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A New Method to Prepare Macroporous Copolymer of Glycidyl Methacrilate and Ethylene Dimethacrilate As Enzyme Carrier[1]

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Abstract—Macroporous copolymer of glycidyl methacrylate and ethylene dimethacrylate containing surface epoxy groups was firstly synthesized with dodecyl alcohol and cyclohexanol as liquid pore-forming agents and nanosize calcium carbonate as solid porogenic agent. The scanning electron microscopy was used to characterize their surface structure. Under the optimum conditions, β-galactosidase from *Aspergillus oryzae* was immobilized on the support obtained above, and the basic property and the kinetic data of the reaction on immobilized enzyme were determined. These data were compared with those obtained for the enzyme immobilized on the support prepared only with the liquid solution as pore-forming agent. Satisfactory results were obtained in enzyme activity, immobilization yield, pH stability, thermal stability, operational stability, and Michaelis constants K_M. The results indicated that the copolymer of glycidyl methacrilate and ethylene dimethacrylate newly prepared was more suitable to immobilize enzyme than the carrier synthesized with traditional method.

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Many attempts have been made over the last years to improve the catalytic activity and operational stability of industrial enzymes. Immobilization is an effective procedure which increases the stability of enzymes and allows their reuse in industrial processes [1]. 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 on phenol-formaldehyde resin and bone powder [2–10]. Some of these methods suffer from low immobilization yields or continuous leakage of enzyme [11].

Nowadays, increasing attention is being devoted to the preparation of polymers containing epoxy groups. 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 even in wet conditions [12, 13]. 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, ether and thioether bonds) with minimal chemical modification of the protein [14].

[1] The article is published in the original.

In this paper, the copolymer of glycidyl methacrylate and ethylene dimethacrylate (GMA-co-EDMA) with macroporous morphology, containing reactive epoxy groups, was firstly synthesized with dodecyl alcohol and cyclohexanol as liquid pore-forming agents and nano-calcium carbonate as solid pore-forming agent simultaneously, and the scanning electron microscopy (SEM) micrographs were done to characterize its surface structure. 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 *trans*-galactosylation reaction to produce galactooligosaccharides [15]. The activity of the immobilized β-galactosidase and the immobilization yield were also investigated in order to examine the suitability of the support obtained to immobilize enzyme. Finally, the kinetic parameters of *o*-nitrophenyl-β-*D*-galactopyranoside reaction on the immobilized enzyme, the values of Michaelis constants K_M, were also determined.

EXPERIMENTAL SECTION

Apparatus and Reagents

Ultraviolet spectrotometer ("T6 New Century"), scanning electron microscope (KYKY-2800W), vacuum desiccator (DZ-6020), digital pH meter (PHS-3C), universal grinder (FW-200) and water constant

temperature oscillator (SHA-B) were used for the study. All aqueous solutions were prepared with twice distilled water.

Glycidyl methacrylate (GMA) was purchased from "Tokyo Chemical Industry Co. Ltd." And ethylene dimethacrylate (EDMA) (98%) 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 all other reagents were of analytical grades.

Preparation of Enzyme and Substrate Solution

β-Galactosidase (0.1500 g) was weighed and added to 25 ml of 0.1 M sodium phosphate buffer. The enzyme solution obtained was stored in the refrigerator for use.

The substrate solution was prepared by dissolving 0.0150 g ONPG in 10 ml of twice distilled water.

Preparation of Macroporous Glycidyl Methacrylate Polymer

The macroporous poly(GMA-co-EDMA) was prepared by suspension copolymerization. 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 degased and homogenized by ultrasonication for 20 min and then 0.32 g of nanosize calcium carbonate was added for another 20 min. Then the mixture was added into a four-necked flask containing 55 ml of polyvinyl alcohol (2%) and 55 ml glutin (0.1%) and mechanically stirred at 55°C under a nitrogen atmosphere. After homogenization, the polymerization was allowed to proceed at 65°C for 3 h and at 85°C for 2 h. Then the polymer particles obtained were washed with water completely. The polymer was kept in ethanol for 24 h to remove the liquid phase porogen and in 0.1 M hydrochloric acid solution for 24 h to get rid of nanosize calcium carbonate, and then dried under vacuum. In addition, the carrier was also prepared only with dodecyl alcohol and cyclohexanol as pore-forming agents according to the procedure above.

