# Stability and Activity of Chymotrypsin Immobilized on Magnetic Nanogels Covered with Carboxyl Groups

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**ABSTRACT:** α-chymotrypsin as model enzyme was anchored to the carboxyl-functionalized magnetic nanogels prepared by *in situ* photochemical polymerization. Furthermore, to explore the optimum immobilization, the effects of immobilization time, pH of the reaction mixture, and proportion of enzyme to the magnetic nanogels were studied. The immobilized enzyme was stable in the presence of enzyme denaturation surfactants, and maintained their activity against protein-digesting enzyme. The immobilized enzyme exhibited hydrolysis activity against the substrate of casein, and cleaved the substrate into small fragments. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 2844–2850, 2009

**Key words:** magnetic nanogel; immobilization; α-chymotrypsin; stabilization; photopolymerization

## INTRODUCTION

Immobilization of enzymes onto solid supports is a well-established area, and holds considerable promising applications in biotechnology. The covalent attachment of enzymes onto solid supports is attractive because the formation of stable linkage can diminish the leakage of enzymes. Furthermore, to produce a covalent bond between enzymes and solid supports, a number of techniques have been proposed for the chemical derivatization of the support surface.[1–5]

The covalent immobilization of enzyme and superparamagnetic behaviors of ultrasmall magnetic nanoparticles allows ready recovery of enzymes from reaction media, and also offers long-term stability for the immobilized enzymes. Especially, nanoparticle could greatly reduce the mass transfer resistance, and hence enhance the catalysis efficiency. As a result, magnetic immobilized enzymes become a hot spot of enzyme research.[6–10] However, the immobilized enzymes still bear some shortcomings. One of the main issues concerning enzyme immobilization is maintaining or eventually enhancing the structural stability of the catalytic macromolecules in view of long-term applications. When enzyme is subjected to the reaction media, its active center is exposed to ambient condition.[11–13] The conformational changes would occur at inadequate condition, accompanying denaturation or permanent loss in activity. To alleviate inactivation during the immobilization process, several approaches have recently been proposed. For noncovalent and site-specific immobilization, monoclonal antibodies, which bind to an enzyme with high affinity without affecting its catalytic activity, were employed.[14] Additionally, porous supports and crosslinking of enzyme were also employed for enzyme immobilization.[15,16]

In our previous work, α-chymotrypsin (CT) was covalently immobilized on the aminated magnetic nanogels.[17] However, the production of magnetic nanogels with reactive amino groups was time-consuming while Hoffman degradation could destroy surface structure of the support. Consequently, magnetic nanogels with carboxyl groups were directly prepared by *in situ* photochemical polymerization without any chemical postsynthesis modification.[18] Herein, CT as model enzyme was covalently bound to the magnetic nanogels with carboxyl groups, and the optimal coupling condition in the immobilization process was explored. The effects of protease and

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surfactants on the stability of enzymes were investigated while the catalytic activity of the immobilized enzyme against protein substrate was measured.

## EXPERIMENTAL

### Materials

Methylacrylic acid (MAA), *N,N′*-Methylene-bis(acrylamide) (MBA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC HCl), and sodium dodecyl sulfate (SDS) were all of analytical grade and bought from Shanghai Chemical Reagents (Shanghai, China). *N*-Benzoyl-L-tyrosine ethyl ester (BTEE) was purchased from Sigma. Casein and trypsin were available from commercial source in China. Other chemicals were of analytical grade and used without further purification. The $Fe_3O_4$ nanoparticles were prepared by partial reduction method, and their diameter was $\sim$ 10 nm with a polydispersity index of 0.084 measured by dynamic light scattering.

### Preparation of carboxyl-functionalized magnetic nanogels

MAA (0.5 mL, 5.9 mmol) and 1% MBA (1 mL, 64.9 μmol) were mixed in 120 mL water, and adjusted to pH 6 with 5*M* NaOH before charging into the quartz flask. The reaction system was bubbled with nitrogen for 10 min. Subsequently, 2 mL magnetofluid (10 mg $Fe_3O_4$ per mL) was dropped into the flask. Thereafter, the mixture was irradiated for 30 min under xenon lamp. In the whole process, the nitrogen was bubbled. At last, the resultant magnetic nanogels were separated with 0.5 T of magnet, and then washed several times with distilled water followed by vacuum-drying, yield, 92%.

