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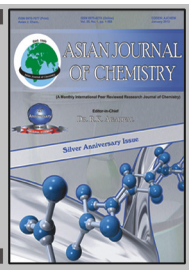

Immobilization of β-Galactosidase from *Aspergillus oryza* on Magnetic Poly(GMA-ST) Beads

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In this study, novel magnetic beads were prepared from glycidylmethacrylate (GMA) and styrene (ST) *via* suspension polymerization with cyclohexanol and lauryl alcohol as porogenic agent. The magnetic poly (GMA-ST) beads were characterized with scanning electron microscope (SEM), magnetic scales and X-ray spectroscopy. The magnetic susceptibility of the porous poly (GMA-ST) beads was 1.25 × 10^{-4} cm^3/g. Under the optimum conditions, β-galactosidase was immobilized on the magnetic poly (GMA-ST) beads described above, the results obtained showed that the activity of the immobilized β-galactosidase reached 412.09 U/g and the activity recovery was 61.32 %. Finally, the basic properties of the immobilized enzyme were determined separately and satisfactory results were obtained in pH stability, thermal stability, operational stability and Michaelis constants K_m.

Key Words: Magnetic beads, Poly(GMA-ST), Immobilized β-Galactosidase, Suspension polymerization.

INTRODUCTION

During the last decade, the immobilization of enzymes has been often used in the production of pharmaceuticals, food and other biological products. Many support materials have been used for enzyme immobilization and magnetic beads for immobilization have received increased attention for industrial manufacturing of enzyme-processed products[1-3], because magnetic particles can be easily separated from reaction medium and stabilized in a fluidized-bed reactor by applying a magnetic fields and they can also reduce capital and operation costs[4].

In present paper, magnetic poly(GMA-ST) beads were synthesized successfully from glycidyl methacrylate and styrene with cyclohexanol and lauryl alcohol as porogenic agent. The resulting carriers were characterized by SEM, magnetic scales and X-ray spectroscopy and then it was employed in the immobilization of β-galactosidase after activation with glutaric dialdehyde. The basic properties 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. Finally, the values of Michaelis constants K_m at 50 ºC was also determined.

EXPERIMENTAL

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 11.2 U/mg solid. Styrene, polyvinyl alcohol (PVA), 2, 2'-azobis (isobutyronitrile) (AIBN) and other reagents were all analytical grades. All the aqueous solutions were prepared by double distilled water.

Ultraviolet spectrotometer (T6 new century), CTP-II magnetic scales, digital pH meter (PHS-3C), vacuum desiccator (DZ-6020), universal grinder (FW-200), ultrasonic cleaning machine, magnetic stirrer and water constant temperature oscillator (SHA-B) were used.

Preparation of magnetic poly(GMA-ST) beads: There were two steps in the preparation of magnetic poly (GMA-ST): In the first step, ferric-poly (GMA-ST) beads were prepared *via* suspension polymerization. Porogenic agent prepared by mixing 2 mL cyclohexanol and 1.5 mL lauryl alcohol was added to a mixture of 4.5 mL glycidylmethacrylate and 1.7 mL styrene in which the free radical initiator (AIBN 0.0395 g) was dissolved. The mixture was degassed and homogenized by ultrasonication for 20 min. Then the mixture was added into a four-necked flask containing 55 mL PVA (2 %) and 55 mL glutin (0.1 %) with $FeCl_3$ (4.46 g) dissolved which was used as a precursor for the thermal iron-oxide precipitation in the beads. The reaction was carried out at 55 ºC in a water bath, equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The polymerization reaction was maintained at 65 ºC for 3.0 h and then at 85 ºC for 2 h. After the reaction, the obtained beads were washed with water completely and then dried under vacuum for 5 h.

In the second step, magnetic beads were prepared by convention co-precipitation reaction of iron oxide in the beads. 2.5 g $FeCl_2$ was dissolved in 100 mL purified water and then was added into a reactor containing 5 g ferric-poly(GMA-ST) beads obtained in the first step in $NH_3 \cdot H_2O$ (20 mL, 25 % w/v). The reactor was also equipped with a mechanical stirrer, nitrogen inlet and reflux condenser at three temperatures (40 ºC, 50 ºC, 90 ºC for 2 h). Then the beads were kept in 50 % ethanol solution for 3 h and then washed with water. Finally, the magnetic poly (GMA-ST) beads were transferred in phosphate butter (pH 8.0; 22.5 mL), containing 2.5 mL of glutaric dialdehyde (50 %). The reaction was carried out at 25 ºC for 6 h under magnetic stirring. The activated beads were washed with distilled water and acetic acid solution (0.1 M, 100 mL) and then dried under vacuum for 5.0 h. The resulting magnetic poly(GMA-ST) beads were used for the immobilization of β-galactosidase.

