



Immobilization of β -galactosidase from *Aspergillus Oryzae* on Macroporous PloyGMA Newly Prepared

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Abstract

The reactive, macroporous and massive epoxy group-containing polyGMA was synthesized simultaneously with methanol aqueous solution and nano-calcium carbonate as porogen by bulk copolymerization. After the polymer was smashed, particles with diameters in the range of 0.30 to 0.45mm 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 was immobilized on the supporter obtained above, the basic property and the kinetic data of all 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 . The conclusion obtained indicated that the ployGMA prepared concurrently with liquid and solid porogen was more suitable to immobilize enzyme than that purely with liquid solution as pore-forming agent.

Keywords: Glycidyl methacrylate, Nano-calcium carbonate, β -galactosidase, Immobilization, PolyGMA

1. Introduction

It is generally accepted that immobilization of enzymes is convenient for improving their performance as industrial biocatalysts. Immobilized proteins may permit recovery of the enzyme, simplify the design and performance of the reactor, etc. Hence the idea of immobilizing the enzyme on a rigid solid support has been of great industrial interest for many years (Katchalski-Katzir, E. 1993; Kennedy, J. F., Melo, E. H. M., & Jumel, K. 1990; Klivanov, A. M. 1983; Rosevear, A. 1984).

There are many protocols for enzyme immobilization described in the literature, but 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.

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 (Serio, Di. M., Maturo, C., Alteriis, De.

E., Parascandola, P., Tesser, R., & Santacesaria, E. 2003; Vaidya, B. K., Ingavle, G. C., Ponrathnam, S., Kulkarni, B. D., & Nene, S. N. 2008).

In hence, 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 (Mateo, C., Torres, R., Fern and ez-Lorente, G., Ortiz, C., Fuentes, M., & Hidalgo, A., *et al.* 2008).

In this paper, the polyGMA with macroporous morphology, reactive epoxy groups was synthesized successfully by buck polymerization of glycidyl methacrylate (GMA) using N, N'-methylene-2-bis (acrylamide)(MBAA) as a crosslinking agent, 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 in the range of 0.30 to 0.45mm were taken as the carrier and characterized by means of SEM method. Then the supporter 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 transgalactosylation reaction to produce galactooligosaccharides (Mozaffar, Z., Nakanishi, K., Matsuno, R., & Kamikubo, T. 1984) and the enzyme activity, the activity yield of the immobilized β -galactosidase was also investigated in order to examine the suitability of the supporter obtained from liquid and solid porogen to immobilize enzyme. Finally, the kinetic data of the immobilized enzyme, the values of Michaelis constants K_m , was also determined.

2. Experimental

2.1 Apparatus and Reagents

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) (99%) was purchased from Shanghai Jinchao Chemical Co. Ltd. β -galactosidase from *Aspergillus oryzae* (11.2U/mg) and o-nitrophenyl- β -D-galactopyranoside (ONPG) were obtained from Sigma. N, N'-methylene-2-bis (acrylamide) (MBAA), Azo-bis-isobutyronitrile (AIBN) and other reagents were all analytical grades.

2.2 Preparation of Enzyme and Substrate Solution

0.1500g of β -galactosidase was weighed and extracted in 25mL 0.1M sodium phosphate buffer (pH 8.0). Then the enzyme solution was obtained and stored in the refrigerator for use.

The substrate solution was prepared by dissolving 0.0150g ONPG in twice distilled water and made up 10mL solution.

2.3 Preparation of Macroporous Glycidyl Methacrylate Polymer

The macroporous polyGMA was prepared by buck copolymerization in a reactor of plastic beaker. The reaction system contains a monomer mixture (GMA 1.4mL, MBAA 2.4000g and acrylamide 0.7500g), initiator(AIBN 0.1%(w/w monomer)), 14mL Methanol aqueous solution as liquid phase porogen(the ratio of inert component to monomer was 10:1), and nano-calcium carbonate (75%w/w monomer) as solid phase porogen. After the mixture was shaken up completely, the reaction was carried out at 70 °C. Then the large piece of solid obtained was smashed and the particles ranging from 0.30 to 0.45mm were taken as the carrier. After being washed with water and ethanol completely, they were kept in acetone for 24h to remove the liquid phase porogen and 0.1M hydrochloric acid solution for 24h to get rid of nano-calcium carbonate, and then dried under vacuum. It is notable that, in the following discussion, the supporter only with liquid phase as porogen was called supporter 1, and the one simultaneously with liquid and solid porogen was called supporter 2.

