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A new method to prepare macroporous poly(GMA-ST) and its application to immobilize β-galactosidase

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ABSTRACT

Two kinds of macroporous poly(GMA-ST) were synthesized by bulk copolymerization using different pore-forming agents, one only with liquid porogenic agent including cyclohexanol and lauryl alcohol and the other simultaneously with a mixture of cylohexanol, lauryl alcohol and nano-calcium carbonate. After the polymer was smashed, particles with diameters ranging 0.15mm to 0.30mm were taken as the carrier, Scanning electron microscopy (SEM) micrographs showed that both kinds of carrier. Under the optimum conditions, β-galactosidase was immobilized on the carrier obtained above, the enzyme activity, the activity yield and the basic property of the all the immobilized enzyme were determined separately, and satisfactory results were obtained in enzyme activity, activity yield, pH stability, thermal stability, and operational stability. The conclusion obtained indicated that the ploy(GMA-ST) prepared concurrently with liquid and solid porogen was more suitable to immobilize enzyme than that purely with liquid solution as pore-forming agents.

Keywords: glycidyl methacrylate, styrene, immobilization, β-galactosidase.

INTRODUCTION

During the last decade, the immobilization of enzymes has been often used in the production of pharmaceuticals, food and other biological products, and it is also essential for their application to industrial processes.

Enzymes can be immobilized on different supports by different methods, including entrapment

in alginate and fiber consisting of cellulose acetate and titanium isoproxide; covalent attachment onto chitosan, polyurethane foam, alginate, gelatin, and bone powder; adsorption onto phenol-formaldehyde resin and bone powder [1-7]. Some of these methods are difficult to perform on an industrial scale. However most of them suffer from low immobilization yields or continuous leakage of enzyme.

In this study, the carriers with macroporous morphology poly(GMA-ST) were synthesized successfully by the bulk copolymerization of glycidyl methacrylate (GMA) and styrene (ST), with different pore-forming agents, one only using a liquid porogenic agent of cyclohexanol and lauryl alcohol and the other simultaneously using a mixture of methanol aqueous solution as liquid pore-forming agents and nano-calcium carbonate as solid one. The resulting carriers were smashed and characterized by SEM, then employed in the immobilization of β-galactosidase. The basic property of the immobilized enzyme including enzyme activity, activity yield, pH stability, thermal stability, operational stability were determined and compared with those of the free enzyme in order to examine the suitability of the carrier obtained from liquid and solid pore-forming agents to immobilize enzyme.

EXPERIMENTAL SECTION

Reagents and apparatus

Glycidyl methacrylate (GMA) (99%) was obtained from Shanghai Jinchao Chemical Co. Ltd; β-galactosidase from *Aspergillus oryzae* and o-nitrophenyl-β-D- galactopyranoside (ONPG) were obtained from sigma. The enzyme activity was 11.2U/mg solid. Styrene and other reagents were all analytical grades. All the aqueous solutions were prepared by twice distilled water.

Ultraviolet Spectrotometer (T6 New Century), Digital pH Meter (PHS-3C), Vacuum Desiccator (DZ-6020), Universal Grinder (FW-200), Ultrasonic Cleaning Machine and Water Constant Temperature Oscillator (SHA-B) were used.

Preparation of the carriers

The reaction was carried out in a plastic beaker, including a mixture of the monomers (GMA 4.5mL and Styrene 1.7mL), initiator (AIBN 0.0395 g), 2mL cyclohexanol and 1.5mL lauryl alcohol as liquid porogenic agent, and 0.2400 g nano-calcium carbonate as solid one. After the mixture was degassed and homogenized by ultrasonication for 20 min, the reaction was carried out at 86℃, then the large pieces of solid obtained was smashed and the particles ranging from 0.15 to 0.30mm were taken as the carrier. After being washed with distilled water completely, the carriers were kept in ethanol for 24h to get rid of liquid porogen and 0.1M hydrochloric acid solution for 24h to remove the solid one- the nano-calcium carbonate, and finally dried in the vacuum oven at 55℃ for use. Note: in this paper, the carrier only with liquid pore-forming agents was called carrier I, and the carrier with both liquid and solid pore-forming agents was called carrier II.

