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Immobilization of *Candida lipolytica* Lipase on Macroporous Beaded Terpolymers with Epoxy Groups

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ABSTRACT: A series of macroporous beaded terpolymers with epoxy groups were synthesized by suspension polymerization with glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), and the third monomers including styrene, methyl methacrylate, *n*-butyl acrylate (BA), butyl methacrylate (BMA), and 2-hydroxyethyl methacrylate for immobilization of *Candida lipolytica* lipase. The effect of various third monomers on loading and activity recovery of immobilized lipase were studied. Terpolymers with BA as the third monomer were found to give the biggest loading of lipase, and the activity recovery of lipase immobilized on poly (GMA-EGDMA-BA) terpolymers reached 79.0%. As the content of BA (%) increasing, the loading of lipase enhanced, but the activity recovery reached 88.5% for the initial stage and decreased to 46.9% at last. The poly(GMA-EGDMA-BA-10) showed an optimal result in lipase immobilization. Lipase immobilized on poly(GMA-EGDMA-BA-10) carriers had broader pH and higher temperature stability. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: terpolymer; third monomer; n-butyl acrylate; lipase

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INTRODUCTION

As a highly effective biocatalyst, enzyme has been widely used in laboratory and industry. However, it is sensitive to temperature, pH, and short in reusability. In recent years, many approaches have been used to improve the catalytic activity and operational stability of enzyme such as adsorption, cross-linked enzyme aggregates, embedding method, and so on, one of the most commonly method is enzyme immobilization.[1–6] Binding to a support, which can keep the enzyme strong, is a good choice for enzyme immobilization. The binding methods included three kinds: physical, ionic, and covalent in nature.[7] Covalent immobilization strategies that assisted the formation between carriers and enzyme had the strongest binding force without affecting its tertiary structure. Macroporous carriers with epoxy groups, which had large surface area, big pore and easy reacting groups could immobilize the enzyme easily and keep most of the catalytic activity. Therefore, it attracted many interests in the covalent immobilization strategies.[8–11]

The investigation of macroporous carriers with epoxy groups referred to the physical structure, component, and immobilized methods. The particles' size, porous radius, pore distribution, and surface area were widely investigated parts in physical structure. Researchers usually searched the optimal porogen and cross-linking agents to get the largest enzyme binding and the best activity recovery.[12–15] It has been found that enzyme can be immobilized well with the pore 3–9 times the size of the enzyme.[16] Performed as the binding bridges between enzymes and carriers, the spacer arm attracted the most eyes in the research of immobilized methods. The length of the spacer arms affected the enzyme immobilization a lot. When the bridge was short, such as ammonia water, it would decrease loading and activity recovery of enzyme. Conversely, the spacer arms like 1,6-diaminohexane and glutaraldehyde would increase the enzymes' activity because of providing long bridge.[17–19] The studies of macroporous carriers with epoxy groups in the component on the enzyme immobilization referred to the various epoxy monomers. There are two main monomers containing epoxy group: glycidyl methacrylate (GMA) and allyl glycidyl ether (AGE). Because of giving bigger pore size, carriers with AGE as epoxy monomer usually performed better in the enzyme immobilization.[20,21]

In the last few years, many researchers have investigated the influence of the chemical surroundings of the macroporous carriers with epoxy groups in enzyme immobilization. Generally speaking, enzyme immobilization is a two-step reaction,[22] the carrier's chemical framework determines the physical adsorption and covalent immobilization of enzyme. To get better results in enzyme immobilization, many attempts have been made to change the carriers' chemical surroundings. Adding methacrylic

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acid to glycidyl methacrylate-divinylbenzene copolymers resulted in good immobilization of glucoamylase.[23] Introducing the tertiary amino groups to GMA-ethylene glycol dimethacrylate (EGDMA) carriers by the reaction of amino and epoxy groups would get good lipase immobilization.[24] Adding the third monomer is a good choice to change the poly(GMA-EGDMA) carrier's chemical framework. Thus, the kind and amount of the third monomer will be the key factor on enzyme immobilization.

