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Preparation and Characterization of Nanoparticles Containing Trypsin Based on Hydrophobically Modified Chitosan

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Trypsin was immobilized on linolenic acid modified chitosan using glutaraldehyde (GA) as cross-linker, which was confirmed by Fourier transform infrared (FTIR) spectra. The chitosan nanoparticles containing trypsin (TR) can be prepared after the sonication of immobilized trypsin. The GA concentration affected both the enzyme activity of the nanoparticle and particle size. Results indicated that the activity of trypsin immobilized onto linolenic acid modified chitosan nanoparticles increased with increasing concentration of GA up to 0.07% (v/v) and then decreased with increasing amount of GA. On the other hand, particle size increased (from 523 to 1372 nm) with the increasing concentration of GA (from 0.03 to 0.1% v/v). The enzyme catalytic characteristics of nanoparticle solution were also studied. The results showed that the kinetic constant value (K_m) of TR immobilized on nanoparticle (71.9 mg/mL) was higher than that of pure TR (50.2 mg/mL). However, the thermal stability and optimum temperature of TR immobilized on nanoparticles improved, which make it more attractive in the application aspect.

KEYWORDS: Chitosan; nanoparticles; trypsin; hydrophobically modified chitosan

INTRODUCTION

The immobilization of enzyme onto the nanoparticle has drawn considerable interest, as it expands the range of potential applications that include nanoscale biosensors and biocatalysts (*1*). In particular, biodegradable polymer nanoparticles having enzyme are expected to be novel diagnostic or therapeutic agents. The developments of nanoparticles made of poly(lactide-*co*-glycolide) (PLGA) and polyisobutylcyanoacrylate (PIBCA) for encapsulation of enzyme offer great promise (*2*, *3*). However, the elaboration of these systems requires the use of aggressive conditions (organic solvents, etc.) that compromise the stability of the encapsulated molecule (*4*). A challenging alternative to these hydrophobic particles is polysaccharide colloidal carriers, among which chitosan has come to be a particularly interesting polymer for the loading and delivery of labile macromolecular compounds (*5*).

Chitosan is a naturally occurring biopolymer made up of β-(1,4)-linked glucosamine units. It is produced by deacetylation of chitin extracted from shells of crabs, shrimps, and krills (*6*, *7*). It has been shown that chitosan is nontoxic and soft compatible in the range of toxicity tests. It has been widely used in the development of chitosan drug control releasing systems including chitosan film, chitosan sponge (*8*−*11*), chitosan bead (*12*), chitosan microbead (microsphere) (*13*), chitosan nanoparticle, etc. (*14*). Calvo et al. first developed the nanoparticles using a tripolyphosphate (TPP) cross-linking method, which were used as protein carrier (*15*). This formulation was evaluated as a carrier for therapeutic peptides such as insulin, vaccine, and proteins (*16*−*18*). In addition, nanopartilces based on poly(ethylene glycol) (PEG)-modified chitosan, which was used as insulin carrier, have been produced. There are no reports about chitosan nanoparticles using enzyme as carriers. In our previous work, chitosan was modified by linolenic acid (*19*), which can form nanoparticles in pH 7.4 phosphate-buffered saline (PBS) buffer after sonication and can also be used as enzyme carrier. On the other hand, chitosan is well-known as an ideal support for enzyme immobilization because of its many features such as hydrophilicity, biocompatibility, biodegradability, and antibacterial properties (*20*, *21*). The presence of amino groups facilitates covalent binding of enzymes by using glutaraldehyde (GA) by formation of the Schiff base (*22*, *23*).

The objective of this research is to prepare nanosized chitosan particles containing trypsin. The nanoparticles were prepared based on trypsin immobilized hydrophobically modified chitosan. Transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR) were used to determine the physicochemical structure of the particle, and laser doppler anemometry was used to determine the size of nanoparticles. Specific activities of trypsin-immobilized nanoparticles were

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also measured and compared with that of free trypsin (TR aqueous solution)

MATERIALS AND METHODS

Materials. Chitosan (low molecular weight) was from Aldrich. Glutaraldehyde was from Showa Chemical Co. Ltd.. Linolenic acids, trypsin, and 1-ethyl-3-(3-dimethylaminopropyyl)carbodiimide (EDC) were purchased from Sigma Chemicals. All other chemicals used in this study were of analytical grade.

