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Preparation and characterization of tannase immobilized onto carboxyl-functionalized superparamagnetic ferroferric oxide nanoparticles

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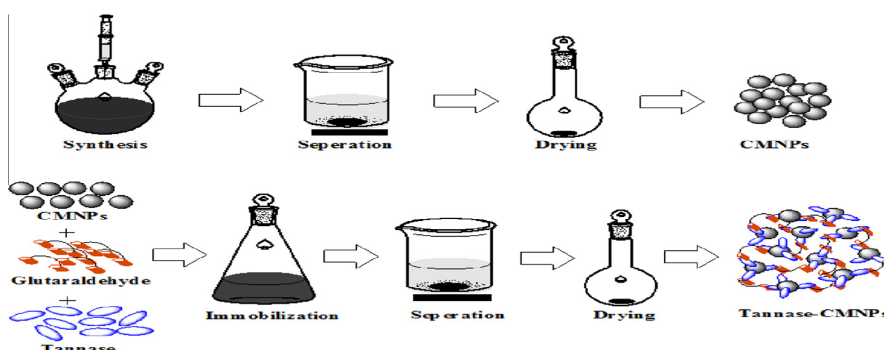
HIGHLIGHTS

- CMNPs was exploited as a novel carrier for tannase immobilization.
- Tannase was directly captured from crude enzyme by the CMNPs.
- The immobilized tannase exhibited excellent magnetic separation and recycling.
- The properties of immobilized tannase was improved than the free enzyme.

GRAPHICAL ABSTRACT



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ABSTRACT

Tannase from *Aspergillus tubingensis* was immobilized onto carboxyl-functionalized Fe_3O_4 nanoparticles (CMNPs), and conditions affecting tannase immobilization were investigated. Successful binding between CMNPs and tannase was confirmed by Fourier transform infrared spectroscopy and thermogravimetric analysis. Vibrating sample magnetometry and X-ray diffraction showed that the CMNPs and immobilized tannase exhibit distinct magnetic responses and superparamagnetic properties. Free and immobilized tannase exhibited identical optimal temperatures of 50 °C and differing pH optima at 6 and 7, respectively. The thermal, pH, and storage stabilities of the immobilized tannase were superior to those of free tannase. After six cycles of catalytic hydrolysis of propyl gallate, the immobilized tannase maintained over 60% of its initial activity. The Michaelis constant (K_m) of the immobilized enzyme indicated its higher affinity for substrate binding than the free enzyme.

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1. Introduction

Tannase (tannin acyl hydrolase, E.C. 3.1.1.20) is an enzyme that catalyzes the hydrolysis of ester and depside bonds in various substrates, such as gallotannins, epigallocatechin-3-gallate, gallic acid esters, and epicatechin gallate, to release gallic acid and glucose (Chávez-González et al., 2012; Jana et al., 2014). Tannase presents extensive application prospects in various fields, including the food, beverage, feed, and pharmaceutical industries; in particular, tannase may be used to produce instant tea, corn liquor, beer, and fruit juice (EL-Tanash et al., 2011; Kumar et al., 2015). While tannase can be extracted from vegetal and animal sources,

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microbial sources are preferred for its industrial production because their biochemical diversity, ease of cultivation, and amenability to genetic modification (Ramos et al., 2011).

Although most of the available research is based on free tannase, the inherent instability of free tannase under different reaction conditions remains a problem that must be solved to improve its industrial applications. To increase the catalytic efficiency of the enzyme and obtain more information on the properties of tannase, purified tannase is often subjected to a purification process (Beniwal et al., 2010). However, this process is a demanding and costly undertaking. As well, free tannase cannot be reused because the free enzyme is difficult to remove from the reaction system; thus, the usage cost of tannase is fairly high.

Immobilized enzymes usually show excellent thermal and pH stability, easy separation, reusability, and good suitability for practical applications. Immobilized biocatalysts have been utilized in a number of practical applications (Atacan and Özacar, 2015; Duarte et al., 2015). For example, lipase has been immobilized onto polyethylenimine-coated magnetic nanoparticles with the assistance of divalent metal chelated ions, and the immobilized enzyme showed better activity than the free enzyme at extreme temperatures and pH (Motevalizadeh et al., 2015). Tan and Lee (2015) reported the immobilization of β-glucosidase on a κ-carrageenan hybrid matrix and used this enzyme to produce reducing sugar from microalgae cellulosic residue. ω-Transaminase was previously immobilized for industrial application, and the immobilized enzyme showed excellent reusability even after 8 cycles of use for 24 h each time (Neto et al., 2015).

