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Covalent binding of α-chymotrypsin on the magnetic nanogels covered by amino groups

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Abstract

A new aminated carrier—magnetic nanogels covered by amino groups, was obtained by Hoffman degradation of polyacrylamide-coated Fe_3O_4 nanoparticles prepared by photochemical polymerization. α-Chymotrypsin (CT) was covalently bound to the magnetic nanogels by use of 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide and *N*-hydroxysuccinimide at room temperature. Immobilization time, pH value of the reaction mixture and proportion of CT to the magnetic nanogels were investigated to obtain the optimum condition for CT immobilization. The maximal specific activity of the bound CT was determined to be 0.93 U/(mg min), 59.3% of free counterpart. The maximal binding capacity was measured to be 102 mg enzyme/g nanogel. Furthermore, the bound CT exhibited good thermal stability, storage stability and reusability.

Keywords: Magnetic nanogels; Immobilized enzyme; Photochemical polymerization; α-Chymotrypsin

1. Introduction

Enzymes, which are usually used as biocatalysts in biochemical processes, are preferred to chemical catalyst because they are more selective and of higher efficiency. Immobilization is usually considered to be an important technique to enhance stability of enzymes. Actually, the enzyme stability was greatly dependent on the immobilization strategy [1,2,9].

Recently, a large number of nano-scaled carriers have been applied in the enzyme immobilization [4–10], and enzyme catalytic biotechnology is explored for its potential application. Enzyme stability is maximized with nano-scaled supports. However, recovery of the nano-scaled immobilized enzymes from the reaction mixture remains difficult. Considering the facile and fast separation of the magnetic particles, magnetic particles are intensively employed as carriers for enzyme immobilization [9,11–15]. Using nano-scaled magnetic nanoparticles as the supports of immobilized enzymes are endowed with the following advantages: (1) higher specific area to favor the binding capacity, (2) lower transfer resistance to solve diffusion problem, and (3) readily recovery from the reaction mixture and lower operational cost. However, enzyme immobilization on the non-porous magnetic particles still bears some shortcomings, for instance, the stability for immobilized enzymes exposition to proteases, inactivation by exposition to organic interface [1–3] and denaturation by gas bubble generated in a stirred system [16].

In general, enzymes are covalently bound to supports by use of the reactive groups on the supports [9,17–20]. Covalent attachment normally leads to improved enzyme stability, often at the cost of partial deactivation due to the conformational restrictions imposed by the covalent bonding of enzyme residues to the support. Nowadays, functional magnetic microbeads can be produced in a number of ways but usually involves the coating of magnetically susceptible particles with synthetic polymers having reactive sites for the affinity ligands attachment [21–23].

Photochemical polymerization is a facile "green" method to prepare core-shell composites with different functional groups. The possible mechanism has been proposed in our previous work [28]. Properties including particle size and polymeric extent of the magnetic nanogels could be conveniently manipulated by variation of monomer concentration, irradiation time and ratio of magnetite nanoparticles to monomer, etc. The magnetic nanogels could be fast gathered from the reaction system. Most important, the reaction medium is free of initiator and stabilizer.

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Hitherto, the magnetic nanogels with amino or hydroxyl groups have been synthesized successfully [24,25].

In this study, α-chymotrypsin (CT) as model enzyme was covalently bound to the aminated magnetic nanogels by use of 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide (EDC) as coupling agent. The factors that affected the immobilization were investigated to obtain the optimum condition for CT immobilization. Furthermore, the reusability, thermal stability and storage stability of the bound CT were studied.

2. Experimental

2.1. Materials

Acrylamide (AM), *N*,*N*′-methylene-bis-(acrylamide) (MBA), 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS) and α-chymotrypsin (CT) were all of analytic grade and purchased from Shanghai Chemical Reagents Corp. BCA protein assay kit was available from Beyotime Corp. Fe_3O_4 nanoparticles were synthesized by partial reduction method according to Refs. [26,27]. They were about 10 nm in diameter, with a polydispersity index of 0.184.

