Immobilization of Lipase on Poly(*N*-vinyl-2-pyrrolidone-co-2-hydroxyethyl methacrylate) Hydrogel for the Synthesis of Butyl Oleate

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ABSTRACT: Lipase from *Candida rugosa* was immobilized by entrapment on poly(*N*-vinyl-2-pyrrolidone-co-2-hydroxyethyl methacrylate) [poly(VP-co-HEMA)] hydrogel, cross-linked with ethylene glycol dimethacrylate (EDMA). The immobilized enzyme was used in the esterification of oleic acid with butanol in hexane. The activities of the immobilized enzyme preparations and the leaching of the enzyme from the hydrogel supports with respect to composition were investigated. The thermal, solvent, and storage stability of the immobilized preparations also were determined. Increasing the percentage VP from 0 to 90, which corresponds to the increase in the hydrophilicity of the hydrogels, increased the activity of the immobilized enzyme. Lipase immobilized onto VP(%):HEMA(%), 90:10 hydrogel had the highest activity. Increasing the hydrophobicity of the hydrogel (increasing the percentage HEMA) seemed to decrease leaching of the enzyme from the support. Immobilized lipase on 100% HEMA hydrogel indicated highest entrapment and lowest leaching by hexane washing. The lipase immobilized on VP(%):HEMA(%), 50:50 hydrogel showed highest thermal, solvent, and storage stability compared to lipase immobilized on other hydrogel compositions as well as the native lipase.

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Enzymes have gained considerable importance as catalysts in organic synthesis because of their high selectivity under milder reaction conditions at near ambient temperature and pressure. Furthermore, enzymes are environmentally friendly (1) as they are generally nontoxic and biodegradable. Thus, enzyme-catalyzed reactions have been used for the preparation of a wide range of natural products, pharmaceuticals, fine chemicals, and food ingredients.

Free enzymes, however, are not always ideal in practical applications because they are often unstable and easily denatured and therefore are unsuitable for use in organic solvents or at high temperatures. In addition, the isolation and production of enzymes and their one-time usage as catalysts are costly. However, these major deterrents may be eliminated by the use of immobilized enzymes. Immobilization of enzymes can be achieved by methods of varied complexity and efficiency (2) on a variety of supports. For example, enzymes can be adsorbed onto insoluble materials, copolymerized with a reactive monomer, encapsulated in gels, cross-linked 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 can imbibe large quantities of water without dissolution of the polymer network. This feature makes them interesting supports for immobilization of enzymes. In addition to providing the water needed for enzyme activity, the hydrogel also can absorb water produced during the esterification reaction, thus increasing the products.

In this work, a new method of immobilization of *Candida rugosa* lipase by entrapment on poly(*N*-vinyl-2-pyrrolidone-co-2-hydroxyethyl methacrylate) hydrogel was carried out. The activities and characteristics of the immobilized lipase preparations were investigated.

MATERIALS AND METHODS

Materials. Lipase from *C. rugosa* (Type VI); monomers *N*-vinyl-2-pyrrolidone (VP) and 2-hydroxyethylmethacrylate (HEMA); and cross-linker ethylene dimethacrylate (EDMA) were obtained from Sigma Chemical Co. (St. Louis, MO). 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 (3Å) before use.

Purification of monomers. VP and HEMA were purified by passing through an aluminum oxide column (2.5 × 10.0 cm) until colorless products were obtained. EDMA was used as received.

Preparation of lipase solution. Commercial lipase from *C. rugosa* (500 mg) was dispersed in distilled water (10.0 mL).

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This mixture was agitated on a vortex mixer, centrifuged at 13,000 rpm for 10 min, and the supernatant used for lipase immobilization.