In the following discussion, the support prepared only with liquid phase as porogen was called support **I**, and the one synthesized simultaneously with liquid and solid porogen was called support **II**.

Method of Immobilization

The immobilization was carried out by adding of polymer particles (0.0500 g) to 0.5 ml of 0.1 M phosphate buffer containing enzyme (6 mg/ml). With gently stirring, the reaction was allowed to proceed at 25°C 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 with the support was attributed to immobilized enzyme.

Enzyme Activity Assay

The activity of the free and the immobilized enzyme were determined according to the references [15, 16] using ONPG as substrate. For determination of free enzyme activity, aliquots of it (0.1 ml) were added to the mixture of 0.9 ml 0.1 M phosphate buffer (pH 5.0) and 0.2 ml ONPG (1.5 mg/ml). After being incubated at 55°C for 2 min, the reaction of ONPG was stopped by the addition of 2 ml 1 M Na_2CO_3 solution, and the amount of ONP was measured directly at $\lambda = 405$ nm. For measuring of the immobilized enzyme activity, 0.0500 g of the immobilized enzyme was soaked in 1 ml of 0.1 M phosphate buffer (pH 5.0), and the reaction was started by adding 0.2 ml ONPG (1.5 mg/ml). After being carried out for 15 min at 55°C the reaction was stopped and analyzed as above. The immobilization yield was calculated as the ratio of immobilized enzyme to enzyme subjected to immobilization. The activity was defined as the amount of enzyme that liberated 1 μmol of product in 1 min at 55°C.

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°C. Enzyme activity was determined after a long duration exposure at various temperatures (50 and 60°C) followed by reaction at 55°C.

The pH-activity dependences in pH range 3.0–9.0 were determined for free and the bounded enzyme at 55°C using ONPG as substrate. The pH stability in the range 2.0–10.0 was determined after 30 min exposure at different pH and 55°C.

Kinetics

The Michaelis constant K_M was calculated for the soluble and the immobilized enzymes by their testing in increasing ONPG concentrations ranging from 0.25 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. At first, 0.0500 g of the immobilized enzyme was taken and soaked in 1 ml of phosphate buffer overnight. After the mixture was incubated at 55°C for 2 min, the reaction was started by adding 0.2 ml of 1.5 mg/ml ONPG solution and then the

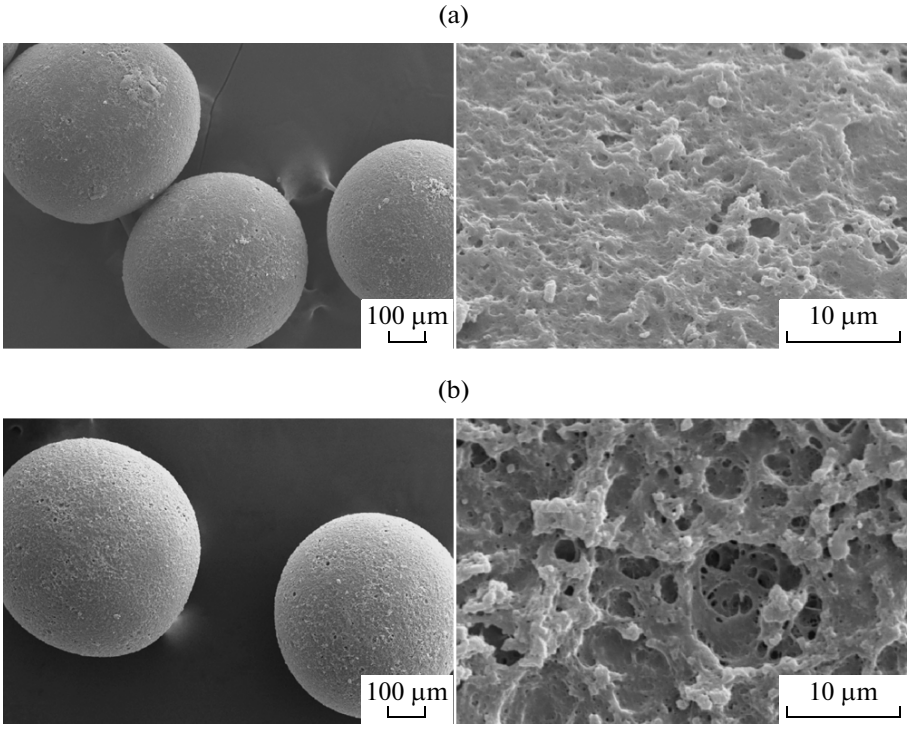

Fig. 1. Scanning electron micrographs of the supports **I** (a) and **II** (b).

