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Poly(glycidyl methacrylate-co-ethylene dimethacrylate) Macroporous Polymer Particles for the Immobilization of β-Galactosidase from *Aspergillus oryzae*

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Macroporous polymer particles containing surface epoxy groups were synthesized using glycidyl methacrylate (GMA) as monomer, ethylene dimethacrylate (EDMA) as crosslinking agent and dodecyl alcohol and cyclohexanol as porogenic agents by suspension copolymerization for immobilization of β-galactosidase. The scanning electron microscopy (SEM) micrographs were also done to characterize their surface structure. Under the optimum conditions, β-galactosidase from *Aspergillus oryzae* was immobilized on the support obtained above. The basic property and the kinetic data of the immobilized enzyme were determined and satisfactory results were obtained in enzyme activity, activity yield, pH stability, thermal stability, operational stability and Michaelis constants K_m. It is concluded that the ploy(GMA-co-EDMA) is suitable to immobilize β-galactosidase from *Aspergillus oryzae*.

Key Words: Glycidyl methacrylate ethylene dimethacrylate, β-Galactosidase immobilization poly (GMA-co-EDMA)

INTRODUCTION

The benefits of using macroporous materials as the support of immobilized enzyme are nowadays recognized. In most case, the use of the appropriate support could improve the enzyme stability activity features. In addition, immobilized proteins may permit recovery of the enzyme, simplify the design and performance of the reactor, affect the stability, pH and temperature optima and Michelis-Menten constant (K_m), *etc*. Hence the idea of immobilizing the enzyme on a rigid solid support has been of great industrial interest for many years.

Many different immobilization methods have been developed including entrapment in alginate and fiber consisting of cellulose acetate and titanium isopropoxide; covalent attachment onto chitosan, polyurethane foam, alginate, gelatin and bone powder, adsorption onto phenol-formaldehyde resin and bone powder. Some of these methods suffer from low immobilization yields or continuous leakage of enzyme[1] and most of them may be difficult to perform on an industrial scale, where long support handling may necessary and some dangerous substances cannot be utilized, problems that are not considered on a laboratory scale, where the experiment is performed by specialized staff under very controlled conditions[2-5].

Among all the materials used to immobilize enzyme, epoxy-activated carriers seem to be almost-ideal systems to develop very easy protocols for enzyme immobilization because epoxy group could exhibit good reactivity under mild conditions and would be very stable at neutral pH values even in wet conditions[6,7]. The recently materials produced from a macroporous copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) are very attractive support because of their mechanical and chemical stability as well as insignificant nonspecific protein adsorption[8]. Thus, this kind of carriers could be stored for long periods of time and the reactions of epoxy groups in carriers with different nucleophilic groups on the protein surface (*e.g.*, amino, hydroxy or thiol moieties) would be suitable to immobilize enzymes by forming extremely strong linkages (secondary amino bonds, ether bonds and thioether bonds) with minimal chemical modification of the protein[9].

In this paper, the poly (GMA-co-EDMA) with macroporous morphology, reactive epoxy groups was synthesized successfully by suspension copolymerization of glycidyl methacrylate (GMA) using ethylene dimethacrylate (EDMA) as crosslinking agent, a mixture of dodecyl alcohol and cyclohexanol as porogenic agent and the scanning electron microscopy (SEM) micrographs were done to characterize its surface structure. Then the support obtained was employed to immobilize β-galactosidase from *Aspergillus oryzae*, which efficiently catalyzes not only the hydrolysis of the β-galactoside linkages of lactose to glucose and galactose but also the *trans* galactosylation reaction to produce galactooligosaccharides[10]. The enzyme activity and the activity yield of the immobilized β-galactosidase were also investigated in order to examine the suitability of the supporter obtained to immobilize enzyme. Finally, the kinetic data of the immobilized enzyme, the values of Michaelis constants K_m, was also determined.

EXPERIMENTAL

Ultraviolet spectrotometer (T6 New Century), vacuum desiccator (DZ-6020), digital pH meter (PHS-3C) and water constant temperature oscillator (SHA-B), universal grinder (FW-200) were used for the study. All the aqueous solutions were prepared by twice distilled water.

Glycidyl methacrylate (GMA) was purchased from Tokyo Chemical Industry Co. Ltd. Ethylene dimethacrylate (EDMA) (98 %) was purchased from New Jersey, USA. β-Galactosidase from *Aspergillus oryzae* (11.2 U/mg) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma. Azo-*bis*-isobutyronitrile (AIBN) and other reagents were all analytical grades.