Preparation of enzyme and substrate solution

0.0300g of β-galactosidase was dissolved in 10mL 0.1M citric acid buffer (pH 4.0), and then kept in the refrigerator at 4℃ for use. The substrate solution was obtained by dissolving 0.0150g

ONPG in 10mL twice distilled water.

Immobilization of β-galactosidase

0.0500g of polymer particles was put in 0.5mL 0.1M citric acid buffer (pH 4.0) containing enzyme (3mg/mL). The reaction was undergone in ultrasonic cleaning machine at 25℃ for 3 hours. After that, the immobilized enzyme was filtered and washed with 0.1M citric acid buffer (pH 5.0) until there was no protein. The enzyme bound on the carrier Ⅰ was called immobilized enzyme Ⅰ, while that bound on the carrier Ⅱ was called immobilized enzyme Ⅱ.

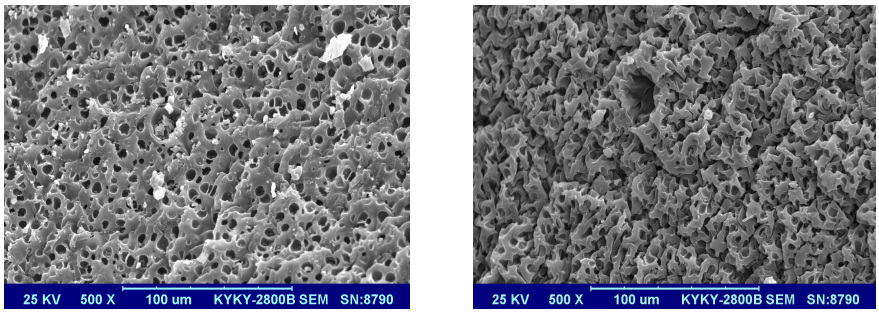
Assay of β-galactosidase activity

Activities of free and immobilized β-galactosidase were assayed by the addition of 0.1mL of free enzyme or 0.0500g of immobilized β-galactosidase in the citric acid buffer (pH 5.0), using 0.2mL of ONPG (1.5mg/mL) as the substrate [8, 9]. After exactly 15 min of incubation at 55℃, the reaction was stopped by adding 2mL of Na2CO3 solution (1M). The absorbance was measured at 405nm. All activity measurement experiments were carried out three times. The activity yield was calculated as the ratio of immobilized enzyme to enzyme subjected to immobilization. One unit of β-galactosidase activity is defined as the amount of enzyme that liberated 1μmol of product per minute under the assay condition.

RESULTS AND DISCUSSION

Comparison of carrier Ⅰ and carrier Ⅱ

The SEM micrographs of the carrier Ⅰ and carrier Ⅱ were illustrated in Fig.1. the photographs from carrier Ⅰ and carrier Ⅱ both exhibited the apparent morphology with macroporous surface. The photos also showed clearly that the carrier Ⅱ, which using both liquid and solid pore-forming agents, had a more porous surface structure than that of the carrier Ⅰ only with liquid pore-forming agents. Carrier Ⅰ and carrier Ⅱ were used to immobilize β-galactosidase under the optimum conditions, and the results were listed in table 1. According to the data presented in Table 1, the activity of the immobilized enzyme on the carrierⅠ reached a maximum of 535.11U/g dry carrier, which was much higher than that of carrier Ⅰ. The obtained enzyme activity was more than 3 times that obtained on the carrier II, which could be explained that the porous surface properties of GMA-ST polymer would favor higher adsorption capacity for the enzyme due to increase in the surface area.



Carrier Ⅰ Carrier II

Fig. 2. SEM photographs of the carrierⅠ and carrierⅡ

Table 1 the immobilization results of β-galactosidase on the carrier Iand the carrier II

Type of carrier	Immobilized enzyme activity (U/g dry carrier)	Activity yield(%)
Carrier I	155.29	23.11
Carrier II	535.11	79.63

Properties of the immobilized enzyme

Effect of temperature

All enzyme activities were determined by ONPG as substrate at various temperatures (40-650C), the results obtained were shown in Fig.2. The optimum temperature of the free and both immobilized enzymes were at 55℃.

