Preparation and Application of Partially Porous Poly(Styrene-Divinylbenzene) Particles for Lipase Immobilization

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ABSTRACT: Partially porous poly(styrene-divinylbenzene) (PS-DVB) particles in the micron size range were prepared by the method of multistep swelling and polymerization involving the use of polymeric porogens. Polystyrene (PS) seeds prepared by dispersion polymerization were expanded in particle size by absorbing styrene and initiator, and then polymerized to form polymeric porogen particles. The newly synthesized PS chains served as the porogens of the PS-DVB particles, resulting from the copolymerization of styrene and divinylbenzene in the swollen polymeric porogen particles. PS-DVB particles with a specific surface area of up to 34 m^2/g and a pore volume of up to 0.15 cm^3/g were obtained. The average pore diameter of PS-DVB particles was in the range of 15–24 nm. An increasing amount of toluene used in the copolymerization step increased the pore volume and specific surface area. Lipase from *Candida rugosa* was immobilized on the prepared PS-DVB by physical adsorption. The optimum temperature for enzymatic activity was increased and the thermal deactivation of enzyme in organic solvent was slowed down by the immobilization. However, compared with soluble enzyme, the immobilized lipase on PS-DVB retained a less activity after the first stage deactivation, suggesting a possible change in the conformation of enzyme molecule by immobilization. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 80: 39–46, 2001

Key words: poly(styrene-divinylbenzene; polymeric porogen; lipase; enzyme immobilization; thermal deactivation

INTRODUCTION

Highly porous and crosslinked poly(styrene-divinylbenzene) (PS-DVB) particles have been extensively studied for over two decades. The methods of suspension polymerization[1] and activated multistep swelling and polymerization[2] can be employed to prepare porous beads of PS-DVB. Applications of porous PS-DVB are in use as the stationary phases for size exclusion and ion-exchange chromatography and the catalytic supports. For biological applications, for example, protein chromatography and enzyme immobilization, the presence of micropores in these particles could lead to diffusional limitation and cause damage to the biomolecules. A use of nonporous particles can solve these problems.[3,4] The draw-

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back of using nonporous particles, however, is a relatively small loading capacity due to the very low surface area of the particles. Therefore, the porous structure with large pores near the periphery of the particles will be much more useful and effective for biological applications. The present paper describes the synthesis of partially porous PS-DVB particles and their applications to the immobilization of lipase.

Lipase (EC 3.1.1.3) is the enzyme that catalyzes the hydrolysis/synthesis of a wide range of soluble or insoluble carboxylic acid esters and amides. The industrial application of lipase-catalyzed reactions includes the hydrolysis of oils and fats, synthesis of fatty acid esters, and production of intermediates for drug synthesis. Several materials have been used for the immobilization of lipase. However, immobilization of lipase on hydrophobic supports by physical adsorption is a very promising method. The adsorbed lipase on hydrophobic supports (e.g., octyl-agarose) shows very enhanced activity and enhanced stereospecifity.[5] The popular hydrophobic polymers used as the supports of lipase immobilization include polypropylene (PP) and polyethylene (PE).[6] Commercially available particles are, for example, low-density polyethylene powder Accurel EP-400 and polypropylene powder Accurel EP-100.[7] According to Montero et al.,[8] lipase can easily be immobilized on microporous PP (EP-100) at room temperature in 1 min. Immobilized lipase (from *Candida rugosa*) can be repeatedly used for hydrolysis of animal fats and a hydrolysis degree of 90% or higher can be achieved.[9] Activity of *Pseudomonas cepacia* lipase in organic media is greatly enhanced after immobilization on PP (EP-100).[10] In the present paper, an alternative hydrophobic polymer, PS-DVB, is employed as the support for lipase immobilization by physical adsorption.

MATERIALS AND METHODS

Materials

Styrene, 2,2′-azobisisobutrionitrile (AIBN), benzoyl peroxide (BPO), sodium dodecyl sulfate (SDS), sodium nitrite, cetyl alcohol, ethanol, and all other organic solvents are of reagent grade or higher. Divinylbenzene (80 %) was purchased from Aldrich and used directly without purification. Polyvinylpyrrolidone (PVP, K-30) was obtained from BASF. Olive oil, olive oil emulsion, and lipase from *C. rugosa* (lyophilized powders containing approximately 20% protein) were obtained from Sigma.

