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Synthesis of urease hybrid nanoflowers and their enhanced catalytic properties

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ABSTRACT

Increasing numbers of materials have been extensively used as platforms for enzyme immobilization to enhance catalytic activity and stability. Although stability of enzyme was accomplished with immobilization approaches, activity of the most of the enzymes was declined after immobilization. Herein, we synthesize the flower shaped-hybrid nanomaterials called hybrid nanoflower (HNF) consisting of urease enzyme and copper ions (Cu^{2+}) and report a mechanistic elucidation of enhancement in both activity and stability of the HNF. We demonstrated how experimental factors influence morphology of the HNF. We proved that the HNF (synthesized from 0.02 mg mL^{-1} urease in 10 mM PBS (pH 7.4) at +4 °C) exhibited the highest catalytic activity of ~2000% and ~4000% when stored at +4 °C and RT, respectively compared to free urease. The highest stability was also achieved by this HNF by maintaining 96.3% and 90.28% of its initial activity within storage of 30 days at +4 °C and RT, respectively. This dramatically enhanced activity is attributed to high surface area, nanoscale-entrapped urease and favorable urease conformation of the HNF. The exceptional catalytic activity and stability properties of HNF can be taken advantage of to use it in fields of biomedicine and chemistry.

1. Introduction

Enzymes, as versatile biological materials, have been commonly utilized in a wide variety of scientific and technical applications including production of biofuels, drug synthesis, chemical reactions in vivo and in vitro, proteomics and industry (beverage, food and textile, etc.). Although free enzymes have unique properties, such as high catalytic activity, reaction specificity and low toxicity, their efficient use is very limited due to their inherent disadvantages associated with instability during storage, loss of catalytic activity after administration into the reaction, high cost and lack of recovery from reaction medium [1–7]. In order to overcome these limitations, several immobilization approaches have been developed. For this intent, various supports (micro and nano size) including silica, magnetic, zeolites, chitosan materials, molecular sieves, polymer stands, silk film and gelatin have been widely used for enzymes immobilization via covalent, non-covalent, entrapment and cross-linking [8–16]. The expectation with enzyme immobilization is to improve both catalytic activity and stability, both of which are critical factors to widely and efficiently use enzyme for many applications. Although immobilized enzymes show enhanced stability in aqueous solution, the most of them exhibit reduced catalytic activity compared to free enzymes most likely due to decreased mobility, unfavorable conformation and mass-transfer limitation when attached on the solid support [17–19]. Thus, the efficient use of immobilized enzyme is mainly obstructed. However, Ackerman et al. reported one of the rare examples, where an increase in catalytic activity was achieved after enzyme immobilization. They simply loaded organophosphorus hydrolase enzyme into carboxylethyl-or aminopropyl groups functionalized mesoporous silica (30 nm pore size) and demonstrated ~200% enhancement in catalytic activity compared to free enzyme [20]. However, this enhancement in activity and stability were not at a satisfactory level since the immobilized enzyme lost almost 15% of its initial activity within 30 days of storage at +4 °C. Furthermore, multiple,

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time-consuming steps and labor-intensive synthetic procedures were also major limitations of this work.

Therefore, there is still a high demand for a new and an alternative enzyme immobilization strategy with improved catalytic activity and stability. Unlike conventional enzyme immobilization methods, a recent exclusive approach in enzyme immobilization was developed by Zare et al. [21]. They created organic–inorganic hybrid nanostructures consisting of protein and metal ions, which resulted in much higher enzyme activity and stability than free enzymes and conventional immobilized enzymes. As a following work, Wang et al. reported multi-enzyme integrated hybrid nanostructures by using glucose oxidase and horseradish peroxidase to utilize functions of these enzymes in one step [22]. Although this system allowed them to get benefit from both enzymes, there were disadvantages associated with long analysis time and reproducibility in synthesis of these hybrid nanostructures. In our previous studies, we synthesized flowerlike hybrid nanomaterials containing horseradish peroxidase (HRP) enzyme and different metal ions with highly enhanced catalytic activity and stability. We also demonstrated that how experiential parameters affect the morphology of flowerlike hybrid nanomaterials and explained the enhancements in the catalytic activity and stability [23,24].

Urease (urea amidohydrolases, EC 3.5.1.5) is a one of the most studied and attractive enzymes in environmental, bioanalytical, and clinical applications [7,25–27]. Urease has been actively used as an efficient biocatalyst for hydrolysis of urea. In addition, urease also acts as a sensor for determination of urea concentrations in several mediums including serum, blood, urine, some alcoholic and non-alcoholic beverages, food products, and environmental wastes. The level of urea in serum and blood is in the range of 2.5–7.5 mM, which is considered as normal and tolerable by human body. An increase and decrease in urea level can induce very serious diseases. High level of urea causes urinary tract obstructions, renal failures, burns and gastrointestinal bleeding while hepatic failures and nephritic syndromes are induced by low levels of urea [28–32].