2.2. Synthesis of polyacrylamide-coated Fe_3O_4 nanoparticles

Polyacrylamide (PAM)-coated Fe_3O_4 nanoparticles were prepared by photochemical polymerization. 0.5 g of AM monomer was dissolved in 120 ml of water, and mixed with 4 ml of 1% MBA solution. The mixture was filtered with 0.45 μm filter before charging into the quartz flak. The reaction system was bubbling nitrogen gas to exclude the air inside the flask for 10 min, and then 2.5 ml of magnetite ferrofluid (8 mg/ml) was added. The reaction system was irradiated under 500 W xenon lamp for 1.5 h. After completion of the synthesis, the resultant nanoparticles were magnetically concentrated and washed several times with water. Finally, the sample was vacuum dried and deposited at desiccator.

2.3. Preparation of magnetic nanogels with amino groups

Twenty milligrams of PAM-coated Fe_3O_4 nanoparticles were dispersed in 100 ml of water and sonicated for 5 min. The mixture was cooled to about −10 °C and then treated by Hoffman degradation. The typical procedures were as follows: a mixture, which consisted of 11.7 ml of sodium hydroxide solution (2.4 wt.%) and 8.6 ml of sodium hypochlorite solution (5.2 wt.%), was cooled at about −10 °C and slowly added into the suspension of the PAM-coated Fe_3O_4 nanoparticles under vigorous stirring and the reaction system was kept at ice-salt bath of −10 °C. After 1.5 h, 58.5 ml aqueous solution of sodium hydroxide (2.4 wt.%), cooled to about −10 °C, was slowly added. The reaction system was kept at ice-salt bath of −10 °C for 0.5 h and then at ice-water bath of 0 °C for 6 h. After completion of Hoffman degradation, the aminated magnetic nanogels were immediately isolated by a magnet and washed several times with distilled water. The aminated magnetic nanogels were dried under vacuum at room temperature.

2.4. Enzyme immobilization

CT was immobilized onto the magnetic nanogels using EDC as coupling agent. The reaction was carried out under different conditions to determine the optimum condition for immobilization, i.e., changes of immobilization time, pH value of the reaction mixture and proportion of CT to the amine-functionalized magnetic nanogels.

For a given pH value of 7.4, EDC·HCl (5 mg) and NHS (6 mg) were dissolved in 3 ml of phosphate buffer solution (50 mM, pH 7.4). And then, the amine-functionalized powder (20 mg) was added into the above mixture. This system was treated by ultrasonic for 10 min at 0 °C. Subsequently, 5 mg of CT was added, and then shaken for 24 h at room temperature. The bound enzyme was collected by an external magnetic field and washed with distilled water for several times. And then, the immobilized enzyme was incubated in PBS (50 mM, pH 7.4) for 30 min. This procedure was repeated for several times until no free enzyme was detected in PBS by BCA protein assay [9]. All the washing solution was pooled and the total protein concentration was measured using BCA protein assay. Finally, the bound enzyme was dispersed in 0.001 M of hydrochloric acid solution. The binding capacity was calculated as:

$$M\,(\text{mg enzyme/g nanogel}) = \left(\frac{m - C_1V_1}{W}\right)$$

where M represented the binding capacity, C_1 and V_1 were the concentration and volume of washing solution after immobilization, respectively, m the weight of enzyme introduced into the immobilization system, and W was the weight of the aminated magnetic nanogels.

2.5. Characterization

The binding of CT on the aminated magnetic nanogels was examined by a Nicolet FT-IR spectrophotometer and a PHILIPS CM120 transmission electron microscopy (TEM). The binding capacity was determined with BCA protein assay kit. A standard curve was constructed with BSA. Polymeric extent was estimated by a simultaneous DTA-TG (Shimadzu, DTG-60M) and DSC apparatus (Shimadzu, DSC-60) by heating the samples from room temperature to 700 °C under N_2 atmosphere at a heating rate of 10 °C min^{-1}.

2.6. Enzyme activity assay

Unit of enzyme activity (U) was defined as: 1 mg of protein will hydrolyze 1.0 μmol of BTEE per min at pH 7.8 at 25 °C. Enzyme activity of the bound CT was determined with a UV–vis spectrophotometer (Shimadzu, Model 1601; Tokyo, Japan).

$$\text{BTEE} + H_2O \xrightarrow{\text{CT}} N\text{-benzoyl-}l\text{-tyrosine} + \text{ethanol}$$

The assay mixture was composed of 1.42 ml of Tris–HCl buffer (80 mM, pH 7.8), 1.4 ml of 1.18 mM BTEE and 0.08 ml

of 2 M $CaCl_2$. After addition of 0.1 ml of enzyme solution, the reaction was carried out at 25 °C for 3 min. The suspension was immediately separated by an external magnetic field of 0.5 T and measured the absorbance of the solution at 256 nm. The specific activity was calculated as follows:

$$\text{specific activity (U/mg min)} = \left(\frac{\Delta A}{0.964 \times \text{Ew} \times 3 \times 3}\right)$$

where ΔA was the absorbance change of the solution at 256 nm, Ew represented the amount of enzyme contained in 0.1 ml of enzyme solution, 0.964 was the molar extinction coefficient of *N*-benzoyl-L-tyrosine at 256 nm.