Preparation of PS-DVB Particles

A multistep swelling and polymerization method involving the use of a polymeric porogen in the copolymerization of styrene and divinylbenzene was employed. In the first step, monodisperse polystyrene seed particles were prepared by dispersion polymerization of styrene (10 mL) in ethanol (80 mL), using AIBN (0.091 g) as the initiator, PVP (1.3 g) as the stabilizer, and cetyl alcohol (0.33 g) as the costabilizer. After nitrogen purging, the reaction mixture was shaken (125 rpm) in a water bath of 60°C for 24 h. The resultant particles were washed with methanol and distilled water, and dried in a vacuum oven. The PS particles obtained from this recipe was nonporous and had an average diameter of 1.8 μm. PS seeds with a larger particle diameter could be obtained by using a higher concentration of PVP and smaller amount of ethanol. The recipe for preparing the PS seeds having an average particle diameter of about 3 μm was 22 mL styrene, 57 mL ethanol, 0.2 g AIBN, and 1.0 g PVP.

The second step is a protocol of swelling and subsequent polymerization. PS seed particles were expanded in particle size by the absorption of styrene. The followed polymerization of absorbed styrene in the swollen particles was an oil-in-water type, seeded suspension polymerization, in which the nonpolar phase, i.e., the particles absorbing both monomer and initiator, were suspended in a polar solution (water). PVP, SDS, and $NaNO_2$ were used to stabilize the suspended particles. The linear PS chains produced from polymerization of absorbed styrene molecules had a smaller molecular weight in comparison with the PS chains of seed particles, and would serve as the porogens, that would be extracted after the copolymerization of styrene and divinylbenzene. The particles containing these newly synthesized PS chains are thus called polymeric porogen particles. For the preparation of polymer porogen particles, PS seed particles (1 g) were swollen with a monomer mixture at room temperature and then polymerized in a shaker at 70°C for 24 h, using BPO as the initiator with an initiator-to-monomer ratio of 5%, w/v. The monomer mixture contained styrene (5 mL or other specified volume), PVP (1 g), SDS (0.25 g), $NaNO_2$ (0.01 g), BPO (0.25 g or 5%, w/v with respect to styrene),

and water (100 mL). At the room temperature, a portion of the PS chains in the seeds was dissolved in styrene as the monomer molecules entered the seed particles. When the temperature increased to 70°C, free radicals generated from BPO attacked the styrene molecules and linear PS chains were produced. These newly produced PS chains were coagulated by van der Waals forces and stayed on the outer layer of the particles. The larger the amount of styrene added per unit mass of PS seeds, the larger the particle size of polymeric porogen particles. However, the resultant polymeric porogen beads were still nonporous.

In the final step, styrene and DVB were absorbed into polymeric porogen particles and then copolymerized within the enlarged particles. This swelling and subsequent polymerization to yield copolymerized PS-DVB particles was very similar to the second step. The protocol involves the use of a comonomer (DVB) and solvent (toluene). DVB was used as the crosslinker that rendered the resultant polymer chains crosslinked. Porous structure of the PS-DVB was achieved through the use of toluene as the solvent of PS chains. During the swelling of polymeric porogen particles with styrene and DVB, the PS chains of the original seeds preferably stayed in the inner and the PS chains produced in the porogen preparation step were at the outer layer of the swollen particles. This is because the PS chains produced in the second step was more shorter and susceptible to be dissolved in toluene. The absorbed styrene and DVB molecules were dissolved in toluene and then polymerized mainly at the outer layer of the particles. To carry out this final step of swelling and polymerization, one gram of polymeric porogen particles was mixed with styrene (5 mL), DVB (1 mL), BPO (0.06 g), PVP (1 g), SDS (0.25 g), $NaNO_2$ (0.01 g), 100 mL water, and 3 mL (or other specified amount of) toluene. After swelling for 24 h at room temperature, the copolymerization was carried out in a shaker at 70°C for 24 h. The resultant copolymerized particles were washed with methanol and water, and dried in a vacuum oven at room temperature for 12 h. To yield porous particles, the dried particles were extracted with toluene for 24 h using Soxhlet apparatus, and then washed with methanol and water. The specific surface area, pore volume and pore diameter of the particles were determined with an ASAP 2000 instrument (Micromeritics Instruments), and calculated with the BET equation using nitrogen as the adsorbate.