reactive mixture was analyzed. Afterward, the solid was filtered and washed thoroughly with distilled water and the above experiment was repeated under the same conditions.

RESULTS AND DISCUSSION

Comparison of Supports I and II

According to the conditions described above, the supports **I** and **II** were obtained separately, and the SEM micrographs of the dried polymer were obtained. The results are presented in Figs. 1a and 1b. SEM micrographs showed that the support **II**, in preparation of which were simultaneously used dodecyl alcohol and cyclohexanol as liquid pore-forming agents and nanosize calcium carbonate as solid one, had a much more porous surface structure than that of the support **I** prepared only with dodecyl alcohol and cyclohexanol as porogen. Both supports were used to immobilize enzyme under their optimum conditions, and the results obtained are listed in Table 1. From the results shown in the Table 1, the activity of the enzyme immobilized on the support **II** reached a maximum of 532.9 U/(g dry carrier). The obtained enzyme activity was approximately 2.5 times value than that acquired on the support **I**. It could be explained that the porous surface properties of GMA polymer would favor higher adsorption capacity for the enzyme due to increase of the surface area.

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

Support	Enzyme activity, U/(g dry support)	Immobilization yield, %
I	228.1	33.94
II	532.9	79.29

Properties of the Immobilized Enzyme

pH optimum and pH stability. Figure 2 showed that the pH profile of the free enzyme have peak at pH 5.0. Similar pH was also found for the immobilized enzymes **I** and **II**. The enzyme activity was determined with ONPG as substrate, at 55°C in various pH buffers (3.0–10.0) for 15 min.

After all the enzymes were exposed to different pH (2.0–10.0) at 25°C overnight, enzyme activity was determined at 55°C and pH 5.0 for all enzymes at 15 min, with ONPG as substrate. As shown in Fig. 3, both immobilized enzymes had wider pH range than that of free enzyme. In the range of 3.0–9.0, the immobilized enzyme activity remained >90%.

Optimum temperature and thermostability. The enzymes activity was determined by ONPG as substrate at various temperatures (40–65°C) at pH 5.0 for

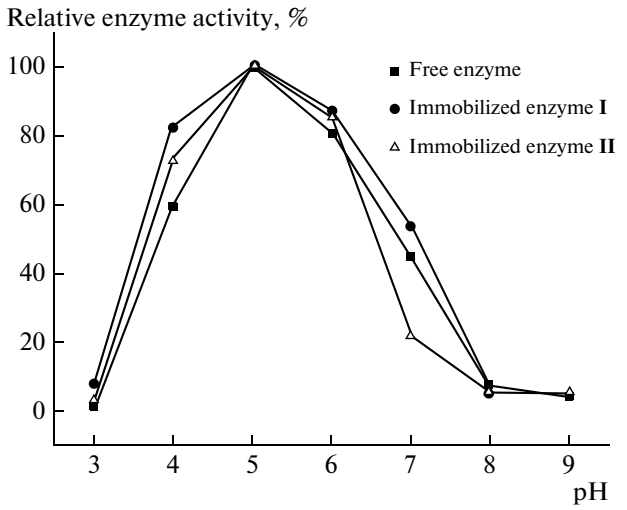

Fig. 2. Influence of pH on immobilized enzyme activity. The activity was determined at 55°C for 15 min.