Preparation of Linolenic Acid (LA)−Chitosan. LA was coupled to chitosan by the formation of amide linkages through the EDC-mediated reaction following the method of Liu et al. (*19*). Chitosan (1 g) was dissolved in 1% (w/v) aqueous acetic acid solution (100 mL) and diluted with 85 mL of methanol. LA was added to the chitosan solution at 0.54 mol/mol glucosamine residue of chitosan followed by a dropwise addition of 15 mL of EDC methanol solution (0.07 g/L) with stirring at room temperature. A 1:1 mole ratio of EDC to LA was used in this study. After 24 h, the reaction mixture was poured into 200 mL of methanol/ammonia solution (7:3, v/v) with stirring. The precipitated material was filtered, washed with distilled water, methanol, and ether, and then dried under vacuum for 24 h at room temperature.

Immobilization of TR. LA-modified chitosan (LACS)−glutaraldehyde (GA) was prepared according to the method of Abdel-Naby et al. (*24*). For the activation of LACS, 0.1 mL GA solution of concentration varying from 3 to 10% (v/v) in PBS buffer (pH 7.4) was added to 10 mL of 1% acetic acid solution that contained 10 mg of LACS and gently stirred for 2 h at room temperature. The solubilized chitosan was precipitated by the addition 1 M NaOH and the solution adjusted to pH 7.4. The precipitate was collected by centrifugation at ∼3000*g* for 30 min at 25 °C. The excess amount of GA was washed off with water until extinction at 220 and 280 nm approached zero and equilibrated with PBS buffer (pH 7.4). Wet activated LACS carrier was suspended into 10 mL of 0.5 mg/mL TR solution for cross-linking. The mixture was stirred at room temperature for 1 h and kept in a refrigerator at 4 °C overnight. The immobilized enzyme was collected by centrifugation and washed three times with distilled water until extinction at 280 nm approached zero.

Preparation of LACS Nanoparticles Containing TR. Ten milligrams of immobilized TR was suspended in 10 mL of PBS buffer (pH 7.4), and the suspension was sonicated using a probe-type sonifier (Utralsonic homogenizer UH-600) at 20 W in an ice bath. The sonication was repeated twice to get an optically clear solution using pulse function (pulse on, 10.0 s; pulse off, 2.0 s). The clear solution of nanoparticles was filtered through a filter (Whatman) to remove unwanted particles.

FTIR Spectroscopy. The IR spectrum of sample was recorded on a Fourier transform infrared spectrometer 430 (Jasco Co., Tokyo, Japan) at room temperature following the method of Shigemasa et al. (*25*). About 2 mg of various samples was mixed with 100 mg of KBr, and prepared pellets were used for studies.

Particle Size Distribution. The average particle size and size distribution were determined by quasielastic laser light scattering with a Malvern Zetasizer (Malvern Instruments Limited) (*26*). Nanoparticle distilled water solution of 3 mL (1 mg/mL) was put into a polystyrene latex cell and measured at a detector angle of 90°, a wavelength of 633 nm, a refractive index of 1.33, a real refractive index of 1.59, and a temperature of 25 °C.

TEM. Specimens were prepared by dropping the sample solution onto a copper grid. The grid was held horizontally for 20 s to allow the molecular aggregates to settle and then at 45° to allow excess fluid to drain for 10 s. The grid was returned to the horizontal position, and one drop of 2% phosphotungstic acid was added to give a negative stain (*27*). The grid was then allowed to stand for 30 s−1 min before excess staining solution was removed by draining as above. The specimens were air-dried and examined using a Philips EM 400 transmission electron microscope at an accelerating voltage of 80.