Lipase Immobilization on PS-DVB

PS-DVB particles (20 mg) were wetted with 60 μL of ethanol and then placed on a plate that was covered with 0.1 mL of phosphate buffer (0.1*M*, pH 7.0), containing 10 mg lipase. Enzyme was immobilized after removal of ethanol in a vacuum oven at room temperature. The enzymatic activity of the immobilized enzyme was determined using olive oil emulsion as the substrate. Incubation of the substrate with enzyme was at 37°C for 20 min. One unit of lipase activity (U) was defined as the amount of enzyme catalyzing the production of 1 μmol free fatty acid per minute.

The stability of lipase at higher temperatures and optimum temperatures for the enzymatic activity were studied by estimating the initial rates of olive oil hydrolysis. The amount of fatty acids released from the hydrolysis of olive oil in isooctane was determined by the method of cupric acetate-pyridine.[11] Incubating the fatty acid standard with cupric acetate-pyridine reagent and measuring the absorbance of the resultant mixture at 715 nm established a calibration curve.

RESULTS AND DISCUSSION

Porous PS-DVB Particles

The polystyrene seeds prepared by the method of dispersion polymerization were compact (nonporous) and monodisperse in particle size distribution. Polymeric porogen beads were prepared in the form of nonporous beads by swelling the PS seeds with a mixture containing the monomer (styrene) and initiator (BPO). The linear PS chains produced from polymerization of absorbed monomers served as the porogens for the preparation of porous PS-DVB particles. Porous structures were formed as the polymeric porogens left their sites within the particles. Figure 1 shows the scanning electron micrographs (SEM) of the prepared PS seeds, polymeric porogen particles, and PS-DVB particles. As expected, the final PS-DVB particles were porous, but polymeric porogen particles were still nonporous.

To 1 g of PS seeds, 4–6 mL of styrene were added in the swelling and subsequent polymerization step for preparing porogen particles. The average diameter of the PS seeds was 1.8 μm and increased to 2.2 μm after the polymeric porogens were introduced. An increase in particle diameter from 1.8 to 2.2 μm represents about a twofold increase in particle volume. The polymeric poro-