2.4 Method of immobilization

The immobilization was carried out by adding an amount of polymer particles (0.0500g) to 0.5mL 0.1M phosphate buffer (pH 8.0) containing enzyme (6mg/mL). With gently stirring, the reaction was allowed to proceed at 25 °C. After 24 hours, the immobilized enzyme was filtered and washed with 0.1M phosphate buffer (pH 5.0) until no protein was detected. The enzyme bound on the supporter 1 was called the immobilized enzyme 1, and that bound on the supporter 2 was called Immobilized Enzyme 2.

2.5 Enzyme activity assay

The activity of the free enzyme and the immobilized enzyme were determined according to the references (Sun, S. F., Li, X. Y., & Nu, S. L., *et al.* 1999; Tu, W. X., Sun, S. F., Nu, S. L., & Li, X. Y. 1998) using ONPG as substrate. For the free enzyme activity, aliquots of it (0.1mL) were added to 0.9mL 0.1M phosphate buffer (pH 5.0). After being incubated at 55 °C for 15min, the reaction was started by adding 0.2mL 1.5mg/mL ONPG. After being carried out for 2min at 55 °C,

the reaction was stopped by the addition of 2 mL 1M Na_2CO_3 solution, and the amount of ONP was measured directly at 405nm. For the immobilized enzyme activity, 0.0500g of the immobilized enzyme was soaked in 1mL 0.1M phosphate buffer. The reaction was started by adding 0.2mL ONPG (1.5mg/mL). After being carried out for 2min at 55 °C, 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 μ mol of product/min at 55 °C.

2.6 Influence of Temperature and pH

The influence of the temperature on the galactosidase activity was determined using ONPG as substrate over the range of 40 °C to 65 °C. Enzyme activity was determined after a long duration exposure to various temperature (50 °C, 60 °C) followed by analysis at the 55 °C.

The pH-activity curve in the range 3.0-10.0 was determined for the free enzyme and the immobilized enzyme at 55 °C using ONPG as substrate. The pH stability in the range 2.0-10.0 was determined after 30min exposure to different pH at 55 °C.

2.7 Kinetics

The Michaelis constant K_m was calculated for the free and the immobilized enzyme by assaying the enzyme in increasing ONPG concentrations ranging from 0.25mg/mL to 1.5mg/mL in phosphate buffer.

2.8 Operational stability of the immobilized enzyme

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

3. Results and discussion

3.1 Comparison of supporter and supporter

According to the conditions described above, the supporter and the supporter were obtained separately, and the Scanning electron micrographs of the dried polymer were obtained using KYKY-2800B scanning electron microscope. The results see Fig.1 and Fig.2. Scanning electron microscopy (SEM) micrographs showed that the supporter, which simultaneously using methanol solution and nano-calcium carbonate as porogen, had a much more porous surface structure than that of the supporter only with methanol solution as porogen. In addition, both supporters were used to immobilize enzyme under their optimum conditions, and the results obtained were listed in Table 1. From the results shown in Table 1, the activity of the immobilized enzyme on the supporter reached a maximum of 519.66U/g dry carrier. The obtained enzyme activity was approximately four times values obtained on the supporter, which could be explained that the porous surface properties of GMA polymer would favor higher adsorption capacity for the enzyme.

3.2 Properties of the immobilized enzyme

3.2.1 pH Optima and pH stability

Fig.3 Showed that the pH profile of the free enzyme peaked at pH 5.0. Similar pH was also found for the immobilized enzyme and the immobilized enzyme. The enzyme activity was determined by ONPG as substrate, at 55 °C in various pH buffers (3.0-10.0) for 2min.

