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Improved performance of immobilized laccase on amine-functioned magnetic Fe_3O_4 nanoparticles modified with polyethylenimine

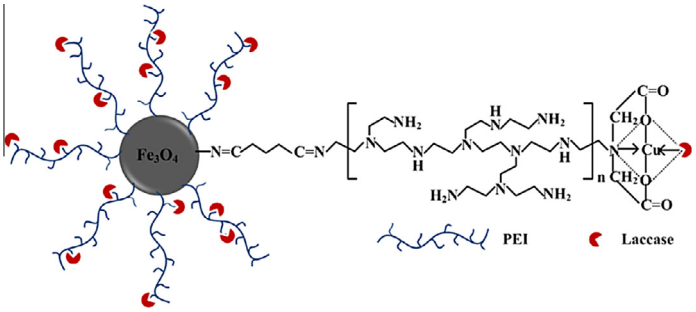
Ting-Ting Xia, Chun-Zhao Liu *, Jian-Hua Hu, Chen Guo *

National Key Laboratory of Biochemical Engineering & Key Laboratory of Green Process and Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, PR China

HIGHLIGHTS

- Polyethylenimine-modified Fe_3O_4 nanoparticles (Fe_3O_4–NH_2–PEI NPs) were chelated with Cu^{2+} to immobilize laccase.
- Fe_3O_4–NH_2–PEI–Cu^{2+} NPs showed the great enzymatic activity recovery during the immobilization process.
- Fe_3O_4–NH_2–PEI–Cu^{2+} NPs retained excellent catalytic activity and operational stability.

GRAPHICAL ABSTRACT



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ABSTRACT

Polyethylenimine (PEI) as a spacer-arm polymer was modified on amine-functioned Fe_3O_4 nanoparticles (Fe_3O_4–NH_2 NPs). PEI modified Fe_3O_4 nanoparticles (Fe_3O_4–NH_2–PEI NPs) were chelated with Cu^{2+} to immobilize laccase through metal affinity adsorption. Meanwhile, PEI un-modified Fe_3O_4–NH_2 NPs acted as a control group. The adsorption capacity of Fe_3O_4–NH_2–PEI–Cu^{2+} NPs was larger than Fe_3O_4–NH_2–Cu^{2+} NPs almost in the whole range of laccase concentration. And the activity recovery of Fe_3O_4–NH_2–PEI–Laccase was also higher than Fe_3O_4–NH_2–Laccase when the laccase concentration was lower than 26 μg/mL. The maximum activity recovery of Fe_3O_4–NH_2–PEI–Laccase (107.41%) was much higher than Fe_3O_4–NH_2–Laccase (42.75%). The corresponding specific activity of Fe_3O_4–NH_2–PEI–Laccase and Fe_3O_4–NH_2–Laccase was separately 101.33 and 74.45 times as large as free laccase product. The enzymatic properties of the two immobilized laccases were both improved and the immobilization realized reuse of laccase. The results showed that Fe_3O_4–NH_2–PEI–Cu^{2+} NPs were more promising than Fe_3O_4–NH_2–Cu^{2+} NPs for purification and immobilization of laccase simultaneously and are potential for large-scale laccase immobilization.

1. Introduction

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) as a multi-copper oxidase can be produced by numerous plants, funguses, and bacteria using simple and inexpensive culture media [1]. Because of its relatively low substrate specificity and high catalytic activity [2], it has gained extensive attention in various fields such as environmental remediation, pulp and paper industry and biosensor [3–5]. However, the industrial applications are limited due to the low stability and poor reusability of free laccase [6].

As is known, the immobilization of laccase can reduce the restrictions by increasing the stability, durability and realizing continuous operations [7]. Up to now, laccase has been successfully immobilized on various kinds of carriers, such as microspheres [8], nanoparticles [9], nanofibers [10] and membrane [11]. Among

* Corresponding authors. Tel./fax: +86 10 82622280.
E-mail addresses: czliu@ipe.ac.cn (C.-Z. Liu), cguo@ipe.ac.cn (C. Guo).

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these carriers, magnetic nanoparticles can be easily and rapidly separated from the reaction medium in an external magnetic field without being subjected to heavy mechanical stress compared with filtration or centrifugation [12] and has been widely investigated [13–15]. The two important criteria for immobilization are immobilization capacity and activity recovery. Some studies use porous magnetic carriers to increase the laccase loading [15–17]. Whereas, the pore diffusion limitations of the substrates and products are inevitable, which may be the rate-controlling step in the catalytic reaction system. Nonporous magnetic nanoparticles are also applied to immobilize laccase because of their larger surface-to-volume ratios [18]. But if the laccase is directly immobilized on the surface of magnetic nanoparticles, there is steric hindrance between the carrier surface and the immobilized laccase [19]. Arica et al. use 1, 6-diaminohexane as a spacer-arm for laccase immobilization [20]. Compared with the spacer-arm of small moleculars, high-molecular-weight polymers, such as polyethyleneimine (PEI), present outstanding advantages as spacer-arm. PEI is a water-soluble cationic polymer, which has high density of primary, secondary and tertiary amino functional groups. It can not only prevent particle agglomeration by the electrostatic repulsive force and steric hindrance [21], but also increase the laccase loading capacity, improve flexibility of immobilized laccase and reduce steric hindrance [22], thus increases enzymatic catalytic efficiency.