Lipase immobilization. Purified monomers, VP and HEMA, of varying weight percentage (wt%) composition were mixed together with 1% EDMA (wt%) in a clean, dry flask. The composition of hydrogels prepared were VP(%)/HEMA(%) [% = wt% of monomer in total weight of (VP + HEMA)]: 0:100, 10:90, 30:70, 50:50, 70:30, 90:10 and 100:0. To these mixtures, dry initiator, α,α′-azoisobutyronitrile (AIBN) (10^{-4} moles) was added and the flasks shaken until the AIBN dissolved. The mixtures were then transferred to a polymerization tube and the solutions degassed with nitrogen for 15 min to remove oxygen. 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 lipase solution (1.0 mL), previously degassed with nitrogen, was added and the polymer solutions shaken until homogeneous solutions were obtained. The solutions in the polymerization tubes were sealed with rubber stoppers and further polymerized at 50°C for about 5 h. The solid polymerized rods were removed from the polymerization tubes, cut into small pieces (0.2–0.4 cm^3), and stored at –4°C prior to use.

Protein assay. Protein content of the hydrogels was determined by using the method of Bradford (4) with bovine serum albumin as standard. For the blank determination, poly(VP-co-HEMA) hydrogel without lipase was used.

Lipase activity. The assay system consisted of poly(VP-co-HEMA)-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 vol/vol, 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, Denmark) to pH 9.5. For the blank determination, poly(VP-co-HEMA) hydrogel without lipase was used. The specific activity of the enzyme was expressed in μmol of free fatty acid used min^{-1} (mg protein)$^{-1}$.

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

Effect of monomers, cross-linker, and the poly(VP-co-HEMA) hydrogels on the activity of free lipase. The purified monomers and monomer mixtures (0.5 mL), crosslinker (EDMA) (0.2 mL), and the poly(VP-co-HEMA) 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-HEMA)-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 four cycles after which residual enzyme activities were determined. Activities are expressed as a percentage of the untreated immobilized preparations.

Thermostability of immobilized lipase. The poly(VP-co-HEMA)-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.

The stability of immobilized lipase in hexane at 40, 50, 60, and 70°C, with respect to incubation time was also investigated. The residual activities are expressed as a percentage of the enzyme activity at zero time.

Stability in organic solvent. The immobilized enzymes were incubated in hexane for between 1 and 12 d at room temperature. Their residual activities were determined at 37°C. The residual activities are expressed as a percentage of the immobilized lipase activity at day 0.

Storage stability of the immobilized lipase. The immobilized enzyme preparations were stored at room temperature (27–28°C), 4, 0, and −80°C for 30 d in sealed vials. After warming preparations to room temperature, the residual activities were determined. The residual activities are expressed as a percentage of the immobilized lipase activity at day 0.

RESULTS AND DISCUSSION

Effect of monomers, crosslinker, monomer mixtures, and poly(VP-co-HEMA) hydrogel polymers on lipase activity. The effect of monomers, crosslinker, and the poly(VP-co-HEMA) hydrogel on the esterification reaction of lipases is shown in Figure 1. Hydrogels of varying composition did not affect the activity of lipase as shown. However, the presence of unreacted monomers, VP and HEMA, in solution form, decreased the activity of the lipase to less than 50%. In contrast, the cross-linking 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 to the monomer mixtures. The results showed that in the presence of hydrogels the enzyme retained more than 90% of the activity. Apparently, the reactive monomers 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 lipase activity.

Activity of immobilized lipases. The esterification results using the poly(VP-co-HEMA)-immobilized lipase are shown in Figure 2. As expected, immobilization of the lipase onto hydrogels showed increased esterification activity compared