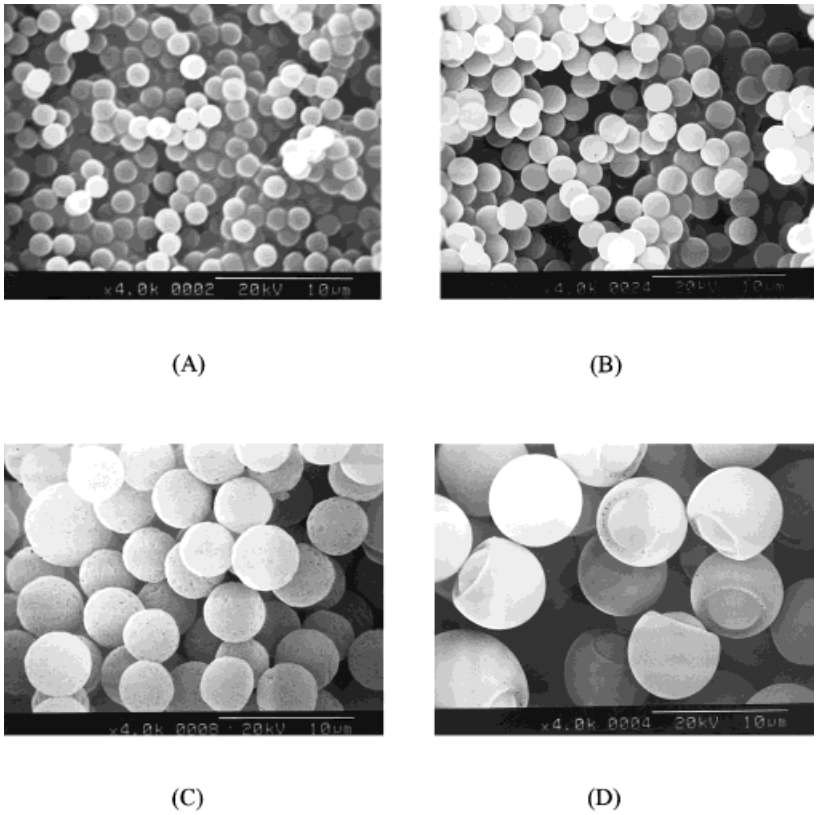

Figure 1 SEMs (4000× magnification) of the PS seeds (A), polymeric porogen particles (B), and PS-DVB particles (C and D). Particles as shown in A–C were prepared by following the recipe for the prepration of 1.8 μm PS seeds and the use of these seed particles. Particles as shown in D were obtained from PS seeds with an average diameter of 3 μm.

gen particles finally became partially porous PS-DVB beads with a particle diameter ranging from 3.4 to 4.5 μm, depending on the amount of styrene used in the polymeric porogen preparation step and the amount of toluene in the final copolymerization step. In general, the larger the amount of styrene added to per unit mass of PS seeds, the larger the particle size of polymeric porogen particles and then the larger the particle size of resultant PS-DVB particles. Since the initiator-to-monomer ratio in the preparation of polymer porogens was kept constant, the molecular size of the linear polystyrene chains was believed to be constant. These porogens resulted in PS-DVB particles with an average pore diameter ranging from 15 to 24 nm. The pore diameter in this range is much larger than the molecular size of an enzyme like lipase; the prepared PS-DVB particles thus mimic as the carriers for enzyme immobilization.

In the final copolymerization step, styrene and DVB polymerized within the enlarged polymeric porogen particles. Porous structure was achieved using a mixture of polymeric porogen and solvent (toluene) in the final copolymerization step. The specific surface area and pore volume of the copolymer particles generally increased with the amount of toluene used in the final copolymerization step. Toluene is a good solvent to linear PS molecules; a large amount of toluene resulted in more pores present in the PS-DVB particles after extraction with organic solvent. Using a toluene volume from 3 to 7 mL based on per unit gram of polymeric porogen particles, the surface area of PS-DVB particles was in the range of 4–34 m^2/g; meanwhile the pore volume varied from 0.02 to 0.15 cm^3/g. The particles (Fig. 2) having the highest values of specific surface area and pore volume (34 and 0.15 m^3/g, respectively) were obtained from the polymeric porogen particles that were prepared with 1 g PS seeds (1.8 μm) and 5 mL styrene, and using 5 mL of toluene in the copolymerization of styrene and divinylbenzene. This recipe resulted in PS-DVB particles having an

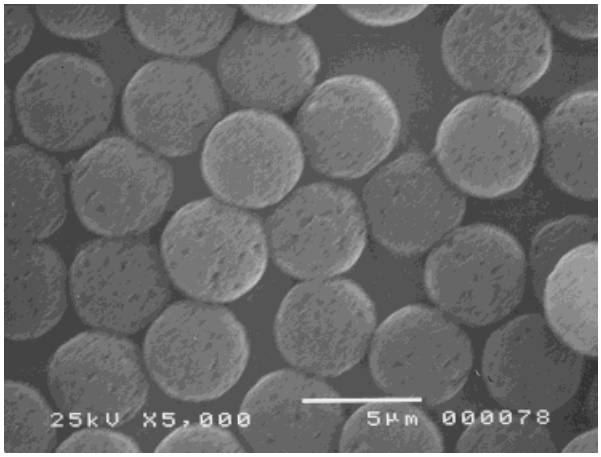

Figure 2 SEM (5000× magnification) of partially porous PS-DVB particles, having a specific surface area of 34 m^2/g and pore volume of 0.15 cm^3/g.

average particle diameter of 3.7 (μm and an average pore diameter of 17 nm. The multiple-step swelling and polymerization method involving the use of polymeric porogens has been employed by Wang et al.[12,13] to prepare monodispersed macroporous PS-DVB beads, starting from the PS seeds (with a particle diameter less than 1 μm) prepared by the emulsifier-free emulsion polymerization method. Porous particles with a medium pore diameter in the range of 10–48 nm were obtained by using a large amount of polymer porogens and employing dibutyl phthalate as the solvent, and used for the separation of polymers in SEC and separation of proteins in reversed-phase mode.