Preparation of enzyme and substrate solution: 0.1500 g of β-galactosidase was weighed and extracted in 25 mL 0.1 M sodium phosphate buffer (pH 7.0) and then the enzyme solution was obtained and stored in the refrigerator for use.

The substrate solution was prepared by dissolving 0.0150 g of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in double distilled water and made up 10 mL solution.

Preparation of poly (GMA-co-EDMA): The macroporous poly (GMA-co-EDMA) was prepared by suspension copolymerization method. In a typical procedure, 3.5 mL of porogenic agent was prepared by mixing 1.5 mL dodecyl alcohol and 2 mL cyclohexanol. The porogenic agent was added to a mixture of monomers (4.5 mL GMA and 3 mL EDMA) in which the free radical initiator AIBN (0.0395 g) was dissolved. The mixture was degassed and homogenized by ultrasonication for 20 min and then it was added into a four-necked flask containing 55 mL PVA (2 %) and 55 mL glutin (0.1 %) and mechanically stirred at 55 ºC under a nitrogen atmosphere. After being homogenized, the polymerization was allowed to proceed at 65 ºC for 3 h and at 85 ºC for 2 h. The resulting particles were washed with water completely and they were kept in ethanol for 24 h to remove the porogenic agent and then dried under vacuum.

Method of immobilization: The immobilization was carried out by adding an amount of polymer particles (0.0500 g) to 0.5 mL 0.1 M phosphate buffer (pH 7.0) containing enzyme (6 mg/mL). With gently stirring, the reaction was allowed to proceed at 25 ºC in water constant temperature oscillator. After 24 h, the immobilized enzyme was filtered and washed with 0.1 M phosphate buffer (pH 5.0) until no protein was detected. The enzyme bound on the supporter was called the immobilized enzyme.

Enzyme activity assay: The activity of the free enzyme and the immobilized enzyme were determined according to the reported methods[11,12] using ONPG as substrate. For the free enzyme activity, aliquots of it (0.1 mL) were added to 0.9 mL 0.1 M phosphate buffer (pH 5.0). After being incubated at 55 ºC for 15 min, the reaction was started by adding 0.2 mL of 1.5 mg/mL ONPG. After being carried out for 2 min at 55 ºC, the reaction was stopped by the addition of 2 mL 1 M Na_2CO_3 solution and the amount of ONPG was measured directly at 405 nm. For the immobilized enzyme activity, 0.0500 g of the immobilized enzyme was soaked in 1 mL 0.1 M phosphate buffer. The reaction was started by adding 0.2 mL ONPG (1.5 mg/mL). After being carried out for 2 min at 55 ºC, the reaction was stopped and analyzed as above. The activity yield was calculated as the ratio of immobilized enzyme to enzyme subjected to immobilization. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of product/min at 55 ºC.

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 ºC. Enzyme activity were determined after a long duration exposure to various temperature (50 and 60 ºC) followed by analysis at the 55 ºC.

The pH-activity curve in the range 3.0-9.0 was determined for the free enzyme and the bound enzyme at 55 ºC using ONPG as substrate. The pH stability in the range 2.0-10.0 was determined after 0.5 h exposure to different pH at 55 ºC.

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 0.25 mg/mL to 1.5 mg/mL in phosphate buffer.

Operational stability of immobilized enzyme: The operational stability of the immobilized enzyme was determined according to the following procedures. 0.0500 g of the immobilized enzyme was taken and soaked in 1.8 mL phosphate buffer overnight. After the mixture was incubated at 55 ºC for 15 min, the reaction was started by adding 0.2 mL 1.5 mg/mL ONPG and then the reactive mixture was analyzed as above. Afterward, the solid was filtered and washed thoroughly with distilled waster and the above experiment was repeated under the same conditions.

RESULTS AND DISCUSSION

Discussion about the support obtained: According to the conditions described above, the support was obtained and the scanning electron micrographs of the dried polymer were obtained using KYKY-2800B scanning electron microscope (Fig. 1). Scanning electron microscopy (SEM) micrographs showed that the support had a much porous surface structure. In addition, the support was used to immobilize enzyme under its optimum conditions and the results obtained were listed in Table-1. From the results shown in Table-1, the activity of the immobilized enzyme reached a maximum of 228.1 U/g dry carrier, which could be explained that the porous surface properties of GMA polymer would favour higher adsorption capacity for the enzyme due to increase in the surface area.