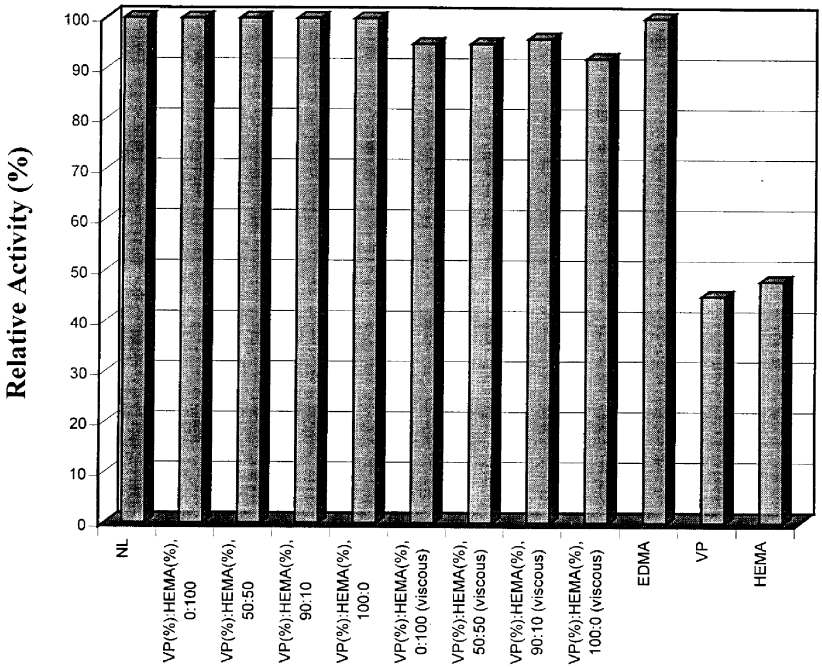

FIG. 1. Effect of monomers, cross-linker, monomer mixtures, and poly(VP-co-HEMA) hydrogel on the activity of lipase. NL, native lipase; VP(%)/HEMA(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10 and 100:0, hydrogels with the respective compositions; VP(%)/HEMA(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0 (viscous), monomer mixtures (50°C) with the respective compositions; EDMA, ethylene dimethacrylate; VP, *N*-vinyl-2-pyrrolidone; HEMA, 2-hydroxyethylmethacrylate. [% = wt% of monomer in total weight of (VP + HEMA)].

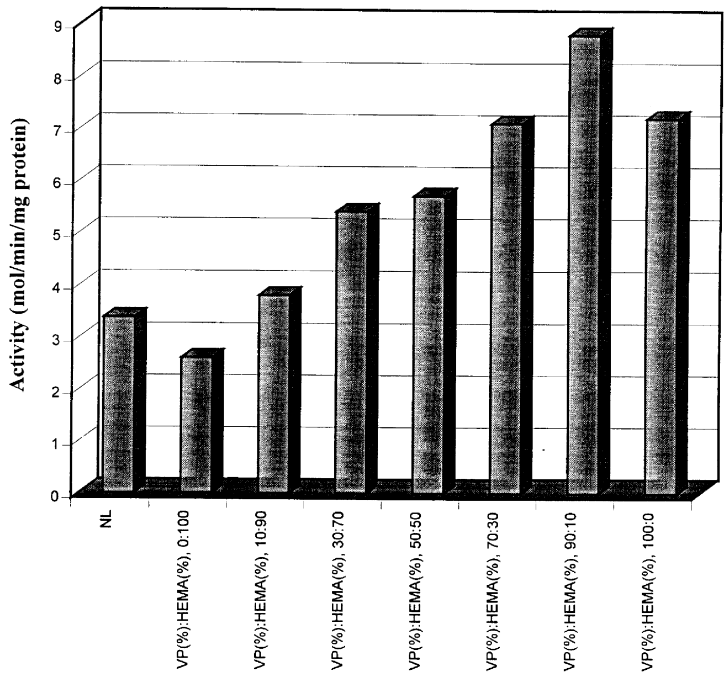

FIG. 2. Esterification activities of poly(VP-co-HEMA)-immobilized lipases. VP(%)/HEMA(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0, hydrogels with the respective compositions. For other abbreviations see Figure 1.

to native lipase, except for the lipase entrapped on 100% HEMA, which showed a decrease in activity. Increasing the percentage VP from 0 to 90, which corresponds to an increase in hydrophilicity of the hydrogel and its equilibrium water content (EWC) (5), seemed to increase the activity of the immobilized enzyme. Lipase immobilized on VP/HEMA hydrogel, 90:10, is the best for the esterification reaction as it gave the highest activity. 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. Alternatively, the hydrophilic support may absorb the water produced in the esterification reaction. This would favor the formation of products, resulting in higher yields. 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 a high concentration of water surrounding the catalytic surface of the lipase using high hydrophilic supports.

The low activity observed for lipase immobilized on 100% HEMA may be due to the decreased EWC of the HEMA polymer. The composition of the monomers in gel formation was important to ensure sufficient water within the matrix. The lipase activity for the 100% HEMA polymer preparation was relatively lower than the activity of lipase on VP/HEMA, 90:10. This may be due to the partial solubility of this hydrogel in water and the lower level of cross-linking, which results in more lipase diffusing out of the gel.