Enzyme Activity Determination. Activities of immobilized and native enzyme preparations were determined in the presence of 0.5 wt % casein in 0.1 mole/L phosphate buffer (pH 7.4) at 37 °C for 20 min, according to the Kunitzs method (*28*). After 20 min, the reaction sample (2 mL) was added to 3 mL of 5% (w/v) trichloroacetic acid solution. After standing at room temperature for 30 min, the solution was centrifuged at 3000*g* for 20 min. The supernatant was transferred to a quartz cuvette, and the absorbance was measured at 280 nm using an HP UV−visible spectrophotometer. The amount of enzyme was calculated in enzyme units. (One unit is defined as the amount of enzyme that produces an ΔA_{280nm} of 0.001/min at pH 7.4 and 37 °C.)

The thermal stability of TR and TR immobilized on LACS nanoparticles was determined by incubation ranging from 20 to 300 min at a temperature of 50 °C. After the incubation at the given time, the preparation was left for 1 h at room temperature before enzyme activity was determined. The optimum temperature of the TR immobilized on LACS nanoparticles was determined under different temperatures (from 25 to 65 °C). The activity obtained at 37 °C was assigned a value of 100%.

Statistical Analysis. The assays were performed at least in triplicate on separate occasions. The data collected in this study are expressed as the mean value ± standard deviation (SD).

RESULTS AND DISCUSSION

FTIR Spectroscopic Study. The structure changes of chitosan after grafting LA with GA or activation with GA were examined by IR spectrometry as shown in **Figure 1**. Curves **a**, **b**, **c**, and **d** are the FTIR spectra of the chitosan (CS), linolenic acid modified chitosan (LACS), glutaraldehyde-activated LACS (LACS-GA), and trypsin-immobilized LACS (LACS-TR), respectively. The characteristic absorption peaks of chitosan were observed at 3000−3800 cm^{-1} (OH, NH_2), 2867 cm^{-1}(−CH stretching), 1656 cm^{-1} (−CONH amide band II), 1554 cm^{-1}(−NH amide band II), 1322 cm^{-1}(−NH amide band III), and 1107 cm^{-1}(C−O stretching vibration). After grafting LA, the absorption of amide band I of chitosan at 1656 cm^{-1} increased, which is due to the formation of an amide linkage between the amide group of chitosan and the carboxyl group of LA (**Figure 1b**). The characteristic absorption peaks of GA activation of LACS were observed at 2936 cm^{-1} (C−H stretching vibration), 1665 cm^{-1} (imine bond), and 1552 cm^{-1} (ethylenic bond), which are due to the reaction between free $-NH_2$ groups of the hydrophobic chitosan and the −CHO groups of GA (**Figure 1c**) (*29*). In **Figure 1d**, the absorption around 3300−3500 cm^{-1} (N−H stretching) increases and two new absorption bands appear at 1664 and 1534 cm^{-1}, which originate from amide band I and amide band II of TR (*30*). These verify the linkage between carrier and TR. FTIR spectra confirm reactions that are shown in **Figure 2**. EDC is a "zero-length" cross-linker, which brings about the branches of an amide linkage between the carboxyl group of linolenic acid and the amino group of chitosan without leaving a spacer molecule (*23*, *31*). The reaction includes two stages: first, the activation of the carboxyl group, leading to the formation of the activated derivative *O*-acyl-isourea; second, the formation of an amide bond with elimination of urea that acts as a thermodynamic drive force (*32*) (**Figure 2a**). GA is capable of reacting via the aldehyde groups with the nucleophilic groups such as the amino groups present on the surface of the enzyme and chitosan derivative, thus forming Schiff bases and Michael adducts, between enzyme−enzyme and enzyme−support bonds, thus enhancing adsorption interaction (*33*) (**Figure 2b**).

Effects of GA Concentration on the Activity of TR Immobilized on LACS Nanoparticles and the Size of Nanoparticles. The activity of TR immobilized on LACS nanoparticles was 7 (IU) when GA concentration was 0.03% v/v and increased with increasing concentration of GA up to a maximum (13.3 IU) at 0.07%. Furthermore, the activity dropped when high GA concentration (from 0.09 to 0.1% v/v) was used (**Figure 3**). At low concentrations of GA, it is probable that