2.7. Thermal stability measurement

Thermal stabilities of free and bound CT were checked by measuring their residual activities after being incubated for 30 min in the temperature range of 35–85 °C. Thermal stabilities of free and bound CT were also examined by assaying their residual activities after being incubated at 45 °C for a required period. All data used in this formula were averages of duplicated experiments.

2.8. Storage stability

Activities of free and bound CT after storage in the hydrochloric acid solution (pH 3) at 4 or 25 °C were determined by measuring the absorbance at 256 nm. The measurements were performed at intervals of a week within a period of 36 days.

2.9. Reusability assay

The reusability of bound CT was examined by conducting the activity measurement of bound CT at 25 °C at time intervals of 30 min. After each activity measurement, the bound CT was separated magnetically and washed several times with PBS. Then, 1.42 ml of Tris–HCl buffer (80 mM, pH 7.8), 1.4 ml of 1.18 mM BTEE and 0.08 ml of 2 M $CaCl_2$ were added to the bound CT in sequence and the next activity measurement was carried out.

3. Results and discussion

3.1. Preparation of the magnetic nanogels covered by amino groups

In this study, magnetic nanogels containing reactive amino groups were obtained by Hoffman degradation of PAM-coated Fe_3O_4 nanoparticles, prepared by photochemical polymerization with quantum-sized Fe_3O_4 nanoparticles as photoinitiator. Since amino-functionalized magnetic nanogels tended to aggregate, Hoffman degradation was optimized in order to obtain magnetic nanogels with smaller particle size. In the experiment, the solution of sodium hydroxide and sodium hypochlorite was slowly added into the reaction mixture, aiming at minimizing aggregation caused by fierce reaction. The reaction was carried out at low temperature to lower the rate of reaction and prevent the magnetic nanogels against aggregation. On the other hand, side reactions such as hydrolysis of amido groups to form alkylacrylureas and alkylureas and the cleavage of the hydrocarbon main chain would take place during the Hofmann degradation of PAM. These reactions could be effectively prevented if the reaction temperature was low enough [37]. As amino groups tended to be oxidized [36], N_2 was hereby bubbled as protective gas throughout the experiment. It was proved that the magnetic nanogels with amino groups were successfully obtained [27].

The amine-functionalized magnetic nanogels were about 25 nm in hydrodynamic diameter. Combined with the result of conductometric titration and the polymeric extent of PAM-coated Fe_3O_4 nanoparticles (10.8%), the amination degree of the aminated magnetic nanogels was determined to be 74.8%, namely, 74.8% of amido groups converted to amino groups. There were some uneliminated carbonyl groups on the magnetic nanogels, otherwise further Hoffman elimination would destroy surface structure of the magnetic nanogels. Magnetic content of Fe_3O_4 was as high as 80%, which guaranteed that the magnetic nanogels were susceptive to external magnetic field.

3.2. Binding of CT on the magnetic nanogels with amino groups

3.2.1. Immobilization

CT was covalently bound on the amine-functionalized magnetic nanogels *via* carbodiimide activation in the PBS (50 mM, pH 7.4). In this coupling reaction, active ester was formed between CT and NHS, and then reacted with amino groups existing on the magnetic nanogels. The immobilization protocol was as illustrated in Fig. 1.

