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Catechol modification and covalent immobilization of catalase on titania submicrospheres

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ABSTRACT

In this article, chemical modification with catecholic derivative in solution and subsequent immobilization of catalase (CAT) on titania submicrospheres (450–500 nm) were described. Catalase was first reacted with 3-(3,4-dihydroxyphenyl) propionic acid activated via 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) coupling chemistry. The above chemically modified CAT bearing catechol groups was then covalently bound to the surface of titania through the facile chelation reaction between the catechol groups and titania. The immobilized CAT retained 60% catalytic activity with a high loading capacity of 500 mg/g titania. Meanwhile, the immobilized CAT displayed enhanced operational stability, thermal stability and storage stability compared with native, modified CAT counterparts. In repeated batches of decomposition of hydrogen peroxide, after 10 and 19 cycles, the immobilized CAT maintained about 90% and 75% of its initial activity, respectively.

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1. Introduction

Enzymes have been considered as versatile biocatalysts for their wide applications in biotechnological processes due to their high activity, selectivity under mild conditions [1,2]. However, enzymes in free form are usually unstable, difficult to reuse, difficult isolated from substrate(s) and product(s), which dramatically restrict their industrial applications. Generally speaking, enzyme immobilization is a powerful tool to bypass these drawbacks and endow enzyme with improved properties for practical applications [3,4]. Methods for enzyme immobilization basically include adsorption, entrapment, encapsulation, cross-linking and covalent binding [5]. Among them, covalent binding is one of the most effective methods with the great potential for long-term and high-shearing operations.

It has been widely recognized that immobilization can stabilize enzyme structure substantially and improve enzyme performance remarkably. Modification of enzyme [6,7] as an alternative strategy may acquire a synergistic effect based on the process in which modification is performed prior to immobilization [8]. Despite the interesting prospects of genetic modification, chemical modification remains a popular method to optimize enzyme properties [9]. The distinct attribute of chemical modification is that it does not require the thorough understanding of the whole enzyme structure and can attach various functional groups onto the native enzyme [10]. Till now, enzymes have been chemically modified by various reagents, such as surfactant [11], phthalic anhydrides [12,13], PEG [14,15] and catechol type polyphenols [16]. It can be conjectured that the approach which couples chemical modification with immobilization may open an efficient avenue to the enhancement of enzyme catalytic properties [17].

Recently, 3,4-dihydroxyphenylalanine (DOPA), a substance found in mussel-adhesive proteins has drawn considerable attention in the creation of multifunctional surface through PEGylation [18], cell adhesive peptide conjugation [19], and DNA immobilization [20] on various surfaces. Additionally, catechols and their derivatives have displayed strong coordination ability toward metal ions, such as titania and iron oxide [21–23]. This coordination (chelation reaction) process could occur spontaneously at room temperature. Therefore, the catechol derivative (3-(3,4-dihydroxyphenyl) propionic acid) with two phenolic hydroxyl groups and a carboxyl group can serve as the enzyme modifier and the conjugation agent for efficient enzyme immobilization.

Many inorganic carriers, such as silica, alumina, zirconia and titania, have good thermal stability, high mechanical and antibacterial properties, thus becoming suitable candidates as enzyme supports [24–27]. The methods for enzyme immobilization on inorganic carriers mainly include adsorption, encapsulation and covalent binding. Among the inorganic carriers, titania is of peculiar interest because of their excellent pH and corrosion resistance, superior mechanical strength, as well as good biocompatibility [26].

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Catalase (EC 1.11.1.6), one of the most common enzymes in plant and animal tissues, has won applications in many industrial fields [28–30]. In recent years, there are many research reports on catalase (CAT) immobilization through covalent binding method. For instance, Alptekin et al. have covalently immobilized CAT onto Eupergit C. The immobilized CAT showed good reusability, thermal stability, and long-term storage stability [31]. Yang et al. have functionalized CAT by (3-aminopropyl)trimethoxysilane (APTS) and then triggered the *in situ* synthesis of the silica nanoparticles for covalent encapsulation of the modified CAT. Both high enzyme loadings and high enzymatic activity recoveries (90%) were achieved [24]. However, at present, there are rare reports dealing with CAT immobilization on titania submicrospheres using covalent binding method.

In this study, CAT was first modified with 3-(3,4-dihydroxyphenyl) propionic acid activated via 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) coupling chemistry. The catechol groups on the modified CAT facilitated the subsequent immobilization onto the surface of titania by facile covalent chelation. The activity and stability of modified and immobilized enzymes were examined and compared with its native enzyme counterpart.