Preparation of enzyme and substrate solution: 0.0500 g of β-galactosidase was dissolved in 10 mL of 0.1 M citric acid buffer (pH 4.0) and then kept in the refrigerator at 4 ºC for use. The substrate solution was obtained by dissolving 0.0150 g ONPG in 10 mL twice distilled water.

Immobilization of β-galactosidase: 0.0500 g of polymer particles was put in 0.5 mL 0.1 M citric acid buffer (pH 4.0) containing enzyme (5 mg/mL). The reaction was undergone in ultrasonic cleaning machine at 25 ºC for 3 h. After that, the immobilized enzyme was filtered and washed with water and 0.1 M citric acid buffer (pH 5.0) until there was no protein.

Activity assays of β-galactosidase: Activities of free and immobilized β-galactosidase were assayed by the addition of 0.1 mL of free enzyme or 0.0500 g of immobilized β-galactosidase in the citric acid buffer (pH 5.0), using 0.2 mL of ONPG (1.5 mg/mL) as the substrate[5,6]. After 15 min of incubation in a shaking water bath at 55 ºC, the reaction was stopped by adding 2 mL of Na_2CO_3 solution (1 M). The absorbance of medium was recorded at 405 nm. All activity measurement experiments were carried out three times. One unit of β-galactosidase activity is defined as the amount of enzyme that liberated 1 μmol of product per minute under the assay condition.

RESULTS AND DISCUSSION

Discussion about the magnetic poly(GMA-ST) beads: The magnetism of the poly (GMA-ST) beads obtained was evidenced by magnetic scales and X-ray spectroscopy. From the results, it could be seen that magnetic susceptibility (K_m) of the beads ould reach 1.25×10^{-4} cm^3/g. X-ray spectroscopy of magnetic poly (GMA-ST) beads presented in Fig. 1 clearly showed that the beads obtained described above contained Fe, which also indicated that magnetism was successfully formed within the structure of poly(GMA-ST) beads.

Meanwhile, scanning electron microscopy micrographs were also done to characterize its surface structure and the result was illustrated in Fig. 2. The photographs showed that the magnetic beads had a porous surface structure, which would be suitable for the immobilization of enzymes and also provide a good transmission for substrate and product during the enzymatic reaction.

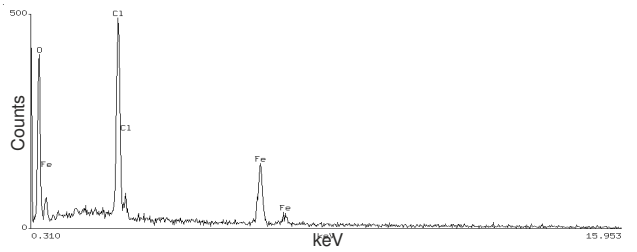

Fig. 1. X-ray spectroscopy of the magnetic poly(GMA-ST) beads; (Accelerating voltage: 25 KeV; take off angle: 36.7739 ; live time: 100 sec; dead time: 14.504)

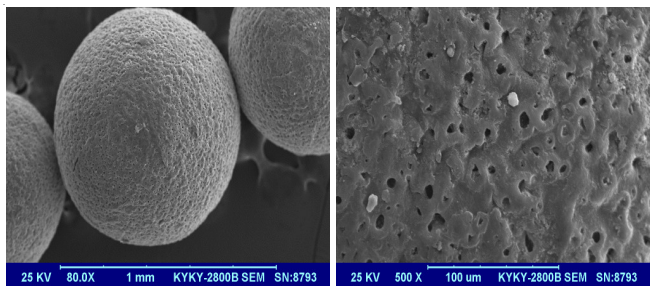

Fig. 2. SEM photographs of the magnetic poly(GMA-ST) beads

Under the optimum conditions, the magnetic beads were used to immobilize β-galactosidase and the results obtained were listed in Table-1. According to the data, the activity of the immobilized enzyme could reach a maximum of 412.09 U/g dry carrier and the activity recovery of the immobilized β-galactosidase was 61.32 %. The obtained enzyme activity was higher than values obtained on the non-magnetic poly(GMA-ST) beads.

