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Using of Silica Particles as Porogen for Preparation of Macroporous Chitosan Macrospheres Suitable for Enzyme Immobilization[1]

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Abstract—Porous chitosan macrospheres were prepared at the first time by using silica particles as porogen, and the optimal weight ratio of silica to chitosan during preparation was determined. Scanning electron microscopy micrographs showed that the support with silica as porogen (support I) had a much more porous surface structure than the support without porogen (support II). Both supports were applied to immobilize β-galactosidase from *Aspergillus oryzae*. Much higher specific activity and yield of galactose hydrolysis products were observed for the support I. Properties of both immobilized enzyme were determined and compared with the free enzyme, satisfactory results were obtained in thermostability, pH arid operational stability, Michaelis constants K_m and in maximum velocity of hydrolysis (V_m). Suggested method allow to prepare chitosan macrospheres as immobilized enzyme carrier with moreporous surface structure and more active reaction groups.

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Application of solid-phase biocatalysts had become important during the last decades. Enzymes could be immobilized on various supports and by different methods [1, 2]. The properties of the immobilized biocatalysts depends on the characteristics of enzyme, support material and of the immobilization method. A suitable support should have high affinity to proteins, reactive functional groups, hydrophilicity, mechanical stability and rigidity. Chitosan is a natural polyaminosaccharide which offered the characteristics above, therefore it was often used for enzyme immobilization [3, 4].

For practical purposes, the carrier with porous surface structure, which would favor higher adsorption capacity for the enzyme, is the most suitable for enzyme immobilization [5, 6]. Thus, the possibility to increase the specific surface area of the support and get more active sites available for the enzyme immobilization has high importance in industry and biotechnology.

The aim of the present paper is to suggest a new process to prepare porous chitosan macrospheres used for enzyme immobilization. Silica particles are usually used as a porogen. In contrast to chitosan, the silica particles are insoluble in acidic media but soluble in alkaline solutions. These opposite properties allow us to use silica particles to control the number of the pores in the chitosan macrospheres. Firstly, the weight ratio of silica to chitosan was determined and the porous chitosan macrospheres were obtained. Secondly, β-galactosidase from *Asp. oryzae*, which efficiently catalyzes not only the hydrolysis of the β-galactoside linkages of lactose to glucose and galactose but also the transgalactosylation reaction to produce galactooligosaccharides [7], was employed to be immobilized on above carriers with glutaraldehyde as crosslinker under the optimum conditions, and the enzyme activities on different supports with and without silica as porogen were compared. Finally, the kinetic data of the immobilized enzyme—the values of Michaelis constants K_m and the maximum velocity of reaction (V_m)—were also determined for both immobilized enzyme and compared with those of the free enzyme.

EXPERIMENTAL PART

Apparatus and Reagents

Ultraviolet Spectrophotometer (T6 New Century), Vacuum Desiccator (DZ-6020), Digital pH Meter (PHS-3C) and Water Constant Temperature Oscillator (SHA-B) were used for the study. All the aqueous solutions were prepared in twice distilled water.

β-Galactosidase from *Aspergillus oryzae* (11.2 U/mg) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from "Sigma." Chitosan (medium molecular weight, M_w = 500000) was acquired from "Jinan Haidebei Marine Bioengineering Co Ltd." Silica 10–40 μm was products of "Jiangyou Co Ltd". Glutaraldehyde, glycerol and other reagents were all analytical grades.

