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Preparation of lactoperoxidase incorporated hybrid nanoflower and its excellent activity and stability

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ABSTRACT

We report a green approach to synthesize lactoperoxidase (LPO) enzyme and metal ions hybrid nanoflowers (HNFs) and investigate mechanism underlying formation and enhanced catalytic activity and stability under different experimental parameters. The HNFs formed of LPO enzyme purified from bovine milk and copper ions (Cu^{2+}) were synthesized at two different temperatures (+4 °C and 20 °C) in PBS (pH 7.4). The effects of experimental conditions, pH and storage temperatures, on the activity and stability of LPO–copper phosphate HNFs were evaluated using guaiacol as a substrate in the presence of hydrogen peroxide (H_2O_2). Optimum pHs were determined as pH 8 and pH 6 for LPO–copper phosphate HNF and free LPO, respectively. LPO–copper phosphate HNF has higher activity than free LPO at each pHs. Activities of LPO–copper phosphate HNF at pH 6 and pH 8 were calculated as 70.48 EU/mg, 107.23 EU/mg, respectively while free LPO shows 45.78 EU/mg and 30.12 EU/mg, respectively. Compared with free LPO, LPO–copper phosphate HNFs exhibited ~160% and ~360% increase in activities at pH 6 and pH 8, respectively. Additionally, LPO–copper phosphate HNFs displayed perfect reusability after six cycles. Finally, we demonstrated that LPO–copper phosphate HNFs can be utilized as a nanosensor for detection of dopamine and epinephrine.

1. Introduction

Although free enzymes are biocompatible, highly water-soluble and show high specificity to their substrates, high cost, short life time and low activity in the wide range of pH and temperature strictly limit their applications in scientific and technical fields [1–5]. To address these limitations of free enzymes, several immobilization methods have been developed. In addition to that, increasing the activity and stability of enzyme is also the major intent of immobilization methods. For achieving that, enzymes have been immobilized on\in various micro (glass, membrane, polymers, gel beads, sol–gel materials, porous silica and monolithic matrix) and nano sized (mesoporous silica, solid and fiber nano particles) supports via adsorption, encapsulation, coating and covalently linking [6–14]. In spite of huge effort on various immobilization methods aforementioned, enzymes have only increased stabilities but most of the immobilized enzymes have showed lower activity compared to that of free enzymes in solution [15–20]. Zhu et al., have found that atom transfer radical polymerization for electron transfer (AGET ATRP) procedure for preparing polymer–protein conjugates have increased stability of HRP used as a model enzyme [21]. Ge et al., have based on a new drug delivery platform which is a hybrid nanoparticle consisting of a hydrophobic polymeric core of PMMA and BSA, have shown tunable size, surface charge, ease of modification, excellent biocompatibility and efficient cell uptake [22]. It is worth noting that when free enzyme is immobilized to supports, the immobilized enzyme has appeared unwelcome situations, such as unfavorable conformation and mass-transfer limitation, lowering activity have appeared [18,19]. Additionally, these conventional immobilization methods

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usually have multi step, time-consuming and complex procedures and need expertise.

Only very few studies reported that immobilized enzyme prepared with conventional immobilization method performed higher activity than that of free enzyme. For instance, Lei et al., have shown immobilized organophosphorus hydrolase in mesoporous silica with different protein loading efficiencies. It showed an increase in activity of ~200% compared to free urease in solution [23]. Zhang et al., have stated that a greatly increased apparent activity in organic solvent using temperature-responsive lipase–Pluronic conjugate for the chemo-enzymatic Synthesis of valrubicin which is an anti-cancer drug [24]. However, multi step, time consuming and complex procedure and lack of reproducibility are main disadvantages of this system. Therefore, there is high need to explore a novel immobilization approach for overcoming disadvantages of free enzyme use and conventional immobilization methods and enhancing enzyme activity and stability, simultaneously. Recently, Ge and co-workers discovered an exciting and inspiring strategy to synthesize hybrid protein–inorganic hybrid nanoflowers with highly increased catalytic activity and stability compared with both free and conventionally immobilized enzymes [22,25–29]. Inspiring from this work, we prepared lactoperoxidase (LPO) hybrid nanoflowers and investigate their formation and enhanced catalytic activity and stability under the different experimental parameters as predicted parameters in previous studies [30–32].

The lactoperoxidase (LPO) which one of the prominent enzymes generally found in several sources, such as bovine milk, saliva and tears [33]. LPO has crucial applications in various fields. For example, LPO protects intestinal tract system of newborn infants against pathogenic microorganisms by catalyzing halides and pseudohalides [34] and it has also role for preservation of raw milk. It is reported that purified LPO has been conventionally immobilized on various external supports however enhancement in activity of LPO was not observed [33–37].