The prepared PS-DVB particles were partially porous. The linear PS molecules of the original seed synthesized by dispersion polymerization were believed to not be extracted from the particles and the PS-DVB particles were possible superficially porous as shown in Figure 1(D). Particles as shown in Figure 1(D) were prepared from PS seeds with a larger particle size (about 3 μm). The particle diameter increased finally to 5.4–6.8 μm after two steps of swelling and polymerization. PS-DVB particles with a surface area of up to 19 m^2/g were obtained. During the swelling of polymeric porogen particles with styrene and DVB, the PS chains of the original seeds were believed to preferably stay in the inner and the PS chains produced for using as the porogens were at the outer layer of the swollen particles. It was possible that the copolymerization of styrene and DVB occurred mainly at the outer layer of the particles. The produced PS-DVB crosslinking chains were coagulated by van der Waals forces and unevenly distributed on the outer layer of the particles. Due to the difference in density and solvencity between the core and shell layers, a portion of the outer layer was eroding away [as shown in Fig. 1(D)], during the extraction of porogens by using toluene. This problem could be more serious when they were extracted with toluene for a longer period or when polymeric porogen particles with a larger particle diameter were used. The SEM picture as shown in Figure 1(D) suggests that the porous structure was present near the periphery of the particles.

As shown in Figure 3, the prepared PS-DVB particles were porous, whereas the PS seeds and polymeric porogen particles were both nonporous. The straight line in Figure 3 stands for the relationship between the specific surface area and the reciprocal of particle diameter for nonporous particles. The total surface areas of the nonporous particles are contributed solely by the external surface of the particles. The slope of this straight line is $6/\rho$, where ρ is the bulk density of polystyrene and is reported to be 1.05 g/cm^3, according to the literature.[14]

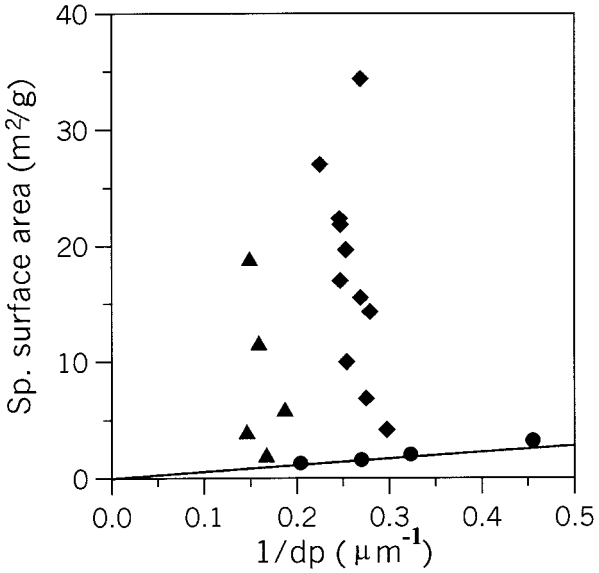

Figure 3 The relationship between the specific surface area and the recipocal of particle diameter for nonporous PS seeds and polymeric porogen particles (●), and porous PS-DVB particles. These PS-DVB beads were prepared staring from PS seeds having average particle diameters of 1.8 (♦) and 3 μm (▲).