### Enzyme immobilization

α-Chymotrypsin (CT) was anchored onto the magnetic nanogels by EDC activation.[19] The reaction was carried out under different conditions to determine the optimum immobilization condition, i.e., the immobilization time, the pH value of reaction system, and the proportion of enzyme to support.

For a given pH of 6.5, EDC HCl (5 mg, 26 μmol) and NHS (7 mg, 61 μmol) were dissolved in 3 mL of phosphate buffer solution (PBS, 50 m*M*, pH 6.5). After that the carboxyl-functionalized powder (20 mg) was added into the above mixture and uniformly dispersed. The reaction was carried out in a water bath of 0°C for 25 min. Thereafter, the magnetic nanogels were gathered by a magnet and washed with PBS (50 m*M*, pH 6.5). The concentrated magnetic nanogels were redispersed in 3 mL of PBS (50 m*M*, pH 6.5), and then 5 mg of CT was added into the immobilization medium. The mixture was shaken for 24 h at ambient temperature. When the reaction was completed, the immobilized enzyme was magnetically collected and washed several times with PBS (50 m*M*, pH 6.5) until no free enzyme was detected in the washing solution by bicinchoninic acid (BCA) protein assay. At last, 1 mL of 10 m*M* ethanol amine was added into the resultant immobilized enzyme, and the reaction was kept for 1 h at ambient temperature. The immobilized enzyme was separated by a magnet and redispersed in pH 3 of hydrochloric acid.

The enzyme loading was determined by measuring the enzyme concentration in the washing solution using BCA protein assay. A calibration curve was established with bovine serum albumin. The enzyme loading was calculated according to the following formula:

$$M\ (\text{mg enzyme/g nanogel}) = (m - C_1V_1)/W \quad (1)$$

where $M$ represented the enzyme loading; $m$ is the amount of enzyme introduced into the immobilization medium; $C_1$ and $V_1$ are the enzyme concentration and the volume of the washing solution, respectively; $W$ is the weight of the magnetic nanogel.

### Activity assay

The unit of enzyme activity (U) was defined as: 1 mg of protein will hydrolyze 1.0 μmol of BTEE per minute at pH 7.8 at 25°C. The activity of the immobilized CT was spectroscopically assayed:

$$\text{BTEE} + \text{H}_2\text{O} \xrightarrow{\text{CT}} \text{Benzoyl-L-Tyrosine} + \text{Ethanol}$$

The assay mixture consisted of 1.42 mL of Tris-HCl buffer (80 m*M*, pH 7.8), 1.4 mL of 1.18 m*M* BTEE, and 0.08 mL of 2*M* $CaCl_2$. After adding 0.1 mL of enzyme solution to the above mixture, the reaction was carried out at 25°C for 3 min. Thereafter, the suspension was immediately separated by a magnet and measured the absorbance increment of the solution at 256 nm. Specific activity was calculated using the following formula:

$$\text{Specific activity [U/mg min]} = \Delta A/(0.964 \times E_w \times 3 \times 3) \quad (2)$$

where $\Delta A$ was the absorbance increment of the solution at 256 nm; $E_w$ represented the amount of enzyme (mg) contained in 0.1 mL of enzyme solution; 0.964 was the molar extinction coefficient (L $mol^{-1}$ $cm^{-1}$) of *N*-Benzoyl-L-Tyrosine at 256 nm.

### Effects of surfactants on the enzyme activity

The effects of the surfactants of sodium dodecyl sulfate (SDS), hexadecyltrimethyl ammonium bromide (CTAB), and Tween 20 on the stability of immobilized CT were investigated. The experiments were conducted in the mixture, which consisted of 1.42 mL of Tris-HCl buffer (80 m*M*, pH 7.8) containing 0.1% surfactants, 1.4 mL of 1.18 m*M* BTEE, and 0.08 mL of 2*M* $CaCl_2$. In the case of SDS, 0.08 mL of 2*M* $CaCl_2$ was replaced by 0.08 mL of water.

### Hydrolysis of casein

Fifty milliliters of casein solution (0.5% (p/v) in 50 m*M* $NH_4HCO_3$) was hydrolyzed with immobilized CT (20 mg) at pH 8.0 at 35°C. The immobilized enzyme was magnetically removed periodically. Herein, the solution was used for determining the degree of hydrolysis.