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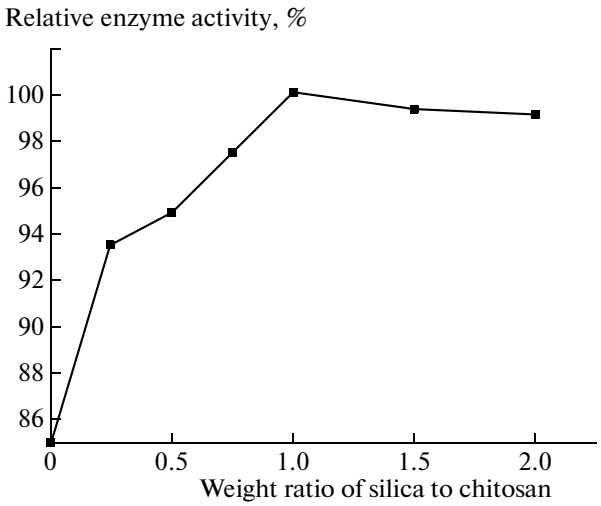

Fig. 1. Effect of weight ratio of silica to chitosan on enzyme activity.

Preparation of Macroporous Chitosan Macrospheres

The chitosan macroporous macrospheres were prepared as follows: a solution of chitosan was first obtained by dissolving 1 g of chitosan in 20 ml of 2% (w/v) aqueous acetic acid solution. Then, 1 g silica particles were added into it, followed by vigorous stirring in order to disperse them uniformly. The viscous solution obtained was placed in a vacuum dryer to remove air bubbles, and then was sprayed drop-wise through a syringe into a gently stirred coagulation liquid containing 15% NaOH and ethanol in a volume ratio of 4 : 1 at a constant rate. The obtained macrospheres were washed and immersed in a 5 wt % aqueous NaOH solution and kept for 2 h at 80°C in order to dissolve the silica particles and to generate a porous macrospheres. Finally, the porous beads were washed with distilled water to remove the remaining NaOH. In order to prevent its shrinkage during drying, the macrospheres was immersed in a 30 vol % aqueous glycerol solution (softening agent) for 1 h and, after the excess glycerol solution was removed, it was placed on a glass plate and allowed to dry. Thus, a strong and flexibly porous chitosan macrospheres without shrinkage was obtained. The chitosan carrier with silica as pore-forming agents was referred to support I while the carrier without silica as porogen was referred to support II.

Immobilization Procedure

Preparation of activated porous chitosan macrospheres by glutaraldehyde 2 g of the chitosan macrospheres 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 macrospheres obtained were rinsed thoroughly and stored for the next use.

Immobilization of β-galactosidase on the activated chitosan macrospheres. The immobilization was carried out by adding of activated porous macrospheres (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 phosphate buffer until no protein was detected.

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 65°C. Enzyme stabilities were determined after a long exposure at various temperature (50 or 60°C), followed by analysis at the 55°C.

The pH–activity curve in the range pH 4.0–9.0 was determined for the free enzyme and immobilized enzymes at 55°C using ONPG as substrate. The pH stability in the range 3.0–11.0 was determined after 30 min exposure at different pH and room temperature.

Operational Stability of Immobilized Enzyme

The operational stability of the immobilized enzyme was determined according to the following procedures. 0.5 g of the immobilized enzyme was soaked in 1.9 ml of phosphate buffer overnight. After the mixture was incubated at 55°C for 2 min, the reaction was started by adding 5 mmol/1 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.

Kinetics

The Michaelis constant K_m was calculated for the soluble and the immobilized enzyme by assaying the enzyme in increasing ONPG concentrations ranging from 0.75 to 7.5 mmol in phosphate buffer.

RESULTS AND DISCUSSION

Effect of Silica on the Structure of the Chitosan Macrospheres

The surface structure of the chitosan macrospheres could be easily controlled by changing the weight ratio of silica to chitosan. During the preparation of the carrier, various amounts of silica were added into the chitosan solution, the results obtained are shown in Fig. 1. It was shown that the activity of the immobilized enzyme rapidly increased at first, then kept nearly constant with the increase of the ratio of silica