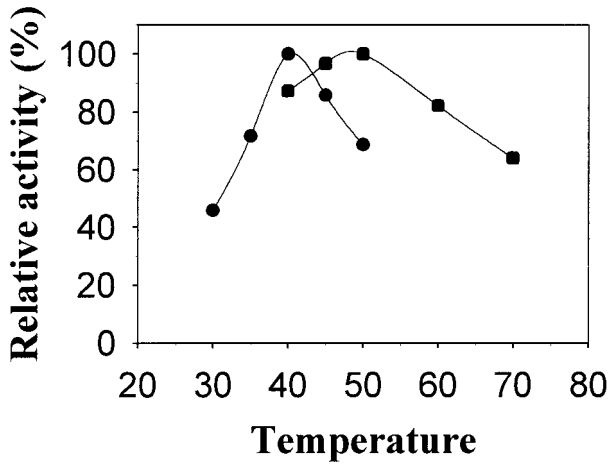

Figure 4 Effect of temperature on the hydrolysis of olive oil catalyzed by soluble (●) and immobilized lipase (■).

Characteristics of Immobilized Lipase

Immobilization of lipase by physical adsorption on the prepared PS-DVB particles (Fig. 2) previously wetted with ethanol was achieved at room temperature. The activity of lipase immobilized on the PS-DVB was 145 U g^{-1} particles, determined using olive oil emulsion as the substrate. The activity of lipase before immobilization was 5.7 U per unit milligram of enzyme solid. Thus, the yield of immobilized activity was 5%. This value is smaller than that of immobilized lipase on commercial porous PP powders wetted with ethanol (15.2 %), but higher than that on the same PP powders without pretreatment of ethanol (3.6%).[8] The difference in binding capacities of enzyme on PS-DVB and PP particles was due to the different chemical structure of these two materials, though they are similar in porosity and specific surface area. The benzene rings in the size chain of the PS-DVB copolymer might involve in the adsorption of lipase molecules via a complicated interaction, which caused a change in protein conformation. The anchored polymer PVP on the PS-DVB particles might also play a role in the process of enzyme immobilization.

The effect of temperature on enzyme activity was studied using olive oil dissolved in isooctane as the substrate. As shown in Figure 4, the temperature optimum of the enzyme reaction increased from 40 to 50°C by immobilization. The shift of optimum temperature from low to high was also observed when lipase was immobilized on PP.[8] Figure 4 also shows that the immobilized enzyme was active in a broad range from 40 to 70°C, whereas the profile for soluble enzyme was relatively narrow. Immobilization increased the stability of enzyme at higher temperatures.

Immobilization also effected the inactivation of enzymes. Deactivation of the lipase can be expressed as the following two-stage series mechanism[15]:

$$E_1 \xrightarrow{k_1} E_2 \xrightarrow{k_2} E_3 \qquad (1)$$

Equation (1) considers enzyme transition from a fully active stage (E_1) to an intermediate less active stage (E_2) and to a final inactive stage (E_3). It has been reported that for lipase, E_3 is fully inactive.[16,17] The following expression ensues:

$$\frac{e}{e_0} = \left(1 + \frac{Ak_1}{k_2 - k_1}\right)e^{-k_1 t} - \frac{Ak_1}{k_2 - k_1}\, e^{-k_2 t} \qquad (2)$$

where e is the enzyme activity at time t, e_0 is the intial enzyme activity, k_1 and k_2 are the first-order transition consatnts between the enzyme stages, and A is the activity ratio between E_1 and E_2.

Table I summerizes the parameter values estimated by fitting the experimental deactivation profiles with Eq. (2), as shown in Figures 4 and 5. The enzyme activity was determined by estimating the initial rate of oilve oil hydrolysis

Table I Estimated Parameters for the Thermal Deactivation of Soluble and Immobilized Lipase

	Soluble			Immobilized		
	45°C	55°C	65°C	55°C	70°C	75°C
k_1 (min^{-1})	0.45	1.0	0.95	0.27	0.42	0.57
$k_2 \times 10^4$ (min^{-1})	2.6	4.0	5.8	6.7	8.1	6.9
A	0.81	0.80	0.74	0.60	0.44	0.40

in isooctane. The result $k_1 \gg k_2$ suggests that the transition from the initial to the intermediate stage is much faster than that from the intermediate to the final inactive stage. The values of k_1 and A were decreased by immobilization. The decrease in k_1 value indicates a slow rate of deactivation, while the decrease in A value suggests that the enzyme at the intermediate stage is less active in the immobilized form. However, the temperture dependency of the first-order deactivation constant k_1 for both free and immobilized enzymes followed the Arrhenius equation. The activation energy for this rate constant was not changed by immobilization, and estimated to be 34 kJ/mol for both free and immobilized lipases.

