Immobilization of Lipase on Poly(*N*-vinyl-2-pyrrolidone-*co*-styrene) Hydrogel

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ABSTRACT: Lipase from *Candida rugosa* was immobilized by entrapment while polymerizing a poly(*N*-vinyl-2-pyrrolidone-*co*-styrene) [poly(VP-*co*-ST)] hydrogel using ethylene dimethacrylate (EDMA) as the crosslinking agent. The immobilized enzymes were used in the esterification reaction of oleic acid and butanol in hexane. The activities of the immobilized enzymes and the leaching ability of the enzyme from the support with respect to the different compositions of the hydrogels were investigated. The thermal, solvent, and storage stability of the immobilized lipases were also determined. The activities were relatively higher when the percent compositions of VP(%):ST(%) were 50:50 and 30:70. The lipase immobilized on VP(%):ST(%) 50:50 showed the highest thermal stability, while lipase immobilized on VP(%):ST(%) 30:70 exhibited the highest solvent stability. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 1404–1409, 2001

Key words: lipase; hydrogels; polymerization; esterification; activation; stabilization

INTRODUCTION

Biocatalysts such as enzymes are often immobilized on various supports to protect them from environmental stresses such as pH, temperature, solvents, salts, self-destruction, inhibitors, and poison[1] and to maintain high enzyme activity and stability. Besides that, as enzymes are immobilized on or in an insoluble support, they may be readily separated from soluble substrates and products. Thus, an enzyme can be recovered or reused in a continuous process.

Enzymes can be immobilized in a number of methods of varied complexity and efficiency[2] and on a variety of supports. For example, enzymes can be adsorbed onto insoluble materials, crosslinked with a bifunctional reagent, covalently bound to an insoluble carrier,[3] or entrapped within an insoluble gel matrix of natural or synthetic resin.[2]

Hydrogels are polymeric materials made from hydrophilic and/or hydrophobic monomers which can be a homopolymer or a copolymer. Their major characteristic is that they have the ability to swell in large quantities of water without dissolution of the polymer network. Enzymes can be entrapped within the hydrogels by mixing the monomers with the biocatalyst solution and subsequently inducing gelation by polymerization. In this way, the enzyme is distributed throughout the matrix. The attractive part of this method is that the enzyme is not actually attached to anything, so it may have the same conformation as that of a free enzyme. Hydrogel, aside from providing the water needed for enzyme activity, can also absorb water produced during the esterification reaction, thus increasing the conversions to the products.

In this work, immobilization of *Candida rugosa* lipase by entrapment on a poly(*N*-vinyl-2-pyrroli-

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done-*co*-2-styrene) [poly(VP-*co*-ST)] hydrogel was carried out. The activities and characteristics of the immobilized lipase preparations were investigated.

EXPERIMENTAL

Materials

Lipase from *C. rugosa* (Type VI), the monomers, *N*-vinyl-2-pyrrolidone (VP) and styrene (ST), and a crosslinker, ethylene dimethacrylate (EDMA), were obtained from Sigma Chemical Co. (St. Louis, MO). The initiator, α,α'-azoisobutyronitrile (AIBN), was from Fluka Chemical (Buchs, Switzerland). All other reagents were of analytical grade. The organic solvents and substrates were dried over molecular sieves (3A) before use.

Purification of Monomers

VP and ST were purified by passing them through an aluminum oxide column (2.5 × 10.0 cm) until colorless products were obtained. EDMA was used as purchased.

Preparation of Lipase Solution

Commercial lipase from *C. rugosa* (500 mg) was dispersed in distilled water (10.0 mL). This mixture was agitated on a vortex mixer and centrifuged at 13,000 rpm for 10 min and the supernatant was used for lipase immobilization.

Lipase Immobilization

Purified monomers, VP and ST, of varying weight percent composition were mixed together with 1% EDMA (wt %) in a clean, dry flask. The compositions of the hydrogels prepared were VP(%):ST(%) (% = wt % of the monomer in total weight of VP + ST) 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0. To these mixtures, a dry initiator, AIBN (10^{-4} mol), was added and the flasks were shaken until the AIBN was dissolved. The mixtures were then transferred to a polymerization tube and the solutions were degassed with nitrogen for 15 min to remove any oxygen present. The mixtures were incubated to polymerize in a 55–60°C water bath. After the polymer solutions became viscous (1–4 h), the polymers were cooled to 50°C and the lipase solution (1.0 mL), which was previously degassed with nitrogen, was added and the polymer solution was shaken until a homogeneous solution was obtained. The solution in the polymerization tube was sealed with a rubber stopper and further polymerized at 50°C for about 5 h. The solid polymerized rods were removed from the polymerization tubes. These rods were cut into small pieces (0.2–0.4 cm^3) and stored at 0°C prior to use.