Covalent binding and adsorption are regarded as two main methods for laccase immobilization on the surface of nonporous nanoparticles [1,23]. Covalent binding can avoid the laccase desorption in the industrial application. However, the laccase conformational changes may occur during the immobilization process, which has negative effects on the activity of immobilized laccase [24]. As for the method of adsorption, the active sites of laccase are not easily destroyed, while most adsorbents are nonselective [25] and the immobilized laccase may be desorbed because of the weak forces between laccase and carriers [18]. Metal-chelated adsorption can overcome the aforesaid problems. Laccase is rich of histidine residues [26], which can form special covalent bonds: coordinate bonds with transition metal ions such as Cu^{2+} and Zn^{2+}. This special strong binding affinity not only can minimize laccase distortion and desorption, but also can immobilize laccase specifically. Therefore, metal-chelated adsorption is selected as a suitable method to realize purification and immobilization of laccase simultaneously [14,27,28].

In this work, PEI as a spacer-arm was modified on amine-functioned Fe_3O_4 nanoparticles (Fe_3O_4–NH_2 NPs). PEI modified Fe_3O_4 nanoparticles (Fe_3O_4–NH_2–PEI NPs) were chelated with Cu^{2+} to immobilize laccase through metal affinity adsorption. The enzymatic properties of free laccase and immobilized laccase were investigated. Meanwhile, in order to prove the advantages of the spacer-arm, Fe_3O_4–NH_2 NPs acting as a control group were conducted the same research as Fe_3O_4–NH_2–PEI NPs.

2. Experimental

2.1. Materials

Laccase from *Trametes versicolor* was provided by our group, and PEI (branched, *Mw* = 1300 g/mol) was purchased from Sigma–Aldrich (USA). Amine-functioned Fe_3O_4 nanoparticles (Fe_3O_4–NH_2 NPs) (the concentration of primary amino groups on the surface is 0.2 mmol/g and the size is 15 nm) were provided by the Beijing GiGNano Biointerface Company. Glutaraldehyde (GA, 50% in water), Ethylenediamine (EDA) and cupric sulfate ($CuSO_4{\cdot}5H_2O$) were supplied by Sinopharm Chemical Reagent Co., Ltd. (Beijing). Sodium chloroacetate ($CH_2ClCOONa$) was obtained from Aladdin (Shanghai). All other chemicals were of analytical grade and were acquired from Beijing Chemical Reagents Company (Beijing).

2.2. Preparation of Cu^{2+} chelated Fe_3O_4 nanoparticles for immobilization of laccase

Modifying Fe_3O_4–NH_2 NPs with PEI and chelating Cu^{2+} on Fe_3O_4–NH_2 NPs and Fe_3O_4–NH_2–PEI NPs are according to the methods reported previously [29]. The yielding products: Fe_3O_4–NH_2–Cu^{2+} NPs and Fe_3O_4–NH_2–PEI–Cu^{2+} NPs were stored in NaAC–HAC buffer (10 mM, pH = 4.0) for future use. Because the isoelectric point (pI value) of laccase from *T. versicolor* is around 4.0 [27] and the maximum adsorption capacity is always obtained at the isoelectric point of protein [30], the laccase adsorption on these two kinds of Cu^{2+} chelated Fe_3O_4 NPs was conducted at pH 4.0 in NaAC–HAC buffer (10 mM). 1.0 mg of the two kinds of Fe_3O_4 NPs were separately added to 10 mL of laccase solution dissolved in NaAC–HAC buffer (10 mM). The initial concentrations of laccase were in the range of 2.5–55.0 μg/mL. All of the experiments were carried out in an oscillator at 25 °C for 30 min. After reaction, the produced Fe_3O_4–NH_2–Laccase, Fe_3O_4–NH_2–PEI–Laccase NPs and the supernatants were separated and collected through a permanent magnet. The NPs were subsequently washed with the above buffer until no laccase was detected in the supernatant. The concentrations of protein in the initial and final laccase solution were measured using the Bradford protein assay method to determine the amount of laccase immobilized on the NPs [31].

2.3. Assays of free and immobilized laccase activity

The activity of free and immobilized laccase was assayed according to the method reported by Wang et al. [28]. A suitable amount of laccase was added to 0.1% (w/v) catechol dissolved in Na_2HPO_4-citrate acid buffer (50 mM) and shaken for 10 min at 150 rpm and suitable pH. The UNICO UV-2000 spectrophotometer (Shanghai, China) measured the increase in the absorbance of supernatant at 450 nm during the process. The molar absorption coefficient of catechol is 2211 M^{-1} cm^{-1}. One unit of laccase activity is defined as the amount of laccase required to oxidize 1 μmol of catechol per minute. The activity recovery of the immobilized laccase is calculated according to the formula (1):

$$R(\%) = (A_i/A_f) \times 100\% \quad (1)$$

where R is the activity recovery of the immobilized laccase (%), A_i is the activity of the immobilized laccase (U) and A_f is the activity of the same amount of free laccase in solution as that immobilized on the NPs (U).

Effects of pH and temperature on the activity of free and immobilized laccase were determined as the relative activity under different ranges of pH (3.0–8.0) and temperature (25–80 °C).

2.4. Determination of properties and kinetic parameters of free and immobilized laccase

The thermal stabilities of the free and immobilized laccase were studied by measuring the residual enzyme activity at 60 °C in Na_2HPO_4-citrate acid buffer (50 mM, pH = 4.0 for free and Fe_3O_4–NH_2–PEI–Laccase NPs, pH = 5.5 for Fe_3O_4–NH_2–Laccase NPs) for 5 h. A sample (100 μL) was withdrawn every 1 h and measured remaining laccase activity. Storage stabilities of immobilized laccase were determined after storage in Na_2HPO_4-citrate acid buffer (50 mM, pH = 4.0 for Fe_3O_4–NH_2–PEI–Laccase NPs, pH = 5.5 for Fe_3O_4–NH_2–Laccase NPs) for 1 month at 4 °C.

The operating stabilities of the immobilized laccase were assessed by using immobilized laccase repeatedly to oxidize cate-