In this article, a series of beaded terpolymers were synthesized by suspension polymerization of GMA, EGDMA, and using various other monomers [including styrene (S), methyl methacrylate (MMA), *n*-butyl acrylate (BA), butyl methacrylate (BMA), and 2-hydroxyethyl methacrylate (HEMA)] as the third monomer for *Candida lipolytica* lipase (triacylglycerol acylhydrolases, E.C. 3.1.1.3) immobilization, in which toluene and *n*-heptane were used as porogen. The composition and structure of the resins were characterized with Fourier Transform Infrared Spectroscopy (FTIR) and Brunauer-Emmet-Teiler (BET) methods. The effects of the third monomer and its content on loading and activity recovery of lipase, as well as the pH, thermal, and storage stability of lipase immobilized on the terpolymers with the optimal content of third monomer were investigated.

MATERIALS AND METHODS

Materials

GMA was obtained from Mitsubishi Chemical (Japan); ethyleneglycol dimethacrylate (EGDMA) was offered by Fushun Anxin Chemical Company Limited (China); olive oil and *Candida lipolytica* lipase were bought from Aladdin Reagent (China); azodiisobutyronitrile (AIBN), BMA, MMA, BA, HEMA, S, pyridine, copper sulfate, and polyvinylpoyrrolidone (PVP K90) were of analytical grade from local suppliers.

Preparation of the Terpolymers

The macroporous resins were synthesized by typical suspension copolymerization procedure. Suspension polymerizations were carried out in a 250 mL cylindrical reactor equipped with a heating coat, a mechanical stirrer, a reflux condenser, and an addition funnel. The initiator (AIBN; 1 wt %) was dissolved in monomers, and then the mixture was diluted with a mixed solvent (toluene and *n*-heptane; 3/1, v/v) taken in 4/3 (v/v) proportions. The dispersion medium was prepared through the dissolution of 1 wt % PVP (K90) and PVA in deionized water, and it was used in a 1/7 (w/w) ratio according to the organic phase. The reaction mixture was stirred at 350 rpm for 0.5 h at 50°C, 4 h at 65°C, 2 h at 75°C, and then for 3 h at 80°C. After cooling, the diluents and any pending monomers from the products were removed by a washing procedure. The spherical beads were suspended in acetone for three times (each time cost about half an hour), and then they were washed by a mass of deionized water. The terpolymers were dried under vacuum at 40°C up to a constant mass. The dry beaded polymers were sieved through 80–110 mesh.

Characterization Methods

The FTIR spectra were recorded by using an IR Prestige-21 FTIR with KBr to form a tablet. The pore radius of the carriers was measured by BET method with a Micromeritics tristar 3000.

The Swelling Characteristics of the Carriers

The carriers were swollen in different agents and the swelling factor St was calculated by the formula of:

$$St = ((So - S)/S) \times 100\%$$

S and *So* were the weights of carriers before and after swelling in reagent, respectively.

Preparation of the Immobilized Lipase

Wetted polymer particles (1.0 g) were added into 40.0 mL 0.1*M* sodium phosphate buffer (pH 7.0) with 10.0 mL lipase solution. The mixture solution was shook in a plate thermostatic oscillator with 200 rpm at 37°C for 24 h. After immobilization, the carriers were washed thoroughly with 0.1*M* sodium phosphate buffer (pH 7.0). The amount of protein immobilization on carriers was examined by the Bradford method.[25] The absorbance of bovine serum albumin with different concentrations was examined to build a standard curve. The concentration of lipase was revised by that standard curve.

Assay of Free and Immobilized Lipase Activity

The activity of free lipase was measured by a colorimetry method.[26] First, 1.0 mL of free enzyme and 5.0 mL 0.1*M* sodium phosphate buffer (pH 7.0) were incubated at 35°C for 10 min. Subsequently, 4.0 mL olive oil emulsion (V olive oil/V 4% PVA, 1/3; emulsify at 2500 rpm for 30 min) was added and incubated for 10 min at the same temperature, followed by addition of 10.0 mL toluene to finish the reaction. The mixture was centrifuged at 3000 rpm, from which 1.0 mL of the upper lays was transferred to a test tube, then added 3.0 mL toluene and 1.0 mL of chromogenic agent (10 g copper sulfate was dissolved in deionized water and filled with deionized water to 100 mL. The solution was filtrated and adjusted to pH 6.1 with pyridine). The mixture in the test tube was completely mixed by putting in a plate thermostatic oscillator at 200 rpm for 10 min. The absorbance was measured at 710 nm.

