Immobilization of a Nonspecific Chitosan Hydrolytic Enzyme for Application in Preparation of Water-Soluble Low-Molecular-Weight Chitosan

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ABSTRACT: A nonspecific chitosan hydrolytic enzyme, cellulase, was immobilized onto magnetic chitosan microspheres, which was prepared in a well spherical shape by the suspension crosslinking technique. The morphology characterization of the microspheres was carried out with scanning electron microscope and the homogeneity of the magnetic materials (Fe_3O_4) in the microspheres was determined from optical micrograph. Factors affecting the immobilization, and the properties and stabilities of the immobilized enzyme were studied. The optimum concentration of the crosslinker and cellulase solution for the immobilization was 4% (v/v) and 6 mg/mL, respectively. The immobilized enzyme had a broader pH range of high activity and the loss of the activity of immobilized cellulase was lower than that of the free cellulase at high temperatures. This immobilized cellulase has higher apparent Michaelis–Menten constant K_m (1.28 mg/mL) than that of free cellulase (0.78 mg/mL), and the maximum apparent initial catalytic rate V_{max} of immobilized cellulase (0.39 mg mL^{-1} h^{-1}) was lower than free enzyme (0.48 mg mL^{-1} h^{-1}). Storage stability was enhanced after immobilization. The residual activity of the immobilized enzyme was 78% of original after 10 batch hydrolytic cycles, and the morphology of carrier was not changed. J Appl Polym Sci 101: 1334–1339, 2006

Key words: cellulase; immobilization; stability; chitosan; microspheres

INTRODUCTION

Water-soluble low-molecular-weight chitosan (LMWC) has received much attention for its interesting biological activities. Water-soluble LMWC prepared from enzymatic hydrolysis with hemicellulase revealed high antitumor activity.[1] LMWC of 1000–5000 Da exhibited superior free-radical scavenging activity.[2] Compared with high-molecular-weight chitosan, oral absorption profiles were observed to increase more than 23 and 25 times with water-soluble LMWC of 3800 Da both *in vitro* and *in vivo* transport experiments, respectively.[3] Moreover, LMWC with average M_w in the range of 5000–10,000 Da was shown to possess strong bactericidal superior biological activities when compared with chitosan.[4] So, it is of increasing interest to degrade chitosan into low-molecular-weight component under appropriate conditions. The methods for preparing LMWC are either chemical or enzymatic.[5] The chemical approach has several defects: harsh conditions of hydrolysis, low yields of the product, chemical modifications of glucose ring, and so on,[6] while the chitinase are unavailable in bulk quantities for commercially viable applications. Recently, several hydrolytic enzymes, such as hemicellulase, papain, pectinases, and neutral protease, were found to catalyze the cleavage of glycosidic linkages in chitosan.[1,7,8] Cellulase is one of the nonspecific enzymes that can hydrolyze chitosan efficiently.[9]

The efficiency of free enzyme is lower commonly and immobilization technology is often used to increase its use efficiency. Magnetic materials have been widely used in immobilization of cells and enzymes[10,11] and in other biotechnologies.[12,13] The use of magnetic particles can also reduce the capital and operation costs. For these reasons, many magnetic materials have been developed using various polymers[10,14] containing magnetic particles. Chitosan has been known as an ideal support material for enzyme immobilization, because of its hydrophilicity, biocompatibility, and antibacterial property.[15] It exhibited a considerable protein-binding capacity and a high recovery of enzyme activity, allowing that the enzyme immobilized thereon remains considerably active.[16] Chitosan can be easily crosslinked by reagents such as glutaraldehyde to form rigid microspheres, and the microspheres could hardly be depolymerized by cellulase.

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The present study describes the immobilization of a nonspecific chitosan hydrolytic enzyme (cellulase) using magnetic chitosan microspheres. The morphological characterization of the microspheres was carried out with scanning electron microscope (SEM) and the homogeneity of the magnetic materials (Fe_3O_4) in the microspheres was determined from optical micrograph. The optimum concentration of the crosslinker and cellulase solution for the immobilization was determined. The properties of immobilized cellulase, kinetic parameters, thermal and storage stability, and operational stability of immobilized cellulase were also investigated in detail.