3.2.2. Confirmation of CT bound to the magnetic nangels with amino groups

The binding of CT onto the magnetic nanogels was confirmed by TEM observation and FT-IR spectrum measurement. In order to keep morphology of the magnetic nanogels, the sample of TEM was freeze dried at liquid nitrogen (−196 °C) and then vacuum dried. As was evident from Fig. 2, CT was layered over the amine-functionalized magnetic nanogels. The bound CT possessed considerable dispersancy. However, aggregation occurred due to interaction between magnetic nanoparticles. Fig. 3 showed the FT-IR spectral characteristics of CT bound to the magnetic nanogels. The peaks of 1637.1 cm^{-1}, 1530.4 cm^{-1} and 1399.4 cm^{-1}, which also existed in the IR spectrum of the bound CT, were the characteristic peaks of CT. Strong absorption band around 578.6 cm^{-1} was ascribed to the Fe–O bond of naked Fe_3O_4. Additionally, the sample of bound CT for FT-IR spectrum measurement was washed with distilled water, and then incubated in PBS until no free enzyme was detected by BCA protein assay before being dried. This procedure guaranteed that no free enzyme was adsorbed on the support. Consequently, the results above demonstrated clearly that CT was bound to the magnetic nanogels successfully.

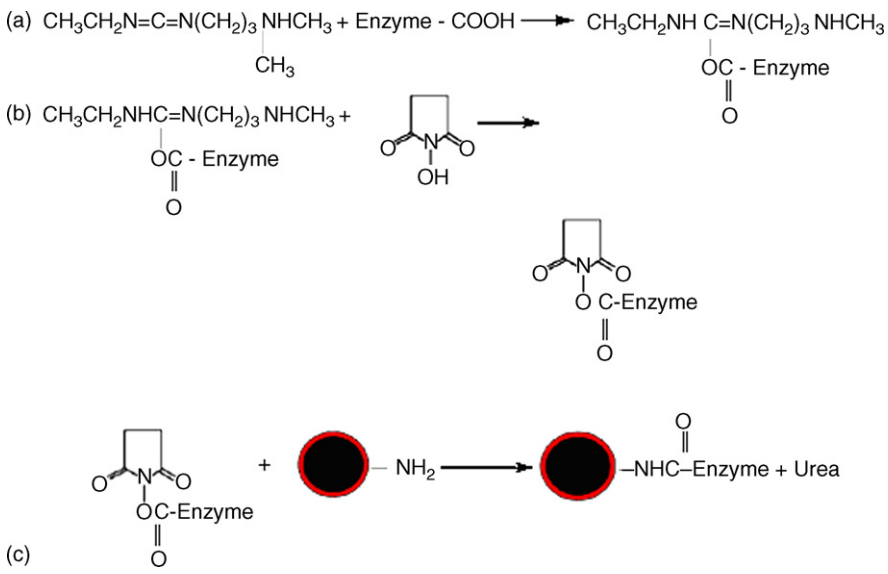

Fig. 1. Schematic presentation of CT immobilization on the magnetic nanogels.

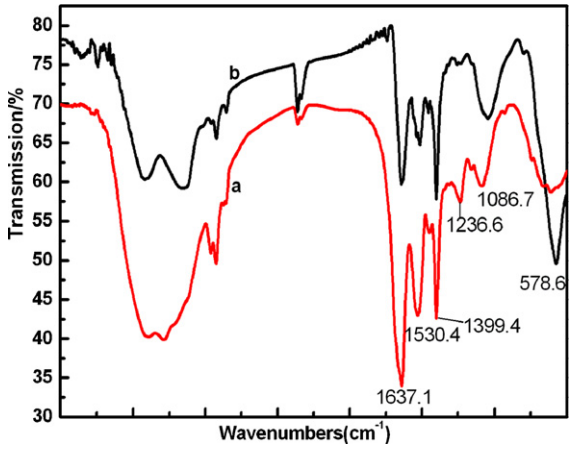

Fig. 2. TEM images of (a) uncoated Fe_3O_4 nanoparticles, (b) magnetic nanogels with amino groups and (c) bound CT.

3.2.3. *Effect of pH value of buffer solution on the immobilization*

It is well known that pH has a crucial importance on the enzymes' properties. For extreme situation (inadequate pH value or long term exposure to medium of inadequate pH), enzymes will permanently loss their activities [32].

Considering hydrolysis of active esters and influence of pH on the activity of enzyme, the coupling reaction was carried out in the pH range of 5.8–8.0. The binding capacity was determined by estimating the enzyme in the washing. As shown in Table 1, when the reaction was performed at pH 8.0, the magnetic nanogels did not show enzyme activity, and no enzyme was detected by BCA protein assay. Therefore, it was reasonable to assume that no enzyme was bound at pH 8.0. At pH 7.4, 24.4% of CT was bound to the magnetic nanogels, while 40.8% of CT was immobilized at pH 5.8. The lower pH value of buffer helped to immobilize enzyme onto the magnetic nanogels. This was in good agreement with the coupling reaction.