### Determination of degree of hydrolysis

The DH was determined by the trinitrobenzene sulfonate (TNBS) method described by Adler-Nissen.[20] The typical procedures were depicted as follows: 0.25 mL of a sample (containing 1% SDS) was mixed with 2.0 mL of PBS at pH 8.2 in a testing tube. Two milliliters of 0.1% TNBS solution was added, and then the testing tube was shaken and placed in a water bath of 50°C for 60 min. During incubation of the testing tubes, the water bath must be covered with aluminum foil. Thereafter, 4 mL of 0.1*M* HC1 was added to terminate the reaction, and the testing tube was allowed to stand at room temperature for 30 min before the absorbance was measured against water at 340 nm.

### Analyses of casein hydrolysates by SDS-PAGE

SDS-PAGE analysis of protein fractions from casein hydrolysates was performed according to the discontinuous buffer system method described by Laemmli.[21] The gels consisted of a 5% acrylamide stacking gel in 0.125*M* Tris-HCl and a 15% acrylamide resolving gel in 0.25*M* Tris-HCl. A running buffer of 25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, and pH 8.8 were used. Protein solution was boiled for 5 min in a sample buffer containing 2-β-mercaptoethanol and then $\sim$ 20 μL/track of solutions containing 0.5 mg/mL of proteins were loaded onto the gel. Electrophoresis was performed at a constant voltage of 80 V for stacking gel and 120 V for resolving gel at room temperature around 25°C. Gels were then stained with Coomassie Brilliant Blue G-250 solution and destained in a solution containing 23% ethanol and 10% acetic acid in double distilled water. The gels were subsequently scanned with Quantity one scanner (Bio-Rad). The molecular weight (MW) of proteolytic enzymes was determined by interpolation using a plot of the log MW of standards versus their relative mobility.[22]

### Stability of immobilized preparation in the presence of proteases

Trypsin was used in the degradation test. 0.1 and 0.5 mg/mL of trypsin was contained in the immobilized enzyme solution (7 mg/mL), respectively. The reaction was performed at 4°C. After a predetermined period of the digestion reaction, the suspension was separated by a magnet and washed with PBS several times, followed by dispersing in a hydrochloric acid solution of pH 3. The recovered activity was assayed by the aforementioned method.

## RESULTS AND DISCUSSION

### Properties of magnetic nanogels covered with carboxyl groups

Polymer-covering magnetic nanogels containing carboxyl groups were obtained by *in situ* photochemical polymerization with quantum-sized $Fe_3O_4$ nanoparticles as photoinitiator.[23,24] In this experiment, xenon lamp was used as an irradiation source to trigger polymerization of monomer on the surface of $Fe_3O_4$ nanoparticles. Pure nitrogen was bubbled as protective gas throughout the synthesis because of the sensitivity of excited free radical to oxygen. It should be noted that vigorous stirring must be avoided because it could enhance the termination rate of polymerization. The particle size and polymeric extent could be tuned by varying the experiment factor, such as irradiation time, irradiation intensity, monomer concentration, etc. The polymer coating containing carboxyl groups on the surface of superparamagnetic $Fe_3O_4$ nanoparticles had been proved in our former work.[25]

The resultant magnetic nanogels were measured to be 38 nm by dynamic light scattering while the polymer content was determined to be 6.7% by thermogravimetric analysis. Furthermore, the magnetic content of $Fe_3O_4$ was calculated to be 84.9% in dried state. In addition, the crystalline structure of the carboxyl-functionalized superparamagnetic nanogels was identical to that of the freshly prepared $Fe_3O_4$. It indicated that xenon lamp irradiation did not result in the photo erosion of $Fe_3O_4$. The saturation of the magnetic nanogels was calculated to be 61.6 emu $g^{-1}$. Meanwhile, the coercivity and remanence were close to zero, suggesting that superparamagnetic behaviors were retained for $Fe_3O_4$ after surface polymer coating.

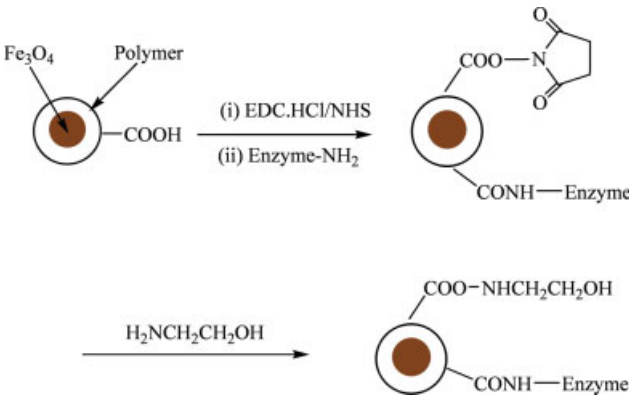


**Figure 1** Schematic illustration of enzyme onto the carboxyl-functionalized magnetic nanogels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Effect of immobilization time on enzyme immobilization