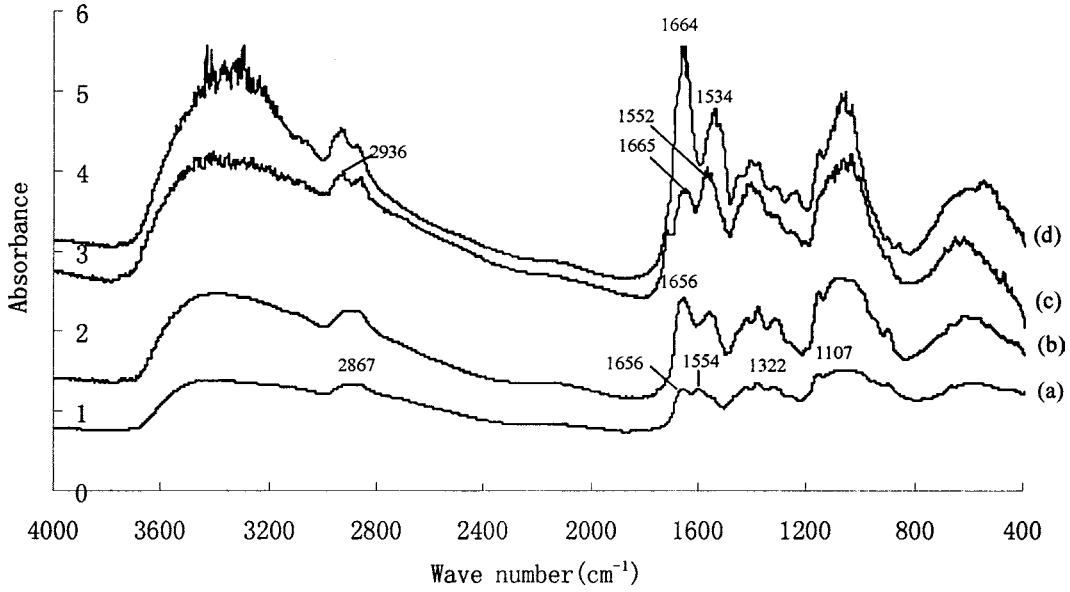

Figure 1. FTIR spectra of (**a**) chitosan (CS), (**b**) linolenic acid modified chitosan (LACS), (**c**) glutaraldehyde-activated LACS (LACS-GA), and (**d**) trypsin-immobilized LACS (LACS-TR).