Protein Assay

The protein content of the hydrogels was determined using the method of Bradford[4] with bovine serum albumin as the standard. For the blank determination, a poly(VP-*co*-ST) hydrogel without lipase was used.

Lipase Activity

The assay system consisted of poly(VP-*co*-ST)-immobilized lipase (0.3 g), oleic acid (2.0 mmol), butanol (8.0 mmol), and hexane (2.6 mL). The mixture was incubated at 37°C for 5 h in a horizontal water-bath shaker at 150 rpm. The reaction was terminated by dilution with acetone–ethanol (1:1 v/v, 3.5 mL). The residual free fatty acid in the reaction mixture was determined by titration, with NaOH (0.2*M*) using an automatic titrator (ABU 90, Radiometer, Copenhagen) to a pH of 9.5. For the blank determination, a poly(VP-*co*-ST) hydrogel without lipase was used. The specific activity of the enzyme was expressed in μmmol of free fatty acid used min^{-1} mg^{-1} protein. The effect of using different organic solvents in the esterification reaction was also carried out.

Gas Chromatography

The reaction products were analyzed periodically on a Shimadzu 8A gas chromatograph using a 30-m polar capillary column Nukol TM (0.32 mm, i.d.) from Supelco Inc. (Australia). Nitrogen was used as the carrier gas, at 1.0 mL/min. The injector and detector temperature was set at 250°C. The initial column temperature was at 110°C. The temperature was increased at 8°C per min to 200°C.

Effect of Monomers, Crosslinker, and Poly(VP-*co*-ST) Hydrogels on the Activity of Free Lipase

The purified monomers and viscous monomer mixtures (50°C; 0.5 mL), the crosslinker (EDMA, 0.2 mL), and the poly(VP-*co*-ST) hydrogels (0.3 g) were placed into separate vials that contained the enzyme assay solution and the native lipase [0.02–0.05 mg (protein equivalent in 0.3 g immobilized lipase)]. The vials were then incubated at

37°C in a horizontal water-bath shaker at 150 rpm for 5 h. The enzyme activities are expressed as a percentage of the activity compared to free lipase.

Lipase Leaching

The poly(VP-*co*-ST)-immobilized lipases (0.3 g) were placed into sealed vials with hexane (4.0 mL). The mixtures were shaken at 30°C for 0.5 h in a horizontal water-bath shaker at 150 rpm. The immobilized lipases were isolated from the organic solvent by filtration through Whatman No. 1 filter paper (one cycle). The above procedure was repeated accordingly up to six cycles, after which the residual enzyme activities were determined. Activities are expressed as a percentage of the untreated immobilized preparations.

Thermostability of Immobilized Lipase

The poly(VP-*co*-ST)-immobilized lipases (0.3 g) were incubated in hexane at various temperatures for 1 h in sealed vials. After incubation, the enzyme mixtures were cooled to room temperature and lipase activity was determined at 37°C. The relative activities are expressed as a percentage of the untreated immobilized lipase.

Stability in Organic Solvent

The immobilized enzymes were incubated in hexane for between 1 and 12 days at room temperature. Their residual activities were determined at 37°C. The residual activities are expressed as a percentage of the immobilized lipase at day zero.

Storage Stability of the Immobilized Lipase

The immobilized enzyme preparations were stored at room temperature (27–28°C) and at 4, 0, and −20°C for 60 days in sealed vials. After warming to room temperature, the residual activities were determined. The residual activities are expressed as a percentage of the immobilized lipase at day zero.

RESULTS AND DISCUSSION

Effect of Monomers, Crosslinker, Monomer Mixtures, and Poly(VP-*co*-ST) Hydrogel Polymers on Lipase Activity

Hydrogels of varying composition did not affect the activity of lipase. The monomer, styrene, in the solution also did not affect the activity of lipase. However, the presence of the monomer, VP, in the solution decreased the activity of the lipase to 50%. In contrast, the crosslinking agent, EDMA, gave 98 % of the residual activity. The effect of the monomer mixtures on the enzyme at 50°C was studied since this was the temperature at which the enzyme was introduced into the monomer mixtures. The results showed that the enzyme retained more than 90% of the activity for all monomer mixtures. Apparently, the reactive monomer, VP, may have a poisoning effect on the enzyme, thus decreasing its activity. However, if all the monomers are completely polymerized, the gel has no effect on the lipase activity.