The activity of immobilized lipase was measured by the same method. The reaction mixture consisted of 50 mg of immobilized enzyme and 6.0 mL of 0.1*M* sodium phosphate buffer (pH 7.0), and the following procedure was the same as that for the free enzyme.

Optimum Temperature and pH of Free and Immobilized Lipase

The optimum temperature of free lipase and immobilized lipase were studied by testing their activities at varying temperature in the range of 30–60°C under pH 7.0, as described in the Section "Assay of free and immobilized lipase activity."

The optimum pH of free lipase was examined by the following method. First, the free lipase was dissolved in 0.1*M* sodium phosphate buffer (in varying pH from 5.0 to 8.5). Then, the activity of free lipase was determined by incubating the lipase under its optimal temperature and the same pH of their solution, as described in the Section "Assay of free and immobilized lipase activity." The optimum pH of immobilized lipase was tested by incubating the immobilized lipase under its optimal temperature at varying pH buffers from 5.0 to 8.5, as described in the Section "Assay of free and immobilized lipase activity" too.

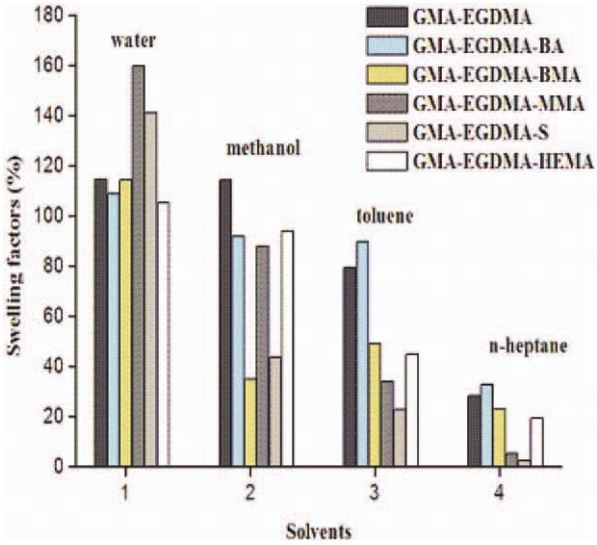

Figure 1. Swelling ability of GMA-EGDMA-M carriers in different agents. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

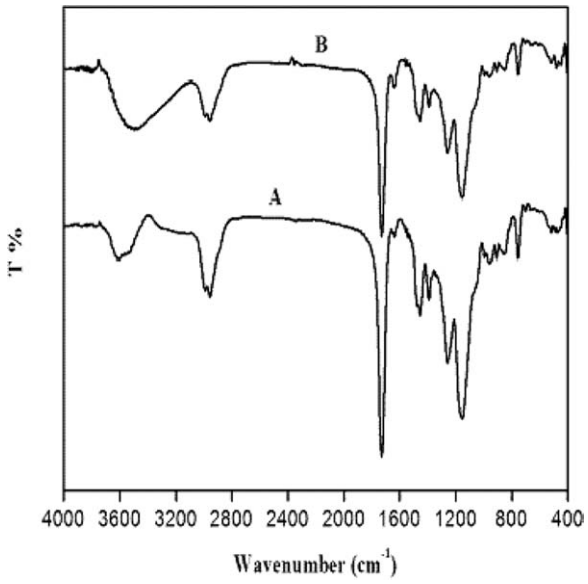

Figure 2. FTIR spectra of GMA-EGDMA-BA (A) carriers and GMA-EGDMA-BA immobilized lipase (B).

