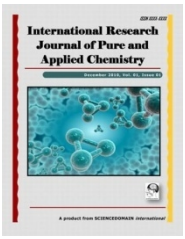

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Preparation of Macroporous Poly (GMA-DVB) and Its Application to Immobilize β-Galactosidase from Aspergillus Oryzae

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Authors' contributions

This work was carried out in collaboration of all authors. Author SS designed the study. All authors contributed in practical work and managed the analysis of the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

The reactive, macroporous glycidyl methacrylate (GMA) and divinyl benzene (DVB) polymer was synthesized with a mixture of cyclohexanol and lauryl alcohol as liquid pore-forming agents and nano-calcium carbonate as solid one via suspension polymerization. The surface structure of the poly (GMA-DVB) beads was characterized with scanning electron microscopy (SEM). Under the optimum conditions, β-galactosidase from Aspergillus Oryzae was bound on the polymer beads obtained above. The activity yield of the immobilized enzyme was 80.35% and the activity of it could reach 540.01 IU/g dry carriers. Meanwhile, the basic properties of the immobilized enzyme, such as optimum temperature, optimum pH, pH stability, thermal stability, operational stability, etc., were investigated.

Keywords: Glycidyl methacrylate; divinyl benzene; suspension polymerization; β-galactosidase; immobilization.

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1. INTRODUCTION

Enzymes can be produced in large quantities, because of the development of the modern genetic engineering. And enzyme biocatalysts have been used more and more widely in the environmental protection in recent years [1-3]. Since the free enzyme can neither be separated from the reaction mixture nor recycled, immobilization of enzyme has attracted the attention [4-7].

In recent years, the research for the enzyme carrier has made great progress at home and abroad. A variety of materials, such as the cellulose, the chitosan and its derivatives, the gel materials, organic synthetic polymers and so on, have been used as carrier materials [8]. Among all the materials, epoxy activated carriers seemed to be almost ideal to immobilize enzyme because epoxy group could exhibit good reactivity under mild conditions and would be very stable at neutral pH values even in wet conditions. Moreover, it's also easy to be modified because of its active epoxy group [9-13].

The porous poly (GMA-DVB) only with cyclohexanol and lauryl alcohol as liquid pore-forming agents has been synthesized in our lab and the study results have been published in reference [14]. However, only micropores were found on the beads obtained above, which was not perfect enough for it as enzyme immobilization supporter. In order to increase the specific surface and improve the pore structure of the carrier, the pore-forming agents including both solid nano-calcium carbonate and organic liquids were used to synthesize macroporous poly (GMA-DVB) by suspension polymerization with glycidyl methacrylate (GMA) as monomer and divinyl benzene (DVB) as cross-linker in this study. Scanning electron microscopy (SEM) was used to characterize the surface structure of the beads and the copolymer obtained was applied to the immobilization of β-galactosidase from Aspergillus Oryzae. Under the optimum conditions, the enzyme activity and the activity yield of the immobilized enzyme were determined. Finally, the basic properties of the immobilized enzyme including optimum temperature, optimum pH, pH stability, thermal stability and operational stability were also researched.

2. EXPERIMENTALS

2.1 Apparatus and Reagents

Vacuum Pump with Circulated Water System (SHZ-D), Vacuum Desiccator (DZ-6020), Ultraviolet Spectrometer (T6 New Century), Digital pH Meter (PHS-3C) and Water Constant Temperature Oscillator (SHA-B) were used.

Glycidyl methacrylate (GMA) was purchased from Shanghai Jinchao Chemical Co. Ltd. Divinyl benzene (DVB) (45%) was acquired from Tianjin Guangfu Fine Chemical Industry Research Institute. β-galactosidase from Aspergillus Oryzae (11.2 U/mg) and o-nitrophenyl-β-D-galactopyranoside (ONPG) were supplied from Sigma. Azobisisobutyronitrile (AIBN) and other reagents were all analytical grade. All the aqueous solutions were prepared with twice distilled water and stored at 4ºC.

