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Enhanced activity and stability of papain by covalent immobilization on porous magnetic nanoparticles

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ABSTRACT

Papain enzyme was successfully immobilized by covalent bonding onto biocompatible Fe_3O_4/SF nanoparticles, which were prepared with the soft template of silk fibroin (SF). The optimized immobilization condition is pH 6.0, hydrolysis time of 60 min, and an enzyme/support ratio of 10.0 mg/g. Compared with free papain, the immobilized papain exhibits a high effective activity, broader working pH and temperature. This immobilized papain can be separated from the solution by the external magnetic field for cyclic utilization, and 70% of initial activity was retained after eight consecutive operations while completely loss of proteolytic activity for the free papain. Furthermore, the immobilized papain maintained 85% of their initial activity after being stored for 28 days. Kinetic parameters, maximum reaction rate (V_{max}) and Michaelis constant (K_m) of immobilized papain, were determined as 4.95 mg/l·min and 0.23 mg/ml, larger than its free counterpart. All the results above indicated that the immobilized papain onto magnetic Fe_3O_4/SF nanoparticles would have potential industrial and medical applications.

1. Introduction

The use of enzymes has been increased in catalytic engineering along with the current demands of sustainable green production technology [1]. Among those enzymes, papain, obtained from papaya latex, exhibits highly effective hydrolysis towards proteins, peptides, and amide links, which has great importance in the areas of medicine, food, and so on. However, soluble papain is easy to denature or deactivate once exposed to the harsh conditions of industrial processes. Moreover, free papain is difficult to be separated from the reactants, resulting in poor recover and recycle [2,3]. To overcome these drawbacks, immobilization of free enzymes on support is an effective way for industrial application.

Up to now, integration of enzymes into nanoparticles has been proved to maintained or even enhanced immobilized enzyme performance [4]. Among them, mesoporous magnetic material has attracted much attention, because of its excellent magnetic responsibility, good biocompatibility, chemically modifiable surface and good reusability [5–8]. However, magnetic Fe_3O_4 nanoparticles were easy to agglomeration in solution, owing to its strong magnetic dipole-dipole attraction. Effective way to solve this problem was to coat magnetite particles with some polymers, containing specific functional groups. Among those polymers, silk fibroin (SF), as a natural protein, has excellent biocompatible, self-assembly performance and substrate recognition properties, providing promising templates for different functional nanoparticles such as calcium carbonate [9], copper oxide [10], silver [11], and iron oxides [12,13] with controllable morphologies. Furthermore, —COOH, —OH groups on the surface of silk fibroin will be convenient for covalent immobilization, resulted in increased thermal and storage stability. In our previous study, magnetic Fe_3O_4/SF nanoparticles were successfully prepared by using one-step and environment-friendly method and proved be good biocompatibility [12].

In the present study, we performed the immobilization of the papain on porous Fe_3O_4/SF nanoparticles through covalent binding, so as to improve the specific activity of the immobilized form of papain and to increase its catalytic efficiency. Various analytical techniques were used to investigate the characterizations and optimal conditions of immobilized papain in terms of optimal pH, temperature, initial papain concentration, immobilization time. Compared with free papain, the stabilities, reusability and catalytic activity for casein degradation together with kinetic parameters were comprehensively investigated.

2. Material and methods

2.1. Materials

Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate trihydrate ($NaAc \cdot 3H_2O$), sodium carbonate (Na_2CO_3) and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd.,

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Shanghai, China. Ethylene glycol (EG), N-Hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), lithium bromide (LiBr), casein, and papain (lyophilized powder, 10 units/mg protein) were bought from Sigma-Aldrich. All the reagents were used without further purification.

2.2. Preparation and characterization of porous Fe_3O_4/SF nanoparticles

Porous Fe_3O_4 nanoparticles were synthesized according to a solvothermal procedure [12]. Firstly, silk fibroin (SF) was prepared according to our previously published procedures. Then, SF solution (7 wt%, 10 ml) was added into the mixture of $FeCl_3 \cdot 6H_2O$ (1.35 g) and $NaAc \cdot 3H_2O$ (5.97 g) in 60 ml EG under stirring for 0.5 h to form a transparent solution. The solution was transferred into a 100 ml Telfon-lined stainless-steel autoclave and heated at 160 °C for 12 h. After cooling to room temperature, the as-prepared Fe_3O_4/SF nanoparticles were collected after a centrifugal process (8000 rpm, 30 min), and washing process with deionized water and ethanol for three times each, followed by drying in a vacuum oven at 60 °C for 12 h. The obtained Fe_3O_4/SF nanoparticles were then calcined in Ar at 350 °C for 3 h to generate porous nanoparticles. Total amino group (-NH_2) levels on the surface of Fe_3O_4/SF nanoparticles were quantitatively determined by using the ninhydrin colorimetric reaction. The amount was about 0.16 mmol/g.

2.3. Immobilization of papain on porous magnetic nanoparticles

Papain was immobilized on the surface of the porous Fe_3O_4/SF nanoparticles (Fe_3O_4/SF NPs) by cross-linking of EDC and NHS. First, 2.0 mg of Fe_3O_4/SF particles was dispersed in the solution of 0.1 M EDC-NHS for 12 min. Then, the suspension was mixed with papain solution and incubated for the immobilization at 25 °C for 12 h. Finally, the Fe_3O_4/SF nanoparticles immobilized with papain (Fe_3O_4/SF-Papain) were separated with an external magnet, rinsed several times with water, and dried at 50 °C under vacuum. The protein concentration of the reaction mixture was measured according to the standard curve of papain. Additionally, the supernatant after each washing step was collected for analyzing unbound enzyme.