EXPERIMENTAL

Materials

Chitosan (CS), as initial material from shrimp shells, was obtained from Yuhuan Biochemical Co. (Zhejiang, China). CS1 ($M_w = 23.5 \times 10^4$, DD = 95.3%) was used to prepare magnetic CS microspheres, CS2 ($M_w = 40 \times 10^4$, DD = 75%) was used as the hydrolysis substrate for cellulase. Bovine serum albumin and Coomassie brilliant Blue G250 were purchased from Sigma Chemical Co. (USA). D-glucosamine HCl was purchased from Seikagaku Corp. (Japan). All other chemicals were of reagent grade. The cellulase was a product of Ningxia XiaSheng Industry Co., Ltd. (China).

Preparation of magnetic CS microspheres[17,18]

Magnetic particles were prepared by coprecipitating Fe^{2+} and Fe^{3+} ions by ammonia solution and treating under hydrothermal conditions. $FeSO_4 \cdot 7H_2O$ (0.1*M*) and $FeCl_3 \cdot 6H_2O$ (0.2*M*) were dissolved in 200 mL distilled water and added to 8*M* solution of NH_4OH under continuous stirring at room temperature. During the reaction process, the pH was maintained at about 10. The precipitates were heated at 80°C for 30 min, then washed several times with distilled water, finally, the magnetic particles were dispersed in slightly alkaline medium (pH 8.9).

The suspension crosslinking technique was used for the preparation of magnetic chitosan microspheres. A 50 mL 4% (w/v) chitosan (CS1) solution was prepared using a 2% (v/v) aqueous acetic acid solution containing a certain amount of magnetic particles. It was then poured into the dispersion medium, which was composed of 50 mL liquid paraffin and an emulsifier (Span-80). The mixture was stirred at 1000 rpm with a mechanical stirrer for 30 min to form water in oil (w/o) dispersion. Later, glutaraldehyde was added into the medium and temperature was raised to 40°C. After 1 h of crosslinking, the magnetic CS microspheres were collected and washed consecutively with aether, ethanol, and distilled water. The microspheres were then dried in an oven at 60°C for further analysis and use.

Morphology of magnetic CS microspheres

The surface morphology of the magnetic CS microspheres was investigated by using SEM (Hitachi, S-750, Japan). Dry microspheres were coated with a thin layer of gold and photographed in the electron microscope. The homogeneity of the magnetic materials (Fe_3O_4) in the mocrospheres was determined from optical micrographs of the microspheres taken with an optical microscope (Olympus, BX51, Japan).

Immobilization of cellulase

Different concentration of cellulase solution was prepared in 0.2*M* NaAc-HAc buffer (pH 5.6), and the immobilization process was achieved by the following approach: 0.4 g magnetic CS microspheres were dispersed in 8 mL 0.2*M* NaAc-HAc buffer (pH 5.6), swelling for 24 h at room temperature. Then, freshly prepared cellulase solution (2 mL) was introduced to the suspension and the mixture was placed in a shaking incubator at 25°C and 150 rpm for 4 h. Then, the microspheres were recovered and washed with 0.2*M* NaAc-HAc buffer (pH 5.6) until protein in the washing could not be detected. The amount of immobilized cellulase on the microspheres was determined by measuring the initial and final concentrations of protein within the adsorption medium using Coomassie brilliant blue as described by Bradford,[19] using bovine serum albumin as the standard.

Measurement of cellulase activity

Twelve milligrams of cellulase was added to 10 mL 1% (w/v) chitosan (CS2) solution prepared by 0.2*M* sodium acetate buffer and incubated in a thermostatic shaker at 55°C for 1 h. The mixture was boiled for 10 min to remove the enzyme. The hydrolysate was filtered, and the reducing sugars resulted from a cleavage of glycosidic linkage was determined by spectrophotometric analysis on the basis of Schales' modified method,[20] with D-glucosamine HCl as standard.

