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# Semi-permeable nanocapsules of konjac glucomannan–chitosan for enzyme immobilization

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ABSTRACT

Carboxymethyl konjac glucomannan–chitosan (CKGM–CS) nanocapsules, spontaneously prepared under very mild conditions by electrostatic complexation, were used for immobilizing L-asparaginase. The matrix has semi-permeability to allow the substrate and product to pass through and to keep L-asparaginase in the matrix to prevent leaking. The cell-like hydrogel matrix was prepared in aqueous system without organic solvents and reagents. The process of the preparation does not denature the enzyme and the activity of the immobilized and native enzyme is very similar. The activity, stability, and characters of the enzyme-loaded nanocapsules were studied. The results indicated the immobilized enzyme has better stability and activity in contrast to the native enzyme. These studies may supply a new material for the immobilization of pH and temperature-sensitive enzyme.



## 1. Introduction

Enzyme immobilization has attracted great interest of chemists and biochemists for its wide application in the fields of fundamental academic research and industrial processes (Taylor, 1991). The essence of enzyme immobilization is to attach the enzyme to a support material which will stabilize the enzyme and maintains enzyme activity (Gemeiner, 1992). There are three main methods to immobilize the enzymes, including (a) chemical immobilization of enzyme in which the enzyme is linked to the matrix by a covalent bond, (b) physical absorption on the surface of a carrier and (c) entrapping the enzyme in a semi-permeable supportive material. The chemical immobilization is steady and can increase the stability of the enzyme and reduce the immune reactions. However, the chemical immobilization always results in a large loss of the enzymatic activity because it usually employs organic reagents and rigorous conditions such as severe pH or improper temperatures incompatible with the enzyme (Vina et al., 2001; Zhang et al., 2004). Physical absorption is less disruptive to the enzyme, but enzyme leakage is often observed due to weak bonding. Entrapment enzyme in semi-permeable matrix is an effective approach because it does not require bonding between the enzyme and the matrix. The basic requirement for an entrapment matrix is the semi-permeability, which could prevent the enzyme from leaving while allowing substrates, products, and other co-factors to pass through. Moreover, the matrix should be compatible with the enzymes and prevent immune recognition. The process for entrapment should also be mild enough so as not to denature the enzyme. By far, the number of entrapment materials for enzyme immobilization is very limited. Most encapsulation systems use sodium alginate (Funduenanu et al., 1999; Velten et al., 1999; Taqieddin and Amiji, 2004), chitosan (CS) (Taqieddin and Amiji, 2004), or polylysine (Lee et al., 1993). The objective of this study is to present a novel hydrogel using carboxymethyl konjac glucomannan (CKGM) for enzyme immobilization.

Konjac glucomannan (KGM), one of the high molecular weight water-soluble natural polysaccharides found in tubers of *Amorphophallus konjac*, is composed of β-(1 → 4) linked D-glucose and D-mannose in the molar ratio 1:1.6 to 1:1.69. It is isolated from the tubers of *A. konjac* plant, the main crop in mountainous areas in China and Japan. KGM has long been used as a health food to reduce the risk of developing diabetes and heart disease (Li et al., 2005; Chen et al., 2006). CKGM is an anionic polymer produced by the carboxymethylation of KGM. It has good water solubility, biocompatibility, bioactivity and excellent gelation ability when mixed with a polymer of opposite charge. Because of these properties,

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CKGM has been proved as a promising biomaterial in the drug delivery systems (Nakano et al., 1979; Wu and Shen, 2001; Wang and He, 2002; Du et al., 2004). To the best of our knowledge, this is the first study to date to investigate the application of CKGM in enzyme immobilization. Chitosan is another natural polymer, which is cationic and produced by *N*-deacetylation of chitin, has attracted many attentions in protein drugs, drug delivery and controlled release (Carrara and Rubiolo, 1994; Huguet et al., 1994; Itoyama et al., 1994; Polk et al., 1994; Gaserod et al., 1999; Albarghouthi et al., 2000). It is an aminopolysaccharide and can gelate with CKGM to form nanocapsules due to electrostatic complexation. A chitosan–alginate core–shell structure applied to immobilize β-galactosidase indicates that it is a good biomaterial for the enzyme immobilization (Taqieddin and Amiji, 2004).