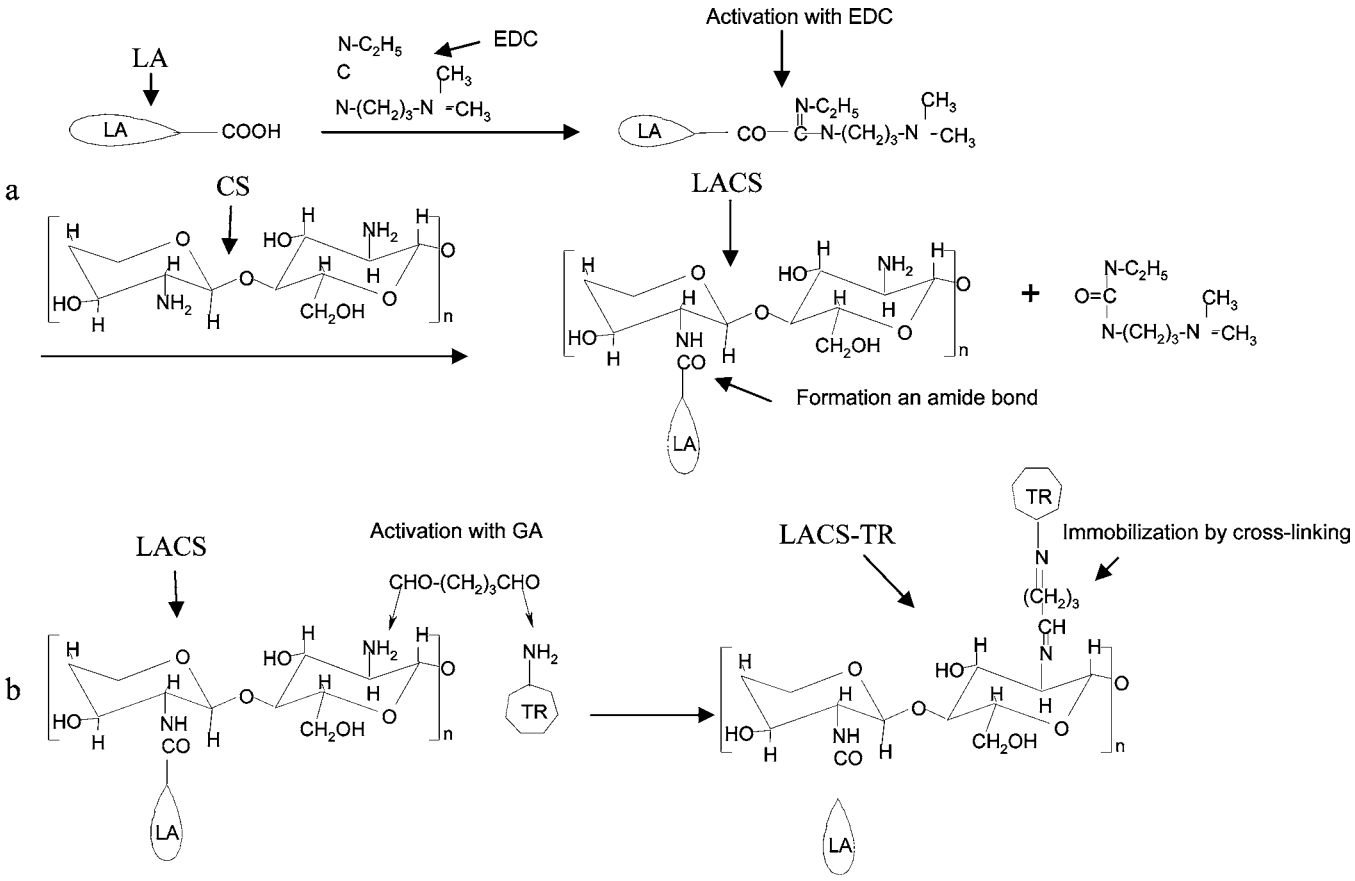

Figure 2. Schematic illustration of linolenic acid (LA) modification of chitosan with EDC (**a**) and immobilization of trypsin (TR) with GA (**b**).

the aldehyde groups present on the support cross-link the amine groups of the chitosan, and the few reversible bonds (Schiff bases) involving the enzyme molecules are not sufficient to avoid their leakage (*28*). At high concentration of GA, the formation of high-density enzyme-support bonds may cause partial structural change during the immobilization. At the intermediate concentration of GA the lower density of bond per enzyme molecule and its high amount adsorbed properly give a less distorted conformation (*34*).

On the other hand, **Figure 4** shows that the particle size increased with increasing GA concentration. At the low concentration of GA, the average particle size was 523 nm; when GA concentration was >0.09%, the average particle size was significantly increased more than 1000 nm. This may due to stiffness of the cross-linked chitosan gel increasing with increasing amount of GA and a dense three-dimensional network structure might have been formed, which leads to be hardly formed nanoparticles after sonication. Furthermore, the prob-

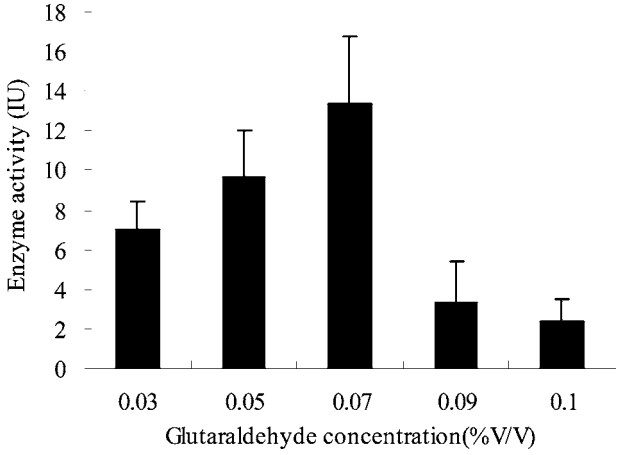

Figure 3. Effect of GA on casein hydrolysis activities of TR immobilized on LACS nanoparticle.