Leaching study in hexane. The effect of hexane washing on lipase activity is shown in Figure 3. Increasing the hydrophobicity of the hydrogel (increasing the percentage HEMA) decreased the leaching effect with hexane. Lipase immobilized on 100% HEMA hydrogel retained its activity during the washing process, whereas lipase immobilized on 100% VP exhibited the lowest retention stability in this experiment. As the HEMA content decreased, physical cross-linking (hydrophobic bonding) may be decreased, thus lowering the entrapment stability.

Thermostability of the immobilized lipase. Immobilization of lipase onto hydrogel seemed to increase its thermal stability compared to free lipase after 1 h incubation (Fig. 4). The immobilized preparations were more thermostable over the temperature range of 40–70°C than the native lipase. The relative activity of the immobilized lipase decreased starting at 50°C with further decrease at 70°C. As the temperature is raised, the EWC of the hydrogel gradually decreases, which is accompanied by the shrinkage of gel matrix. This reduced

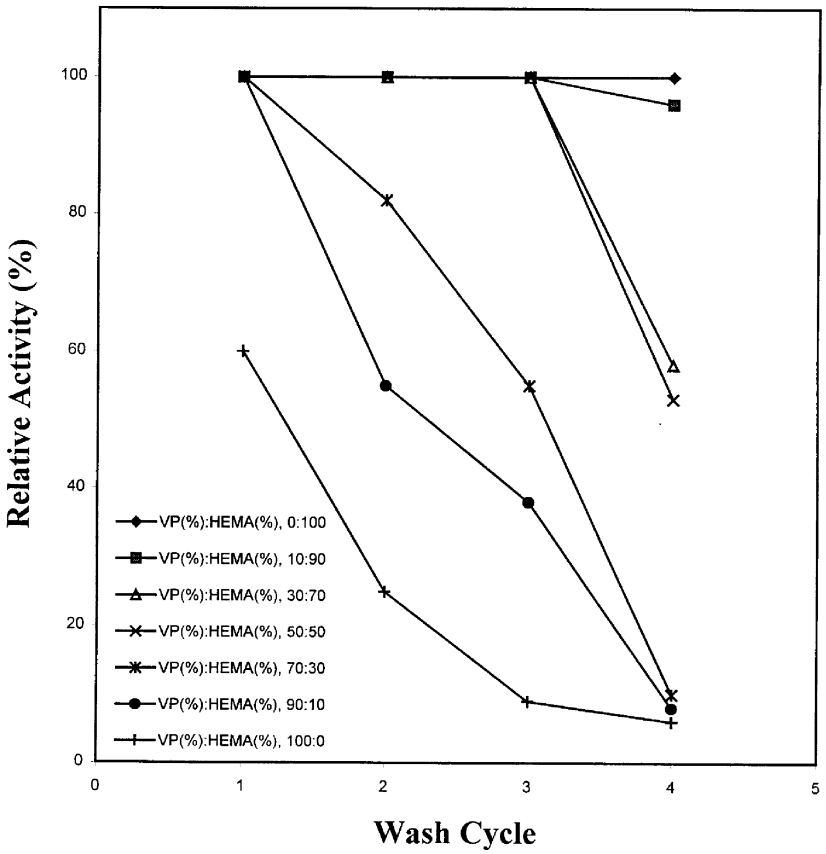

FIG. 3. Leaching study of poly(VP-co-HEMA)-immobilized lipases by hexane washing. VP(%)/HEMA(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10 and 100:0, hydrogels with the respective compositions. For abbreviations see Figure 1.