Immobilized enzyme (0.4 g microspheres) and 10 mL 1% (w/v) chitosan (CS2) solution reacted in the same condition for 1 h, then, separated the immobilized enzyme from the reaction system to terminate the reaction, and the reducing sugars of hydrolysate was determined.

The activity assays were carried out over the pH range from 4.4 to 6.0 and temperature range from 40 to 70°C to determine the pH and temperature profiles of free and immobilized enzyme. The activity of pH profiles was determined at various pH values at 55°C, and the activity of temperature profiles was determined at different temperatures at pH 5.6.

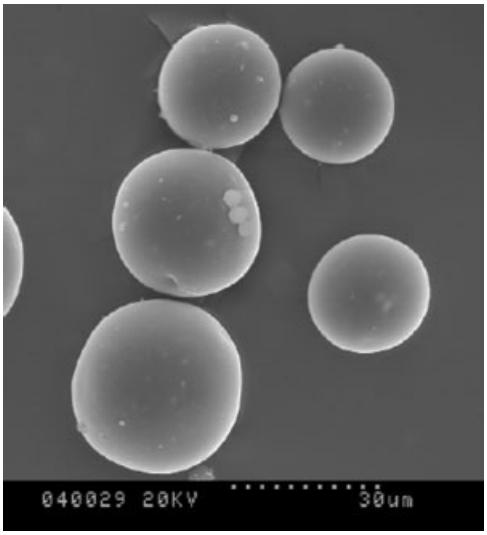

Figure 1 SEM micrograph of magnetic CS microspheres.

The effect of substrate concentration on the activity of free and immobilized cellulase was studied by increasing the concentration of CS2 substrate from 0.25 to 2.5% in the optimum conditions. The apparent Michaelis–Menten constant (K_m) and the maximum apparent initial catalytic rate (V_{max}) were calculated from the experimental data, following the Lineweaver–Burk plot method.

Enzyme stability

The storage and thermal stability of free and immobilized cellulase was determined by measuring the residual activity of enzyme after storage in a NaAc-HAc buffer (0.2*M*, pH 5.6) at 4, 25, and 55°C. Activity of samples was performed at optimum conditions.

The operational stability of immobilized cellulase in the study was evaluated in a repeated batch process. After each reaction run, the immobilized cellulase was removed and washed with NaAc-HAc buffer (0.2*M*, pH 5.6) to remove the residual substrate within the microspheres. They were then reintroduced into fresh reaction medium and the enzyme activity was determined.

RESULTS AND DISCUSSION

Morphology of magnetic CS microspheres

The morphology of magnetic chitosan microspheres was investigated using SEM and optical microscope. Figure 1 is the SEM micrograph of magnetic CS microspheres, as can be seen, the microspheres were well shaped and regular with a rather smooth surface. The homogeneity of the magnetic materials (Fe_3O_4) in the CS microspheres was evaluated from the optical micrograph. Figure 2 shows an optical micrograph of magnetic CS microspheres. The black particles in CS microspheres were magnetic materials (Fe_3O_4), and it can be seen that a homogenous distribution of Fe_3O_4 particles was achieved in the microspheres.

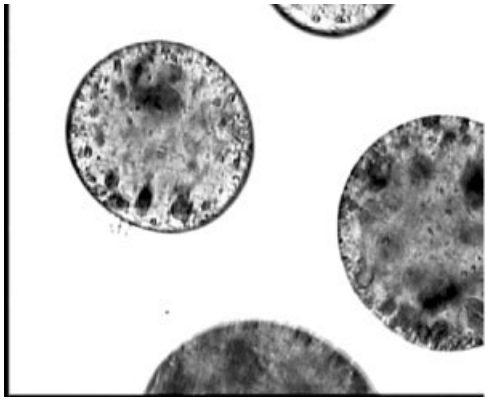

Figure 2 Optical micrograph of magnetic CS microspheres.