CT was covalently attached to the support by EDC activation.[25] The immobilization protocol was schematically illustrated in Figure 1. By assaying the unbound enzyme in the washing elution, the effect of immobilization time on the binding capacity was studied. As seen in Figure 2, the maximal enzyme loading was determined to be 37.5 mg enzyme/g nanogels. Meanwhile, the enzyme loading increased with an increase of immobilization time, and the amount of CT immobilization onto magnetic nanogels reached a plateau value when the immobilization time was above 9 h. It might be related to the hydrolysis of active ester in the aqueous solution as well as depletion of reactive groups for immobilization. As the immobilization time prolonged, the residual active ester in the reaction mixture for enzyme immobilization decreased. Therefore, the enzyme loading nearly kept the same when the immobilization time was above 9 h. However, the activity of the immobilized preparation decreased with increment of enzyme loading for the immobilized enzyme. It might be correlated with the steric repulsion of enzyme. Consequently, a balance between enzyme loading and enzyme activity should be considered for the immobilized enzyme.

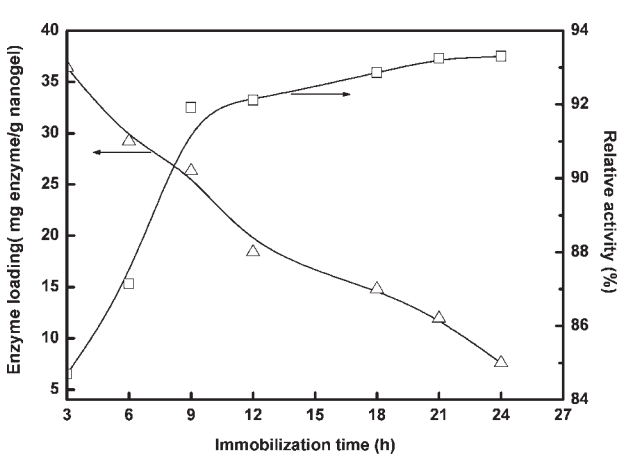


**Figure 2** The time course of CT immobilization on the magnetic nanogels. The immobilization was carried out in 3 mL of PBS (pH 6.5, 50 m*M*) containing 5 mg of CT and 20 mg of EDC-activated magnetic nanogels at room temperature.

### Dependence of pH on enzyme immobilization

The pH condition was a crucial importance to the properties of immobilized enzyme. For extreme situation (inadequate pH or long-term exposure to medium of inadequate pH), the enzymes will permanently loss their activities.[26] Considering the hydrolysis of active esters and the influence of pH on the activity of enzyme, the coupling reaction was carried out in the pH range of 5.8–8.0. As shown in Table I, when the reaction was performed at pH 8.0, the magnetic nanogels did not show enzyme activity. The amount of enzyme in the washing solution, calculated by BCA protein assay, was comparative to the free enzyme joined into the reaction. As a result, it could be concluded that no enzyme was bound to magnetic nanogels in the case of pH 8.0. Only 7.4% CT was coupled at pH 7.4 while the largest extent of enzyme binding (19.3%) was achieved at pH 5.8. The buffer with lower pH value helped to immobilize enzyme onto the magnetic nanogels.

Although maximal enzyme loading was obtained at pH 5.8, only 22.4% of the offered activity was retained (Table I). The maximal remaining activity for enzyme was observed when the coupling reaction was performed at pH 7.4. The loss in enzymatic activity might arise from the alternations in the properties of enzyme, such as conformational distortion, mass transfer resistance, etc. The optimum pH for CT binding was observed at pH 6.5, the enzyme loading and activity for the immobilized enzyme could enhance the catalysis efficiency in practical applications, and especially in large-scaled production. In addition, the leaking experiment revealed that no enzyme leaked out from the support. The

**TABLE I**
**Effect of pH on the Coupling of CT to the Magnetic Nanogels with Carboxyl Groups in 3 mL of PBS Containing 5 mg of CT**

| pH of buffer | Extent of binding (%) | Enzyme loading (mg enzyme/ g nanogel) | Residual activity (%) |
|---|---|---|---|
| 5.8 | 19.3 | 48.3 | 22.4 |
| 6.5 | 15.0 | 37.5 | 82.5 |
| 7.4 | 7.4 | 18.4 | 93.0 |
| 8.0 | 0 | 0 | – |

