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A Novel Process to Prepare Chitosan Macrospheres without Shrinkage and its Application to Immobilize β -Galactosidase

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Abstract: A new process for the preparation of chitosan macrospheres, which was simple and practicable, was suggested in this paper and various chitosans with different molecular weight were used as materials to immobilize β -galactosidase and the chitosan macrospheres with the lowest molecular weight (500 000) was selected as enzyme immobilization carrier based on the highest enzyme activity. In order to overcome the shrinkage of chitosan during drying, the wet macrospheres obtained was treated by 30% glycerol solution for 1 h before drying and the results showed that the dried chitosan macrospheres obtained could keep almost the same structure as its wet form, which was very important for chitosan as enzyme carrier in industry. Finally, β -galactosidase from *Aspergillus oryzae* was immobilized on above dry chitosan macrospheres and a satisfactory result of the immobilized enzyme was obtained in enzyme activity yield, pH stability, thermal stability, operational stability, Michaelis constants K_m and the maximum velocity (V_m).

Keywords: Chitosan macrospheres, β -Galactosidase, Glycerol, Immobilization, Glutaraldehyde

Introduction

Application of solid-phase biocatalysts had become important during the last decades. Enzymes could be immobilized on various supports and by different methods. The properties of the immobilized biocatalysts were influenced by the characteristics of enzyme, support material and the immobilization method. A suitable support should have high affinity to proteins, reactive functional groups, hydrophilicity, mechanical stability and rigidity. Chitosan was a natural polyaminosaccharide which offered the characteristics above; therefore it was often used for enzyme immobilization^{1,2}.

Chitosan microspheres with size falling into millimeter range were often used to immobilize enzyme for practical purposes and it was often prepared by the precipitation method³, the usual operation was to add fresh chitosan solution to a curing solution using a syringe by manpower, which waste a lot of time and manual labor. Except that, the microspheres obtained shrunk greatly during drying because a lot of water was lost, thus the wet chitosan was often used and it was generally stored in aqueous solution, which would largely limit the industrial applications of chitosan. How to prevent its shrinkage during drying? No report was found to our knowledge except that the chitosan was coupled with other materials to form a complex product with a complicated reaction process⁴.

In this paper, a novel process, which would save a lot of time and manpower, was offered to prepare chitosan microspheres and chitosan with different molecular weight was made into microspheres to immobilize β -galactosidase, the one with the highest enzyme activity was chosen as the enzyme carrier. Besides, the strong and the flexible chitosan microspheres without shrinkage during drying were obtained with glycerol solution as treatment agent and β -galactosidase from *Asp. oryzae* was employed to be immobilized on the above carrier with glutaraldehyde as crosslinker. Finally, the kinetic data of the immobilized enzyme, the values of Michaelis constants K_m and the maximum velocity (V_m) were also determined.

Experimental

0.05 g of β -Galactosidase was weighed and extracted in 100 mL 0.1 M sodium phosphate buffer (pH 6.0), then the enzyme solution was obtained and stored in the refrigerator for use. The substrate solution was prepared by dissolving 0.0751 g ONPG in twice distilled water and made up 50 mL solution.

Preparation of chitosan microspheres

1 g of Chitosan with different molecular weight was accurately weighed and dissolved in acetic acid (2%, w/v) to form 5% solution (w/v). After the viscous solution obtained was placed in a vacuum dryer to remove air bubbles, it was placed into the syringe. After the water tap was opened, the air in the condenser was compressed, and the chitosan solution was extruded by the compressed air to spray drop-wise into a gently stirred coagulation liquid containing 15% NaOH and ethanol in a volume ratio of 4:1 at a constant rate. The obtained microspheres were filtered and washed with distilled water until neutrality. Then the microspheres were divided into two groups, one group was stored in a refrigerator at 4 °C (support I) and the other was dried in the air (support II).

The chitosan microspheres treated with glycerol solution

The obtained chitosan microspheres were immersed in a 30% aqueous glycerol solution for 1 hour. After the excess glycerol solution was removed, the obtained chitosan microspheres were divided into two groups. One group was washed with distilled water for three times and stored in a refrigerator for use (support III) and the other was dried in a vacuum desiccator for 8 h at 85 °C and then stored at room temperature for use (support IV).