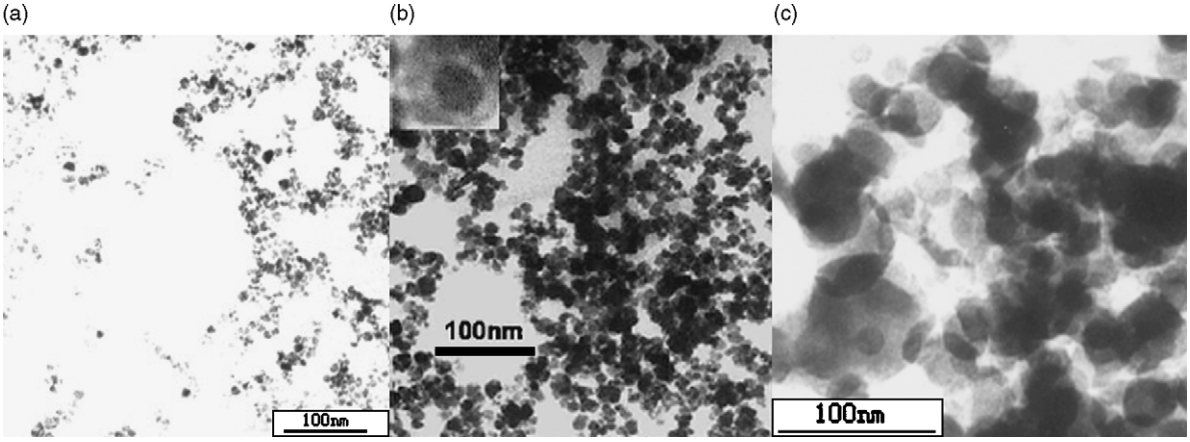

Fig. 3. FT-IR spectra of (a) free CT and (b) bound CT.

Table 1
Effect of pH on the binding of CT on the magnetic nanogels with amino groups in 3 ml of PBS containing 5 mg of CT

pH of buffer solution	Extent of binding (%)	Binding capacity (mg protein/g nanogels)	Specific activity U/(mg min)
5.8	40.8	102	0.47
6.5	38.0	95	0.56
7.4	24.4	61	0.93
8.0	0	0	–

Maximal specific activity was observed when the immobilization was carried out at pH 7.4. Although maximal binding capacity was obtained at pH 5.8, only 29.9% of the specific activity of the enzyme was retained (Table 1). The loss in enzymatic activity might be contributed to the alternations in the properties of the enzyme such as changes in conformation (including the changes caused by pH and immobilization), transfer limitation and so on. The optimum pH for CT binding was observed at pH 6.5, which was in agreement with the results that the coupling reaction carried out at slightly acidic pH increased the percentage binding of enzyme [34].

3.2.4. Effect of ratio of CT to the magnetic nanogels on the immobilization

Effect of ratio of CT to the magnetic nanogels with amino groups was investigated. The reaction was performed in 3 ml of PBS (pH 6.5, 50 mM) for 24 h at room temperature. As anticipated, with the higher proportion of CT to the magnetic nanogels, the binding capacity was higher. However, the increase extent of binding capacity decreased when the ratio was above 0.3 (Fig. 4). It was probably related to the limited amino groups on the surface of magnetic nanogels exposed to active ester existed in the reaction mixture.

3.2.5. Effect of immobilization time on the immobilization

By assaying the unbound enzyme in the washing solution, effect of immobilization time on the binding capacity was studied. As indicated in Fig. 5, the maximal binding capacity was

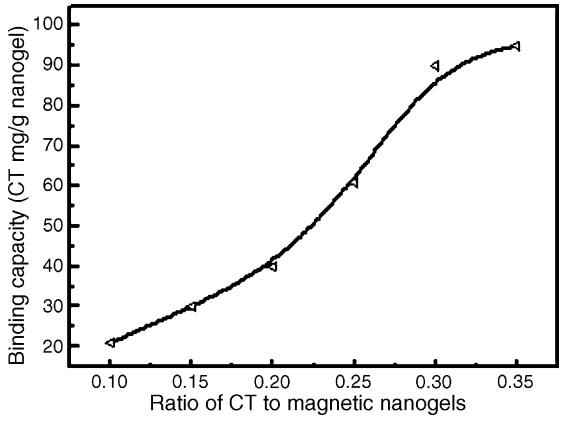

Fig. 4. Effect of ratio of CT to the magnetic nanogels on the binding capacity. The reaction was carried out in 3 ml of PBS (pH 6.5, 50 mM) containing 20 mg of amine-functionalized magnetic nanogels at room temperature.