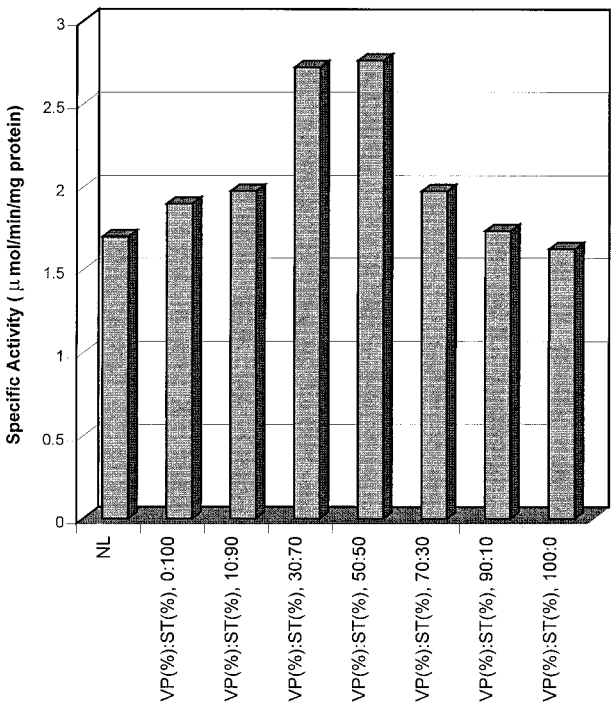

Figure 1 Esterification activities of poly(VP-*co*-ST) immobilized lipases. NL, native lipase; VP(%)/ST(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0, hydrogels with the respective compositions [% = wt % of monomer in total weight of (VP + ST)].

Activity of Immobilized Lipases

The esterification results using the poly(VP-*co*-ST) hydrogel-immobilized lipase are shown in Figure 1. As expected, immobilization of the lipase onto hydrogels increased the esterification activity of the lipase. The activities of hydrogel-immobilized lipases at a lower percent VP (0–10%) were relatively lower. The lipase immobilized on the VP(%):ST(%) hydrogel, 30:70 and 50:50, gave the highest activity. Subsequently, the

Table I Esterification Activities of Poly(VP-*co*-ST)-immobilized Lipases in Various Organic Solvents

Immobilized Lipase	Relative Activity (%)[a]			
	Acetone	CCL_4	Hexane	Heptane
VP(%):ST(%) 0:100	34.3	36.0	100	93.2
VP(%):ST(%) 30:70	12.8	60.1	100	95.6
VP(%):ST(%) 50:50	71	100	100	100
VP(%):ST(%) 70:30	9.0	21.6	100	97.6
VP(%):ST(%) 100:0	20.7	80.6	100	100
Native lipase	9.0	70.3	100	92.3

[a] Activity is expressed as percent of the lipase activity in hexane. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing butanol and oleic acid. VP(%)/ST(%), 0:100, 30:70, 50:50, 70:30, and 100:0, hydrogels with the respective compositions.

activity was decreased at a composition of the hydrogel higher than % VP of 50. The composition of the monomers in the gel formation apparently was important so as to assure sufficient water within the matrix. This may be due to the available water surrounding the enzyme in the hydrogel. Sufficient water is needed to maintain the three-dimensional conformation of the lipase to retain active catalysis. The balance in the structure in these hydrogels may provide the right amount of water for the lipase to function efficiently. The low activity observed for lipase immobilized on the more hydrophobic hydrogel (high ST content) may be due to the decreased equilibrium water content (EWC)[5] of the polymer, while the low activity observed for lipase immobilized on the more hydrophilic hydrogel (high VP content) may be due to a high EWC of the polymer. The data obtained are in agreement with the results of Kosugi and Suzuki,[6] who reported that the activity of an entrapped lipase depends on the concentration of the water surrounding the catalytic surface of the lipase.

Effect of Different Organic Solvents on the Esterification Activity

In all cases, immobilized lipases were generally active in all organic solvents tested (Table I). The activities were much higher in organic solvents which were nonpolar. This is in agreement with the results reported by Laane et al.[7] The lower activities of lipase in the less polar solvents could be due to that the more polar solvents stripped the essential water layer around the enzyme, which is important for enzyme catalysis.