Immobilization procedure

Preparation of activated chitosan microspheres by glutaraldehyde

2 g of Chitosan microspheres were washed and immersed in distilled water for 12 h. After being dried with filter paper, the beads were placed in a 50 mL vessel containing 20 mL of 0.1% glutaraldehyde (pH 8.0) and the reaction was carried out by shaking it for 2 h at 30 °C, then the

activated microspheres obtained were rinsed thoroughly and stored for the next use.

Immobilization of β -galactosidase on activated chitosan microspheres

The immobilization was carried out by adding an amount of activated microspheres (0.1 g) to 0.1 M phosphate buffer (pH 6.0) containing enzyme (0.5 mg/mL). With gently stirring, the reaction was allowed to proceed at 4 °C, after 16 h, the immobilized enzyme was filtered and washed with 0.1 M phosphate buffer (pH 6.0) until no protein was detected.

Enzyme activity assay

The activity of the free and the immobilized enzyme was determined according to the references^{5,6} using ONPG as a substrate. For the free enzyme activity, aliquots of it (0.1 mL) were added to the mixture of 1.8 mL 0.1 M phosphate buffer (pH 6.0) and 0.1 mL ONPG (5 mM), after being incubated at 55 °C for 15 min, the reaction of ONPG was stopped by the addition of 2 mL 1 mol/L Na₂CO₃ solution and the amount of ONP was measured directly at 405 nm. For the immobilized enzyme activity, 0.5 g of the immobilized enzyme was soaked in 1.9 mL 0.1 M phosphate buffer, the reaction was started by adding 0.1 mL ONPG (5 mM). After being carried out for 15 min at 55 °C, the reaction was stopped and analyzed as above. The activity yield was calculated as the ratio of immobilized enzyme to enzyme subjected to immobilization. One unit of activity was defined as the amount of enzyme that liberated 1 μ mol of product/min at 55 °C.

Influence of temperature and pH

The influence of temperature on the galactosidase activity was determined using ONPG as substrate over the range of 40 to 70 °C. Enzyme stabilities were determined after a long duration exposure to various temperature (50 °C, 60 °C), followed by analysis at the 55 °C. The pH activity curve in the range 4.0-9.0 was determined for the free enzyme and the bound enzyme at 55 °C using ONPG as substrate. The pH stability in the range 3.0-11.0 was determined after 30 min exposure to different pH at room temperature.

Kinetics

The Michaelis constant K_m and the maximum velocity V_m were calculated for the soluble and the immobilized enzyme by assaying the enzyme in increasing ONPG concentrations ranging from 0.75 mM to 7.5 mM in phosphate buffer.

Operational stability of immobilized enzyme

The operational stability of the immobilized enzyme was determined according to the following procedures. 0.5 grams of the immobilized enzyme was taken and soaked in 1.9 mL phosphate buffer overnight, after the mixture was incubated at 55 °C for 2 min, the reaction was started by adding 5 mM ONPG (0.1 mL, pH 6.0, dissolved in the same buffer as described above) for 15 min at 55 °C, and then the reactive mixture were analyzed. Afterward, the solid was filtered and washed thoroughly with distilled water and the above experiment was repeated under the same conditions.

Results and Discussion

Process to make chitosan microspheres

As we all know, macro-, micro- and nanosized chitosan particles were all suitable to immobilize enzyme, but for practical purposes, chitosan microspheres were often used and it was often prepared by precipitation method. The operation was generally carried out by dropping the chitosan solution through a syringe into a gently stirred coagulation by

manpower⁷, which was simple but waste a lot of time and manual labor. In our paper, an improvement of the precipitation method was carried out using compressed air instead of manpower, which was much faster and convenient than the traditional operation.

Effect of various chitosan molecular weights

Various chitosans (500 000, 1 000 000, 2 000 000) with nearly the same structure were used as materials to immobilize β -galactosidase, the results obtained were listed in Table 1. From Table 1, it could be seen that activity and the recovery of the immobilized enzyme bound on the chitosan with 500 000 molecular weight was the highest, which perhaps could be explained by the following facts: with the increase of the molecular weight, the chitosan molecular chain become long and the interaction of them was strengthened in the macrospheres, therefore the surface of the chitosan macrospheres become much closer, and the pores of the chitosan macrospheres were decreased or shrunk, which was not good to immobilize enzyme. So, it could be seen, when the chitosan was selected as enzyme immobilization carrier, the molecular weight of the chitosan was also very important in order to get the optimum immobilization effect. In our paper, the chitosan with 500 000 molecular weight was selected in the later study.