TABLE-1
IMMOBILIZATION RESULTS OF β-GALACTOSIDASE ON THE MAGNETIC BEADS

Carrier	Immobilized enzyme activity (U/g dry carrier)	Activity yield (%)
Magnetic poly(GMA-ST) beads	412.09	61.32
Non-magnetic poly(GMA-ST) beads	266.15	39.62

Properties of the immobilized β-galactosidase

Effect of pH and temperature on the catalytic activity: The effect of pH values of free and immobilized enzymes was determined in 3-9 pH range and the results were shown in Fig. 3. 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 ºC in various pH buffers (3-9) for 15 min.

The effect of temperature (40-65 ºC) on the free and immobilized enzymes activities were investigated by using ONPG as substrate as shown in Fig. 4. The optimum temperature of both free and immobilized enzymes were at 55 ºC.

pH and thermal stability: 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. Fig. 5 shows that the immobilized enzymes hold a better adaptability.

The thermal stability of free and immobilized enzymes could be seen in Fig. 6. After incubation at 50 ºC for 8 h,

63.1 % of immobilized β-galactosidase remained active, while the remaining activity of the free enzyme was 27.5 %. Therefore, the immobilization remarkably enhanced the heat resistance of β-galactosidase.

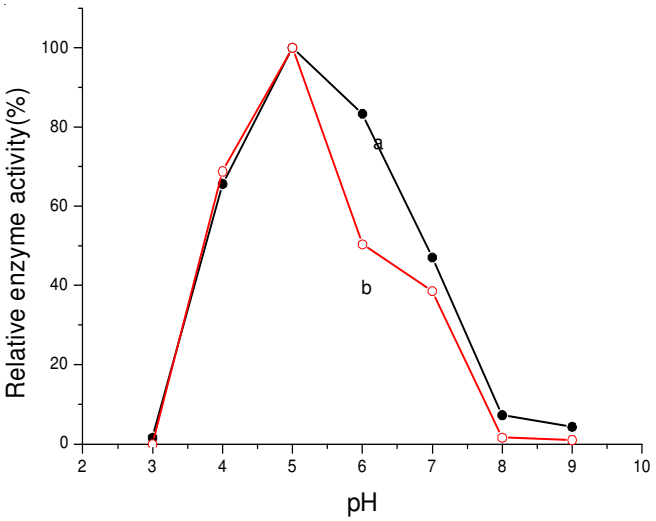

Fig. 3. Effect of pH on the activity of free (a) and immobilized (b) enzymes

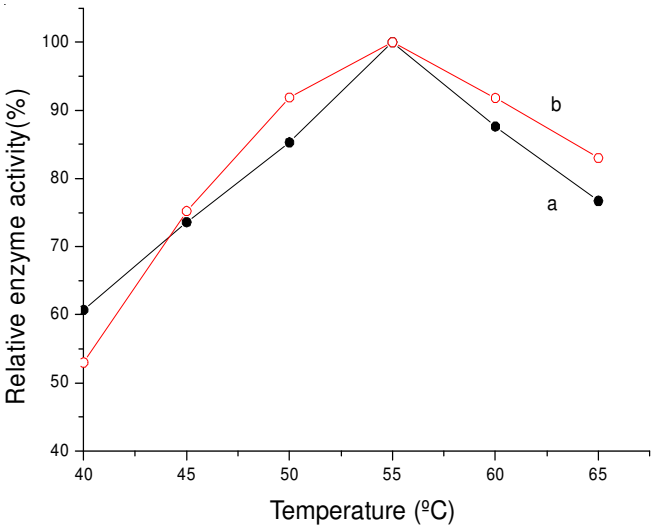

Fig. 4. Effect of temperature on the activity of free (a) and immobilized (b) enzymes