To the best of our knowledge, herein, we present synthesis, catalytic activity and stability of hybrid nanoflower (HNF) formed of urease enzyme and copper ions (Cu^{2+}) and a mechanistic elucidation of enhancement in the catalytic activity and stability HNF. In this study, we also demonstrate how reaction parameters influence the morphology of HNF and evaluate catalytic activity and stability of HNF by hydrolysis of urea based upon their morphologies and storage temperatures.

2. Experimental

2.1. Chemicals and materials

Urease from jack bean, albumin from bovine serum (BSA) (lyophilized powder), Copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) and urea were purchased from Sigma–Aldrich. Salts (NaCl, KCl, Na_2HPO_4, $KHPO_4$, $CaCl_2{\cdot}2H_2O$, $MgCl_2{\cdot}6H_2O$) were also obtained from Sigma–Aldrich. Ultrapure water (18.2 MΩ; Millipore Co., USA) was used in all experiments.

2.2. Synthesis of urease based hybrid nanoflowers

The HNF was synthesized using a modified method [21]. In a standard synthesis protocol, 60 μL aqueous $CuSO_4$ from a freshly prepared stock solution (120 mM) in the biology-grade water was added to 9 mL of 10 mM phosphate buffered saline (PBS) solution (pH 7.4) containing 0.02 mg mL^{-1} of urease. The mixture was vigorously agitated and then incubated at +4 °C for 3 days. After incubation, the blue color precipitation (indication of HNF formation) was observed at the bottom of reaction tube. In order to stop reaction, the mixture was centrifuged at 10,000 rpm for 15 min. The HNF was washed with water and centrifuged at least 3 times to remove unreacted components. Finally, collected HNF was dried under vacuum at room temperature. Note that the HNF was also synthesized with various concentrations of urease, Cu^{2+} ion and different pH values in PBS solution to observe their effects on the morphology of HNF. The synthesis of HNF under different experimental parameters was occurred at only +4 °C for 3 days.

2.3. Instrumentation and characterization

In order to calculate encapsulation yield (EY) of urease in HNF, the urease concentration in the supernatant was measured with Bradford protein method using BSA as a standard. The weight of HNF powder and EY value was utilized to calculate the weight percentage (WP) of enzyme in HNF. The EY of HNF (from 0.02 mg mL^{-1} urease and 0.8 mM Cu^{2+} ions at +4 °C) was calculated as ~99.2% while WP was found as ~19%. The EY and WP of each HNF were calculated accordingly prior to activity and stability measurements. First, the EY of HNFs synthesized (from 0.02 mg mL^{-1} urease and 0.8 mM Cu^{2+} ions at +4 °C) at pH 6, 8 and 9 are almost the same as ~98.15%, ~99.1% and ~99.4%, respectively while WP of all HNFs is around ~19%. Second, EY and WP of HNFs synthesized (from 0.8 mM Cu^{2+} ions in pH 7.4 at +4 °C) using 2.5, 0.5, 0.05 and 0.01 mg mL^{-1} urease were calculated as ~99.2%, ~99.4%, ~99.3% and ~99.5%, respectively while WP of HNFs are determined to be ~29%, ~24%, ~21% and ~16%, respectively. The activity and stability of the HNF formed as a function of the pH of the PBS solution and concentrations of urease enzyme were determined by UV–vis spectrophotometer (HITACHI).

The dry HNF powders were put on carbon tape covered stub, then it was coated with gold by a sputter coater. Finally, images of HNF were generated by scanning electron microscopy (SEM) (ZEISS EVO LS10). In addition to SEM, elemental analysis of the HNF was carried out using energy-dispersive X-ray (EDX) (ZEISS EVO LS10) to determine weight and atomic percentage of Cu^{2+} in the HNF. The crystal pattern of $Cu_3(PO_4)_2{\cdot}3H_2O$ was identified with X-ray diffraction analysis (XRD) (BRUKER AXS D8). Firstly, the HNF was dried at 80 °C; XRD was applied to the dry HNF for crystal analysis. The dry free urease (black line) and HNF (red line) were also analyzed with FTIR to prove the formation of HNF.

2.4. Enzyme activity measurement

The HNF and free urease (1 mg mL^{-1} identical amount) were dissolved in PBS solution (pH 7.0) in two different reaction tubes. To the each tube, 0.8 mL urea (3 mg mL^{-1}) was added and then the resulting both tubes were incubated for 30 min. After incubation, 4 mL deionized water and 100 μL Nessler's reagent were added to the each tube and then NH_3 produced through hydrolysis of urea form each tube was spectrophotometrically determine at 425 nm. Enzyme activity measurements were performed at room temperature (RT = 20 °C) and +4 °C. In the study, one unit of enzyme activity was defined as the amount of enzyme that changes the absorbency 0.01 unit per min. It is known that K_M (Michaelis constant) is an important characteristic for an enzyme which shows the affinity of the enzyme to the substrate and the robustness of the enzyme-substrate complex [33]. For this reasons, the K_M values of free urease and HNF were determined. To determine the K_M, a certain concentration of free urease and HNF were incubated with five different concentrations of urea (0.3, 0.45, 0.60, 0.75, 1.05 mM). Thus, the K_M values were calculated from the plot of $1/V$ versus $1/[S]$ by the method of Lineweaver and Burk [34].