Table 1. Effect of various chitosan molecular weights

Chitosan	Molecular weight $\times 10^4$	Immobilized enzyme activity, U/g dry support	Recovery of immobilized enzyme, %
C ₁	50	71.43	60.35
C ₂	100	66.19	55.55
C ₃	200	62.33	52.02

Effect of glycerol solution on chitosan macrospheres

It was found that the chitosan macrospheres shrunk greatly during drying (about from 2.2 mm to 0.9 mm), and the diameter of the dry macrospheres was nearly fixed after being immersed in distilled water or buffer solution for enough time, which meant the structure of the chitosan macrospheres was destroyed during drying. Another experiment was also carried out to test above viewpoint; both wet and dry chitosan particles were taken and used to immobilize β -galactosidase under the same conditions, the enzyme activity of the latter was far lower than the former. Therefore, if dry chitosan macrospheres were needed, the wet ones must be treated further before drying.

In our experiments, glycerol solution was selected as treatment agent, and the effects of glycerol concentration and treatment time on the chitosan macrospheres were also investigated. Firstly, the treatment time of glycerol solution ranging from 0.5 to 2.0 h was discussed and the dry chitosan macrospheres treated by above solution were used to immobilize β -galactosidase, the results obtained were presented in Figure 1. From Figure 1, it could be seen that the enzyme activity of the immobilized enzyme increased firstly then decreased with the increase of treatment time and 1.0 h was found to be the best based on the highest enzyme activity. Then, various concentrations of glycerol solution from 10% to 60% were also examined, the dry carrier obtained was also used to bound enzyme under the same conditions (see Figure 2). Figure 2 shows that the activity of the immobilized enzyme was the highest when the glycerol concentration was 30%, which might be explained by the following facts, the structure of the chitosan macrospheres could not be kept if the glycerol concentration was lower and the hole of the carrier could be blocked up if the concentration of the treatment solution was much higher. So 30% glycerol solution was selected in the next experiments.

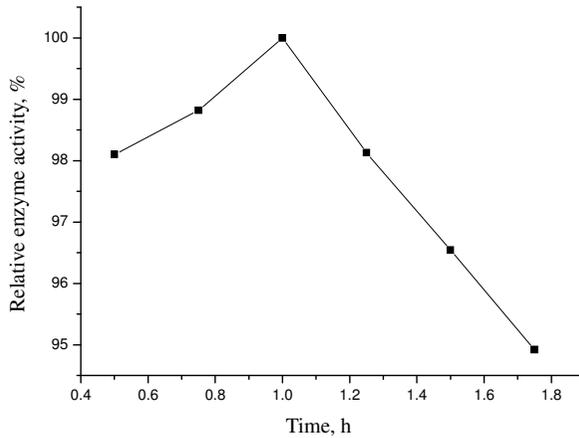


Figure 1. Effect of glycerol treatment time on the chitosan macrospheres.

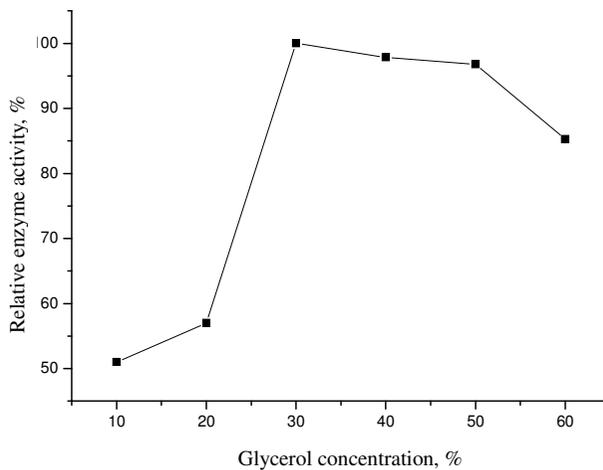


Figure 2. Effect of glycerol concentration on the chitosan macrospheres.

According to above optimum conditions, the chitosan macrospheres were treated by glycerol solution, and it was found that the diameter of the chitosan macrospheres with glycerol treatment kept nearly constant before and after drying at 85 °C, but those without glycerol treatment shrunk greatly during drying at the room temperature. Meanwhile, SEM was done for both dry chitosan particles with and without glycerol treatment in order to understand the morphology of them, and the results obtained were shown in Figure 3 (a) (b). From the Figure 3 (a) (b), it could be seen that the surface of the carrier with glycerol treatment was loose and the apparent pores existed, but the carrier without glycerol treatment was close and nearly no pore was found. Meanwhile, four kinds of supports were taken and employed to immobilize enzyme, and the results obtained were listed in Table 2. From Table II, it could be seen that the enzyme activity of the wet macrospheres, the wet with glycerol treatment and the dried with glycerol treatment were almost the same, however, the enzyme activity of the dried support without glycerol treatment was just 10.29% of the wet support.