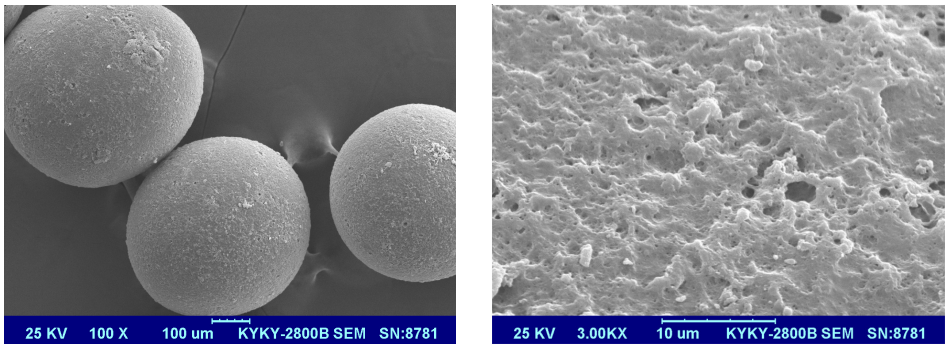

Fig. 1. Scanning electron micrographs of the support

TABLE-1
IMMOBILIZATION RESULTS OF β-GALACTOSIDASE ON THE SUPPORTER

Supporter	Immobilized enzyme activity (U/g dry supporter)	Activity yield (%)
Support	228.1	33.94

pH Optima and pH stability: Fig. 2 showed that the pH profile of the free enzyme peaked at pH 5.0. Similar pH was also found for the immobilized enzyme. The enzyme activity was determined by ONPG as substrate at 55 ºC in various pH buffers (3.0-9.0) for 2 min.

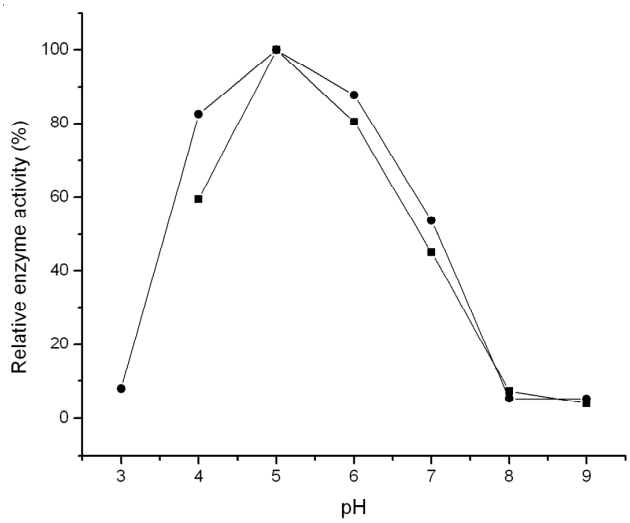

Fig. 2. Influence of pH on the enzyme reaction
(-■- Free enzyme; -●- Immobilized enzyme)

After all the enzymes were exposed to different pH (2.0-10.0) at 25 ºC overnight, enzyme activity was determined at 55 ºC pH 5.0 for all the enzymes for 2 min, with ONPG as substrate. As shown in Fig. 3, the immobilized enzyme had a wider pH range than that of the free enzyme. In the range of 3.0-9.0, the immobilized enzyme activity remained > 92 %.

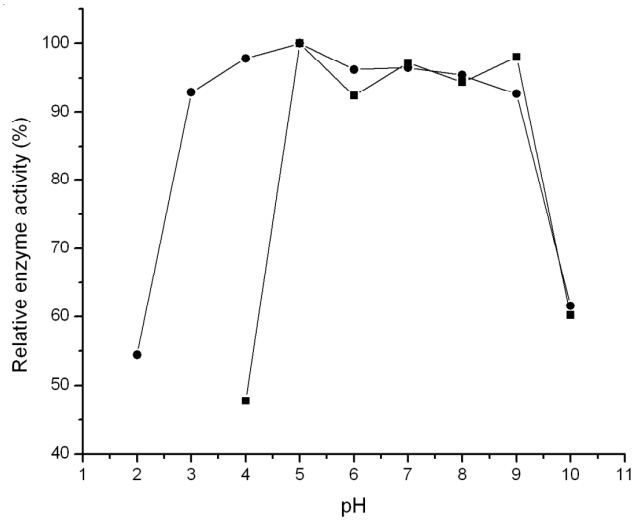

Fig. 3. pH stability of the free enzyme and the immobilized enzyme
(-■- Free enzyme; -●- Immobilized enzyme)

Optimum temperature and thermostability: As seen in Fig. 4, just like that of the free enzyme, enzyme activity was determined by ONPG as substrate at various temperatures (40-65 ºC) at pH 5.0 for 2 min. The temperature optimum of the immobilized enzyme was also 55 ºC.