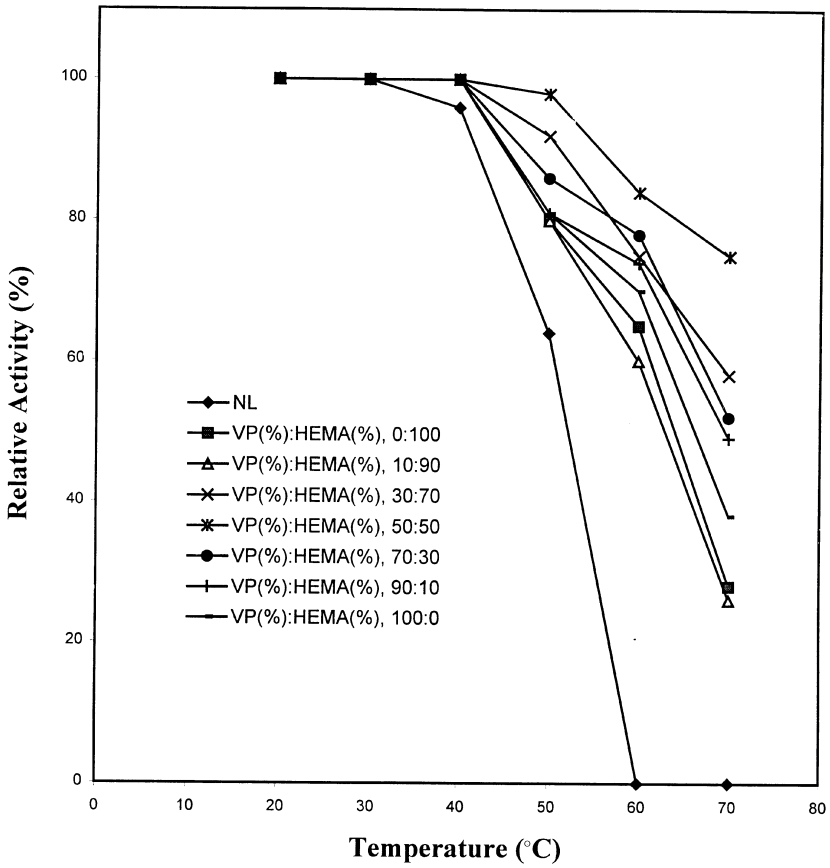

FIG. 4. Thermostability of poly(VP-co-HEMA) immobilized lipases incubated for 1 h in hexane. VP(%):HEMA(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0, hydrogels with the respective compositions. For abbreviations see Figure 1.

pore size in the hydrogel results in a decrease in the diffusion of substrate (7), thus resulting in a decrease in esterification. The decreased activity of the immobilized lipase at higher temperature also may be due to decreased hydrophobic interactions between the lipase and hydrogel.

The lipase, immobilized on VP/HEMA, 50:50 exhibited the highest resistance to thermal denaturation. The balance in hydrophobic and hydrophilic sites in this hydrogel seemed to stabilize the enzyme to heat-induced denaturation. The hydrophilic sites (VP) offer the advantages of high water content and softness, whereas the hydrophobic sites (HEMA) give rigidity and toughness to the hydrogel. In addition, the balance in structure in this hydrogel may provide the right amount of water for the lipase to function efficiently. Hydrogels whose percentage composition varied from VP/HEMA, 50:50, showed lower stability. The decrease in activity of immobilized lipase with respect to temperature may also be due to the nonhomogeneous cross-linking of the polymer network noted at high contents of VP.

Figure 5 shows the residual activity of lipase-immobilized hydrogel of composition VP/HEMA, 50:50, incubated at various temperatures with respect to time. Incubation at 40°C for 3 h did not decrease the activity of the immobilized lipase whereas at 50, 60, and 70°C incubation for 3 h decreased the relative activity of the immobilized lipase. Increasing the incubation time at higher temperature also changed the appearance of the gel, indicating that water may be evaporating from the matrix. Cantarella *et al.* (8) found that a hydrophilic matrix protects enzymes against thermal and chemical deactivation compared to a hydrophobic matrix.