After all the enzymes were exposed to different pH (2.0-10.0) at 25 °C overnight, enzyme activity was determined at 55 °C, pH 5.0 for all the enzyme for 2min, with ONPG as substrate. As shown in Fig.4, both the immobilized enzymes had a wider pH range than that of the free enzyme. In the range of 3.0-9.0, the immobilized enzyme activity remained >95%.

3.2.2 Optimum Temperature and Thermostability

As seen in Fig.5, just like that of the free enzyme, enzyme activity was determined by ONPG as substrate at various temperature (40-65 °C) at pH 5.0 for 2min. The temperature optimum of both the immobilized enzymes was 55 °C.

Fig.6 and Fig.7 showed that the immobilized enzymes were more stable than the free enzyme. At 50 °C, after 8h, the remaining activity of the Immobilized enzyme was 79%, the immobilized enzyme was 80.4%, and the free enzyme was 59.9%. At 60 °C, over a period of the same time, the residual activity of the free enzyme was 47.8%, whereas that of the immobilized enzyme was 62% and the immobilized enzyme was 64.2%. Results showed that both the immobilized enzymes had better thermostability than that of the free enzyme.

3.2.3 Operational stability of the 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.8 and it was shown that the immobilized β -galactosidase and the immobilized β -galactosidase were 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 enzymes obtained was very good.

3.2.4 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 enzymes 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 (Arica, M. Y., Hasirci, V. 1993).

4. Conclusion

In this paper, the reactive, macroporous polyGMA was synthesized simultaneously with methanol aqueous solution and nano-calcium carbonate as porogenic agents by bulk copolymerization, and the particles with diameters in the range of 0.30 to 0.45mm were taken as carrier after the polymer was smashed. Scanning electron microscopy (SEM) micrographs showed that the supporter, which simultaneously using liquid and solid materials as porogen, had a much more porous surface structure than that of the supporter only with liquid solution as porogen. Under the optimum conditions, β -galactosidase was immobilized on the supporter described above and the enzyme activity of the immobilized enzyme was much higher than that of the immobilized enzyme, which showed that the supporter was more suitable to immobilize enzyme because of its increase in specific surface. Meanwhile properties of the free and both the immobilized enzymes were determined and compared, and satisfactory results of both the immobilized enzymes were obtained in pH stability, thermal stability and operational stability. Finally, K_m values for the free, the immobilized enzyme and the immobilized enzyme were obtained respectively. 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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Table 1. The immobilization results of β -galactosidase on the supporter and the supporter

Type of supporter	Immobilized enzyme activity (U/g dry supporter)	Activity yield (%)
Supporter	144.55	21.51
Supporter	519.66	77.33

Table 2. Kinetic parameters of immobilized enzyme, and free enzyme

Temperature()/ K_m (mmol/L)	40	50	60
immobilized enzyme	22.79	18.71	24.65
immobilized enzyme	20.65	15.56	19.53
free enzyme	10.50	8.282	11.85