L-Asparaginase was chosen as a model enzyme for our experiments as it has been widely used to treat malignant tumors, particularly acute lymphoblastic leukemia (ALL) (Campbell et al., 1967). The growth of malignant tumor cells depends on the ingestion of exogenous L-asparagine in most of the patients with ALL. Tumor cells will languish without ingestion of exogenous L-asparagine whereas normal cells can synthesize L-asparagine themselves. L-Asparaginase can effectively decrease the content of L-asparagine in blood by converting it to L-aspartic acid and ammonia. Although it has high therapeutic efficacy, there are still some problems that exist in the therapeutic field, such as immunological response and side effects (i.e. fever, skin rashes, allergic reactions and even anaphylactic shocks) (Mathe et al., 1970; Jaffe et al., 1973). In order to improve enzyme efficiency and reduce the immune response and toxicity, the native L-asparaginases were often chemically modified or administered by a drug delivery system. Chemical methods suffer from severe reactive conditions and always result in a large loss of enzymatic activity (Holle, 1997; Balcão et al., 2001; Zhang et al., 2004). Polyethylene glycol (PEG) conjugated L-asparaginase named pegaspargase came into commercial use but a correlation of pancreatitis associated with pegaspargase (Alvarez and Zimmerman, 2000) was reported recently. Loading L-asparaginase into a drug delivery system could reduce the side effects and increase the enzyme efficiency (Alpar and Lewis, 1985; Naqi et al., 1988; DeLoach et al., 1990; Kravtzoff et al., 1996). However, immunological response and toxicity still exist when L-asparaginase is released into the blood (Gaspar et al., 1998; Wolf et al., 2003).

Herein we reported a novel CKGM–CS immobilization system which entraps L-asparaginase in semi-permeable CKGM–CS nanocapsules preventing the enzyme from leaking into the blood. In order to better understand the immobilized enzyme, characterizations, including encapsulation efficiency, leakage of the enzyme, particle size, and transmission electron microscopy (TEM) experiments were conducted. The activity and stability of the enzyme under different temperatures and pH conditions were carried out to determine the difference in activity and stability between the native enzyme and the immobilized enzyme.

## 2. Materials and methods

### 2.1. Materials

CS and CKGM, with a viscosity-average molecular weight of 60 and 20 kDa, respectively, were procured from Sichuan Dikang Sci & Tech Pharmaceutical Co. Ltd. (Chengdu, China). CS was refined by initially dissolving in dilute acetic acid solution. Following centrifugation, it was then precipitated with aqueous NaOH, washed with deionized water, and dried at 60 °C under reduced pressure. The degree of deacetylation of CS was 95% as determined a titration method. CKGM was purified by initially dissolving in deionized water. Following centrifugation, it was precipitated with ethanol, and dried at 60 °C under reduced pressure. The degree of substitution of CKGM was 45% as determined by standard methods (Smith, 1967). L-asparaginase from *E. coli* with absolute activity of 1271.3 U/mg (the absolute activity of the enzyme was determined by literature method (Shirfrin et al., 1974)) was purchased from Changzhou Bio-Pharma Qianhong Co., China. Other reagents were analytical pure and used directly without further purified.

### 2.2. Preparation of the nanocapsules, encapsulation procedure

CKGM anionic solutions of different concentrations and CS cationic solutions of different concentrations were prepared according to the particular experiment needed. The L-asparaginases was added to the cationic solutions with a given concentration. The nanocapsules were prepared by dropping the CKGM solution with L-asparaginase into the CS solution through a needle, while sonicating. To ensure the semi-permeability to the L-asparaginase and adducts of the complex matrix, the molecular weights and viscosity of the polymer were screened. The experiments resulted in selecting CS and CKGM with a viscosity-average molecular weight of 60 and 20 kDa, respectively. All of the procedures were carried out at 25 °C unless otherwise noted. Brief procedures: 15 ml of CKGM aqueous solutions (containing 2.0 mg L-asparaginase) in different concentrations [0.01, 0.02, 0.04, 0.06, 0.08, 0.1% (w/v)] were added dropwise into 15 ml of CS aqueous solutions (dissolved in 0.05% (v/v) acetic acid) with different concentrations [0.01, 0.02, 0.04, 0.06, 0.08, 0.1% (w/v)] for 30 min through a needle with an inner diameter 0.3 mm, while sonicating with an ultrasonic sonicator at 160 W. After 5 min sonication to ensure the formation of the capsules and to keep them from sticking together, the capsules were collected by centrifugation at 16,000 rpm (TGL-20M, Saite Centrifuge Co., Shanghai, China). The collected capsules were dispersed by the sonicator at 160 W for 5 min and rinsed with Tris buffer three times (pH 8.6, 4 ml × 3), and then separated by centrifugation at 16,000 rpm. The capsules were kept at −18 °C until use in subsequent experiments. The supernatants of the centrifugation were collected for the measurements of the encapsulation efficiency.