Immobilization of cellulase

Effect of concentration of glutaraldehyde on cellulase immobilization

Different concentration of glutaraldehyde ranging from 1 to 5% (v/v) was used for magnetic CS microsphere samples preparation to determine the optimum concentration of glutaraldehyde. Each sample (0.4 g) was used to immobilize 12 mg of cellulase. After immobilization, the activity of each immobilized enzyme was determined and the amount of protein loaded to the microspheres was calculated. As indicated in Table I, the amount of loaded protein and enzyme activity increased with increasing concentration of glutaraldehyde. When the concentration of glutaraldehyde was more than 4%, the amount of loaded protein had no obvious changes, while the enzyme activity decreased. The optimal concentration of glutaraldehyde for immobilization was 4%.

Effect of added cellulase concentration on cellulase immobilization

Two milliliters of a different concentration of cellulase solution was added to 0.4 g of magnetic CS micro-

TABLE I
Effect of Concentration of Glutaraldehyde on Cellulase Immobilization

Concentration of glutaraldehyde (%)	Relative activity[a] (%)	Loaded protein (mg)
1	70.5	5.9
2	83.7	7.1
3	92.2	8.2
4	100	9.0
5	96.3	9.1

[a]The maximum activity of immobilized cellulase was defined as 100%.

TABLE II
Effect of Added Enzyme Concentration on Cellulase Immobilization

Enzyme concentration (mg/mL)	Relative Activity[a] (%)	Loaded protein (mg)
1	21.8	1.6
3	52.2	4.8
5	83.3	7.6
6	96.9	9.0
7	97.3	9.5
10	100	12.1

[a]The maximum activity of immobilized cellulase was defined as 100%.

sphere suspension (pH 5.6). After immobilization, the activity of each immobilized enzyme was determined and the amount of protein loaded to the microspheres was calculated. The results are shown in Table II. With the increase of the cellulase concentration, both the enzyme activity and loaded protein increased rapidly. But there was little advantage in using enzyme more than 6 mg/mL. In this case, increasing the cellulase concentration to 10 mg/mL, the enzyme activity was only about 3% higher than that of adding 6 mg/mL cellulase solution. Although the added enzyme concentration increased, the immobilized enzyme activity did not increase accordingly, denoting the constant number of active sites of the enzyme on the surface of the microspheres. Thus, the additional costs would seldom be justified, the maximum concentration of added cellulase solution in this study was 6 mg/mL.

Properties of immobilized cellulase

Effect of pH on the activity of immobilized cellulase

The effect of pH on the activity of free and immobilized cellulase was studied at various pH values at 55°C. The reactions were carried out in 0.2*M* NaAc-HAc buffer, and Figure 3 shows the relative activity trends as a function of pH. Both enzymes showed an optimum pH of 5.6, but the immobilized enzyme has a broader pH range of high activity. The loss of the activity of immobilized cellulase was lower than that of the free cellulase at more acid pH. Microenvironment phenomena may be responsible for the stability of immobilized enzyme at low pH, given that the enzyme is bound to a polycation carrier.

Effect of temperature on the activity of immobilized cellulase

The temperature dependence of the free and immobilized cellulase activity was studied in 0.2*M* NaAc-HAc buffer (pH 5.6) in the temperature range 40–65°C and the temperature profiles of free and immobilized cellulase are showed in Figure 4. Optimum temperature