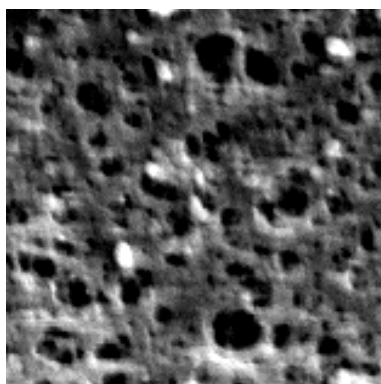


Figure 1. Supporter

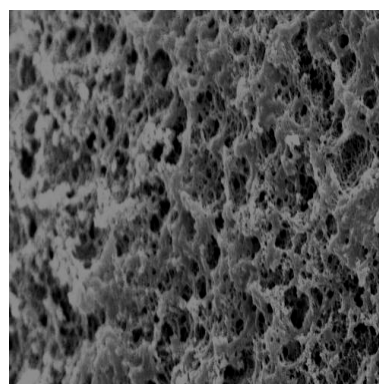


Figure 2. Supporter

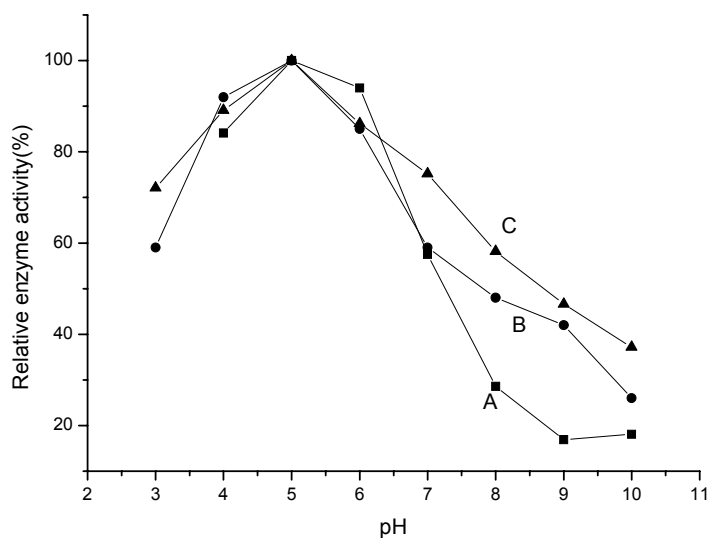


Figure 3. Influence of pH on the enzyme reaction
(A: Free enzyme; B: Immobilized enzyme ; C: Immobilized enzyme)

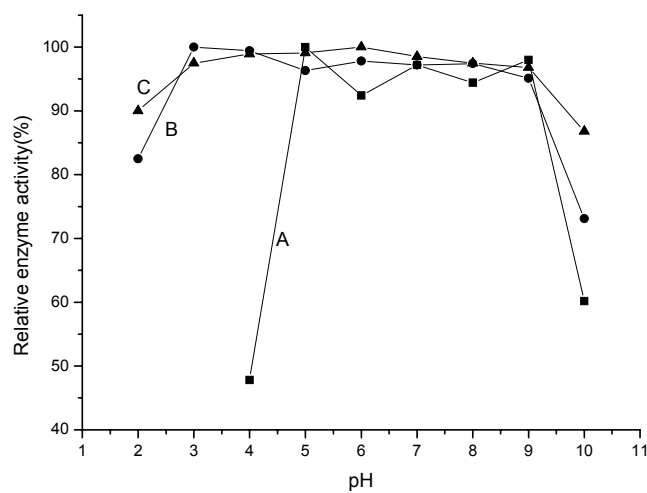


Figure 4. The pH stability of free enzyme and immobilized enzymes
(A: Free enzyme; B: Immobilized enzyme ; C: Immobilized enzyme)

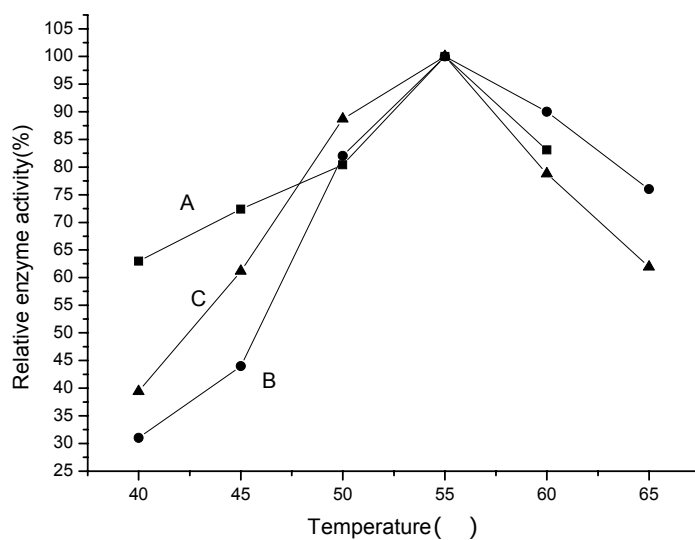


Figure 5. Influence of temperature on the enzyme reaction
(A: Free enzyme; B: Immobilized enzyme ; C: Immobilized enzyme)