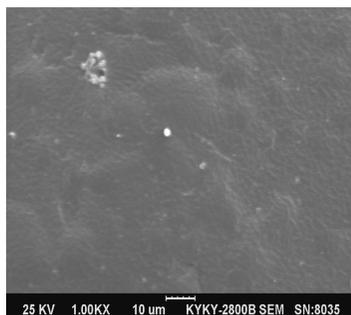


Figure 3 (a). Scanning electron micrographs of the supports II

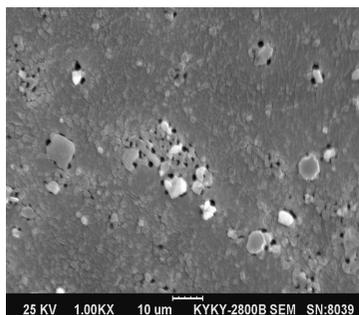


Figure 3 (b). Scanning electron micrographs of the supports IV

Table 2 Comparison of immobilized enzyme activity of different chitosan supports.

Type of supports	Immobilized enzyme activity (U/g dry support)
I (the wet support)	71.43
II (the dried support without glycerol treatment)	7.35
III (the wet support with glycerol treatment)	71.29
IV (the dried support with glycerol treatment)	69.61

Based on above facts, we could see that chitosan macrospheres could be prevented shrinkage with glycerol treatment and kept nearly the same structure as its wet form. In the references reported using chitosan as immobilization carrier^{8,9}, wet macrospheres often employed in order to avoid its shrinkage during drying. It was not very good because the storage of wet form was inconvenient. Chitosan was also coupled with other inorganic materials¹⁰, which could prevent chitosan macrospheres from shrinking, but materials obtained were complex and the reaction process was usually very complicated. So the method developed here was very important for chitosan as enzyme immobilization carrier in industry.

Properties of the immobilized enzyme

pH Optima and pH stability

Figure 4 shows that the pH profile of the free enzyme peaked at pH 5.0, while the immobilized enzyme peaked at pH 6.0, a pH unit shift from the pH optimum for the soluble enzyme, which might be induced by the alteration of the microenvironment of the enzyme due to immobilization on support. Similar effects were also found for the immobilization of other enzymes on chitin and chitosan supports¹¹. The enzyme activity was determined by ONPG as substrate, at 55 °C in various pH buffers (4.0-9.0) for 15 min.

After all the enzymes were exposed to different pH (3-11) at room temperature for 30 min, Enzyme activity was determined at 55 °C, pH 6.0 for the immobilized enzyme and pH 5.0 for the free enzyme for 15 min, ONPG as substrate. As shown in Figure 5, the immobilized enzyme had a wider pH range than that of the free enzyme, in the range of 3-10, the immobilized enzyme activity remained > 94.7%.

Optimum temperature and thermostability.

Enzyme activity was determined by ONPG as substrate at various temperature (40-70 °C) at pH 6.0 for 15 min. As seen in Figure 6, the immobilized enzyme temperature optimum was 55 °C, just like that of the free enzyme.

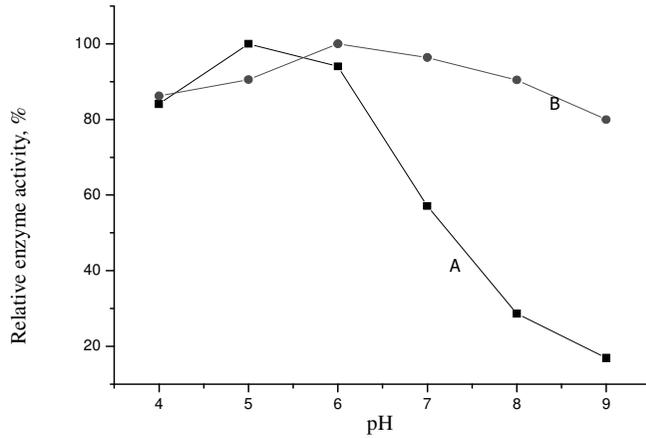


Figure 4. The pH activity profiles for free enzyme (-■-) and immobilized enzyme(-●-).