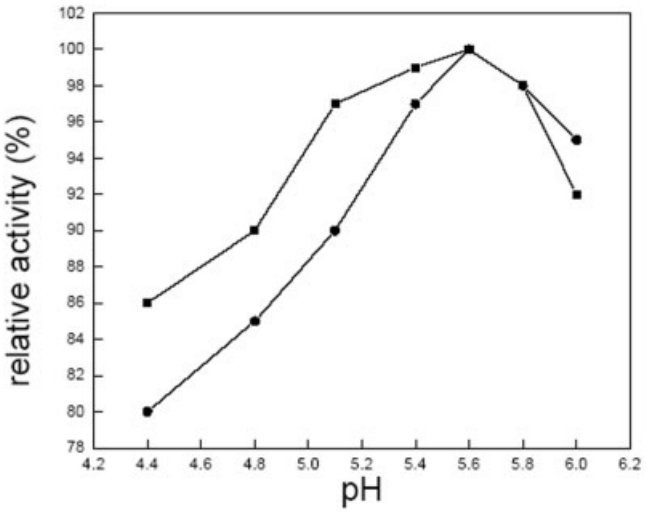

Figure 3 Effect of pH value of CS2 solution on the activity of free cellulase (●) and immobilized cellulase (■).

was found at 55°C for free and immobilized enzyme. Figure 4 showed that the loss of the activity of immobilized cellulase was lower than that of the free cellulase at high temperatures. The support has a protecting effect at the high temperatures at which enzyme deactivation takes place. Immobilization of cellulase in CS beads may reduce the conformational flexibility of the immobilized enzymes, which makes the immobilized enzymes require a higher temperature to form the proper conformation to recognize and bind the substrate molecules. Thus, the relative activity of free cellulase was higher than that of immobilized cellulase at low temperatures. The increase in the enzyme rigidity was reflected by an increase in stability towards denaturation by raising the temperature also.[21]

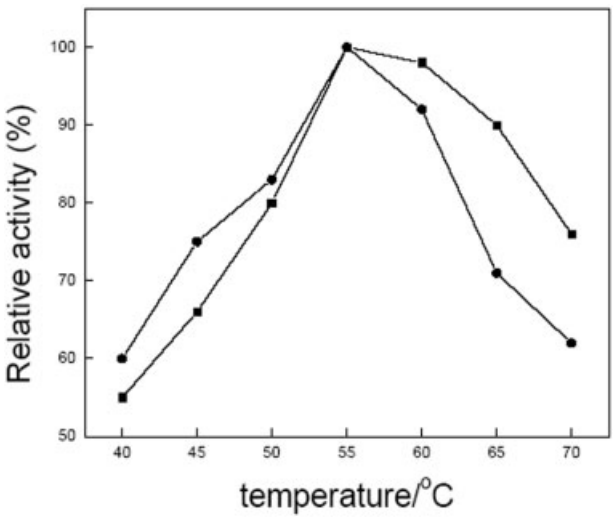

Figure 4 Effect of temperature on the activity of free cellulase (●) and immobilized cellulase (■).

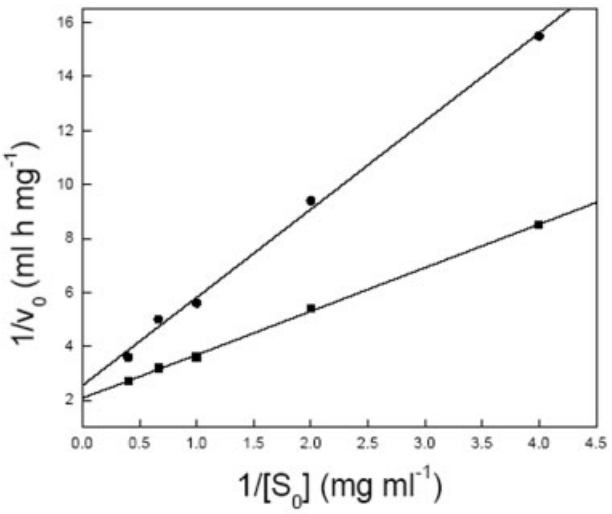

Figure 5 Lineweaver–Burk plots for free cellulase (■) and immobilized cellulase (●).