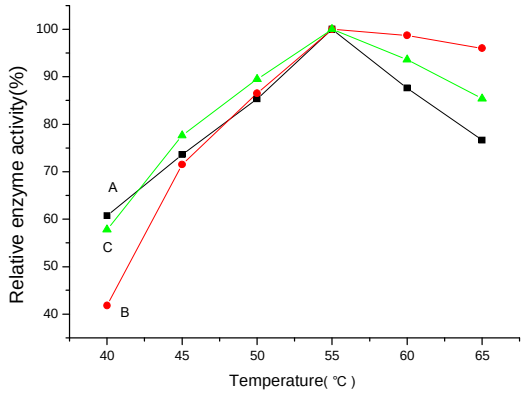


Fig. 2. Effect of temperature on the activity of the free and immobilized enzymes (A: Free enzyme; B: Immobilized enzyme Ⅰ; C: Immobilized enzyme Ⅱ)

Thermal stability

It was well known that free enzyme is not stable and its activity would also decrease gradually during use. The Thermal stability of immobilized enzymes conferred to a good performance as could be seen in Fig. 3 and Fig. 4. After incubation at 40℃ for 8h, more than 77% of immobilized β-galactosidase remained active, while the remaining activity of the free enzyme was 50.9%. At 50°C, over a period of the same time, the residual activity of the free enzyme was 27.5%, whereas that of the immobilized enzyme Ⅰ was 70.7% and the immobilized enzyme Ⅱ was 58.7%. Therefore, the immobilization remarkably enhances the heat resistance of β-galactosidase.

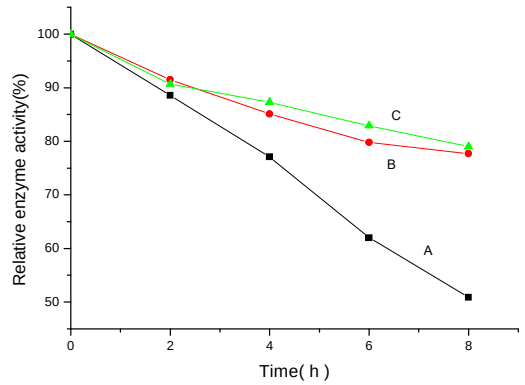


Fig. 3. Effect of temperature on the stability of free and immobilized enzymes at 40℃ (A: Free enzyme; B: Immobilized enzyme Ⅰ; C: Immobilized enzyme Ⅱ)

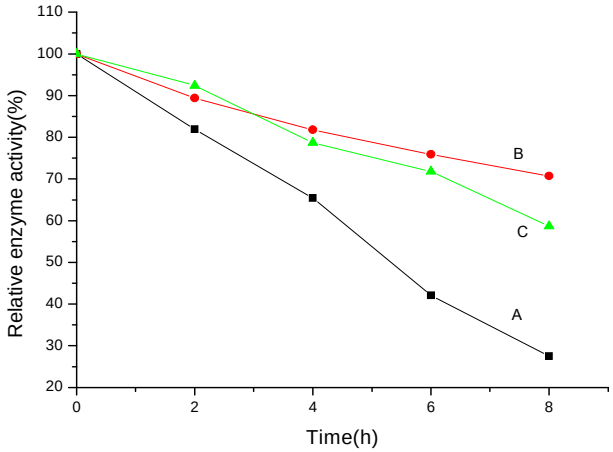


Fig. 4. Effect of temperature on the stability of free and immobilized enzymes at 50℃
(A: Free enzyme; B: Immobilized enzyme Ⅰ; C: Immobilized enzyme Ⅱ)

pH optima

The effect of pH values of free and both immobilized enzymes were determined in 3.0-10.0 pH range, and the results were shown in Fig. 5. The maximum value of relative activity was observed at pH 5.0 for all the enzymes including free and immobilized enzymes.

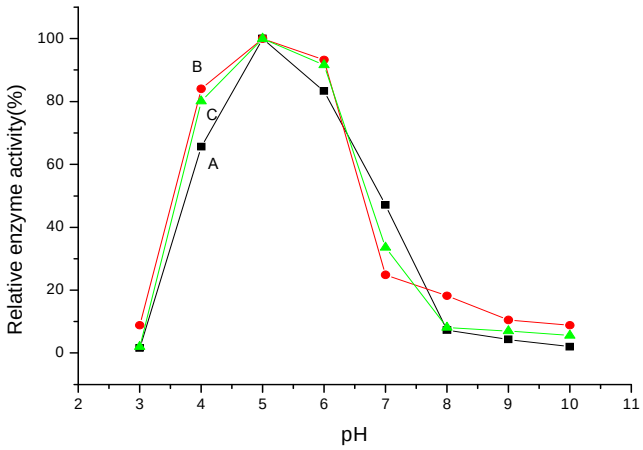


Fig. 5. Effect of pH on the activity of free and immobilized enzymes
(A: Free enzyme; B: Immobilized enzyme Ⅰ; C: Immobilized enzyme Ⅱ)