Stability in organic solvent. The stability of the immobilized lipase preparations in hexane were also investigated (Fig. 6). Lipase immobilized on VP/HEMA, 30:70, 50:50, and 70:30, retained their activities for the first 3 d, whereas only the immobilized lipase on VP/HEMA, 50:50, retained its activity after 8 d. The immobilized lipase on VP/HEMA, 50:50, exhibited the highest half-life (11.50 d), whereas lipase immobilized on VP/HEMA of other compositions and native lipase had lower solvent stability. The native lipase exhibited the least stability in organic solvent indicating that active protein structure was denatured by the organic solvent. The solvent stability of lipase immobilized on VP/HEMA, 0:100, 10:90, 90:10, and 100:0, were low. Hydrogels of high hydrophobicity and high hydrophilicity seemed to be least effective in protecting the enzymes from unfavorable contact with the organic solvent

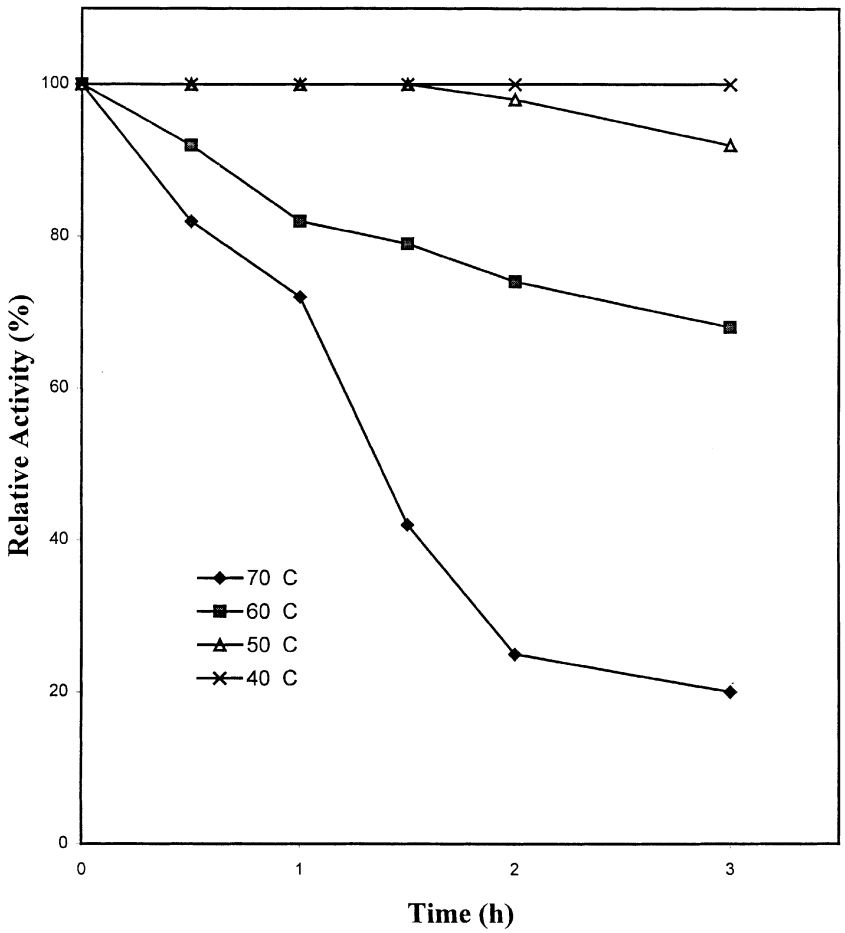

FIG. 5. Thermostability of poly(VP-co-HEMA) immobilized lipases (VP/HEMA, 50:50) with respect to time at 40, 50, 60, and 70°C. For abbreviations see Figure 1.

Storage stability of the immobilized lipase. The stability of the various immobilized lipases incubated in hexane for 30 d under different storage conditions is shown in Table 1. All immobilized lipase preparations and the native lipase showed full catalytic activity after storage at −80°C. Immobilized lipases retained their full activity when stored at 0°C, whereas the native lipase showed 30% of activity. At 4°C, the lipase immobilized on the more hydrophobic hydrogels showed an increased storage stability compared to the native lipase, with the lipase immobilized on VP/HEMA hydrogel 50:50 having the best stability. However, lipase immobilized on the more hydrophilic hydrogels showed lower stability even when compared to native lipase. When stored at room temperature, the immobilized lipases, except VP/HEMA 50:50, had decreased storage stability compared to native lipase (34% of activity). As observed in thermal treatment, lipase immobilized on hy-