2.2 Preparation of Poly (GMA–DVB) Beads

The porous poly (GMA–DVB) beads were synthesized by suspension polymerization in a four-neck round bottom flask equipped with a thermometer, reflux condenser, stirrer and

nitrogen inlet tube. The composition of the discontinuous organic phase was as follows: GMA (4 ml) as monomer, DVB (2.3 ml) as cross-linker, 0.0395 g of AIBN as initiator, lauryl alcohol (1.5 ml) and cyclohexanol (2 ml) as liquid porogen and 0.3600 g of nano-calcium carbonate as solid porogen. The continuous phase comprised of 55 ml PVA (3%) and 55 ml glutin (0.2%). Typical procedure: the degassed organic phase was prepolymerized for 24 h in water constant temperature oscillator at 40ºC and then was added into a four-neck round bottom flask with the continuous phase which had been stirred at 55ºC under nitrogen. After sustaining at 55ºC for 0.5 h, the polymerization reaction was maintained at 65ºC for 3.0 h and then at 85ºC for 2.0 h. Finally, the synthesized poly (GMA–DVB) beads were separated from the reaction medium and soaked in ethanol for 24 h to remove the liquid porogenic agent and the unreacted monomers. Then it was immersed in hydrochloric acid solution (0.1 M) for 24 h to get rid of the solid nano-calcium carbonate. After that, it was washed completely with ethanol and distilled water and dried in the vacuum oven at 55ºC overnight.

2.3 Preparation of Enzyme and Substrate Solution and Enzyme Immobilization

The enzyme solution was obtained by dissolving 0.0400 g of β-galactosidase from Aspergillus Oryzae in 10 ml citric acid buffer (0.1 M, pH 4.0) and the substrate solution was prepared by immersing 0.0150 g of ONPG in twice distilled water. All solutions were kept in the refrigerator at 4 ˚C for use.

The immobilization of enzyme was conducted by putting 0.0500 g of poly (GMA–DVB) beads into 0.5 ml enzyme solution (3 mg/ml) in ultrasonic cleaning machine at 25ºC for 3.0 h. And then the enzyme immobilized beads obtained was filtered and washed completely with citric acid buffer (0.1 M, pH 4.0) to remove the physically bound enzyme.

2.4 Activity Assay of β-galactosidase

The activities of free and immobilized enzyme were determined according to the references [14,15]. For the free enzyme, 0.1 ml of it was added into 0.9 ml citric acid buffer (0.1 M, pH 5.0) and then 0.2 ml of ONPG (1.5 mg/ml) was added into the mixture above to start the reaction at 55ºC in water constant temperature oscillator. After exactly 15 min of incubation, 2.0 ml of Na_2CO_3 solution (1.0 M) was added into the reaction system to stop the reaction and the absorbance of reaction solution was measured at 420 nm. For the determination of the activity of the immobilized enzyme, the same assay measures were applied to it. 0.0500g of β-galactosidase immobilized beads were soaked in 1.0 ml buffer solution used above and then all the steps were conducted as same as that proceeded on the free enzyme. The ratio of the activity of immobilized enzyme to that of free enzyme subjected to immobilization was calculated as the activity yield. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate ONPG per minute at 55ºC.

3. RESULTS AND DISCUSSION

3.1 Discussion about Macroporous Poly (GMA-DVB) Beads

The SEM micrographs of the polymer beads were obtained using KYKY-2800B scanning electron microscope. The results were presented in Fig. 1. From the SEM micrograph, two kinds of pores were found on the polymer beads obtained in this study and the maximum aperture could attain up to about 10 μm after 507 times enlargement. However, only one kind of pore was observed on the polymer beads synthesized using only liquid porogen in

reference [14] and its aperture was about 0.2 μm after 3.00 k times amplification. It was clearly shown that the carriers obtained using both liquid and solid porogens had much more porous surface structure than the carriers only using liquid porogen. Under the best conditions, the carriers obtained here were used to immobilize β-galactosidase. The activity yield was 80.35% and the activity of the immobilized enzyme could reach 540.01 U/g dry carriers. Whereas the enzyme activity of the immobilized enzyme whose carriers used only liquid porogen was 247.20 U/g dry carriers [14]. All the results described above suggested that the carriers obtained much more specific surface due to the addition of nano-calcium carbonate as porogen, which would favour higher immobilization capacity for the enzyme.