### 2.3. Activity of the immobilized and free L-asparaginase

The activities of the immobilized and the free L-asparaginase were performed with a slightly modified method according to Shirfrin et al. (1974) described previously. This concise procedure contains the following steps as described next. Step 1: after 0.1 ml free L-asparaginase or immobilized enzyme (net enzyme, approximately 2.0 mg in 10.0 ml buffer solution with different pH) was added to the temperature-stabilized mixture containing 0.1 ml 189 mM L-asparagine solution, 1.0 ml buffer and 0.9 ml deionized water in a container with a magnetic bar, the mixture was incubated at the assigned temperature for 30 min. Then 0.1 ml 1.5 M trichloroacetic acid was added to interrupt the enzymatic reaction. The mixture was centrifuged for 2 min at 10,000 rpm to separate the solution. Step 2: 0.2 ml of supernatant from step 1, 4.3 ml deionized water and 0.5 ml Nessler's reagents were added to a suitable container with a stirbar. The absorbance value of the solution at 436 nm was recorded on a spectrophotometer (APL-UV-2000, Shanghai, China). The absolute activities of the immobilized and free enzyme were calculated with the standard curve plotted by the $\Delta A_{436\,nm}$ of the standard versus the ammonia concentration (the standard curve was plotted using a standard $(NH_4)_2SO_4$) solution) from the

equation below.

$$\text{Units/mg enzyme} = \frac{\mu\text{mole of } NH_3 \text{ liberated} \times 2.20}{0.2 \times 30 \times 0.1 \times \text{used enzyme in mg/ml}}$$

### 2.4. Determination of L-asparaginase leakage from the capsules and the selective-permeability of the matrix

In order to evaluate L-asparaginase diffusion out of the complex matrix, the procedure is designed as follow: the nanocapsules prepared from Section 2.2 were dispersed homogeneously by sonicator at 160 W for 5 min and kept in 2.0 ml Tris buffer at 4 °C for 72 h. Then, the mixture was separated by centrifugation at 16,000 rpm. The clear supernatant was collected and the mass of L-asparaginase it contained was determined. The percentage of leakage from the nanocapsules was calculated using the follow equation:

$$\text{Percentage of leakage of the nanocapsules (EE) (\%)} = \frac{\text{mass of L-asparaginase detected in the supernatant}}{\text{mass of L-asparaginase encapsulated}} \times 100$$

### 2.5. L-Asparaginase encapsulation efficiency

The free and immobilized L-asparaginase were separated by centrifugation of the suspension at 16,000 rpm for 20 min. In order to determine the encapsulation efficiency of the nanocapsules, it was necessary to detect the free enzyme in the supernatant. Measurements were carried out by the standard method (Shirfrin et al., 1974). The supernatant was diluted to 50.0 ml and measured by UV spectrometer. The amount of the free L-asparaginase in the clear supernatant was determined. The L-asparaginase encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation efficiency (EE) (\%)} = \frac{\text{mass of L-asparaginase used in formulation} - \text{mass of free L-asparaginase}}{\text{mass of L-asparaginase used in formulation}} \times 100$$

### 2.6. Activity recovery of the immobilized L-asparaginase

To determine the effect of the immobilization process on the enzyme, the activity recovered enzyme was conducted. The loading amount of the enzyme was fixed at 2.0 mg. The enzyme was applied to different concentration ratios of CKGM–CS systems and the absolute activity of the immobilized enzyme was calculated. The activity recovery of the immobilized enzyme was calculated from the equation listed below:

$$\text{Activity recovery (\%)} = \frac{\text{the absolute activity of immobilized L-asparaginase used in formulation}}{\text{the absolute activity of the native L-asparaginase (1271.3 U/mg)}} \times 100$$

### 2.7. Characterization of the nanocapsules

#### 2.7.1. Sizes

The samples of this experiment were prepared as outlined in Section 2.2. The size of the nanocapsules in aqueous solutions were measured by dynamic light scattering (DLS; BI-9000AT, BI-200SM, Brookhaven Instruments Co., USA) at 25 °C using a Coherent innove 304 laser electronic source at the wavelength 514.5 nm; the scattering angle is 90°.