2. Experimental

2.1. Materials

Catalase (hydrogen peroxide oxidoreductase; 250 kDa; 3.43×10^3 units/mg protein; EC.1.11.1.6), 2-(N-morpholino) ethanesulfonic acid sodium salt (MES) and Tris(hydroxymethyl) aminomethane (Tris) were purchased from Sigma–Aldrich chemical company. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Co., Ltd. 3-(3,4-Dihydroxyphenyl) propionic acid (98+%) was purchased from Alfa Aesar. Acetone, ethylene glycol, tetrabutyl titanate (TBT, >98%, 340.36 Da), hydrogen peroxide (H_2O_2, 30%), acetic acid, sodium acetate, sodium dihydrogen phosphate dehydrate ($NaH_2PO_4{\cdot}2H_2O$) and sodium phosphate dibasic dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$) were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). All other chemicals were of analytical grade. Millipore water with a resistivity of 15.0 MΩ cm was used throughout the study.

2.2. Synthesis of titania submicropheres

Titania (TiO_2) submicropheres were fabricated according to the method as reported in literature [32]. Typically, 0.02 mol 6.8 ml tetrabutoxytitanium (TBT) was dissolved in 100 ml ethylene glycol in a round flask under N_2 gas protection and then magnetically stirred rapidly for 20 h at 30 °C. The above resultant transparent solution was then poured into acetone solution with ~0.3 wt% of water quickly. After aging for about 1 h, the white precipitate was collected by centrifugation and washed with water and ethanol alternatively to remove any residual ethylene glycol. Finally, TiO_2 submicrospheres were obtained with a size of 450–500 nm and a BET surface area of 257.7 $m^2\ g^{-1}$. After drying at 80 °C for 24 h, the TiO_2 submicrospheres were stored at 4 °C before use.

2.3. Chemical modification and immobilization of catalase

First, 20.0 mg 3-(3,4-dihydroxyphenyl) propionic acid was dissolved in 15.0 ml MES buffer solution (100 mM, pH 6.5). 36.0 mg NHS and 60.0 mg EDC were then added to the above solution, successively. Reaction proceeded for 1 h at room temperature. Second, CAT solution was prepared in 10.0 ml pH 6.5 MES buffer solution with a protein concentration of 5.0 mg/ml. The solution was then added to the first reaction solution and allowed further reaction for 4 h at 4 °C. Then, the unreacted chemicals and byproducts were removed from the above obtained solution by extensive dialysis through a regenerated cellulose membrane (cutoff molecule weight 8000–14400). The resultant modified CAT solution was stored at 4 °C before use.

50.0 mg TiO_2 submicropheres were dispersed in 5 ml MES buffer solution (100 mM, pH 6.5) under ultrasonic treatment for 15 min, and then added to the above modified CAT solution. After stirred vigorously for 0.5 h, the catalase–titania conjugates (TiO_2–CAT) were separated by centrifugation and washed thoroughly with Milli-Q water. The immobilized enzyme was lyophilized and stored at 4 °C before use.

2.4. Characterizations

TEM observation was performed on a JEM-100CXIIinstrument. FTIR spectra were obtained on a Nicolet-6700 spectrometer. The secondary structure of the free enzyme was determined using a Jasco J810 CD spectrophotometer in the far-UV region (185–260 nm) equipped with a 5 mm quartz cell. The specific surface area of the titania submicropheres was determined by nitrogen adsorption–desorption isotherm measurements performed on a Tristar 3000 gas adsorption.

2.5. Enzyme loading capacity and activity assay

The amount of immobilized CAT was determined by measuring the initial and final concentrations of CAT with washing solution using Coomassie Brilliant Blue reagent, following the Bradford's method [33]. The loading capacity was determined according to Eq. (1):

$$M\left(\frac{\text{mg enzyme}}{\text{g support}}\right) = \frac{(m - C_1V_1)}{W} \quad (1)$$

where M (mg/g) represented the loading capacity; m (mg) was the amount of CAT introduced into the immobilization medium; C_1 (mg/ml) and V_1 (ml) were the enzyme concentration and volume of the washing solution, respectively; W (g) was the weight of the TiO_2 submicropheres.

The activity of catalase was determined by the decomposition of hydrogen peroxide to water and oxygen [34]. Briefly, the H_2O_2 reaction solution (19.58 mM) was prepared in 100 ml Tris–HCl buffer solution (50 mM, pH 7.0). A certain amount of TiO_2 submicrospheres with 0.1 mg bound CAT were added into 20 ml reaction solution at 25 °C. After 3 min, the decrease in absorbance at 240 nm of H_2O_2 was recorded spectrophotometrically. By comparison, the same amounts of native and modified enzyme (0.1 mg) were used in the reaction system. One unit of CAT was defined as the decomposition of 1 μmol hydrogen peroxide per minute at 25 °C and pH 7.0. The relative activity (%) was calculated by comparing the activity of modified or immobilized CAT with its native form under equal amount of enzyme (Eq. (2)).

$$\text{Relative activity}\,(\%) = \frac{\text{modified or immobilized enzyme activity}}{\text{native enzyme activity}} \times 100 \quad (2)$$

2.6. Effect of pH and temperature on enzyme activity

Optimum pH for native, modified and immobilized CAT was determined by measuring the enzyme activity in buffers of different

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