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Immobilization of *Aspergillus Oryzae* *β*-Galactosidase on Newly Prepared Porous Poly(GMA-ST)

SUN SUFANG* and XU XIAOBING

College of Chemistry and Environmental Science
Hebei University, Baoding 071002, China
sunsufang@hbu.edu.cn

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Abstract: The macroporous and reactive carriers polyGMA-ST was synthesized simultaneously with a mixture of cyclohexanol and lauryl alcohol as liquid pore-forming agents and nano-calcium carbonate as solid one by bulk copolymerization. After the polymer was smashed, particles with diameters ranging 0.15 mm to 0.30 mm were taken as the carrier and the Scanning electron microscopy (SEM) micrographs were done to characterize its surface structure. Under the optimum conditions, *β*-galactosidase *Aspergillus oryzae* was immobilized on the supporter obtained above, its enzyme activity could reach to 535.11U/g dry carrier and the activity recovery of the immobilized *β*-galactosidase was 79.63%. Meanwhile, the basic property and the kinetic data of the immobilized enzyme were determined and compared with those of the free enzyme and satisfactory results were obtained in pH stability, thermal stability and operational stability. The conclusion obtained here indicated that the ploy(GMA-co-ST) prepared concurrently with liquid and solid porogen was suitable to immobilize enzyme.

Keywords: Glycidyl methacrylate(GMA), Styrene(ST), Immobilize, *β*-galactosidase

Introduction

In the present study, the immobilization of enzymes onto insoluble carriers has been an active research topic in enzyme technology and it is also essential for their application to industrial processes[1,2]. This is because free enzyme is lack of long-term stability and difficult to be recovered and recycled from the reaction mixture, making the reuse of the enzyme impossible. However, enzyme immobilization provides easy recovery and reuse of the enzyme and many other advantages. Carriers which play an important role in the utility of an immobilized enzyme should provide a large surface area being suitable for enzymatic reactions[3].

β-galactosidase (*β-D*- galactosidase galactohydrolase, EC 3.2.1.23) from *Aspergillus oryzae* was immobilized by many 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 ontophenol-formaldehyde resin and bone powder[4-12]. 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[13].

In this study, the carriers with macroporous morphology were synthesized successfully by the bulk copolymerization of glycidyl methacrylate (GMA) and styrene (ST), simultaneously with a mixture of cyclohexanol, lauryl alcohol 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 supporter obtained from liquid and solid pore-forming agents to immobilize enzyme.

Experimental

Glycidyl methacrylate (GMA) (99%) was obtained from Shanghai Jinchao Chemical Co. Ltd; *β*-galactosidase from *Aspergillus oryzae* (11.2U/mg solid) and *o*-nitrophenyl-*β-D*-galactopyranoside (ONPG) were obtained from sigma. Styrene and other reagents were all of 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 enzyme and substrate solution

0.0300 g of *β*-galactosidase was dissolved in 10 mL 0.1 M citric acid buffer (pH 4.0) and then kept in the refrigerator at 4 ^{o}C for use. The substrate solution was obtained by dissolving 0.0150 g ONPG in 10 mL twice distilled water.

Preparation of the carriers

The reaction was carried out in a plastic beaker, including a mixture of the monomers (GMA 4.5 mL and Styrene 1.7 mL), initiator (AIBN 0.0395 g), 2 mL cyclohexanol and 1.5 mL 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 ^{o}C, then the large pieces of solid obtained was smashed and the particles ranging from 0.15 to 0.30 mm were taken as the carrier. After being washed with distilled water completely, the carriers were kept in ethanol for 24 h to get rid of liquid porogen and 0.1 M hydrochloric acid solution for 24 h to remove the solid one- the nano-calcium carbonate and finally dried in the vacuum oven at 55 ^{o}C for use.

Method of immobilization

0.0500 g of Polymer particles were put in 0.5 mL 0.1 M citric acid buffer (pH 4.0), which contained enzyme (3 mg/mL). The reaction was conducted in ultrasonic cleaning machine at 25 ^{o}C for 3 h. After that, the immobilized enzyme was filtered and washed with 0.1 M citric acid buffer (pH 5.0) until there was no protein.

Assay of β-galactosidase activity

Activities of the free and immobilized *β*-galactosidase were assayed according to the references[14,15], using *o*-nitrophenyl-b-D-galactoside (ONPG) (1.5 mg/mL) as the substrate. For the free enzyme activity, 0.1 mL of free enzyme was added to 0.9 mL of the citric acid buffer (0.1 M, pH 5.0). The reaction was started by adding 0.2 mL ONPG (1.5 mg/mL). After exactly 15 min of incubation at 55 ^{o}C, the reaction was stopped by adding 2.0 mL of Na_2CO_3 solution (1 M), and the amount of ONP was measured directly at 405 nm. For the immobilized enzyme activity, 0.0500 g of the immobilized enzyme was soaked in 1.0 mL of the citric acid buffer (0.1 M, pH 5.0). The reaction was carried out and analyzed as above. 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 1μ mol of product per minute under the assay condition.