The Store Ability of Free and Immobilized Lipase

The free lipase and immobilized lipase were stored at 4°C. The store stability of free and immobilized lipase were evaluated by determining their activity recovery at their optimal pH and thermal conditions up to 15 days.

RESULTS AND DISCUSSION

Effect of the Third Monomers on Lipase Immobilization

Effect of the Third Monomers on Swelling Properties. As shown in Figure 1, the swelling properties of the poly(GMA-EGDM-M) polymer particles in different agents were examined. The poly(GMA-EGDMA-MMA) terpolymers exhibited the highest polarity, while the poly(GMA-EGDMA-BA) showed the least polarity. In water and methanol the swelling factors of the carriers did not show disciplinary results, while good uniform results were demonstrated in toluene and *n*-heptane. The poly (GMA-EGDMA) resins had moderately polarity, and its swelling factor was dependent on the third monomers in low polar agents, but independent of the third monomers in high polar agents. Among the 6 carriers in low polar agents, the poly (GMA-EGDMA-BA) carrier presented the best swelling factors.

Table I. Influence of the Third Monomer on Loading and Activity Recovery of Lipase

Resins	Loading of lipase (mg protein/g resin)	Activity recovery of lipase (%)	Pore size (nm)
GMA-EGDMA	0.38	71.6	11.6
GMA-EGDMA-BA	0.87	79.0	13.2
GMA-EGDMA-BMA	0.48	65.0	11.1
GMA-EGDMA-MMA	0.32	80.5	11.2
GMA-EGDMA-S	0.45	73.5	16.4
GMA-EGDMA-HEMA	0.28	82.9	14.0

Effect of the Third Monomers on Loading and Activity Recovery of Lipase Immobilization. The influence of the third monomers on loading and activity recovery of lipase were showed in Table I, where the trend was clearly that the third monomers influenced the amount of immobilized enzyme and the activity recovery dramatically. Lipase is a hydrophobic enzyme and it facilitates carriers with low polarity. However, carriers with hydrophilic groups will result in good activity recovery of immobilized lipase. Among the six carriers, the poly (GMA-EGDMA-BA) carrier presented the lowest polarity. Therefore, the poly(GMA-EGDM-BA) resins had the highest lipase immobilization and the poly(GMA-EGDM-HEMA) resins had the best activity recovery. After the alkyl was introduced into the carriers, the loading of lipase enhanced. But, the poly (GMA-EGDM-BA) carriers immobilized about two times of lipase compared with poly(GMA-EGDM-BMA) carriers. Therefore, adding methyl group to the structural units of the poly (GMA-EGDMA) carriers would decrease the loading of lipase.[20,21]

Effect of the Content of BA on Lipase Immobilization

The Characterization of the Poly(GMA-EGDMA-BA) Carriers. The FTIR spectra of the poly(GMA-EGDMA-BA) carriers and its immobilized lipase were presented in Figure 2(A, B). Both of the IR spectrums gave the characteristic peaks at 1259, 906, and 850 cm^{-1} due to stretching vibrations of the epoxy group from GMA. The absorption peaks present at 1728, 1390, and 993 cm^{-1} was ascribed to stretching vibrations of C=O and —CH_2—CH_2—CH_3 of ester and *n*-propyl, which indicated that the occurrence of polymerization. The characteristic peak of —NH— group gave characteristic peak at 3470 cm^{-1}, it was clearly that line B had this absorption peaks. This group was from the reaction of lipase and epoxy group (—NH—CH_2—O—). Therefore, the absorption peak at 3470 cm^{-1} in line B could indicate that the immobilization of lipase had occurred.