#### 2.7.2. Transmission electron microscopy

The morphology of the CKGM–CS nanocapsules was examined by TEM (H-600IV, Hitachi Co., Japan). About 5 μl of the nanocapsules solution was placed on a copper grid and stained with phosphotugstic acid. Observation was done at an accelerating voltage of 80 kV.

## 3. Results and discussion

### 3.1. Encapsulation efficiency of the nanocapusles

The enzyme immobilization potential of the CKGM–CS nanocapsules was investigated with a L-asparaginase loading of 2.0 mg. Table 1 reveals that encapsulation efficiency was highest (68.0%) when the ratio of CS to CKGM was 1 and the concentration was fixed at 0.01%. At a fixed concentration of CKGM (or CS), the encapsulation efficiency of L-asparaginase decreased with increasing the concentration of the other material.

### 3.2. Sizes of the nanocapsules

The particle size of CKGM–CS, prepared at varying CKGM and CS concentrations, was evaluated by dynamic light scattering (DLS) and the results are displayed in Table 1. The data shows that the effective size of the sample increased from approximately 300–3000 nm with an increase in either the CKGM or CS concentration. These results may be due to an increased number of entwisted molecular units at higher polymer concentrations leading to the

**Table 1**
The diameter, encapsulation efficiency, leakage efficiency and activity recovery of the nanocapsules

| Entry | Diameter (nm) | Encapsulation efficiency (%) | Leakage efficiency (%)[a] | Activity recovery (%)[b] |
|---|---|---|---|---|
| $C_1K_1$[c] | 325 | 68.00 | 0.90 | 103.5 |
| $C_1K_2$ | 413 | 50.53 | 0.50 | 98.3 |
| $C_1K_4$ | 645 | 48.87 | 0.25 | 95.3 |
| $C_1K_6$ | 1903 | 51.70 | 0.25 | 100.7 |
| $C_1K_8$ | 1220 | 43.56 | 0.00 | 97.3 |
| $C_1K_{10}$ | 1746 | 5.61 | 0.25 | 99.2 |
| $C_2K_1$ | 503 | 60.52 | 0.00 | 101.0 |
| $C_4K_1$ | 939 | 57.07 | 0.45 | 102.2 |
| $C_6K_1$ | 946 | 53.68 | 0.15 | 104.1 |
| $C_8K_1$ | 1672 | 38.37 | 0.75 | 93.4 |
| $C_{10}K_1$ | 3193 | 29.66 | 0.50 | 99.2 |

[a] Average value for two measurements.
[b] Average value for three measurements.
[c] For convenience, the nanocapsule produced by 0.01% CS and 0.01% CKGM were labeled $C_1K_1$ and the others by analogy.

increase in particle size observed. The DLS measurements also showed a monodisperse distribution in all of the above CKGM–CS systems.

### 3.3. Determination of L-asparaginase leakage from the capsules and the semi-permeability of the matrix

Selective permeability is the most important property for enzyme immobilized capsules. The system should prevent the L-asparaginase diffusion into the surrounding solution while allowing penetration of substrate. Many reported systems of encapsulation or entrapment often suffer from enzyme leakage. Table 1 lists the data of leakage of L-asparaginases for different CKGM–CS systems in 72 h. It can be seen that the leakage efficiency is very low for all of the samples. The largest value of leakage efficiency is only 0.90%, which can be considered negligible. Further, the activities of the immobilized and free L-asparaginase are very similar. This indicates that L-asparagine can pass through the matrix and be decomposed by the immobilized enzyme and all the results proved that the matrix has selective-permeability to both the substrate and enzyme.