Determination of optimum temperature, pH

The optimum temperature of free and immobilized *β*-galactosidase was determined over the range of 40 to 65 ^{o}C. The optimum pH was determined using ONPG as substrate for 15 min at 55 ^{o}C under the variety of pH (0.1M, pH3-10).

Results and Discussion

Discussion about the support obtained

The SEM micrographs of the dried polymer were obtained by using KYKY-2800B scanning electron microscope, which were illustrated in Figure 1. As seem from it, the apparent morphology with macroporous surface was observed, which would be suitable for the immobilization of enzymes and also provide a good transmission for substrate and product during the enzymatic reaction. Under the optimum conditions, the carrier was used to immobilize *β*-galactosidase and the results obtained were listed in Table 1. According to the data presented in it, the activity of the immobilized enzyme on the carrier reached amaximum of 535.11U/g dry carrier and the activity recovery of the immobilized *β*-galactosidase could attained 79.63%.

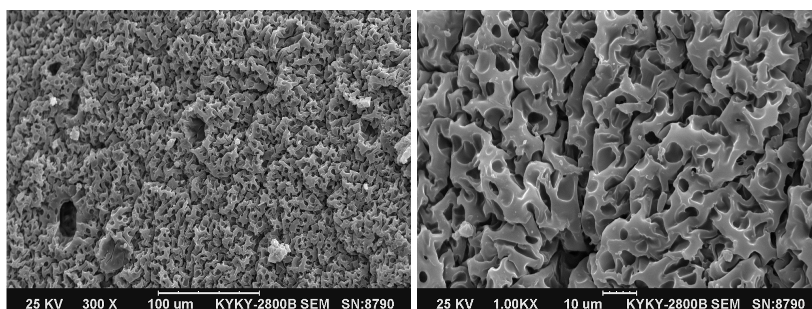

Figure 1. SEM photographs of the carrier, 300×and 1,000×magnification.

Table 1. The immobilization results of *β*-galactosidase on the carrier

Carrier	Immobilized enzyme activity U/g dry carrier	Activity yield, %
Carrier	535.11	79.63

Properties of the immobilized enzyme

pH optima

pH values of free and immobilized enzymes were determined in 3.0-10.0 pH range, as shown in Figure 2, the maximum value of relative activity was observed at pH 5.0 for both free and immobilized enzymes. The enzyme activity was determined by ONPG as substrate, at 55 ^{0}C in various pH buffers (3.0-10.0) for 15 min.

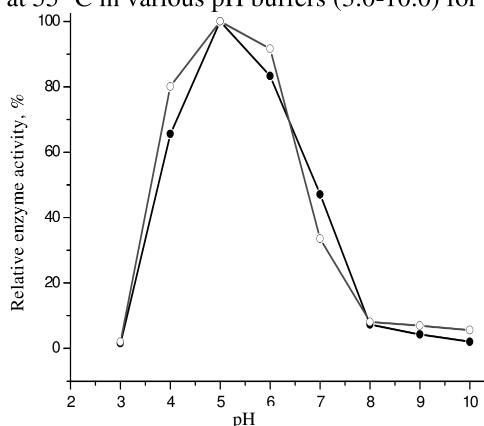

Figure 2. Effect of pH on the activity of free (a) and immobilized (b) enzymes

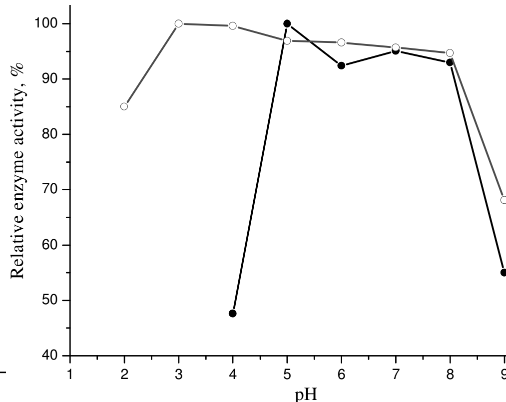

Figure 3. Effect of pH on the stability of free (a) and immobilized (b) enzymes

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 Figure 3 illustrated that the immobilized enzymes hold good adaptability comparing to that of free enzyme.

Optimum temperature

All enzyme activities were determined by ONPG as substrate at various temperatures (40-65 ^{o}C), the reseults obtained were shown in Figure 4. The optimum temperature of both free and immobilized enzymes were at 55 ^{o}C.

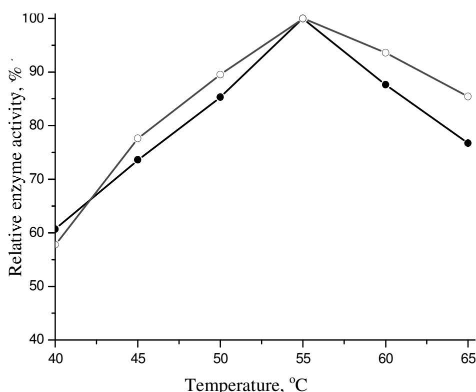

Figure 4. Effect of temperature on the activity of free (a) and immobilized (b) enzymes