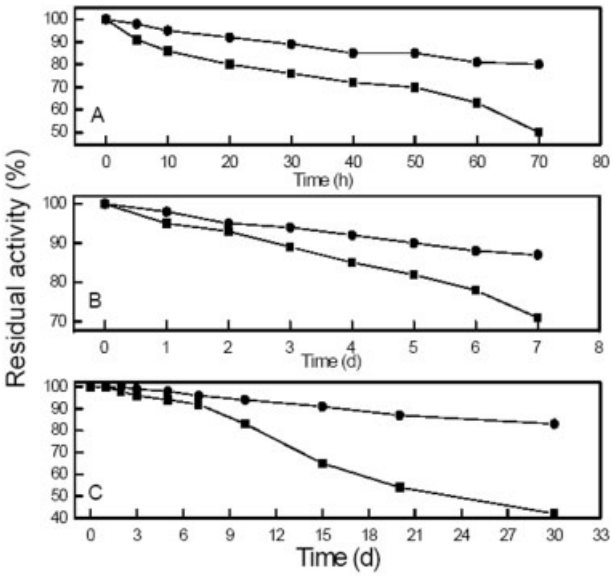

Figure 6 Percentage of enzymatic residual activity as a function of time for free cellulase (■) and immobilized cellulase (●) at 55°C (A), 25°C (B) and 4°C (C).

Kinetic studies

Kinetic of the hydrolytic activity of free and immobilized cellulase was investigated using various initial concentration (0.25–2.5%) of CS2 as substrate. These data were plotted according to the method of Lineweaver–Burk (Fig. 5), and the apparent Michaelis–Menten constant K_m and maximum apparent initial catalytic rate V_{max} were determined. As can be observed, immobilization showed a heavy effect on the K_m and V_{max}. The value of K_m was found to be 0.78 mg/mL whereas the V_{max} was calculated as 0.48 mg mL^{-1} h^{-1} for free cellulase. The value of K_m and V_{max} for immobilized cellulase was 1.28 mg/mL and 0.39 mg mL^{-1} h^{-1}, respectively. The increase in K_m after immobilization indicates that the immobilized cellulase has an apparent lower affinity for its substrate than what the free cellulase has, which may be caused by the steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding, or diffusional resistance to solute transport near the particles of the support.[22]

Enzyme stability

Thermal and storage stability of immobilized cellulase. For storage and application of the immobilized enzyme systems in the preparation of water-soluble LMWC, it is important to examine the stability as a function of temperature. Free and immobilized cellulase was stored in a NaAc-HAc buffer (0.2*M*, pH 5.6) at 4, 25, and 55°C, and the activity measurement was carried out during storage. As shown in Figure 6, there was a very small change in the activity of free and immobilized cellulase within 7 days at 4°C. Later, the activity of free cellulase decreased rapidly, the residual activity of free cellulase was 42% of original, while the immobilized cellulase lost only about 17% of its activity for up to 30 days [Fig. 6(C)]. At 25°C, free cellulase lost about 30% its activity within 7 days, whereas immobilized enzyme lost about 13% its activity [Fig. 6(B)]. Increasing the temperature to 55°C clearly showed the difference in the stability profiles of the free and immobilized enzyme, the immobilized cellulase is more stable than the native one [Fig. 6(A)]. After 70 h, the activity of free cellulase decreased to 49% of original, whereas the residual activity of immobilized enzyme was 80% of original. The decrease in activity was explained as a time-dependent natural loss in enzyme activity, and this was prevented to a significant degree by immobilization.