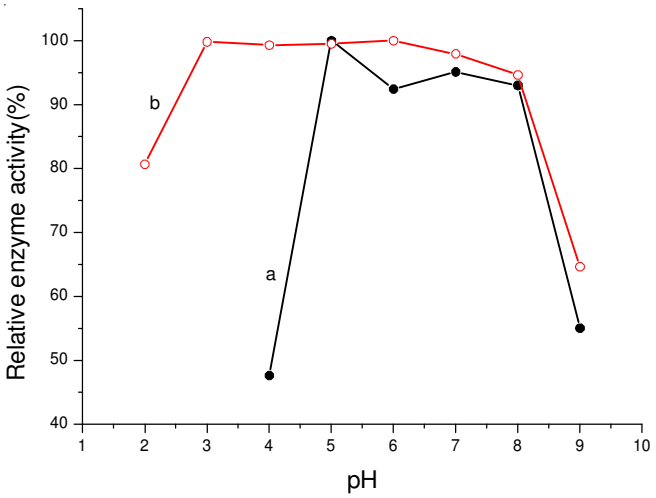

Fig. 5. Effect of pH on the stability of free (a) and immobilized (b) enzymes

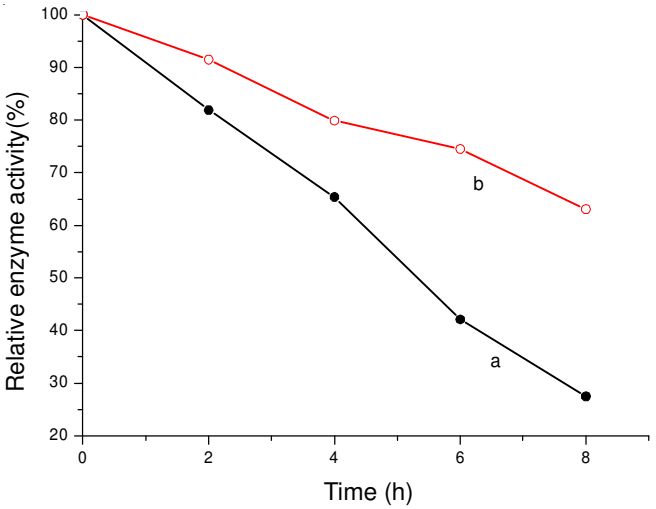

Fig. 6. Effect of temperature on the stability of free (a) and immobilized (b) enzymes at 50 ºC

Operational stability of immobilized enzyme: The experiment was repeated 8 times by using the procedures mentioned above with the same immobilized β-galactosidase at the same initial concentration of ONPG. The results are summarized in Fig. 7 and it shows that the immobilized β-galactosidase was still retained above 97 % of the original activity after 8 times reuses meaning that almost no enzyme was dissociated from the surface of the magnetic poly(GMA-ST) beads in the course of the reaction. So the operational stability of the immobilized enzyme obtained was very good.

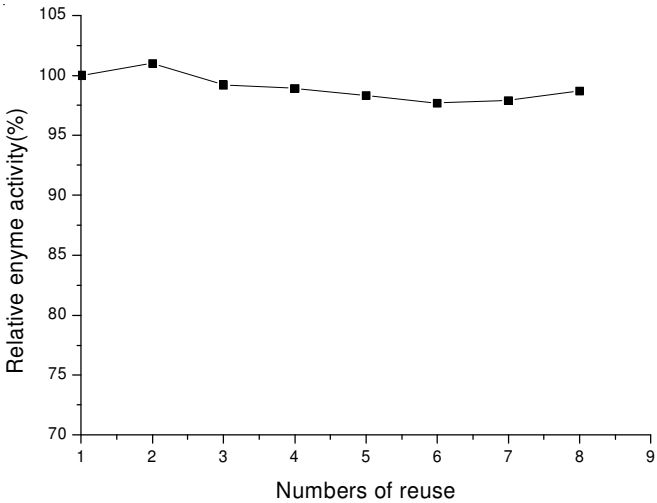

Fig. 7. Operational stability

Kinetic parameters: The initial hydrolytic reaction rates of substrate (ONPG) were measured at different concentrations with the free and immobilized enzymes at 50 ºC. The kinetic parameters of free and immobilized enzymes are presented in Table-2. As shown in it, the value of K_m for the immobilized enzyme was higher than that of the free enzyme. It was probably caused by the lower accessibility of the substrate to the active site of the immobilized enzyme[7].

Conclusion

In this paper, the poly(GMA-ST) magnetic beads were prepared from glycidylmethacrylate (GMA) and styrene (ST)

TABLE-2
KINETIC PARAMETERS OF FREE AND IMMOBILIZED ENZYME AT 50 ºC

Form of enzyme	K_m (mmoL/L)
Free β-galactosidase	8.43
Immobilized β-galactosidase	20.57

via suspension polymerization with cyclohexanol and lauryl alcohol as porogenic agent. The results obtained from magnetic scales and X-ray spectroscopy showed that magnetism was successfully formed within the structure of poly(GMA-ST) beads and the SEM micrographs show that the magnetic beads have a porous surface structure. β-galactosidase from *Aspergillus oryzae* was immobilized on the magnetic beads and a high enzyme binding was acquired and the basic properties of the immobilized enzyme were determined and compared with those of the free enzyme and the satisfactory results were obtained in pH stability, thermal stability, operational stability and Michaelis constants K_m.

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