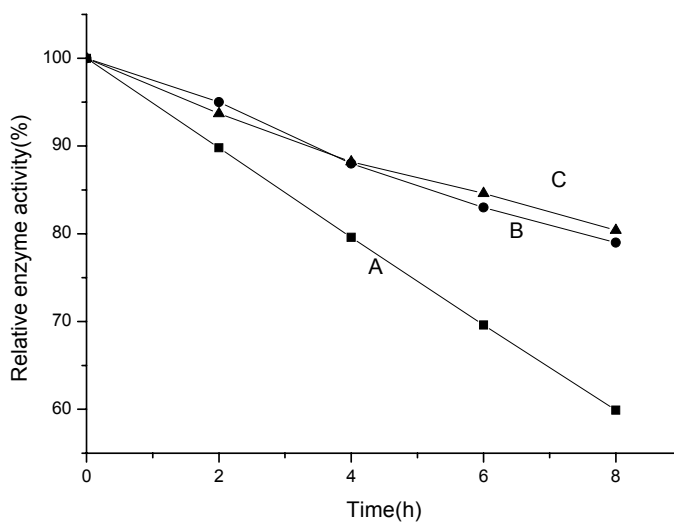


Figure 6. Thermostability of different enzyme at 50
(A: Free enzyme; B: Immobilized enzyme ; C: Immobilized enzyme)

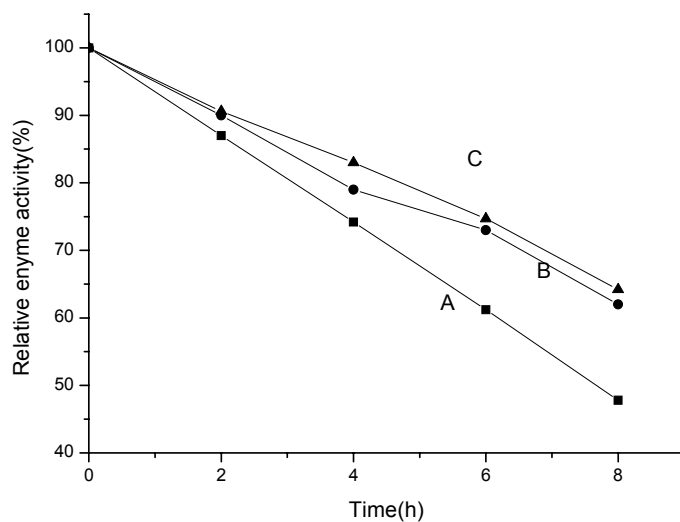


Figure 7. Thermostability of different enzyme at 60
(A: Free enzyme; B: Immobilized enzyme ; C: Immobilized enzyme)

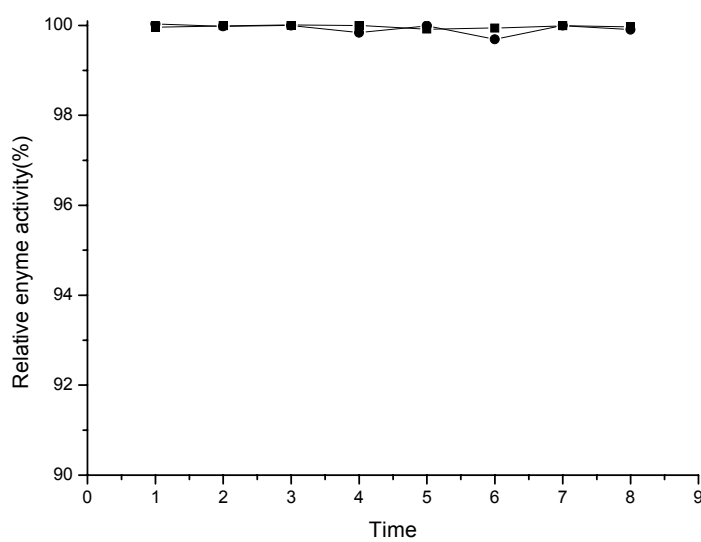


Figure 8. Operational stability