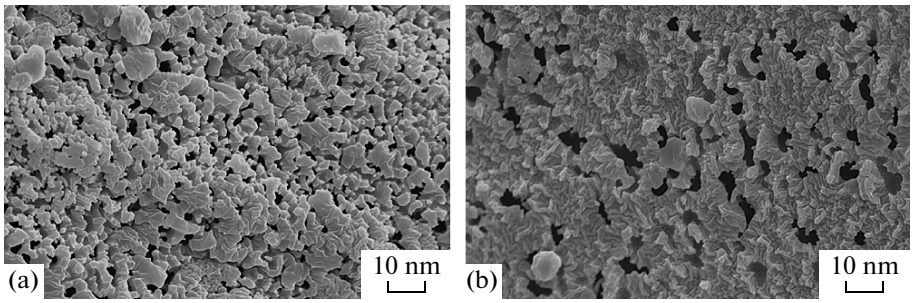

Fig. 2. Scanning electron micrographs of the supports I (a) and II (b).

to chitosan from 0.25 : 1 to 2 : 1. When the ratio was 1 : 1, the enzyme activity attained its maximum, so 1 : 1 weight ratio of silica to chitosan was used in the later investigation.

In order to show the, effect of silica on the surface structure of the chitosan macrospheres, the scanning electron micrographs were carried out for the support I with silica as porogen and the support II without any pore-forming agent, separately. The results are shown in Figs. 2a and 2b. It is seen that the support I had a much more porous surface structure than, the support II. In addition, both supports were used to immobilize enzyme under its optimum conditions. The results obtained are listed in the Table 1. The activity of enzyme immobilized on the support I reached a maximum of 78.88 U/(g of dry carrier). The free enzyme activity was much higher than that on the support I. The reason could be that the porous surface properties of the chitosan macrospheres favored to higher adsorption capacity for the enzyme due to increase in the surface area.

pH Optimum and pH Stability

Figure 3 shows that the pH profile of the free enzyme has maximum at pH 5.0, while for both immobilized enzyme the maximum is at pH 6.0. pH shift from the optimum for the soluble enzyme 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 supports [8, 9]. The enzyme activity was determined with 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 and pH 6.0 for the immobilized enzyme and pH 5.0 for the free enzyme for 15 min with ONPG as substrate. As shown in Fig. 4, 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 more then 94.8%.

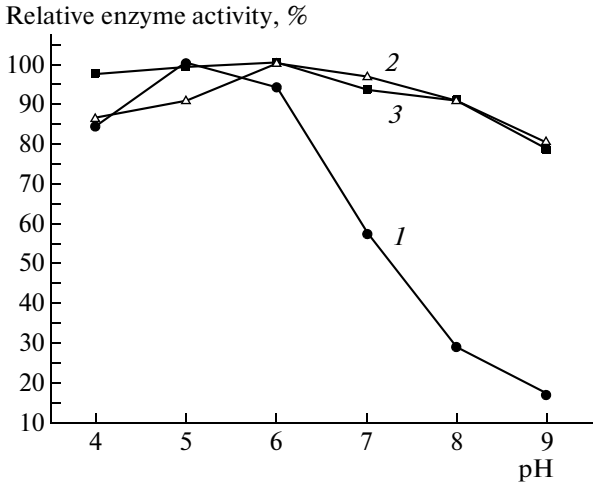

Fig. 3. Effect of pH on enzyme activity: *1*—free enzyme, *2*—enzyme immobilized on support II, *3*—enzyme immobilized on support I.

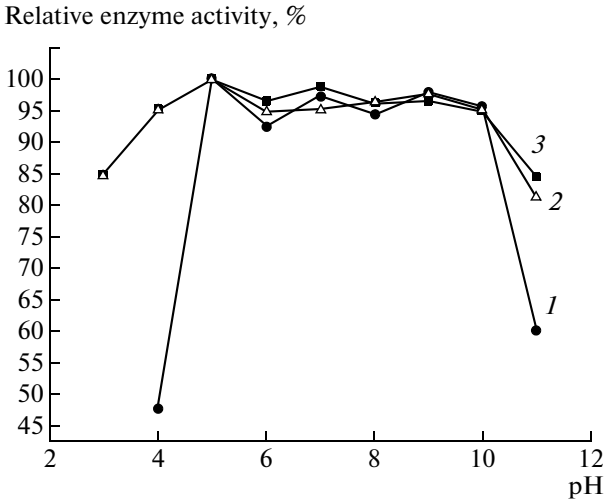

Fig. 4. pH stability: *1*—free enzyme, *2*—enzyme immobilized on support II, *3*—enzyme immobilized on support I.