Crystal structure of the products above was characterized by X-ray diffraction (XRD), which was recorded on X'Pert-Pro diffractometer (PANalytical, Holland) with Cu Kα radiation in the 2θ range from 10 to 90° at 40 kV and 40 mA. The magnetic properties of the products were characterized by vibrating sample magnetometry (VSM) in an applied magnetic field, sweeping from −20 kOe to 20 kOe at 300 K. Additionally, morphology of the papain immobilized nanoparticles was determined using a field emission scanning electron microscope (SEM, SU8010, Hitachi, Japan).

The papain concentration of the reaction mixture was measured with Bradford method [14]. The papain immobilization yield (Y) and efficiency (E) were calculated as follows [1]:

$$Y\% = \frac{R_0 - R_1}{R_0} \times 100\%; E\% = \left(\frac{A_1}{A_0}\right) \times 100\%.$$

where, R_0 and R_1 are the total papain content and that of supernatant after immobilization, respectively; additionally, A_1 and A_0 is the activity of the immobilized and free enzymes, respectively.

2.4. Enzyme activity assay

Papain activity was determined according to the literature [15]. Typically, papain was added into a substrate solution, containing 2 ml casein aqueous solution (1 mg/ml) in phosphate buffer (pH = 7.0), and immediately stirred for 5 h at 50 °C. Absorbance of the supernatant at 277 nm was then determined by UV–vis spectrophotometer. Experiments were repeated no less than three times.

2.5. Kinetic parameters determination of free and immobilized papain

Kinetic study was performed on selected samples in the substrate concentration range of 0.05 to 1.0% (w/v). The Michaelis constant (K_m) and the maximum reaction rate (V_{max}) were calculated from a Lineweaver-Burk plot for the Michaelis-Menten equation as follows [16,17].

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

where, v is the initial rate of the reaction, [S] is the concentration of the substrate, K_m is the Michaelis constant, and V_{max} is the maximum reaction rate.

3. Results and discussion

3.1. Characterization of papain-immobilized Fe_3O_4/SF nanoparticles

Porous magnetic nanoparticles have been used for the adsorption/purification of biomoleculars due to their unique properties, such as excellent magnetic responsibility, high surface area, good biocompatibility, large enzyme loading capacity, and so on. In this work, porous Fe_3O_4/SF nanoparticles were prepared through a silkfibroin-assisted hydrothermal method. The amount of total amino group on the surface of Fe_3O_4/SF nanoparticles was about 0.16 mmol/g. Papain was covalently immobilized onto Fe_3O_4 nanoparticles which were activated with EDC/NHS. Fig. 1 showed the corresponding characteristics of the prepared papain-immobilized nanocomposites. From Fig. 1b, it could be seen that the papain-immobilized nanocomposites had typical peaks of Fe_3O_4/SF. Furthermore, magnetic property of the prepared Fe_3O_4-papain nanocomposite was characterized by VSM in an applied magnetic field, sweeping from −20 kOe to 20 kOe at 300 K. As showed in Fig. 1c, the prepared Fe_3O_4-papain nanocomposite had typical superparamagnetic property.

3.2. Optimized conditions for immobilization of papain onto porous Fe_3O_4/SF nanoparticles

Papain enzyme was immobilized onto porous Fe_3O_4/SF nanoparticles with the cross-linker EDC through the Schiff base reaction [1,18]. In order to get optimum immobilization yield and efficiency, the effects of several factors were studied. Firstly, the effect of papain/Fe_3O_4/SF nanoparticles ratio on the immobilization was investigated. As shown in Fig. 2a, after immobilizing for 6 h, the highest immobilization yield of 78% and efficiency of 66% were obtained when the papain to Fe_3O_4/SF nanoparticles ratio was 10 mg/g. The possible reason might be attributed to the fact that high papain/Fe_3O_4/SF nanoparticles ratio increased the access chances of papain onto the Fe_3O_4 surface. However, a higher papain/Fe_3O_4/SF nanoparticles ratio would result in the aggregation of papain enzyme onto the Fe_3O_4/SF, hiding some active sites [1]. Therefore, the optimum papain/Fe_3O_4 ratio of immobilization was 10.0 mg/g.

Based on this optimum papain/Fe_3O_4/SF nanoparticles ratio, the proper immobilization time was then determined by detecting the papain content of supernatant at varied time from 30 min to 12 h. Fig. 2b indicated that the immobilization yield reached 62% after 1 h, and then steadily increased to 82% when the immobilization time was increased to 10 h. However, the efficiency showed the maximal value of 82% at 1 h, and then decreased subsequently. Therefore, the optimum immobilization time was 1 h.

Finally, the effect of pH on the immobilization of papain onto Fe_3O_4/SF was also studied according to the immobilization yield and efficiency under different pH conditions (Fig. 2c), showing the maximal immobilization yield of 89% at pH 4.0, and highest immobilization efficiency of 65% at pH 6.0. The possible reason for this might be the immobilization process. Papain enzyme was firstly adsorbed onto the Fe_3O_4/SF