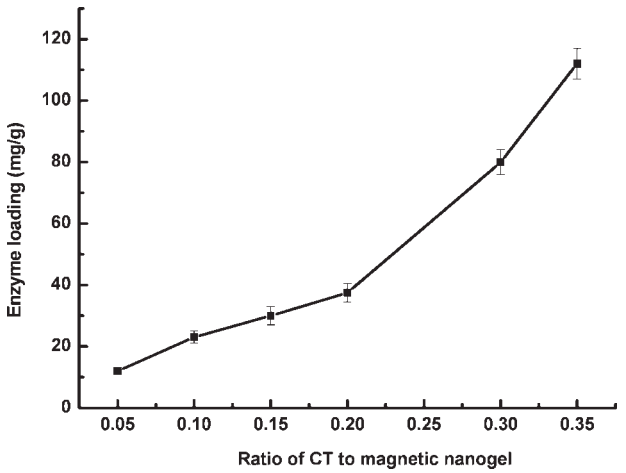


**Figure 3** Effect of ratio of CT to the magnetic nanogels on the enzyme loading. The reaction was carried out in 3 mL of PBS (pH 6.5, 50 m*M*) containing 20 mg of magnetic nanogels at room temperature.

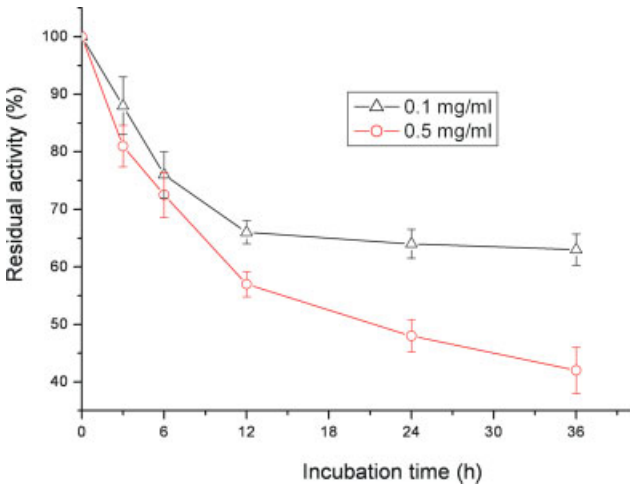


**Figure 4** Stability of immobilized CT against trypsin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

multipoint covalent immobilization held the enzyme in a stable position.

### Ratio of enzyme to magnetic nanogels

Effect of the proportion of CT to the magnetic nanogels on the enzyme loading was investigated. The reaction was performed in 3 mL of PBS (pH 6.5, 50 m*M*) for 24 h at ambient temperature. As expected, when the ratio of CT to the magnetic nanogels was higher, the enzyme loading was higher (Fig. 3), which suggested that the immobilization should also be carried out at higher ratio of enzyme to magnetic nanogels. However, when the ratio was above 0.3, the viscosity of the reaction mixture was greatly enhanced with the increase of enzyme concentration. At this time, a part of the magnetic nanogels or enzyme was conglutinated onto the inner surface of the cuvette. It was difficult to accurately calculate the enzyme loading after the coupling reaction completed. As a result, we select the ratio of 0.25 for the immobilization process of enzyme.

### Effects of surfactants on the enzyme activity

Cationic surfactant CTAB, anionic surfactant SDS, and nonionic surfactants Tween 20 were chosen for studying the effects of surfactants on the stability of free and immobilized enzyme. As depicted in Table II, the immobilized enzyme preparation expressed 93.1% of the offered activity in the presence of 1% of SDS, while the free enzyme just remained 50.8% of activity. In the case of CTAB, the immobilized enzyme had a 1.5-fold of original activity, while free enzyme retained 71.0% of original activity. For Tween 20, the free enzyme nearly kept its initial activity, yet activity of the immobilized enzyme increased to 150.1%. The change in activity for free enzyme and immobilized enzyme might be the outcome of the enhanced stability for enzyme nanoparticles against aggregation. On the other hand, the interaction between surfactants and active site of enzyme should be considered. They would be able to shift the lipase conformation equilibrium toward the open form.[27–30] In this sense, surfactants acted as activators for CT.