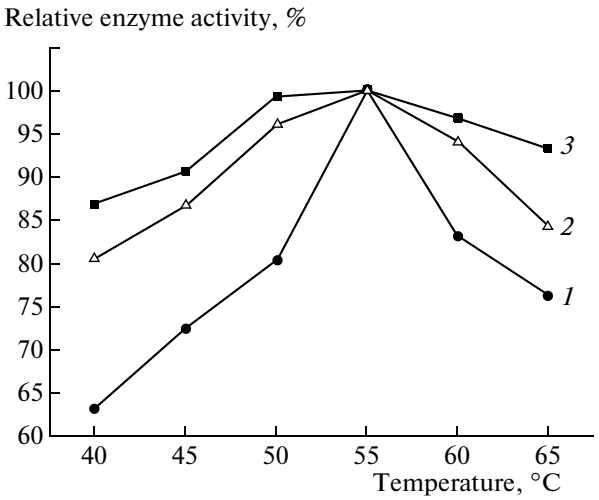

Fig. 5. Effect of temperature on enzyme activity: *1*—free enzyme, *2*—enzyme immobilized on support II, *3*—enzyme immobilized on support I.

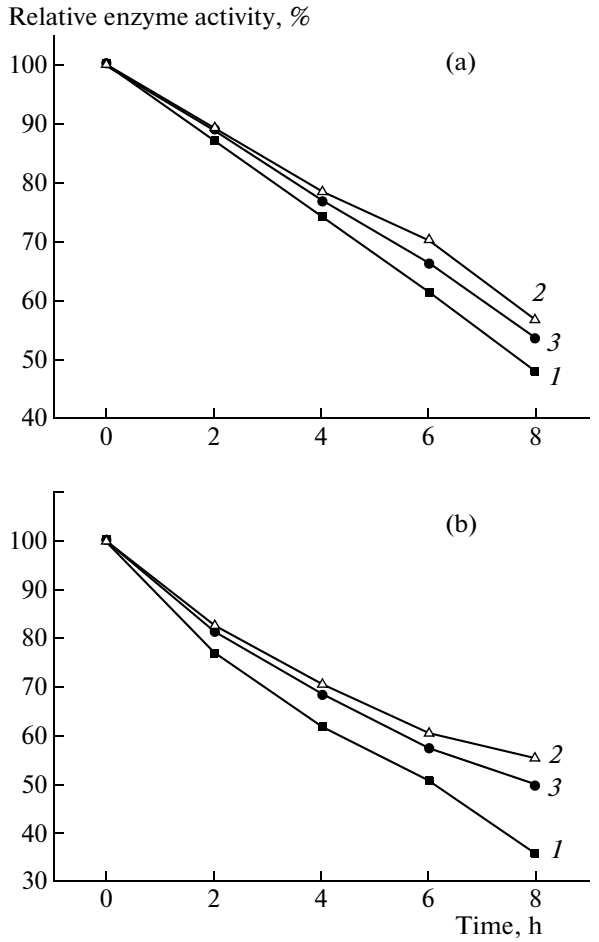

Fig. 6. The stability of β-galactosidase at 50 (a) and 60°C (b): *1* —free enzyme, *2* —enzyme immobilized on support I, *3* —enzyme immobilized on support II.