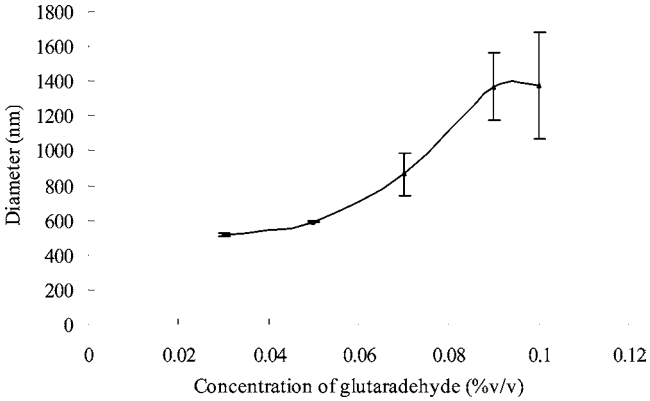

Figure 4. Size of LACS nanoparticles containing TR variation in relation to glutaraldehyde concentration.

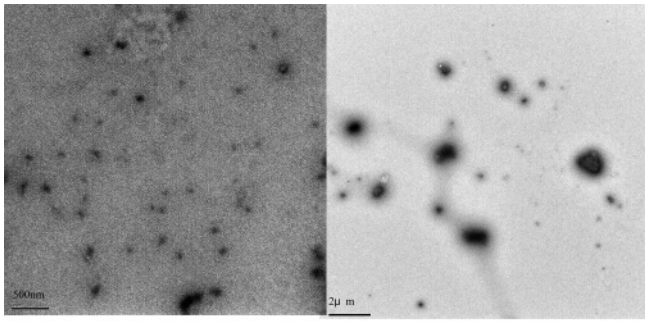

Figure 5. Transmission electron micrographs of LACS nanoparticles containing TR: (**a**) particles were prepared as GA final concentration was 0.03% (v/v); (**b**) particles were prepared as GA final concentration was 0.1% (v/v).

ability of interparticle cross-linking through GA also increases with the increased concentration of cross-link agent (*35*).

TEM observations (**Figure 5**) also confirmed this phenomenon. At the low concentration of GA, particles appear as smaller aggregates (**Figure 5a**), whereas at high concentration, much larger particles were observed, which appear as highly dense aggregates (**Figure 5b**). These results are consistent with those from a prior study involving chitosan nanoparticles that were prepared in an AOT/*n*-hexane reverse micellar system cross-linked with GA (*36*).

These results indicate that GA can be used to prepare TR-containing chitosan nanoparticles, but the cross-linker level must

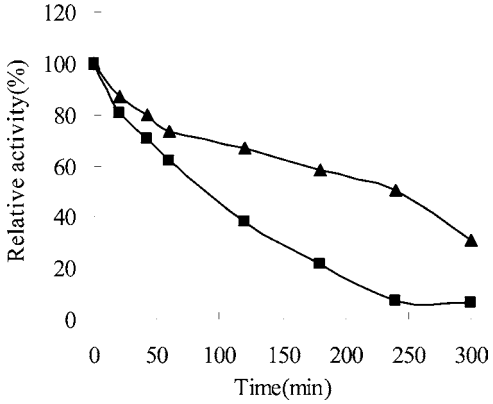

Figure 6. Time dependence of thermal stability of free enzyme (■) and immobilized TR (▲). The free enzyme and immobilized TR were preincubated in PBS buffer (7.4) at 50 °C for different time intervals and assayed under standard conditions.