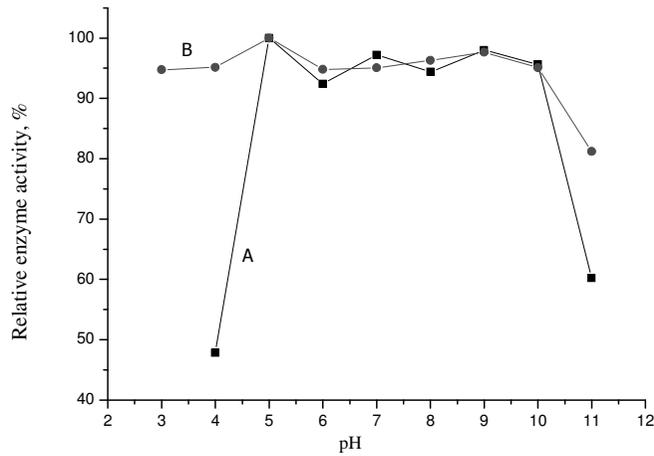


Figure 5. The pH stability of free enzyme (-■-) and immobilized enzyme (-●-).

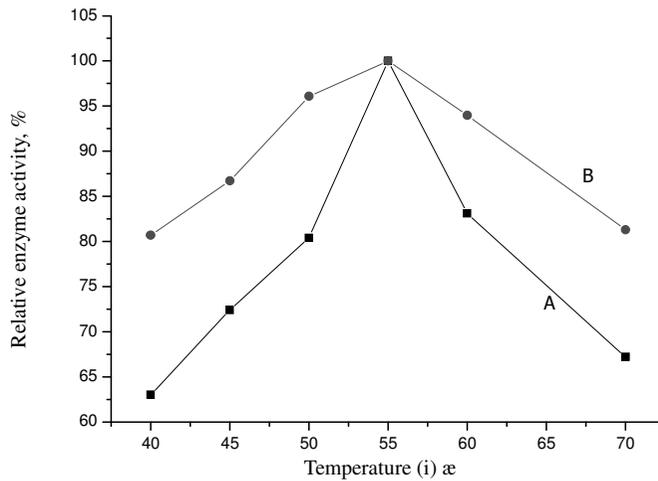


Figure 6. Temperature/activity profiles for free enzyme (-■-) and immobilized enzyme (-●-).

Figure 7 shows that the immobilized enzyme was more stable than the free enzyme. At 50 °C, after 8 h, the remaining activity of the former was 70.5%, but the latter's was 47.8% (Figure 7a). At 60 °C, over a period of the same time, the residual activity of the free enzyme was 40.9%, whereas that of the bound enzyme was 54.8% (Figure 7b). Results showed that the immobilized enzyme had better thermostability than the free enzyme.

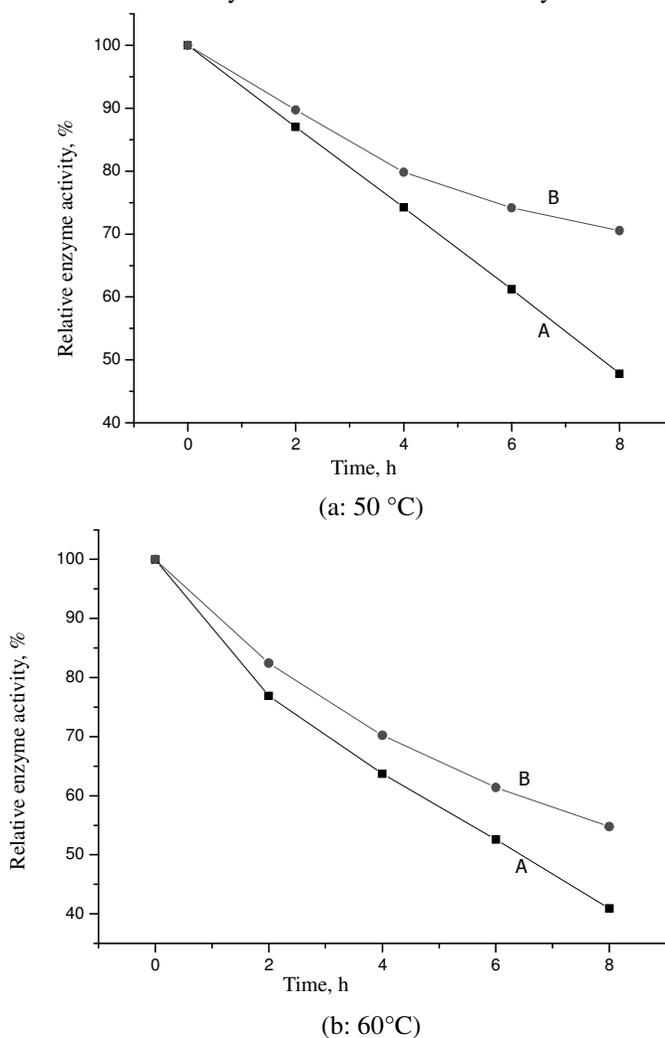


Figure 7. Thermal stability of free enzyme (-■-) and immobilized enzyme (-●-).