Leaching Study in Hexane

A study on the effect of hexane washing on lipase activity was carried out. All hydrogels retained their activity even after six cycles of the washing process. This is in contrast with our earlier work[8] using VP and HEMA, whereby part of the lipase activity was lost after four cycles of washing. A combination of VP and ST seemed to be more suitable for lipase immobilization as compared to VP and HEMA.

Thermostability of the Immobilized Lipase

Immobilization of lipase onto the hydrogel seemed to increase its thermal stability compared to free lipase after 1-h incubation (Fig. 2). The immobilized preparations were more thermostable over the temperature range of 40–70°C than was the native lipase. The relative activity of the immobilized lipase decreased starting at 50°C for native lipase and VP(%):ST(%) 100:0 and at 60°C for the other immobilized lipases, with a further decrease at 70°C. As the temperature is increased, presumably the EWC of the hydrogel gradually decreases, which is accompanied by the shrinkage of the gel matrix. This reduced pore size in the hydrogel results in a decrease in the diffusion of the substrate,[9] thus resulting in a decrease in esterification. The decreased activity of the immobilized lipase at higher temperature also may be due to denaturation of the lipase.

The lipase, immobilized on VP(%):ST(%) 50:50, exhibited the highest stability. The hydrophobic and hydrophilic sites in this hydrogel seemed to stabilize the enzyme to heat-induced denaturation. The hydrophilic sites (VP) offer the advantages of a high water content and softness, whereas the hydrophobic sites (ST) give rigidity and toughness to the hydrogel. Hydrogels of the percent composition from VP(%):ST(%) 100:0, showed lower stability. The decrease in the stability of the immobilized lipase with respect to temperature may also be due to the inhomogeneous crosslinking of the polymer network which was noted at high contents of VP.

Stability in Organic Solvent

The stability of the immobilized lipase preparations in hexane also were investigated (Fig. 3). The lipases immobilized on VP(%):ST(%) 0:100, 30:70, 50:50, and 70:30 retained their activities for the first 8 days. Subsequently, at the 12th day of incubation, the stability was decreased, with

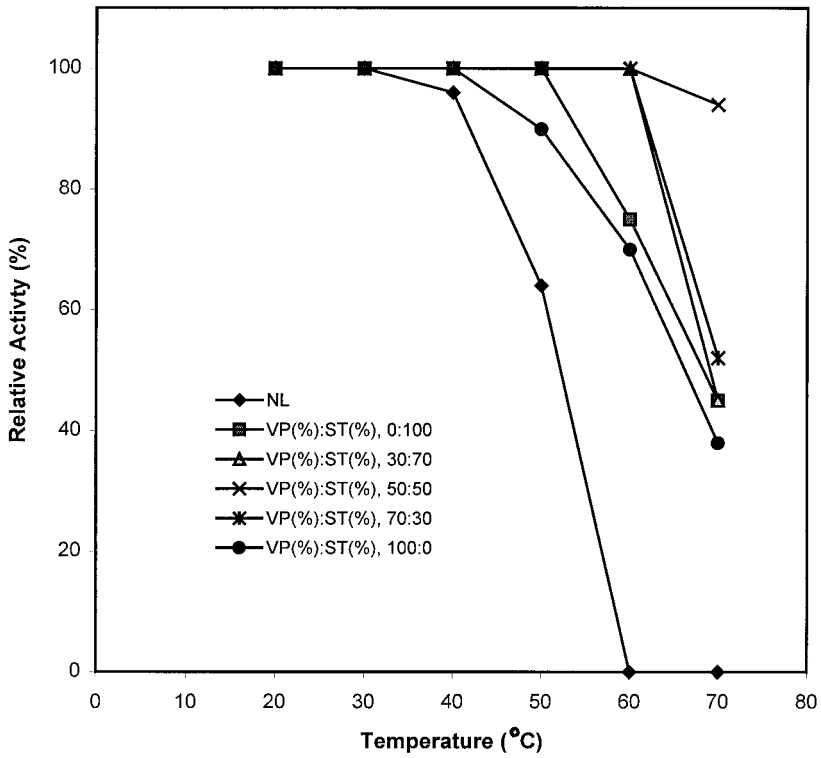

Figure 2 Thermostability of poly(VP-*co*-ST) immobilized lipases incubated for 1 h in hexane. NL, native lipase; VP(%)/ST(%), 0:100, 30:70, 50:50, 70:30, and 100:0, hydrogels with the respective compositions.

immobilized lipase on VP(%):ST(%) 0:100 and 30:70 (high ST content) exhibiting the highest solvent stability followed by immobilized lipase on VP(%):ST(%) 50:50, 70:30, and 100:0. The native lipase exhibited the least stability in the organic solvent, indicating that an active protein structure was denatured by the organic solvent. Immobilization of lipase on all compositions of hydrogels seemed to increase the stability of lipase in hexane. Hydrogels of high hydrophobicity (high percent ST content) seemed to be most effective in protecting the enzymes from unfavorable contact with the organic solvent as compared to hydrogels of low hydrophobicity.