Operational stability of immobilized cellulase. The operational stability of the immobilized enzyme is an important feature for its potential application in indus-

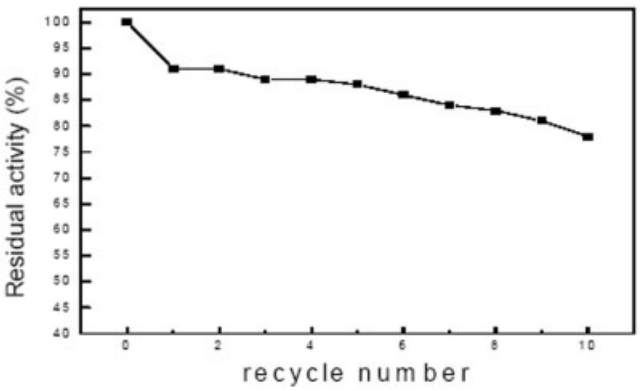

Figure 7 Residual activity of immobilized cellulase after each batch reaction.

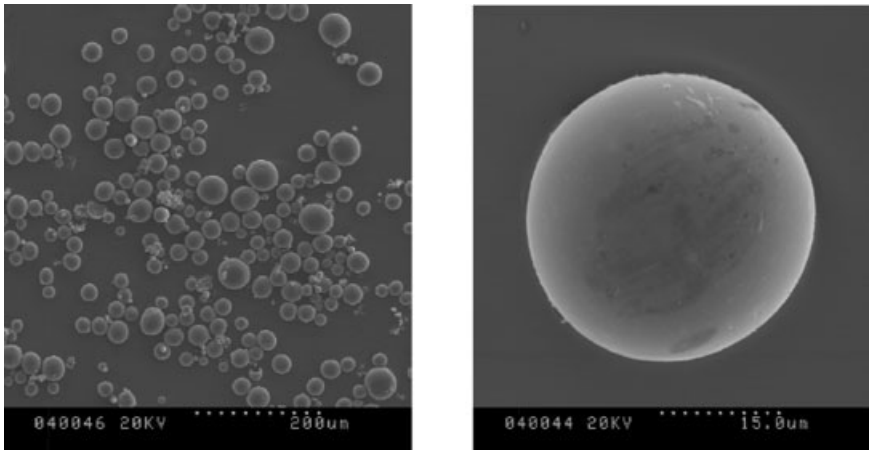

Figure 8 SEM micrographs of immobilized cellulase after 10 batch hydrolytic cycles.

try. By magnetic separation, the immobilized cellulase was recovered and recycled for the hydrolysis of CS. The operational stability of the immobilized cellulase in the study was evaluated in a repeated batch process. Immobilized enzyme (0.4 g) was added to 10 mL of 1% CS2 solution (pH 5.6) in a thermostatic shaker at 55°C, and the reaction was repeated 10 times. During each hydrolytic cycle, a portion of the hydrolysate was taken out at various intervals and mixed with concentrated alkali. No precipitate was produced, indicating that the CS2 had been depolymerized into water-soluble LMWC. The reaction was terminated by separating the immobilized enzyme from the reaction system. After each reaction, the immobilized enzyme activity was determined and compared with the first run (activity defined as 100%). Figure 7 shows the effect of repeated use on activity of immobilized enzyme. The cellulase immobilized on magnetic CS microspheres showed good operational stability, and the residual activity was 78% of original after 10 batch hydrolytic cycles.

Figure 8 showed the SEM micrograph of immobilized enzyme after 10 batch hydrolytic cycles, as can be seen, the morphology of carrier did not change, the microspheres were still well shaped and regular, and they were suitable for the immobilization of cellulase and repeated batch hydrolytic reactions.

CONCLUSIONS

Our aim here is to immobilize a nonspecific chitosan hydrolytic enzyme with high stability, so that it can be reused many times for preparation of water-soluble LMWC. The immobilized cellulase had a broader pH range of high activity and was more stable at high temperatures. After 70-hour storage at 55°C, the residual activity of the immobilized enzyme was 80% of original. The operational stability of the immobilized enzyme is an important feature for its potential application in industry. The activity of the immobilized cellulase, in the repeated use, did not decrease significant, the residual activity was about 78% of the first use after 10 batch hydrolytic cycles. Morphology of the used immobilized enzyme indicates that the CS microspheres are suitable for the immobilization of cellulase and repeated batch reaction.

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