Operational stability

The experiment was repeated 10 times by using the procedures mentioned above with the same immobilized enzyme at the same initial concentration of ONPG. The results are summarized in Figure 8 and it was shown that the immobilized β -galactosidase was used for 10 times without significant loss in activity, meaning that almost no enzyme was dissociated from the surface of the chitosan in the course of the reaction, so the operational stability of the immobilized enzyme obtained was very good.

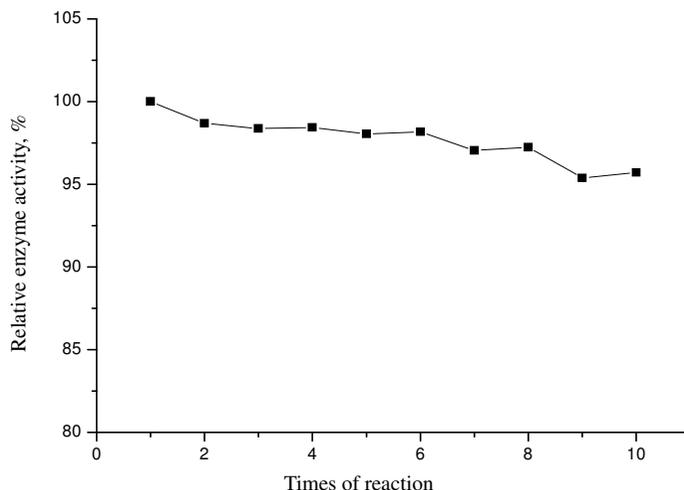


Figure 8. Operational stability of the immobilized enzyme

Kinetic parameters.

Lineweaver-Burk plot for the free and immobilized enzymes using ONPG as substrate was made and the values for K_m and V_m calculated from those graphs were shown in Table 3. From Table 3, it could be seen that the value of K_m for the immobilized enzyme was approximately 2-fold higher than that of the free enzyme for the same substrate and the V_m value of the immobilized enzyme was about 1.5 times lower than that of the free enzyme, which was probably caused by the immobilization procedure and by the lower accessibility of the substrate to the active site of the immobilized enzyme¹².

Table 3. Kinetic parameters of native and immobilized enzymes.

Derivative	K_m , m M	V_m , μ M/min
Free enzyme	10.2	0.278
Immobilized enzyme	18.8	0.183

Conclusion

A new process to make chitosan macrospheres was offered in this paper, it was much more convenient and faster than the traditional operation, and various chitosan with different molecular weight was used as materials to immobilize β -galactosidase, the results showed that the molecular weight of the chitosan should be considered when we selected chitosan as enzyme immobilization carrier and the chitosan with the lowest molecular weight, *i.e.* 500 000, was employed in our study based on the highest enzyme activity. More importantly, it was inconvenient for us to store the wet chitosan macrospheres in industry, but how to prevent it from shrinking during drying? A simple method was presented here, after being treated with 30% glycerol solution for 1h, the chitosan macrospheres could be dried nearly without shrinkage and kept almost the same structure as its wet form. Considering the inconvenience of the storage of the wet form, the dry chitosan macrospheres treated by glycerol solution were more applicable to immobilize enzyme. Finally, β -galactosidase from *Aspergillus oryzae* was immobilized on above dry chitosan macrospheres, properties of the

free and the immobilized enzyme were compared, a satisfactory results of the immobilized enzyme were obtained in enzyme activity yield, pH stability, thermal stability and operational stability. In addition, K_m values for free and immobilized enzyme were 10.2 and 18.8 mM, respectively, and V_m values for them were 0.278 and 0.183 $\mu\text{M}/\text{min}$ respectively. The conclusion obtained here was very useful for industrial application of chitosan microspheres as enzyme immobilization carrier, and the procedure developed were simple, fast and inexpensive.

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