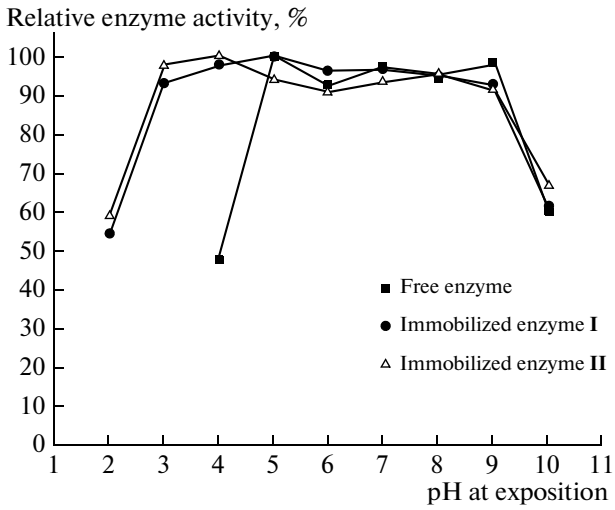

Fig. 3. The pH stability of free and immobilized enzymes. The enzymes were exposed to pH 2.0–10.0 at 25°C overnight. Enzymes activity was determined at 55°C and pH 5.0 for 15 min.

15 min (Fig. 4). The temperature optimum for both immobilized enzyme was 55°C.

Figures 5a and 5b showed that the immobilized enzyme was more stable than the free one. At 50°C, after 3 h, the remaining activity of the immobilized enzyme **I** was 53.38%, the immobilized enzyme **II** 62.73%, and the free enzyme 50.94%. At 60°C, over the same time, the residual activity of the free enzyme was 17.71%, whereas that of the immobilized enzyme **I** was 43.00% and the immobilized enzyme **II** 50.40%. Results showed that both immobilized enzyme had better thermostability than that of the free enzyme.

Operational stability of immobilized enzymes. The experiment was repeated 6 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. 6 and it is seen that the immobilized β-galactosidase (**I** and **II**) was used for 6 times without significant loss in activity. It means that almost no enzyme was removed from the surface of the glycidyl methacrylate carrier in the course of reaction. So the operational stability of the immobilized enzyme obtained is very good.

Kinetic parameters. Lineweaver–Burk dependences for the free and immobilized enzymes using ONPG as substrate were plotted and the values of K_M calculated from those graphs are shown in Table 2. From Table 2, it could be seen that the parameters K_M for the immobilized enzymes are higher than that for the free enzyme. It is probably caused by the influence of immobilization procedure and by the lower accessibility of the active site of the immobilized enzyme to substrate [17].

Thus, in this paper the macroporous poly(GMA-co-EDMA) was firstly synthesized with dodecyl alcohol and cyclohexanol as liquid pore-forming agents and nanosize calcium carbonate as solid one simultaneously by the method of suspension copolymerization. The SEM micrographs showed that the support **II**, in preparation of which simultaneously were used liquid and solid materials as porogens, had a much more porous surface structure than the support **I** prepared only with liquid solution as porogen. Under the optimum conditions, β-galactosidase from *Aspergillus oryzae* was immobilized on the supports described above and the enzyme activity on the support **II** was much higher than that on the support **I**. Therefore support **II** is more suitable to immobilize enzyme because of its higher specific surface. The properties of the free and both immobilized enzymes were deter-

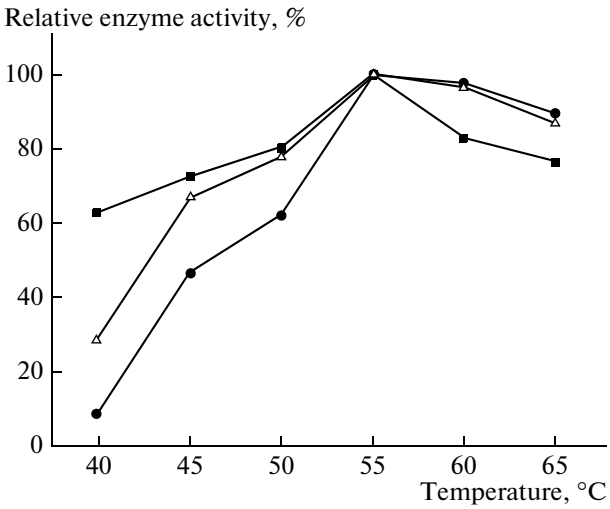

Fig. 4. Influence of temperature on reaction on immobilized enzymes: *1*—free enzyme, *2*—immobilized enzyme **I**, *3*—immobilized enzyme **II**.