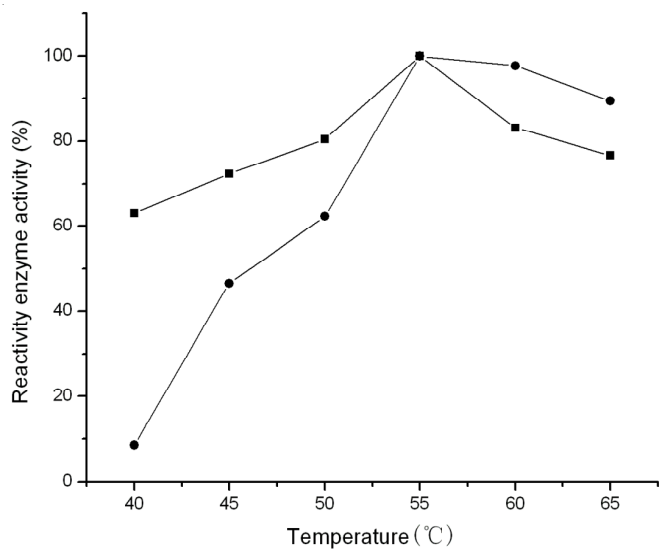

Fig. 4. Influence of temperature on immobilized reaction
(-■- Free enzyme; -●- Immobilized enzyme)

Figs. 5 and 6 showed that the immobilized enzyme was more stable than the free enzyme. At 50 ºC, after 3 h, the remaining activity of the the immobilized enzyme was 53.38 % and the free enzyme was 50.94 %. At 60 ºC, over a period of the same time, the residual activity of the free enzyme was 17.71 %, whereas that of the immobilized enzyme was 43.00 %. Results showed that the immobilized enzyme had better thermostability than that of the free enzyme.

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 are summarized in Fig. 7 and it was shown that the immobilized β-galactosidase was used for 6 times without significant loss in activity, meaning that almost no enzyme was dissociated from the surface of the glycidyl methacrylate carrier 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 the immobilized enzymes using ONPG as substrate was made and the values of K_m calculated from those graphs were shown in Table-2. From Table-2, it could be seen that the values of K_m for the immobilized enzyme were larger than that of the free enzyme, which was probably caused by the immobilization procedure and by the lower accessibility of the substrate to the active site of the immobilized enzyme[13].

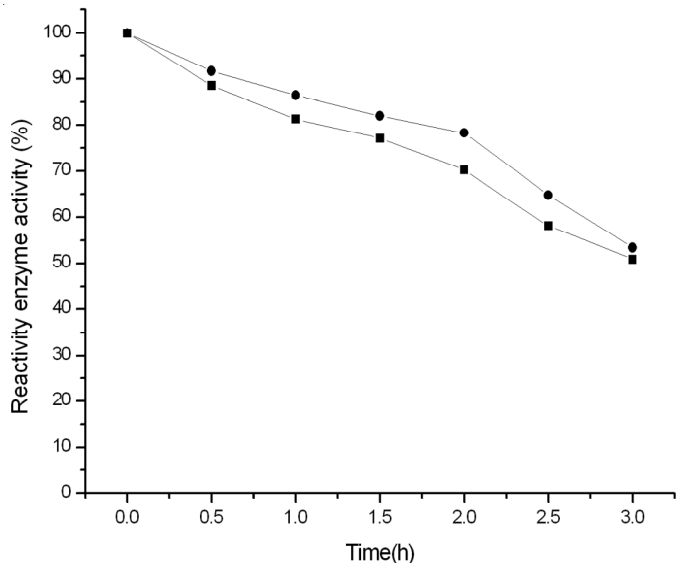

Fig. 5. Thermostability of different enzyme at 50 ºC
(-■- Free enzyme; -●- Immobilized enzyme)