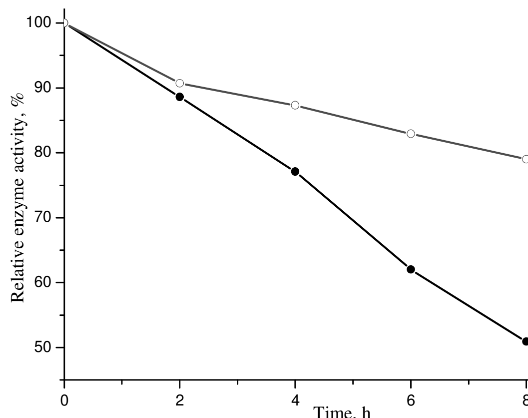

Figure 5. Effect of temperature on the stability of free (a) and immobilized (b) enzymes at 40 ^{o}C

Thermal stability

The thermal stability of immobilized enzymes conferred to a good performance as could be seen in Figure 5 & 6. After incubation at 40 °C for 8h, 79% 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 was 58.7%. Therefore, the immobilization remarkably enhanced the heat resistance of *β*-galactosidase.

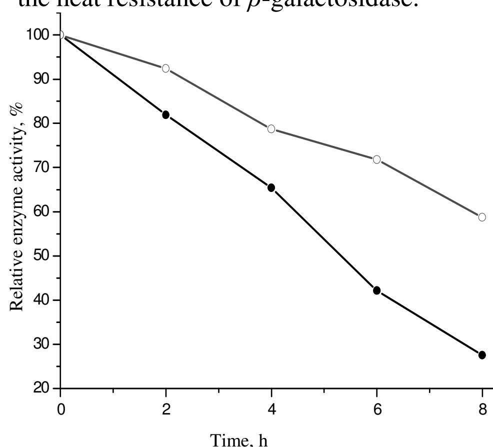

Figure 6. Effect of temperature on the stability of free (a) and immobilized (b) enzymes at 50 °C

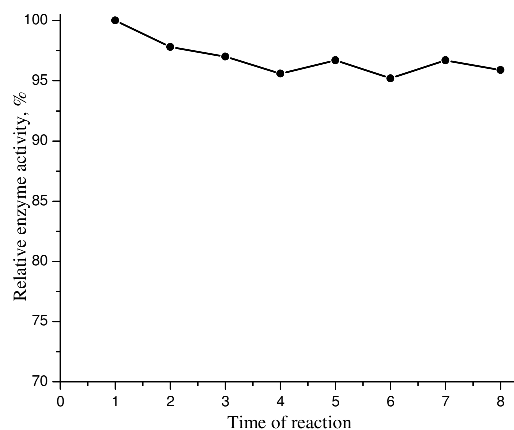

Figure 7. Operational stability

Operational stability of immobilized enzyme

The experiment was repeated 8 times by using the procedures mentioned above with the same immobilized enzyme at the same initial concentration of ONPG. The results were shown in Figure 7 and it was shown that the immobilized *β*-galactosidase was still retained above 95% of the original activity after 8 times reuses meaning that almost no enzyme was dissociated from the surface of the carrier in the course of the reaction, so the operational stability of the immobilized enzyme obtained was very good.

Conclusion

The synthesis of carriers with macroporous structures was carried out by using glycidyl methacrylate and styrene as monomer simultaneously with a mixture cyclohexanol, lauryl alcohol and nano-calcium carbonate as pore-forming agents by bulk copolymerization and the particles with diameters in the range of 0.15 to 0.30 mm were taken as carrier to immobilize enzyme after the polymer was smashed. SEM micrographs showed that the carrier exhibited the apparent morphology with macroporous surface. Under the optimum conditions, *β*-galactosidase was immobilized on the supporter described above and the enzyme activity bound on the supporter was 535.11U/g and the activity yield was79.63 %, Meanwhile properties of the free and the immobilized enzyme were determined, satisfactory results of the immobilized enzyme were obtained in pH stability, thermal stability and operational stability. So it could be seen, the macroporous polyGMA-ST newly made here was suitable as enzyme carrier because of the porous structure obtained.

Acknowledgment

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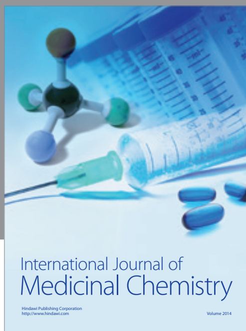

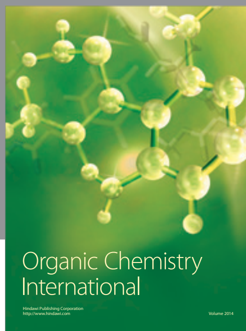

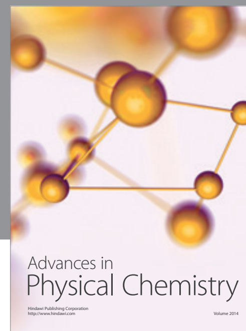

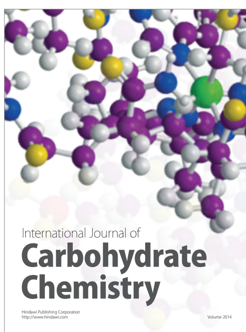

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