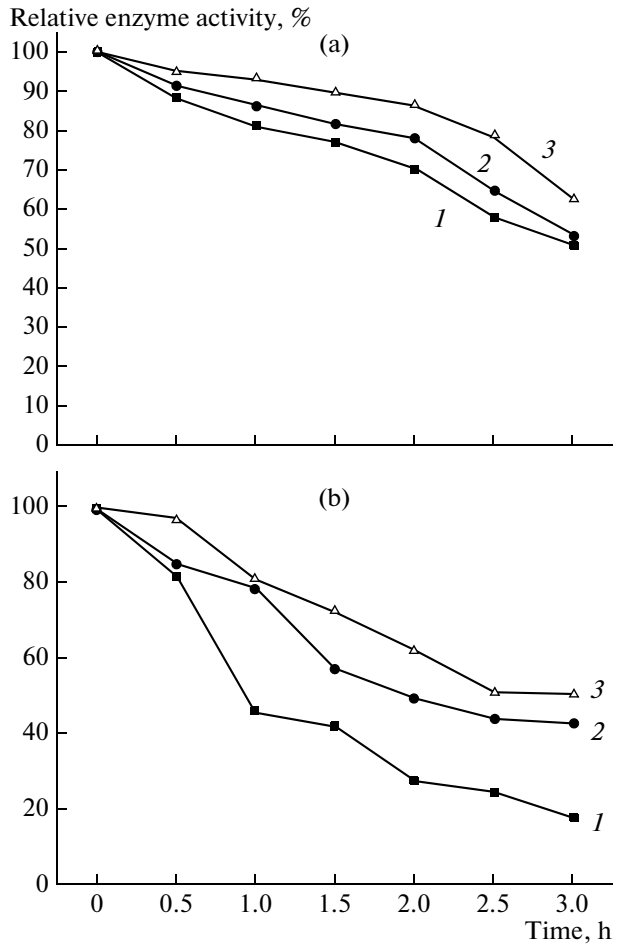

Fig. 5. Thermostability of different enzymes at 50 (a) and 60°C (b): *1*—free enzyme, *2*—immobilized enzyme **I**, *3*—immobilized enzyme **II**.

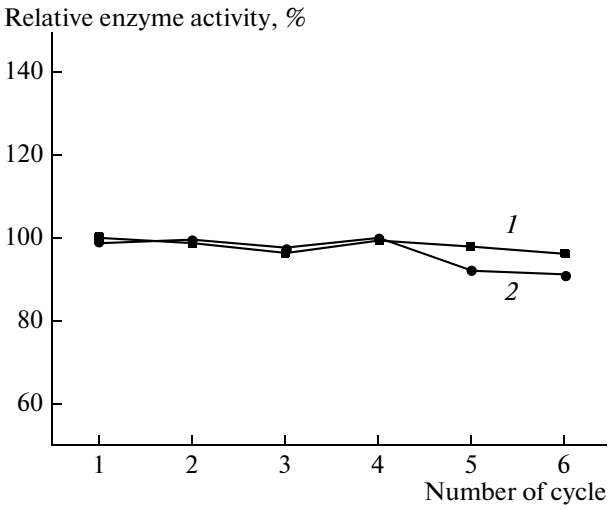

Fig. 6. Operational stability: *1*—immobilized enzyme **I**, *2*—immobilized enzyme **II**.

mined and compared, and satisfactory results of both immobilized enzymes were obtained in pH, thermal and operational stability. Finally, K_M values for the free enzyme, the immobilized enzymes **I** and **II** were obtained. It was shown, that the polymer as enzyme immobilization carrier, which usually was prepared with liquid solution as porogen by traditional method, could also be got using solid and liquid materials as porogens. The new method allows getting more porous surface structure and more activated reaction group. The results obtained are very useful for industrial application of polymer as enzyme immobilization carrier.

Table 2. Kinetic parameters of the reaction on immobilized enzymes **I**, **II** and free enzyme

Enzyme	K_M, mmol/l	
	40°C	50°C
Immobilized enzyme **I**	24.17	22.30
Immobilized enzyme **II**	19.78	18.37
Free enzyme	10.50	8.282

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