**TABLE II**
**Effect of Surfactants on the Stability of Free and Immobilized Enzyme**

| Surfactant | Immobilized enzyme (%) | Free enzyme (100%) |
|---|---|---|
| None | 100 | 100 |
| SDS | 93.1 | 50.8 |
| CTAB | 146.8 | 71.0 |
| Tween 20 | 150.1 | 105.8 |

### Stability of immobilized preparation in the presence of proteases

Enzyme stability measurements were conducted in the protease solution, and free CT was used as control. The stability tests for immobilized CT were performed for 0, 3, 6, 12, 24, and 36 h. As seen in Figure 4, the activity of the immobilized enzyme

**TABLE III**
**DH of Casein Hydrolyzed by Immobilized CT**

| Digestion time (h) | DH (%) |
|---|---|
| 1 | 5.3 |
| 3 | 12.3 |
| 5 | 16.8 |
| 10 | 22.7 |

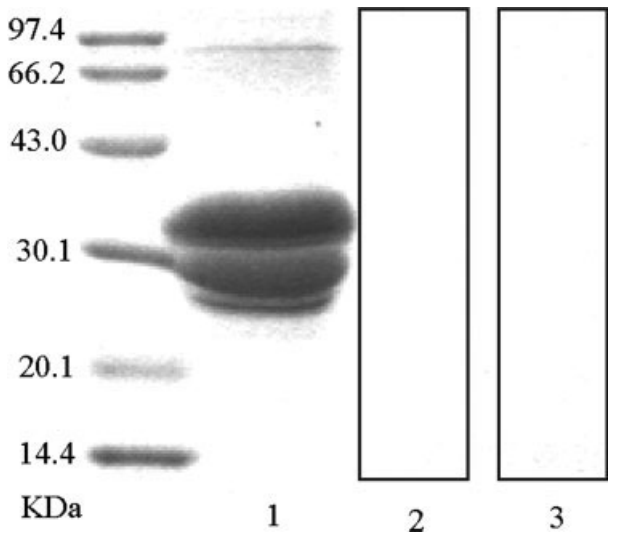


**Figure 5** SDS-PAGE analysis of casein hydroylsate. Lane 1: 0.5% of casein; Lanes 2 and 3: after being digested for 3 and 10 h, respectively.

decreased with the increase of incubation time. The loss in activity might derive from the fact that protein can be cleaved to small fragments (<10 K), which exhibited no enzymatic activity, in the presence of protease. At the same time, free CT nearly lost all initial activity after an incubation period of 6 h. At this time, however, the immobilized enzyme retained 62.8% of initial activity after being incubated in 0.1 mg/mL of trypsin for 36 h, while the immobilized enzyme incubated in 0.5 mg/mL of protease solution just expressed a relative activity of 40.9%. The proteolysis resistance of immobilized enzyme was attributed to multipoint covalent binding of enzyme to the support. Meanwhile, the structural rigidity of enzyme molecules was enhanced for crosslinking, as well as aggregation of immobilized enzyme. It might significantly enhance protein stability in response to temperature, solvent, and other denaturants.

### Hydrolysis of casein

It is well known that α-chymotrypsin can be preferentially hydrolyzed by the following peptide bonds, such as Tyr-Xaa, Trp-Xaa, Phe-Xaa, and Leu-Xaa in carboxyl-terminal position, resulting in the generation of small fragments with amino groups. During each hydrolysis, samples were drawn at regular intervals, and carried out the DH determination according to the procedure described in Experimental section. As shown in Table III, DH of the casein continuously increased with the hydrolysis proceeding. After a digestion period of 10 h, the DH value reached 22.7%. SDS-PAGE electrophoresis was also conducted to corroborate the activity of the immobilized enzyme against casein. Herein, we chose the casein hydrolysates, and digested by the immobilized enzyme for 3 and 10 h, respectively, for electrophoresis measurement. As seen in Figure 5, no hydrolysate was retained in the gel for the two samples above (Lanes 2 and 3). It indicated that casein was successfully cleaved into small fragments by the immobilized enzyme, namely the immobilized CT exhibited enzymatic activity against casein.

## CONCLUSIONS

α-chymotrypsin (CT) was covalently bound to the magnetic nanogels with carboxyl groups by use of EDC/NHS. The covalent immobilization held the enzyme in a stable position, namely enzyme leaking out from the support was not observed for the immobilized enzyme preparation. It was worth noting that the immobilized CT maintained their activity in the presence of surfactants. It was attributed to the structural rigidity of the immobilized enzyme, which provided stability against protein-digesting enzyme. Furthermore, the immobilized enzyme exhibited activity against casein and successfully cleaved the protein to small fragments.

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