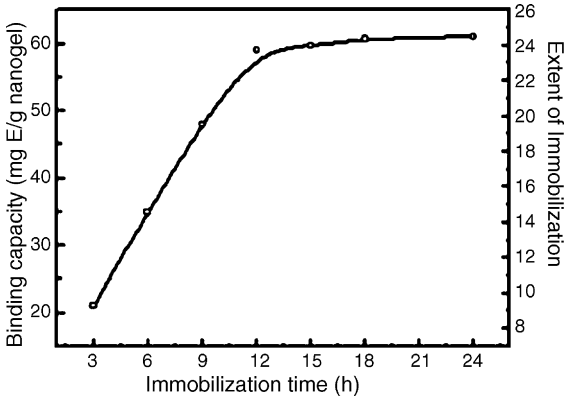

Fig. 5. The time course of CT immobilization on the magnetic nanogels. The immobilization was carried out in 3 ml of PBS (pH 7.4, 50 mM) containing 5 mg of CT and 20 mg of amine-functionalized magnetic nanogels at room temperature.

determined to be 61 mg enzyme/g nanogels; the binding capacity increased with increasing the immobilization time; the extent of CT immobilization on the magnetic nanogels almost kept the same when the immobilization time was above 12 h. This might be related to hydrolysis of active ester in the aqueous solution. With the immobilization time prolonged, the residual active ester in the reaction mixture for enzyme immobilization decreased. Therefore, the binding capacity nearly kept the same when the immobilization time was above 12 h.

3.3. Properties of the bound CT

3.3.1. Thermal stability

Thermal stability of immobilized enzyme and free enzyme preparations was checked by measuring their residual activities after predetermined thermal inactivation. As seen from Fig. 6a, almost no activity was retained for free enzyme when incubation temperature was above 75 °C. However, the activity of the bound CT still had a residual activity of 88.7% at 85 °C. Furthermore, residual activity of the immobilized enzyme after an incubation period of 4.5 h at 45 °C was as high as 92.5% (Fig. 6b), which was higher than that of the free enzyme (85.6%). This resulted suggested that thermostability of CT became higher than that of free CT at high temperature, in good agreement with the results previously reported [29–31]. This might be due to the covalently bound enzyme being protected from conformational changes caused by heat.

3.3.2. Storage stability

In order to investigate the industrial practicability of an immobilized enzyme, the loss in enzyme activity, known as storage stability, is an important parameter to be taken into account. As presented in Fig. 7, free CT stored at 25 °C lost its all-initial activity within 22 days, while free enzyme stored at 4 °C retained 83.5% of its activity after a storage period of 36 days. The immobilized enzyme stored at 4 °C nearly kept their all-initial activity, yet the immobilized enzyme stored at 25 °C only lost about 10% of its activity after a 36-day storage. The covalent immobilization definitely held the enzyme in a stable position in comparison