### 3.4. The activity recovery of the L-asparaginase-loaded nanocapsules

The enzymatic activities of the immobilized and free L-asparaginase were determined by a routine spectrophometic method (Shirfrin et al., 1974) and are listed in Table 1. It is shown that the activity of the immobilized enzyme is consistent with that of the free enzyme, whether the concentration of CKGM or CS was high or low. These results indicate that the process of the preparation of the nanocapsules has no adverse effects on the enzyme, and the CKGM–CS capsule is not a barrier for the influx of L-asparagine and the release of ammonia. It is known that the acidic conditions have a deleterious effect on the L-asparaginase. However, the procedure of using the low acetic acid concentration (acetic acid 0.05%, v/w) for 30 min does not adversely affect the activity of the enzyme.

### 3.5. Morphology studies for the nanocapsules

TEM with phosphotugstic acid staining was employed to successfully confirm the morphology of the CKGM–CS nanocapsules (Fig. 1). In contrast with regular negative staining effects, the particles shown have a dark core. This is due to the fact that the staining reagent penetrates through the CKGM–CS membrane and reacts with the enzyme, which gives further proof of the semi-permeability of the matrix. The particles are shown to exist as discrete individual spheres along with few partial cohesive spheres. The particle size of TEM is ranged from 100–300 nm.

### 3.6. Activity of encapsulated L-asparaginase

#### 3.6.1. Effect of the reaction temperature on the activities of the immobilized and free L-asparaginase

The activities of the free and immobilized L-asparaginase were measured by maintaining the reaction medium in a thermal bath at a constant temperature from 25 to 75 °C. As shown in Fig. 2, the activity profiles of both the free and immobilized enzyme were very similar below 45 °C. The absolute activity of the immobilized L-asparaginase reaches its highest value 1635.9 U/mg at 55 °C. The free enzyme shows maximum activity (1574.6 U/mg) at 65 °C. Above the optimum temperature, both free and encapsulated enzymes gradually deactivated with increasing temperature.

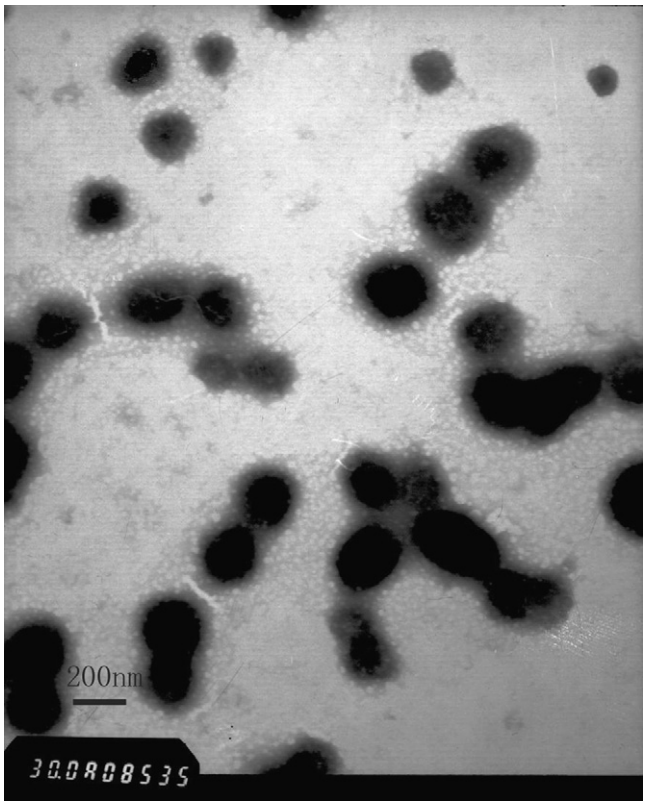


**Fig. 1.** TEM photograph of the L-asparaginase-encapsulated nanocapsules.

#### 3.6.2. The thermal stability of the immobilized and free L-asparaginase

For storage and application of L-asparaginase in the physiological environment, it is important to examine the stability at higher temperatures as a function of time. The immobilized and free L-asparaginases were incubated at a fixed temperature of 60 °C and the absolute activities were measured, respectively. Fig. 3 showed a significant decrease of activity for both enzyme systems with the increasing incubation times, but stability profiles of the free and immobilized L-asparaginase were clearly different. After 90 min incubation, the absolute activity of the immobilized enzyme

