, including electrochemical deposition (Li et al., 2011), electroless plating (Kawasaki et al., 2010), hydro-thermal method (Lai et al., 2011), polyol method (Yin et al., 2012), thermal evaporation (Feng et al., 2010), and sol-gel methods (Senthil et al., 2011). All of these are relatively complex and not highly effective for enzyme immobilization. Recently, a new method was created (Gao et al., 2017a,b) using core-shell magnetic organosilica nanoflowers as supports for *Candida antarctica* lipase B covalent immobilization. This method can be operated under low temperature conditions; however, the carrier still requires several preparatory steps, and covalent binding during immobilization will cause irreversible distortion of the enzyme (Balcão and Vila, 2015; Cantone et al., 2013; Moritz et al., 2016). Therefore, a cheap, convenient, and effective method is required.

Recently, numerous studies have reported growing interest in the use of organic-inorganic hybrid nanoflowers (Ge et al., 2012a,b; Hua et al., 2016; Ke et al., 2016; Lee et al., 2017; Li et al., 2017a, 2016; Wu et al., 2017; Zhang et al., 2016). These nanoflowers can be used to produce various immobilized enzymes through nucleation, growth, and completion under mild conditions. Cui and Jia (2017) described the different hybrid nanoflowers based on the metal ions and biomolecules involved, with copper(II), calcium(II), manganese(II), zinc(II), cobalt (II), and iron(II) ions can be used to form complexes with biomolecules. In our previous work, a $Ca_3(PO)_4$-protein hybrid nanoflower was used for resolution of 1-phenylethanol, with the enzyme activity of the immobilized lipase 3.1-fold higher than that of free lipase (Ke et al., 2016). The $Cu_3(PO_4)_2$-lipase hybrid nanoflower exhibited 460% and 200% higher activity than native lipase and a conventional inorganic-lipase hybrid nanoflower, respectively, through interfacial activation of the lipase (Cui et al., 2016). Additionally, a $Zn_3(PO_4)_2$-lipase hybrid nanoflower exhibited enhanced enzyme activity and operational stability as compared with free lipase (Zhang et al., 2016). However, the enzyme activities of inorganic-lipase hybrid nanoflowers are limited by the interactions between supports and proteins. During crystal nucleation and growth, the proteins are either partially or completely embedded in the crystals (Cui and Jia, 2017). Furthermore, nanoflowers, especially the petals, are considered too soft for many applications (Liang et al., 2015). Meanwhile, Carbon nanotubes (CNTs) have been used to increase mechanical strength for the development of stable nanohybrids. Su et al. (2016) reported a CNT-modified polyethyleneimine (PEI) microcapsule, where the addition of CNTs to PEI decreased the positive charge of the microcapsules, resulting in enhanced lipase activity. Oxidized multi-walled CNTs were subsequently used to immobilize *Burkholderia cepacia* lipase (BCL) through simple adsorption, with the immobilized BCL substantially improving resolution of 1-phenylethanol as compared with that observed with free lipase (Ke et al., 2014). CNTs can also be modified with magnetic particles and dendrimers, and proteins can be connected to the polymers by covalent bonds (Fan et al., 2016, 2017a,b). These studies suggest that CNTs can be applied to enhance the ability of supports and provide binding sites for proteins. Li et al. (2017a) synthesized a self-assembly hybrid nanocomposite with copper phosphate, laccase, graphite oxide, and CNTs; however, according to their design, laccases were encapsulated in copper phosphate, with CNTs acting as the interlayer spacer between graphite oxide sheets increasing the accessibility of the encapsulated laccase. In summary, hybrid nanoflowers are limited biocatalysts in chiral resolution reactions. On the other hand, the immobilized lipase, CNTs-lipase, has be proved to be a good catalyst for chiral resolution reactions. The combination of these two materials and lipase may produce a novel biocatalyst. In the present study, we are planning to combine the high loading capability of nanoflowers with the optimal performance of oxidized CNTs. Additionally, we also trying to combined lipases adsorbed onto CNTs and those encapsulated in precipitated phosphate as hybrids using immobilization methods.

2. Materials and methods

2.1. Materials

BCL and (*R, S*)-1-phenylethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). *n*-Hexane and vinyl acetate were purchased from Shenshi Chemical Industry (Shanghai, China). $CaCl_2$, $FeSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $K_2HPO_4{\cdot}3H_2O$, and KH_2PO_4 were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). High-performance liquid chromatography (HPLC)-grade organic solvents were purchased from TEDIA (Fairfield, OH, USA). CNTs were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China).

2.2. Fabrication of $Ca_3(PO_4)_2$/protein and $Fe_3(PO_4)_2$/protein hybrid nanostructures and $Cu_3(PO_4)_2$/protein hybrid nanoflower

Phosphate buffer (PB; 0.1 mM; pH 7.4) was prepared using $K_2HPO_4{\cdot}3H_2O$, KH_2PO_4, and distilled water, followed by the addition of 3 mL $CaCl_2$ (200 mM) [or 1 mL $FeSO_4$ (120 mM) or 1 mL $CuSO_4$ (120 mM)] into 150 mL PB containing 0.1 mg/mL protein and incubation at 25 °C for 24 h. The solids were separated by centrifugation at 8000 rpm for 10 min and washed with the PB three times. Supernatants were collected to obtain residual protein content using the Bradford method, with bovine serum albumin used as the protein standard (Bradford, 1976). The precipitants were dried in a vacuum dryer and stored at 4 °C for later use. The synthesis process is described in Fig. 1a.

2.3. Fabrication of the CNT/protein hybrid nanostructure

The pretreatment method of crude MWNTs was introduced by (Pan et al., 2006). Modified CNTs (100 mg) were dispersed in 3 mL PB (pH 7.4) to a final protein concentration of 1 mg/mL. The suspension was maintained in a 37 °C bath for 4 h with shaking at 200 rpm. The precipitant was collected by centrifugation, and protein content in the supernatant was determined. After freeze-drying in a vacuum dryer, the immobilized lipase-CNT/protein complex was obtained. The synthesis process is described in Fig. 1b.

2.4. Fabrication of $Ca_3(PO_4)_2$/CNT/protein and $Fe_3(PO_4)_2$/CNT/protein hybrid nanostructures and $Cu_3(PO_4)_2$/CNT/protein hybrid nanoflower

The first step for immobilization of all phosphate/CNT/protein complexes were the same as those described for fabrication of the CNT/protein hybrid; however, before freeze-drying, in the second step for