TABLE 1
The Effect of Storage Condition on the Esterification Reaction by Immobilized Lipase After 30 Days

	Relative activity (%)[a]			
Immobilized lipase	RT	4°C	0°C	−80°C
VP(%)/HEMA(%), 0:100	7	79	100	100
VP(%)/HEMA(%), 10:90	8	80	100	100
VP(%)/HEMA(%), 30:70	33	83	100	100
VP(%)/HEMA(%), 50:50	63	90	100	100
VP(%)/HEMA(%), 70:30	15	30	100	100
VP(%)/HEMA(%), 90:10	3	21	100	100
VP(%)/HEMA(%), 100:0	15	72	100	100
Native lipase	34	50	67	100

[a]Activity is expressed as percentage of the lipase activity at day 0. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing butanol and oleic acid. VP, *N*-vinyl-2-pyrrolidone; HEMA, 2-hydroxyethylmethacrylate; RT, room temperature.

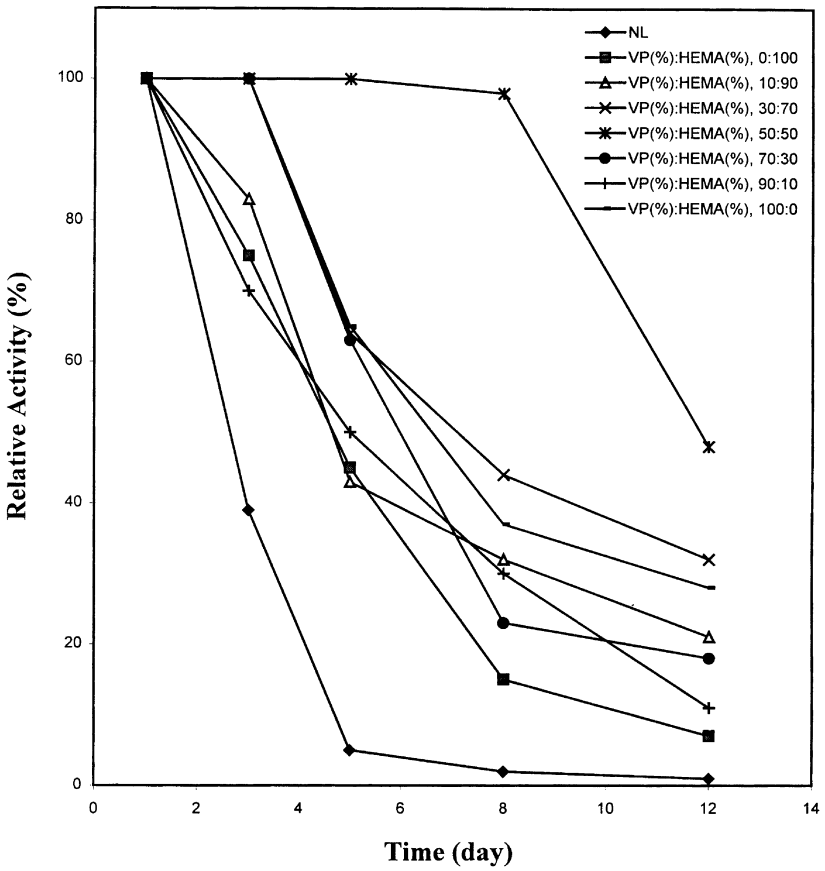

FIG. 6. Stability of poly(VP-co-HEMA) immobilized lipases incubated in hexane for 12 days at room temperature. NL, native lipase; VP(%):HEMA(%), 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0, hydrogels with the respective compositions.

drogel with the composition VP/HEMA 50:50 also exhibited the highest storage stability. The decrease in the relative activity of the immobilized lipases of other compositions as compared to native lipase at room temperature may be attributed to the presence of water in the immobilized lipases introduced during the immobilization procedure (9).

Our results indicate that immobilization of lipase on hydrogels may be suitable for industrial applications. Their increased activity in organic solvents could lead to their use in the increased esterification of fats and oils. Such increased stability is favorable for commercial applications. The simplicity of the technique suggests that it may have wide usage for biologically active proteins that stimulate their use in industrial processes.

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