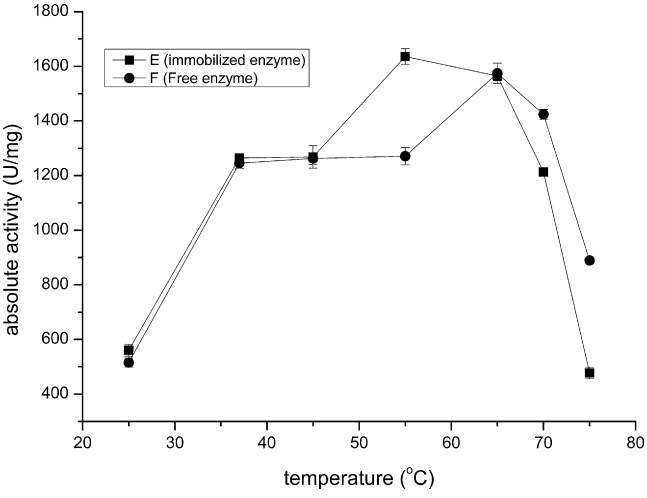


**Fig. 2.** Effect of temperature on the activity of the free and immobilized L-asparaginase.

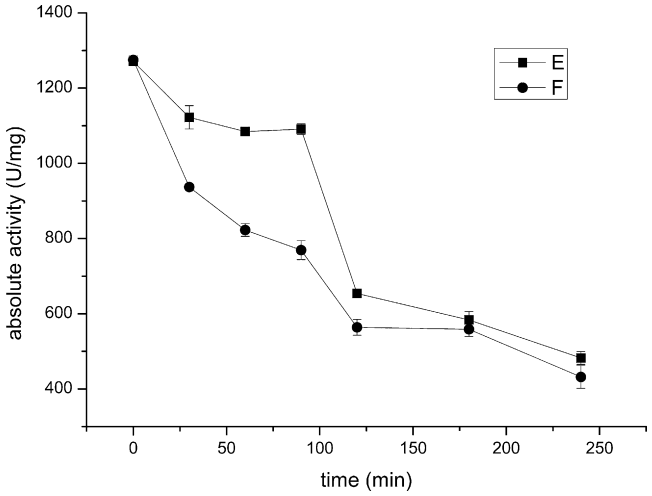


**Fig. 3.** Thermal stability of the free and immobilized L-asparaginase.

remained 77%, while the free enzyme was only 60%. The increased stability observed with immobilized enzyme was probably due to the CKGM–CS reducing the mobility of enzyme and shielding it from the effects of environment. But after 120 min, the absolute activity of the immobilized enzyme was very similar to that of the free enzyme.

### 3.6.3. Effect of pH on the activities of the immobilized and free L-asparaginase

The effect of pH on activity of the free and immobilized L-asparaginase was determined by adjusting the reaction medium at a fixed pH value ranging from 5 to 12. As shown in Fig. 4, the free L-asparaginase showed maximum activity at pH 8.6, while the optimal pH of the immobilized enzyme was much wider, in a range of 7.0–10.0. These results indicate the immobilized L-asparaginase in CKGM–CS system stabilized the enzymatic activity over a broader pH range.

### 3.6.4. The pH stability of the immobilized and free L-asparaginase

It is well known that the acidic environment is very deleterious to the L-asparaginase. In a weakly acidic environment (pH 6), the absolute activity of immobilized and the free L-asparaginase decreased with increasing incubation time (Fig. 5). But the immobilized enzyme was clearly stabilized over the free enzyme. After