Table II. Effect of Different Amount of BA on Loading and Activity Recovery of Lipase

Resins	Loading of lipase (mg protein/g resin)	Activity recovery of lipase (%)	Pore size (nm)
GMA-EGDMA-BA-5	0.87	79.2	13.2
GMA-EGDMA-BA-8	1.09	88.5	13.2
GMA-EGDMA-BA-10	1.26	79.0	13.1
GMA-EGDMA-BA-15	1.48	65.0	13.2
GMA-EGDMA-BA-20	2.07	46.9	13.3

Effect of the Content of BA. Effect of the content of BA on loading and activity recovery of lipase was showed in Table II. According to the data presented in Table II, as the amount of BA increased, the loading of lipase would enhance; however, the activity recovery reached 88.5% for the initial stage and decreased to 46.9% at last. As the cross-linking agent and the pore-forming agent that determined the pore radium remained uniform among the carriers, the pore radium had little changes. When the amount of BA increased, the carriers would have more hydrophobic groups and the adsorption of lipase would increase. Therefore, the content of BA determined the loading of lipase. When the content of immobilized lipase got bigger, the lipase would go deep into the carriers besides the surface. Because of low polarity, it was difficult for the olive oil emulsion to get in the internal surface. Therefore, the activity recovery would increase at first and then decreased. From Table II, we could see that the poly(GMA-EGDMA-BA-10) had better enzyme loading and activity recovery of lipase. It represented the optimal supports for lipase immobilization in this case.

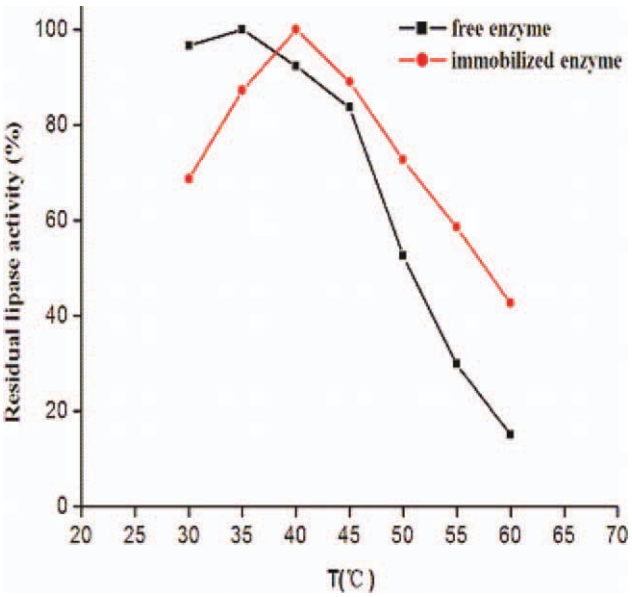

Figure 3. Effect of temperature on the activity of free and immobilized lipase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

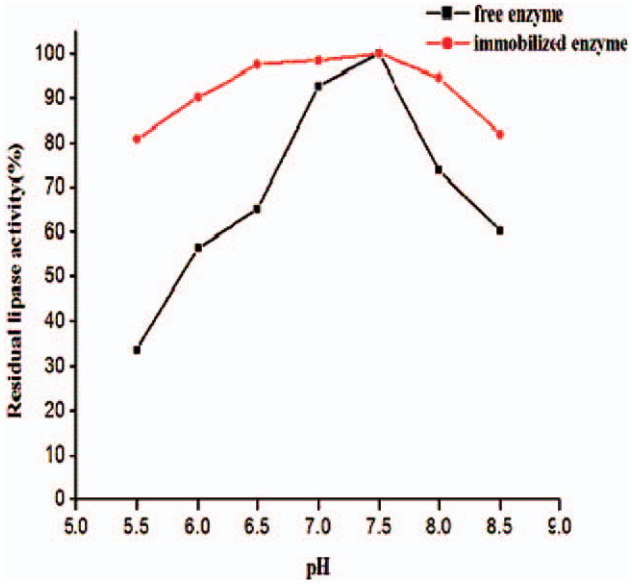

Figure 4. Effect of pH on the activity of free and immobilized lipase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Lipase Immobilization Parameters

Temperature Stability. The stability of free lipase and lipase immobilized on poly(GMA-EGDMA-BA-10) particles were determined by measuring residual activity in the range of 30–60°C. The activity recovery of free and immobilized lipase at different temperature were represented in Figure 3. The optimal temperature of immobilized lipase was 5°C higher than that of free lipase. At 50°C, the free lipase retained only 52.5% residual activity while immobilized lipase retained 72.7%. Similarly, the free lipase retained only 15.0% residual activity while immobilized lipase was found to retain 42.6% at 60°C. Thus, the