Optimum Temperature and Thermostability

The effects of temperature on the activity of free and immobilized β-galactosidase are shown in Fig. 5. Enzyme activity was determined with ONPG as substrate at various temperatures (40–70°C) at pH 6.0 for 15 min. As seen in Fig. 5, the immobilized enzymes temperature optimum was 55°C, just like that of the free enzyme.

Figure 6 shows that the immobilized enzyme is more stable than free one. At 50°C, after 8 h, the remaining activity of the immobilized enzyme II was 53.5% and immobilized enzyme I was 56.6%, but the free enzyme was 47.8%. At 60°C, after the same time, the residual activity of the free enzyme was 35.6%, whereas that of the immobilized enzyme II was 49.8% and immobilized enzyme I was 55.4%. The results showed that both immobilized enzymes had better thermostability than the free enzyme.

Operational Stability of Immobilized Enzymes

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 summarized in Fig. 7 shown that the immobilized β-galactosidase may be used for 10 times without significant loss in activity. It means 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.

Kinetic Parameters

Lineweaver–Burk plot for the free and immobilized enzymes using ONPG as substrate was made and

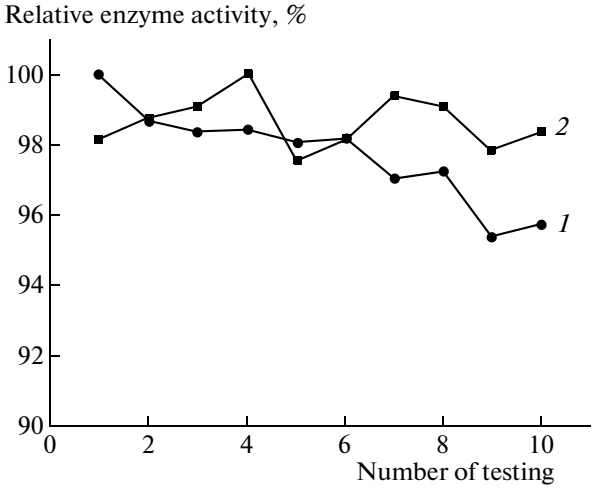

Fig. 7. Operational stability of enzyme immobilized on support II (*1*) and on composite support I (*2*).

Table 1. Immobilization of β-galactosidase on the supports I and II

Type of support	Immobilized enzyme activity, U/(g of dry support)	Recovery of immobilized enzyme, %
Support I	78.88	65.74
Support II	63.42	60.35

Table 2. Kinetic parameters of free and immobilized enzymes

Form of enzyme	K_m, mmol/l	V_m, μmol/min
Free	10.2	0.278
Immobilized on support II	18.8	0.183
Immobilized on support I	23.4	0.251

the values of K_m and V_m calculated from those graphs are shown in Table 2.

The value of K_m for the enzyme immobilized on the support I is higher than that of the free enzyme and enzyme immobilized on the support II. The V_m value of both immobilized enzyme is lower than that of the free enzyme, and it is probably caused by the immobilization procedure and by the lower accessibility of the substrate to the active site of the immobilized enzyme [10].

Thus, in this study, the porous chitosan macrospheres at the first time were prepared by using silica particles as porogen. Scanning electron microscopy micrographs showed that the support with silica as porogen had a much more porous surface structure than the support without pore-forming agents. In order to test the suitability of the support newly prepared, β-galactosidase from *Aspergillus oryzae* was immobilized on it and the results obtained were compared with that obtained on the support without porogen. Much higher specific enzyme activity and yield of hydrolysis products were obtained for the former. Finally, the characteristics of enzymes immobilized on the supports I and II separately, such as pH optima and pH stability, optimal temperature and thermostability, Michaelis constants K_m and maximum velocity of the reaction V_m, were determined and compared with characteristics of the free enzyme. It was shown that the new method of preparing of porous chitosan macrosphere is very successful and very useful for industrial application of chitosan macrospheres as enzyme immobilization carrier because it allow to obtain porous surface structure and more activated reaction groups.

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