The small values of k_2 for both soluble and immobilizaed lipases mean a good stabilization of the enzymatic state E_2. The activity of the intermediate state (E_2) was signficantly afected by the immobilization and temperature. In comparison with soluble enzyme, the temperature effect on the activity of the adsorbed enzyme was more significant. At 55°C, the A value decreased from 0.8 to 0.6 by immobilization, suggesting a conformational change of the adsorbed enzyme molecules.

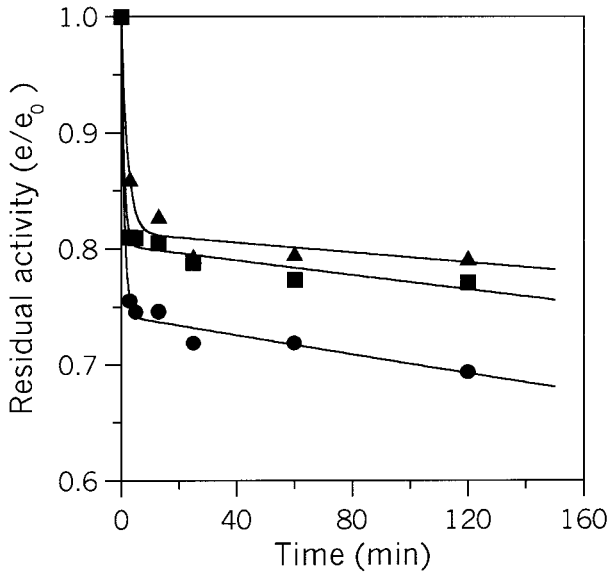

Figure 5 The time courses for deactivation of free lipase at the temperatures of 45 (●), 55 (■), and 65°C (▲). Solid curves represent the best curve-fitting results according to the deactivation model.

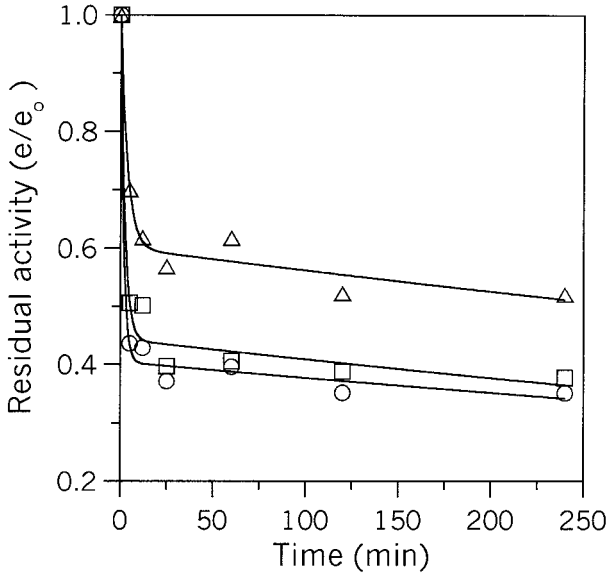

Figure 6 The time courses for deactivation of immobilized lipase at the temperatures of 55 (△), 70 (□), and 75°C (○). Solid curves represent the best curve-fitting results according to the deactivation model

CONCLUSION

Porous PS-DVB particles with a specific surface area of up to 34 m^2/g and a pore volume of up to 0.15 m^3/g were prepared by the multistep swelling and polymerization method. These particles were like to be superficially porous, based on the SEM observation. The porosity of the copolymer beads was significantly controlled by the concentrations of monomer and initiator for preparing polymeric porogen particles and the amount of toluene used in the copolymerization of styrene and divinylbenzene. The presence of polymeric porogen led to porous PS-DVB copolymer particles, which mimic as the carriers for enzyme immobilization. Lipase was successfully immobilized onto the prepared porous PS-DVB particles by physical adsorption. The optimum temperature for the enzymatic reaction increased from 40 to 50°C by immobilization. The activity ratio between the initial and intermediate stages of the enzyme was reduced by immobilization, suggesting that there was a conformational change to the enzyme molecule due to the immobilization.

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