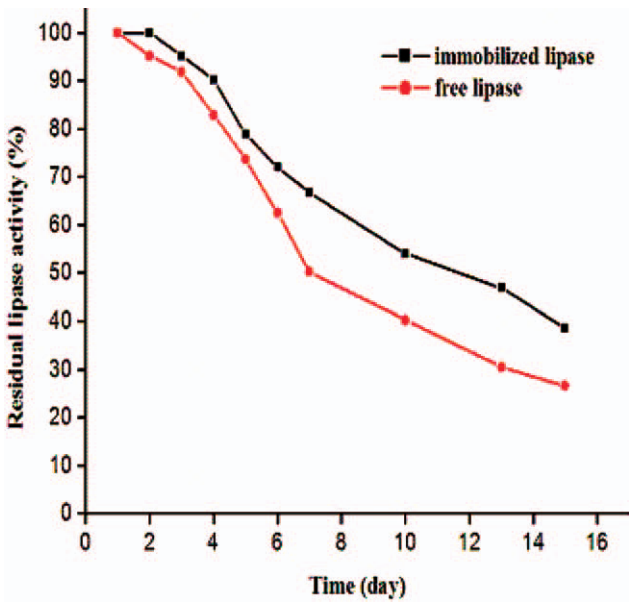

Figure 5. Storage stability of free and immobilized lipase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

described immobilization lipase afforded better temperature stability as compared with the free lipase.

pH Stability. Effect of pH on the residual activity of free lipase and lipase immobilized on poly(GMA-EGDMA-BA-10) particles were studied from pH 5.5 to 8.5, as displayed in Figure 4. Lipase immobilized on poly(GMA-EGDMA-BA-10) particles could keep most of their activity over a broader pH range compared with free lipase. In the range of pH 6.5–8.0, the activity recovery of immobilized lipase changed only a little, which appeared to the free lipase at pH 7.0 and 7.5. The free lipase retained 33.7 and 60.2% residual activity at pH 5.5 and pH 8.5, respectively, whereas at the same pH conditions immobilized lipase retained 80.8 and 82.0% residual activity, respectively. This indicated that the immobilization procedure improved the stability of lipase significantly in the acidic region.

Storage Stability. The storage stability of lipase immobilized on poly(GMA-EGDMA-BA-10) particles was measured and compared with free lipase in Figure 5. The storage stability of the immobilized lipase was a little better than free lipase. After 15 days of storage at 4°C, lipase immobilized on poly(GMA-EGDMA-BA-10) carriers retained 38.6% residual activity, but the free lipase retained only 26.5% residual activity. Nevertheless, the storage stability of immobilized lipase did not increase significantly, which is not quite consistent with the previous studies.[17,27] The possible explanation is that upon the addition of BA, it decreased the polarity of the carriers which could increase the adsorption of lipase but the amount of covalent binding of lipase changed a little. Therefore, the drop of lipase was fast.

CONCLUSION

A series of macroporous beaded terpolymers with GMA, EGDMA, and the third monomers (S, MMA, BA, BMA, and HEMA) were synthesized by suspension polymerization for lipase immobilization, in which toluene and *n*-heptane were used as porogen. Introducing the alkyl to the carriers, the loading of lipase enhanced, while the hydrophilic groups could improve the activity recovery of lipase. As the third monomer BA using in the poly(GMA-EGDMA) carriers, the binding and expression of lipase were improved dramatically. The binding amount of lipase was enhanced with increasing the content of BA in the terpolymers, but the activity recovery only increased for the initial stage and then decreased. The poly(GMA-EGDMA-BA-10) carriers showed an optimal result in lipase immobilization. Comparing with free lipase, lipase immobilized on poly(GMA-EGDMA-BA-10) carriers had a broader pH stability, and the optimal temperature of immobilized lipase increased about 5°C. Owing to little changes made to the covalent immobilization of lipase as the adsorption of lipase increased, the storage ability of the immobilized lipase had not significant increase.

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