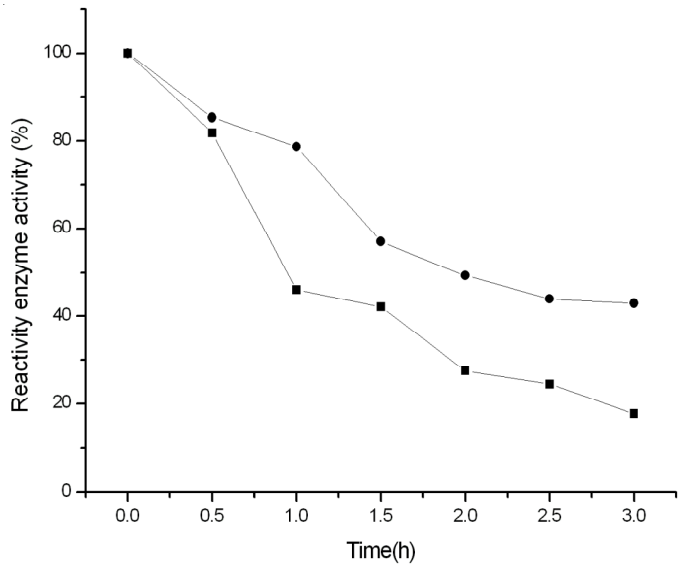

Fig. 6. Thermostability of different enzyme at 60 ºC
(-■- Free enzyme; -●- Immobilized enzyme)

TABLE-2
KINETIC PARAMETERS OF THE IMMOBILIZED ENZYME AND THE FREE ENZYME

Temperature (ºC) / K_m (mmoL/L)	40	50
Immobilized enzyme	24.17	22.300
Free enzyme	10.50	8.282

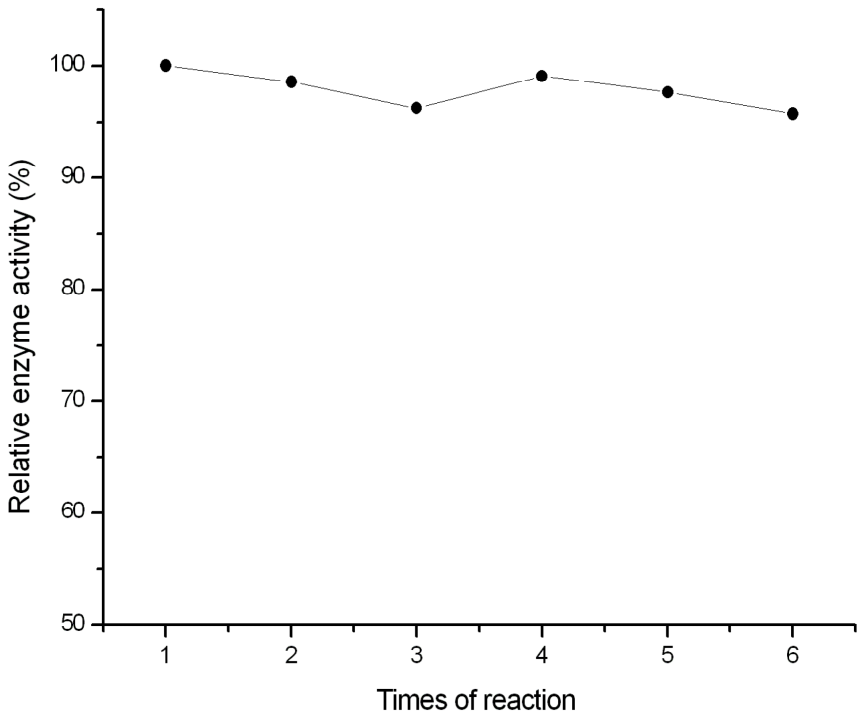

Fig. 7. Operational stability of the immobilized enzyme

Conclusion

In this work, the reactive, macroporous poly (GMA-co-EDMA) was synthesized with dodecyl alcohol and cyclohexanol as porogenic agents by suspension copolymerization and the scanning electron microscopy micrographs showed that the support had a much more porous surface structure. Under the optimum conditions, β-galactosidase from *Aspergillus oryzae* was immobilized on the support described above and the immobilized enzyme activity was high, which showed that the supporter was suitable to immobilize enzyme because of its increase in specific surface. Meanwhile properties of the free and the immobilized enzymes were determined and compared, satisfactory results of the immobilized enzyme were obtained in pH stability, thermal stability and operational stability. Finally, K_m values for the free and the immobilized enzyme were obtained, respectively. All the results described above showed that the support obtained is valuable as enzyme immobilization carrier for industrial application.

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