pH stability

All kinds of enzyme including the free and immobilized enzymes were exposed to different pH (2.0-9.0) at room temperature overnight, and then the enzyme activities were determined with ONPG as substrate. The curve presented in Fig. 6 illustrated that the immobilized enzymes hold good adaptability comparing to free enzyme at 3.0-8.0 pH region.

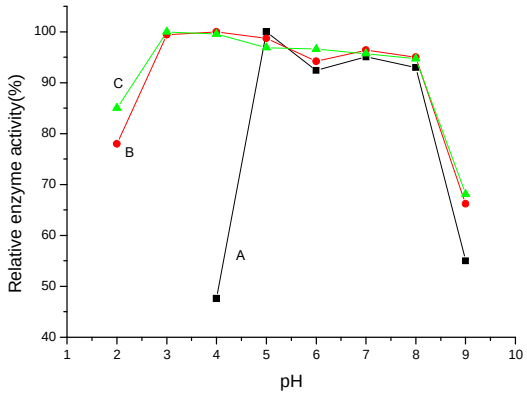


Fig. 6. Effect of pH on the stability of free and immobilized enzymes
(A: Free enzyme; B: Immobilized enzyme Ⅰ; C: Immobilized enzyme Ⅱ)

Operational stability of immobilized enzyme

The experiment was repeated 8 times with the same immobilized enzyme Ⅱ at the same initial concentration of ONPG. The results were summarized in Fig.7 and it was shown that the immobilized β-galactosidase was still retained above 95% of the original activity without significant loss in activity, meaning that almost no enzyme was dissociated from the surface of the Poly(GMA-ST) carrier in the course of the reaction, so the operational stability of the immobilized enzyme obtained was very good.

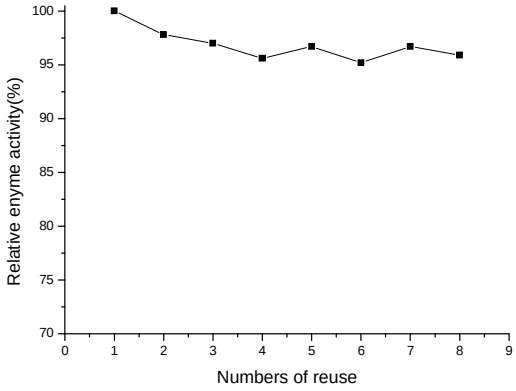


Fig. 7. Operational stability

CONCLUSION

In this paper, the macroporous Poly(GMA-ST) was synthesized by using glycidyl methacrylate and styrene as monomer by bulk copolymerization with different pore-forming agents, one only using a liquid porogenic agent of cyclohexanol and lauryl alcohol and the other simultaneously using a mixture of methanol aqueous solution as liquid pore-forming agents and nano-calcium carbonate as solid one. After the polymer was smashed, particles with diameters ranging 0.15mm to 0.30mm were taken as the carrier. SEM micrographs showed that the carrier Ⅱ, which simultaneously using liquid and solid materials as porogen, had a much more porous surface structure than carrier Ⅰ only with liquid solution as porogen. Under the optimum conditions,

β-galactosidase was immobilized on the carrier described above and the enzyme activity bound on the carrier Ⅱ was much higher than (more than 3 times) that bound on the carrier Ⅰ , which showed that the carrier Ⅱ was more suitable to immobilize enzyme because of its increase in specific surface. Meanwhile, properties of the free and both immobilized enzyme were determined and compared, satisfactory results of both immobilized enzyme were obtained in pH stability, thermal stability and operational stability. So it could be seen, the polymer as enzyme immobilization carrier, which usually was prepared with liquid solution as porogen by traditional method, could also be got well using solid and liquid materials as porogen, which was used to get more porous surface structure and more activated reaction group. It was useful for industrial application of polymer as enzyme immobilization carrier.

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