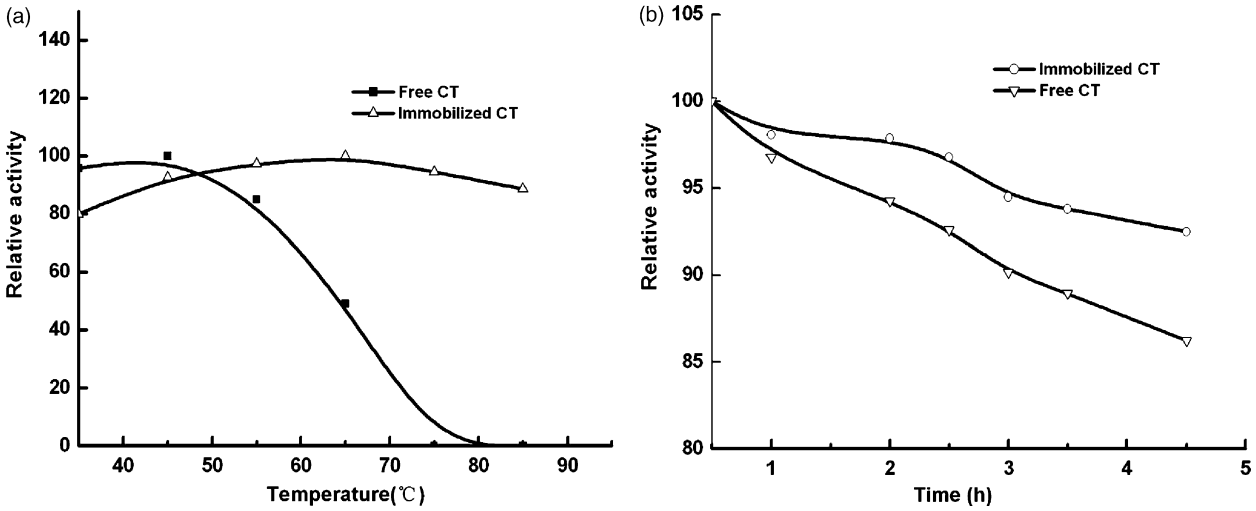

Fig. 6. Thermal stabilities of free and immobilized CT (a) incubated in the temperature range of 35–85 °C for 30 min and (b) incubated at 45 °C.

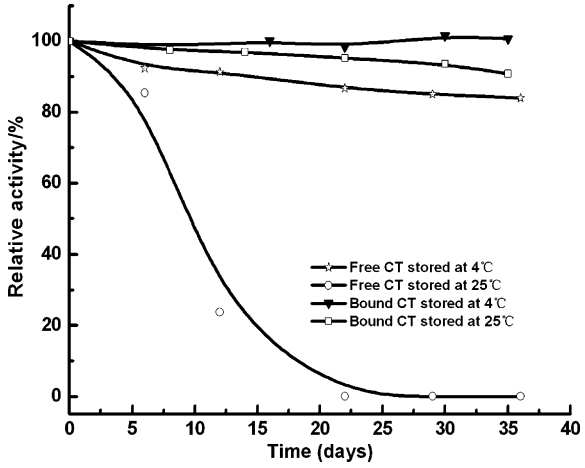

Fig. 7. Storage stabilities of free and bound CT.

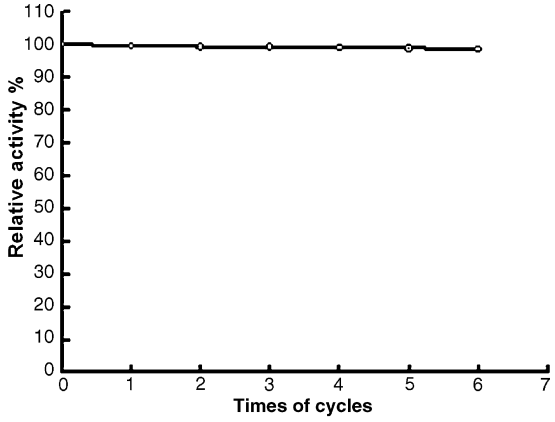

Fig. 8. Reusability of bound CT.

to the free counterpart [33]. On the other hand, hydrophobic group containing supports minimized possible distortion effects imposed from aqueous medium on the active site of the immobilized enzyme [34,35]. The experiment revealed that storage stability of the bound CT was improved in comparison to free CT.

3.3.3. Reusability

Reusability of immobilized enzymes was important for their practical application. As shown in Fig. 8, the activity of bound CT had no significant loss after being reused six times within 2 h. This indicated the resultant bound CT had excellent reusability, which was desirable for applications in biotechnology.

4. Conclusions

The magnetic nanogels, covered by hydrophilic coating layer containing reactive amino groups were obtained by Hoffman degradation of PAM-coated Fe_3O_4 nanoparticles prepared by photochemical polymerization. CT was covalently bound to the magnetic nanogels *via* carbodiimide activation at room temperature. The optimal conditions for enzyme immobilization depended on the immobilization time, the pH of the reaction mixture and the ratio of enzyme to magnetic nanogels. Lower pH value of buffer helped to immobilize enzyme onto the magnetic nanogels. Meanwhile, more loss in activity of the bound CT was observed. Higher proportion of CT to the magnetic nanogels helped to enhance the binding capacity. With increasing the immobilization time, the binding capacity increased, and the extent of CT immobilization on the magnetic nanogels almost kept the same when the immobilization time was above 12 h. After being bound, CT had a maximal residual activity of 59.3% of the free enzyme, and exhibited good thermal stability, storage stability and reusability.

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