Storage Stability of the Immobilized Lipase

The stability of the various immobilized lipases incubated in hexane for 60 days under different storage conditions was studied. All immobilized lipase preparations and the native lipase showed full catalytic activity after storing at −20°C. Immobilized lipases also retained their full activity when stored at 0°C, whereas the native lipase showed 67% of activity. At 4°C, lipase immobilized on the more hydrophobic hydrogels, VP(%):ST(%) 0:100, 30:70, and 50:50, retained its full activity. However, lipase immobilized on the less hydrophobic hydrogels, VP(%):ST(%) 70:30 and 100:0, and native lipase showed less stability with a relative activity of 76, 53, and 50%, respectively. When stored at room temperature, the immobilized lipases had a decreased storage stability [VP(%):ST(%): 0:100 (70% activity); 30:70 (69% activity); 50:50 (71% activity); 70:30 (44% activity); 100:0 (47% activity], with native lipase having the least stability (34% of the activity). The decrease in the relative activity of the immobilized lipases at room temperature (RT) may be attributed to the presence of water in the immobilized lipases introduced during the immobilization procedure.[10]

CONCLUSIONS

Our results indicated that immobilization of lipase on poly(VP-*co*-ST) hydrogels increased its activity as well as its thermal, solvent, and storage stability. These enhanced properties of the immobilized lipase may have potential for use as biocatalysts in the oleochemical industries. The

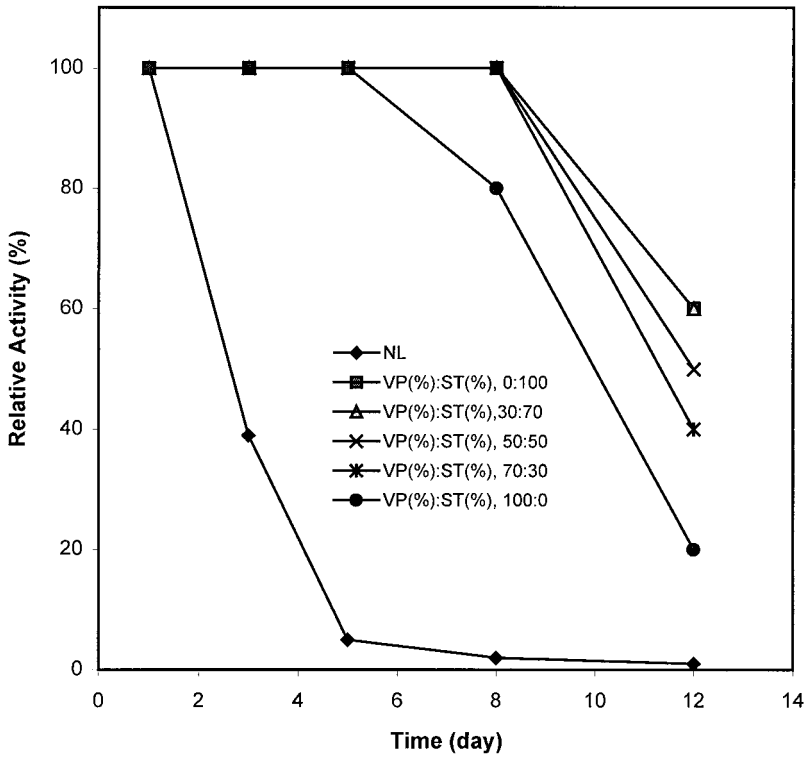

Figure 3 Stability of poly(VP-*co*-ST) immobilized lipases incubated in hexane for 12 days at room temperature. NL, native lipase; VP(%)/ST(%), 0:100, 30:70, 50:50, 70:30, and 100:0, hydrogels with the respective compositions.

simplicity of the technique suggests that it may be applied for other biologically active proteins for use in their respective processes.

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