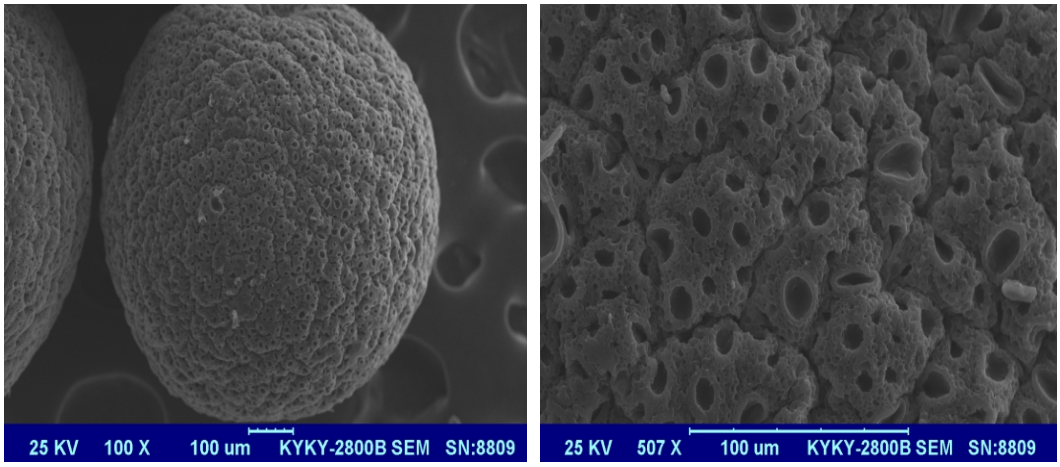

Fig. 1. The SEM micrographs of the carrier obtained

3.2 Properties of The Immobilized Enzyme

3.2.1 Optimum temperature and optimum pH

The activities of the free enzyme and the immobilized enzyme were determined at various temperatures (40ºC, 45ºC, 50ºC, 55ºC, 60ºC, 65ºC) at pH 5.0 with ONPG as substrate. The results were presented in Fig. 2. It could be seen from the Fig. 2 that the optimum temperature of the free enzyme and the immobilized enzyme were all 55ºC. This could be explained by that the optimum temperature for enzyme catalytic reaction was mainly affected by the following two aspects. One is that the mass transfer speed of enzyme and substrate accelerates in pace with the rise of the temperature. The other is that the active groups of enzyme tend to be damaged at high temperatures. From the results it could conclude that the integrated influences of the two aspects on free enzyme and immobilized enzyme were basically the same, so the same optimum temperature was observed.

The optimum pH was tested by determining the free enzyme and the immobilized enzyme activities with ONPG as substrate in the pH range from 3.0 to 10.0 at 55ºC. As shown in Fig. 3. that the optimal pH for both kinds of enzymes were pH 5.0. The results obtained here were consistent with those of Zhou HX [16] and Cochrane FC [17] who had reported that if there were charges in the carriers, the optimal pH of the immobilized enzyme would be shifted. Because the poly (GMA–DVB) carriers used in the study were nonionic, the optimal pH of the immobilized enzyme was not changed.

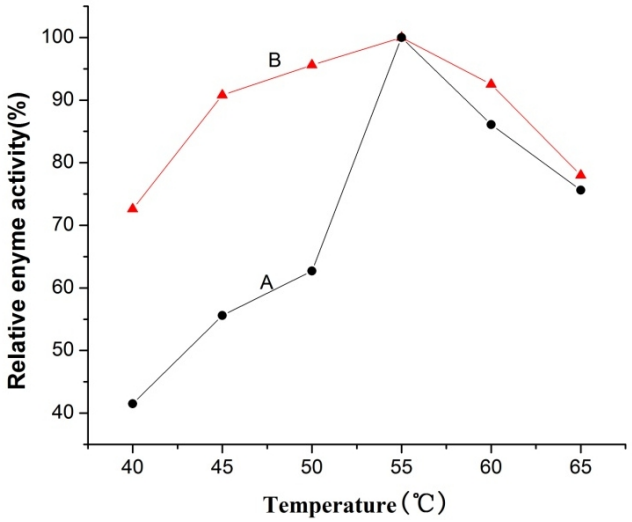

Fig. 2. Effect of temperature on the activity of the free and the immobilized enzyme (A: Free enzyme; B: Immobilized enzyme)