Many immobilization studies have been performed on tannase by using various materials, including ion-exchange resins (Kumar et al., 2015; Sharma et al., 2008), calcium alginate (Erzheng et al., 2010; Srivastava and Kar, 2010; Yao et al., 2014), chitin (EL-Tanash et al., 2011), sepharose (Sharma et al., 2002), and chitosan (Abdel-Naby et al., 1999), as supports for enzyme immobilization. However, novel support materials must be explored to obtain tannase with high activity, stability, and reusability under economically viable conditions. Among the carrier materials currently available, magnetic nanoparticles (MNPs) have received substantial interest because they can be readily separated from solution by an external magnet and are recyclable (Motevalizadeh et al., 2015). Thus, MNPs or functionalized MNPs have been widely used to immobilize several enzymes, such as lipase, phosphor lipase A1, and amylase (Eslamipour and Hejazi, 2015; Motevalizadeh et al., 2015; Verma et al., 2013; Yu et al., 2013).

In the present study, crude tannase from *Aspergillus tubingensis* was directly immobilized onto carboxyl-functionalized Fe_3O_4 MNPs (CMNPs) without purification using glutaraldehyde as a cross-linking agent. The CMNPs and tannase-CMNPs were then comparatively investigated through transmission electron microscopy (TEM), scanning electron microscopy (SEM), vibrating sample magnetometry (VSM), X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA), and zeta potential and particle size analyses. The immobilization process was optimized, and the properties of free and immobilized tannase were systematically investigated. Finally, the catalytic efficiency, thermostability, reusability, and storage of the immobilized tannase were assessed and compared with those of free tannase.

2. Methods

2.1. Materials

Propyl gallate and rhodanine were procured from Tokyo Chemical Industry Co., Ltd. (Japan). All other chemicals used in this work were of analytical purity and obtained from Sinopharm Chemical Reagent Ltd., Corp. (China).

2.2. Synthesis of CMNPs

CMNPs were synthesized using the conventional co-precipitation method (Chen et al., 2012). $FeCl_3$ as a precursor was mixed with $NH_4{\cdot}OH$ aqueous solution containing Fe^{2+} ions to form Fe_3O_4. The CMNPs were prepared by oxidizing the C=C bond of oleic acid coated on the surface of the nanoparticles into —COOH by $KMnO_4$ solution in situ, extracted using a permanent magnet, freeze-dried under vacuum for 48 h, and then stored at 4 °C.

2.3. Preparation of crude tannase

A. tubingensis CICC 2651 was obtained from the China Center of Industrial Collection and used in this study. Tannase production was conducted by solid-state fermentation, and the crude enzyme ($1.0\ U\ mL^{-1}$) was prepared according to a previously described method (Wang et al., 2013b).

2.4. Immobilization of tannase onto CMNPs

Tannase immobilization was performed using glutaraldehyde as the coupling agent to ensure covalent attachment of tannase to the CMNPs. About 20 mg of CMNPs was dispersed in citrate buffer (50 mM, pH 5.0); then, $1.75\ U\ mL^{-1}$ tannase and glutaraldehyde were added to this mixture. Shaking was performed at 22 °C and 220 rpm for up to 4 h. CMNPs bound with tannase were separated by magnetic decantation, washed thrice, and resuspended in citrate buffer (50 mM, pH 5.0). The immobilized tannase was either stored at 4 °C after vacuum freeze-drying or immediately used for activity analysis and physical characterization.

The specific activity of the immobilized enzyme and enzyme recovery were calculated using the following relation:

$$\text{Specific activity (U/g carries)} = C_1 \cdot V_1 / W \tag{1}$$

$$\text{Activity recovery } (\%) = C_1 \cdot V_1 / (C_0 \cdot V_0) \cdot 100\% \tag{2}$$

where C_0 and V_0 are the initial enzyme concentration and total volume of the enzyme immobilization system, respectively, C_1 and V_1 are the immobilized tannase concentration and the volume of the resuspended liquid, respectively, and W is the weight of the MNPs.

2.5. Enzyme activity assay

The activity of free tannase was estimated as described previously (Sharma et al., 2000). Propyl gallate was used as a substrate to produce gallic acid, which was then combined with alcoholic rhodanine to form a chromogen. The absorbance of chromogen was detected at 520 nm after completing the operation steps. The same assay medium was used to determine the activity of immobilized tannase, except that the immobilized tannase was magnetically concentrated once the reaction was completed. One unit of tannase activity is defined as the amount of enzyme required to release one micromole of gallic acid per minute under defined conditions.

2.6. Effects of process parameters on tannase immobilization

Single-factor experiments were applied to study the factors influencing tannase immobilization with increasing glutaraldehyde concentration (0.5–5.0%), enzyme amount ($0.1–2.5\ U\ mL^{-1}$), immobilization temperature (22–34 °C), rotation speed (60–220 rpm), pH (3.0–8.0), immobilization time (0–26 h), and CMNP

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