Herein, we report the synthesis of hybrid nanoflower (HNF) consisting of LPO purified from bovine milk and copper metal ions (Cu^{2+}) via a green approach and mechanism of the HNF formation and of their catalytic activity and stability enhancement. In this particular study, we also investigate how experimental conditions, such as pH values and storage temperatures, influence LPO–copper phosphate HNF formation and the catalytic activity and stability.

2. Experimental

2.1. Chemicals and material

Lactoperoxidase (LPO) (donor: hydrogen peroxide oxidoreductase, E.C.1.11.1.7) purified from bovine milk. Bovine serum albumin (BSA) (lyophilized powder), guaiacol, dopamine hydrochloride, epinephrine and copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) were purchase from Sigma–Aldrich. Salts (NaCl, KCl, Na_2HPO_4, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $MgCl_2{\cdot}6H_2O$) for buffer solution were also purchased from Sigma–Aldrich. In all experiments, ultrapure water (18.2 M Ω; Millipore Co., USA) was used.

2.2. Purification of LPO from bovine milk

The LPO was purified from bovine milk using a reported method. Briefly, the fat in bovine milk was removed via centrifugation at 3500 rpm at +4 °C for 15 min. After centrifugation, to the fresh raw skimmed bovine milk, amberlite CG 50 NH_4^+ resin [equilibrated with 5 mM sodium acetate solution (pH 6.8)] was added at a rate of 4.4 g/150 mL [34,38]. The resin was washed with distilled and deionised water and sodium acetate solution (20 mM, pH 6.8). The bound proteins were eluted with 0.5 M sodium acetate solution at pH 6.8. The obtained eluate was passed through the Sepharose 4B-L-tyrosine-sulphanamide affinity column to collect the purified enzyme [39].

2.3. Synthesis and characterization of LPO–copper phosphate hybrid nanoflowers

LPO–copper phosphate HNFs were prepared using a modified reported methods [28–32,40]. First, $CuSO_4$ stock solution was prepared in ultrapure water. Then, certain volume of that solution was added to phosphate buffer saline solution containing 0.02 mg mL^{-1} LPO. The resulting mixture was vigorously shaken for 30 s sand it was splitted into two reaction tubes. One of the reaction tube was incubated without disturbing at +4 °C for 3 days and other tube was incubated under the same conditions but at room temperature (RT = 20 °C). After incubation, the blue color precipitates (evidence of HNF formation), as products, at the bottom of each reaction tube were collected and washed by centrifugation at 10,000 rpm for 15 min. The washing process was repeated at least 3 times. The collected precipitates were dried 50 °C under vacuum. The LPO–copper phosphate HNF was characterized prior to enzyme activity and stability measurements.

The encapsulation yield of LPO in HNFs was determined by measuring the LPO concentration in the supernatant via Bradford protein assay. The BSA and LPO both were used as standards and almost the same LPO yield (~94%) was obtained. Scanning electron microscopy (SEM) (ZEISS EVO LS10) was used for imaging morphology of LPO–copper phosphate HNFs. The dry LPO–copper phosphate HNFs were deposited on SEM stub, and then it was coated with gold source by using a sputter coater prior to SEM operation. Energy-dispersive X-ray (EDX) (ZEISS EVO LS10) was utilized for the weight and atomic percentage analysis of Cu element in HNF. The crystal structure of LPO–copper phosphate HNF was elucidated using X-ray diffraction analysis (XRD) (BRUKER AXS D8). 20 mg of HNFs powder was dried at 80 °C and used for XRD pattern. The chemical structures of free LPO and LPO–copper phosphate HNFs were analyzed with a Fourier Transform Infrared Spectroscopy (FTIR) spectrum (PerkinElmer Spectrum 400). The activities of LPO–copper phosphate HNFs were tested with UV–vis spectrophotometry (HITACHI).

2.4. Enzyme activity measurement

The identical concentrations of free LPO and LPO–copper phosphate HNF were used for activity experiments. The activities of free LPO and LPO–copper phosphate HNF were determined against guaiacol or 2,2-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) diammonium salt (ABTS) as a model substrate [30,31,41–44]. A standard protocol was used for activity experiment. First, the same amount of free LPO and LPO–copper phosphate HNF were separately dissolved in 1 mL of PBS (pH 6.8, 0.1 M KH_2PO_4, 25 °C) and each mixture was splitted to reaction tubes. To each tube, 1 mL of 22.5 mM hydrogen peroxide (H_2O_2) and 1 mL of 45 mM guaiacol were added. The final concentrations of H_2O_2 and guaiacol in the tubes were determined as 7.5 mM and 15 mM, respectively. Two set of reaction tubes were inbubated at +4 °C and rest tubes were incubated at 20 °C. The change in absorbance values of oxidized guaiacol were monitored at 470 nm at 25 °C using a UV–vis spectrophotometer. One activity unit of lactoperoxidase (EU) was defined as the amount of lactoperoxidase required to catalyze 1 μmol of substrate per minute under the specified reaction conditions.