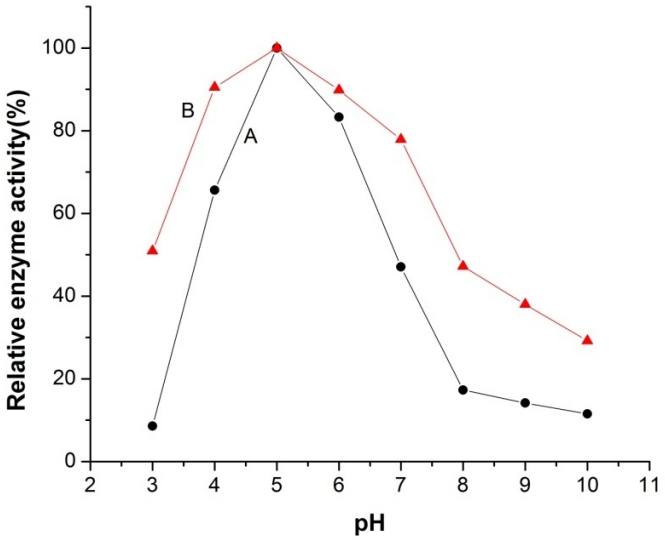

Fig. 3. Effect of pH on the activity of the free and the immobilized enzyme (A: Free enzyme; B: Immobilized enzyme)

3.2.2 Thermal stability and pH stability

After the free enzyme and the immobilized enzyme were exposed to various temperatures (50ºC, 60ºC) for a long time (0-8 h). Their activities were determined at different time intervals (0 h, 2 h, 4 h, 6 h, and 8 h). The results obtained could be seen in Fig. 4 and Fig. 5. Whether the enzymes were exposed to 50ºC or 60ºC, the thermal stability of the bound enzyme was better than that of the free one. After incubation at 50ºC for 8 h, more than 79.6% of the immobilized enzyme remained active. At 60ºC, over a period of the same time,

the residual activity of the immobilized enzyme was 64.9 %, whereas that of the free enzyme was only 48.2%. The results showed that the immobilized enzyme had better thermal stability than the free enzyme. This was mainly caused by the following two aspects. Firstly, once the enzyme was immobilized on the carriers, the conformational mobility of the molecules would be restricted, which could result in that the enzyme protein molecules were not easy to expand. So that the active site of the enzyme would not be easy to be damaged [18]. Secondly, the carrier particles had some shielding effect on the external heat, which could reduce the influence of heat on the enzyme protein molecules, so the activity of the immobilized enzyme was not easy to be destroyed.

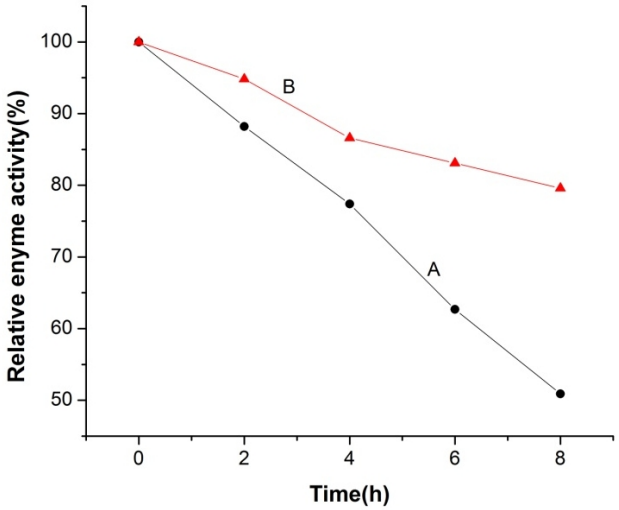

Fig. 4. Effect of temperature on the stability of free and the immobilized enzyme at 50ºC (A: Free enzyme; B: Immobilized enzyme)