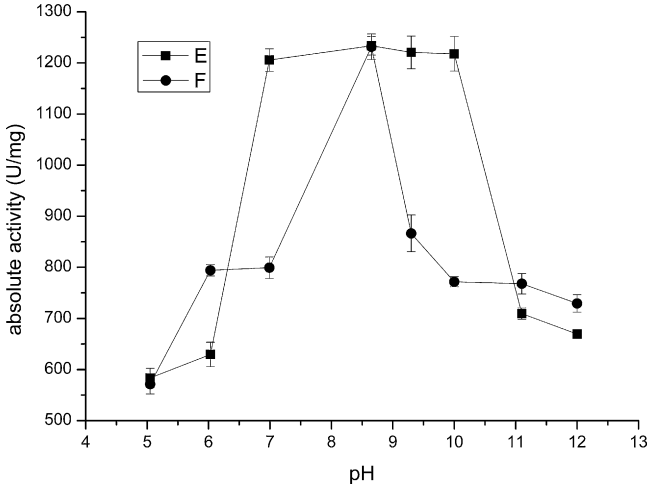


**Fig. 4.** Effect of pH on the activity of the free and immobilized L-asparaginase.

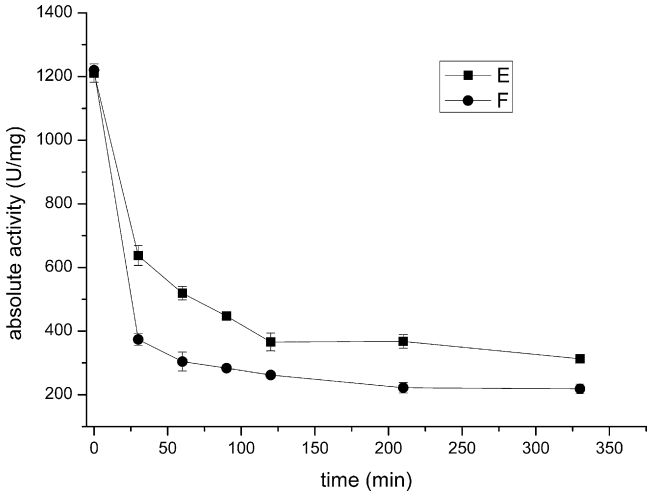


**Fig. 5.** pH stability of the free and immobilized L-asparaginase (pH 6).

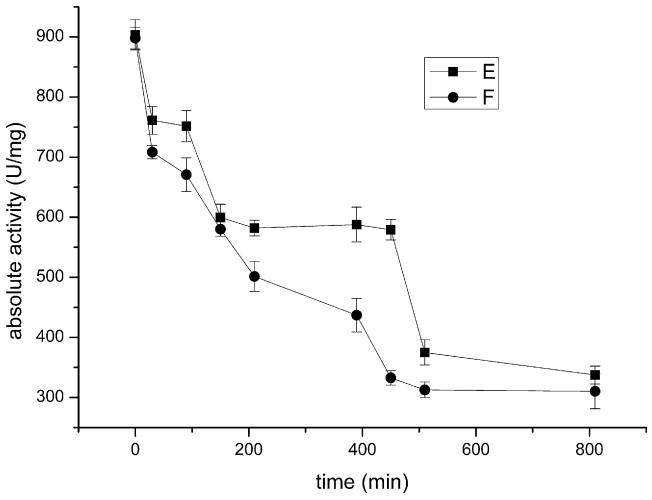


**Fig. 6.** The in vitro half-time of the immobilized and free L-asparaginase in buffer (pH 8.6) solution.

330 min in the pH 6 solution, the remaining activity of the immobilized and native enzyme was 25.8 and 17.9%, respectively. This result is likely attributed in part to the fact that the three-dimensional structure of the immobilized enzyme is fixed within the capsule, which stabilized the active centre (metal ion) and denaturing of the enzyme by $H^+$ is more difficult than the native enzyme.

### 3.6.5. In vitro half-time of the immobilized and free L-asparaginase

Solutions of the immobilized and free enzyme were homogenized and transferred to buffer (pH 8.6) at 37 °C to measure the half-activity time of both enzymes. As shown in Fig. 6, the free enzyme reached the half-activity time at about 400 min. The immobilized enzyme had an increased half-activity time about 500 min in solution. The significant increment of the immobilized enzyme is helpful for the storage of enzyme.

## 4. Conclusions

In this paper, CKGM–CS nanocapsules were prepared as a novel biocompatible matrix system for L-asparaginase immobilization. The preparation of the nanocapsules was completely conducted in

water and the immobilized L-asparaginase maintained the original activity of the free enzyme. The encapsulation efficiency reached 68.0% when both the concentrations of CKGM and CS were 0.01% and the particle size was in a range 100–300 nm. Compared with the free L-asparaginase, the immobilized enzyme system showed significantly higher thermostability and had preferable resistance to acid and alkaline environments. This study illustrated that the nanocapsules have semi-permeability and can be used to immobilize thermal and pH-sensitive enzymes.

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