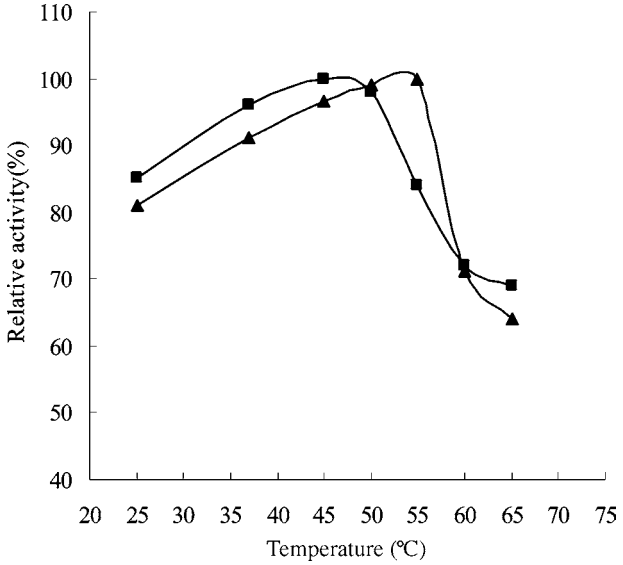

Figure 7. Effect of reaction temperature on free enzyme (■) and immobilized TR (▲). The free enzyme and immobilized TR were preincubated in PBS buffer (7.4) at different temperatures ranging from 25 to 65 °C, maintaining other standard conditions.

be selected to balance the need to retain enzymatic activity with the desire to achieve nanosize particles.

Catalytic Characteristics. To analyze the catalytic activities, TR immobilized on LACS nanoparticles was prepared at a GA final concentration of 0.05% (v/v). **Figure 6** shows relative activities of free TR and TR immobilized on LACS nanoparticles against time. The activity of the immobilized TR decreased slowly with time as compared with that of free TR. We further examined the effect of the temperature on the relative activity of the TR. **Figure 7** shows that compared to free TR, immobilized TR exhibited an increase in the temperature at which hydrolysis of casein was maximal.

The improved thermal stability and the optimum temperature may come from the multipoint attachment of TR with carrier that results in enhanced resistance to unfolding in the thermal treatment (*37*). On the other hand, the cross-linkers are capable of increasing the conformational rigidity of the enzyme and raising the activation energy of the thermal denaturation reaction (*38*).

The Michaelis−Menten kinetics of the hydrolytic activity of the free and immobilized TR was investigated using various

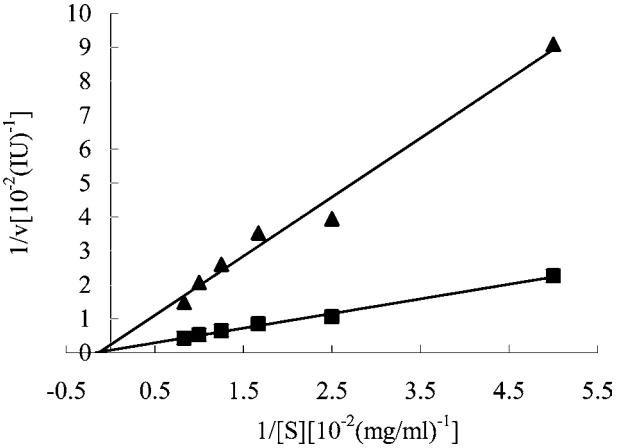

Figure 8. Kinetic parameters of free enzyme (■) and immobilized TR (▲).

initial concentrations of casein as the substrate. The Michaelis constant, K_m, was evaluated from double-reciprocal plots and is shown in **Figure 8**. The calculated value of the kinetic constant K_m of immobilized TR was 71.9 mg/mL, which was higher than that of free TR (50.2 mg/mL). For the enzymes immobilized with GA, the increase in K_m is typical of passage from a free enzyme to an immobilized one (*39*). This increase in K_m upon immobilization on matrix may be due to limitation on mass transfer of the substrate through the matrix (*40*) or steric effect arising from the structural rigidity of the entire enzyme structure, which was distorted after covalent immobilization (*41*). In the case of the TR immobilized on LACS nanoparticles, the increase in K_m may be mainly due to the reduced flexibility of the TR structure during immobilization process because nanosize catalysis composites significantly reduced the mass-transfer limitation compared to other enzyme stabilization methods (*42*).

In summary, trypsin could be immobilized on LACS using GA as cross-linking agent. The immobilized trypsin at certain GA concentrations (from 0.03 to 0.07% v/v) can form nanoparticles after sonication, which still have catalytic activities. The kinetic constant value (K_m) of TR immobilized on nanoparticles (71.9 mg/mL) was higher than that of pure TR (50.2 mg/mL), indicating that the immobilized process slightly decreased the affinity of trypsin on nanopartilces to substrate (casein). On the other hand, this formation can improve the thermal stability and optimum temperature of trypsin, which make it more attractive in the application aspect.

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