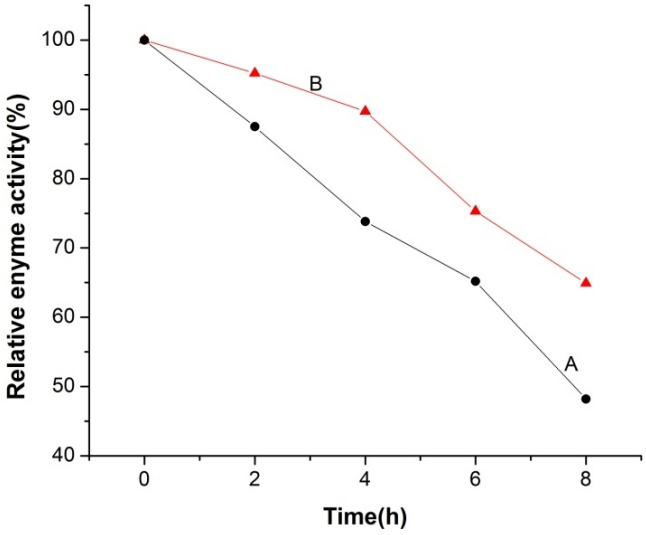

Fig. 5. Effect of temperature on the stability of free and the immobilized enzyme at 60 ˚C (A: Free enzyme; B: Immobilized enzyme)

The enzyme activities were determined at pH 5.0 in the thermostat oscillator (55ºC) after the free enzyme and the immobilized enzyme were soaked in different pH (2.0-9.0) buffer solution at room temperature for 30 min. The results were presented in Fig. 6. It could be illustrated that the pH stability range of the immobilized enzyme was 3.0-8.0, whereas that of the free enzyme was 5.0-8.0. So it could be seen that immobilized enzyme had a better resistance to acid than the free enzyme. This mainly relied on the immobilized method and the structure and property of the carriers [19-21].

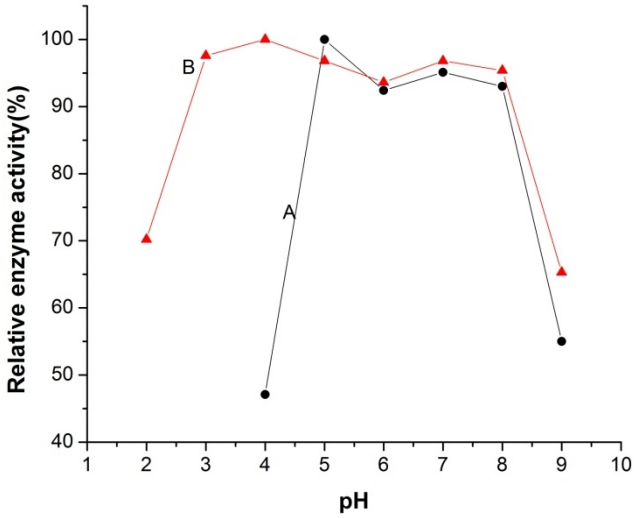

Fig. 6. Effect of pH on the stability of free and immobilized enzyme (A: Free enzyme; B: Immobilized enzyme)

3.2.3 Operational stability of immobilized enzyme

The experiments were repeated 8 times with the same immobilized enzyme. The results were shown in table 1. It was presented that the activities of the immobilized enzyme had not significant loss after the immobilized enzyme being used 8 times, which meant that almost no enzyme molecules dissociated from the carriers during the reaction process. So it could be seen that immobilized enzyme retained good operation stability in the process of reaction.

Table 1. Operational stability

Numbers of reuse	1	2	3	4	5	6	7	8
Relative enzyme activity (%)	100	98.9	99.1	98.2	98.0	99.2	98.5	98.9
Relative standard deviation (%)	0.638							

4. CONCLUSION

The reactive, macroporous polymer of glycidyl methacrylate (GMA) and divinyl benzene (DVB) was synthesized with a mixture of cyclohexanol and lauryl alcohol as liquid pore-forming agents and nano-calcium carbonate as solid one by suspension polymerization. SEM micrographs obtained showed that the carriers, which simultaneously using liquid and solid porogens had much more porous surface structure than the carriers only using liquid porogen. The carriers prepared were used to immobilize β-galactosidase from Aspergillus

Oryzae under the optimum conditions. The activity yield of the immobilized enzyme was 80.35% and the activity of it could reach 540.01 IU/g dry carriers. Both values were much higher than the enzyme bound on the carriers with only liquid porogen. Meanwhile, the basic properties including the pH and temperature optima, the pH and thermal stabilities were also determined and compared with those of the free enzyme and the results showed that the immobilized enzyme had better stability in temperature and pH than the free enzyme. Operational experiments showed that the link between the enzyme and the carrier was very firm, and the immobilized enzyme could be used repeatedly. All these results described above indicated that the supporter prepared with two kinds of porogen here, which had more porous surface structure and more active epoxy group exposed was very useful for industrial application as enzyme carrier.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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