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Lipase Immobilized on Poly(VP-co-HEMA) Hydrogel for Esterification Reaction

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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, and divinylbenzene was 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 was also determined. Increasing the percentage of composition of 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 on VP(%):HEMA(%) 90:10 exhibited the highest activity. Lipase immobilized on VP(%):HEMA(%) 50:50 showed the highest thermal, solvent, storage, and operational stability compared to lipase immobilized on other compositions of hydrogels as well as the native lipase.

Index Entries: Lipase; immobilization; hydrogel; esterification; stability.

Introduction

Immobilization of enzyme has been known to make enzymes more suitable in reactions in organic solvents, operate at relatively higher temperature, as well as be easily separated from the reaction mixture and thus

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easy in retaining enzymes in bioreactors, enabling continuous operation of enzymatic processes *(1)*. Many different methods of immobilization of enzymes are available, each involving a different degree of complexity and efficiency *(2)*. The various methods used can be subdivided into physical method, in which a weaker interaction between enzyme and support or mechanical containment of the enzyme by support is utilized, and chemical method, in which a covalent bond between the enzyme and the support is formed.

Entrapping enzyme within an insoluble matrix support, a physical method, has an important feature whereby the enzyme is not actually attached to anything. Thus, none of the steric problems associated with covalently or electrostatically binding an enzyme on to a polymer may be encountered. Hydrogel, a polymeric material that can be a homopolymer or copolymer made from hydrophilic and/or hydrophobic monomers, may be suitable as a matrix to entrap enzyme. Hydrogel can imbibe large quantities of water without dissolution of the polymer network, making it an interesting support for immobilization of enzyme. Besides providing the water needed for enzyme activity, hydrogel can absorb water produced during the esterification reaction, thus increasing the yield of the products. Moreover, the hydrophobicity and hydrophilicity of the hydrogel can be controlled by varying the amount of monomers and/or the crosslinker used.

In the present study, we immobilized *Candida rugosa* lipase by entrapment on poly(*N*-vinyl-2-pyrrolidone-co-2-hydroxyethyl methacrylate) (poly[VP-co-HEMA]) hydrogel using divinylbenzene (DVB) as crosslinker. We also investigated the activities and characteristics of the immobilized lipase.

Materials and Methods

Materials

Lipase from *C. rugosa* (Type VII) was obtained from Sigma, St. Louis, MO. Initiator, α,α,'-azoisobutyronitrile (AIBN) was from Fluka Chemika (Steinheim, Switzerland). The monomers *N*-vinyl-2-pyrrolidone (VP) and 2-hydroxyethyl methacrylate (HEMA) were purchased from Fluka Chemica, and the crosslinker DVB was obtained from Merck-Schuchardt (Darmstadt, Germany). All other reagents were of analytical grade. The organic solvents and substrates were dried over molecular sieves (3 Å) before use.

Purification of Monomers

VP and HEMA were purified by using an aluminum oxide column (2.5 × 10.0 cm) until colorless solutions were obtained. DVB was used as supplied by manufacturers, without further purification.

Preparation of Lipase Solution

Lipase solution was prepared by dissolving commercial lipase from *C. rugosa* (500 mg) in distilled water (10.0 mL). This mixture was agitated

on a vortex mixer until dissolved. The solution was then centrifuged at 13,000 rpm for 10 min. The supernatant was used for immobilization of lipase.

Immobilization of Lipase

Purified monomers VP and HEMA of different compositions were mixed together with 1% DVB (wt%) in a clean, dry flask. The composition of hydrogels prepared were 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0 VP(%):HEMA(%) (% = wt% of monomer in total weight of [VP + HEMA]). To this mixture, dry initiator, AIBN (10^{-4} mol), was added and the flask was shaken until the AIBN was dissolved. The mixture was then transferred to a polymerization tube, and the solution was degassed with nitrogen for 15 min in order to remove oxygen. The mixture was then incubated to polymerize in a 55–60°C water bath. After the polymer solution became viscous (1–4 h), the polymer was cooled to 50°C, and lipase solution (1.0 mL), which had been previously degassed with nitrogen, was added to the polymer solution and shaken until homogeneous solution was obtained. The solution in the polymerization tube was sealed with a rubber stopper and further polymerized in a 50°C water bath 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 –4°C prior to use.

Protein Assay

The amount of protein was determined by using the method of Bradford *(3)* with bovine serum albumin as standard.

Activity Assay

The reaction 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 with continuous shaking at 150 rpm. The reaction was terminated by dilution with acetone:ethanol (1:1 [v/v], 3.5 mL). The remaining free fatty acid (FFA) in the reaction mixture was determined by titration with NaOH (0.2 *M*) using an automatic titrator (ABU 90, Radiometer, Copenhagen) to an end point of pH 9.5. In the blank determination, poly(VP-co-HEMA) hydrogel without lipase was used instead of the poly(VP-co-HEMA)-immobilized lipase. The specific activity of the enzyme was expressed in micromoles of FFA used/(minute · milligrams of protein).

Gas Chromatography Analysis

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

Effect of Monomers, Crosslinker, and poly(VP-co-HEMA) Hydrogel on Lipase Activity

The purified monomers and viscous monomer mixtures (0.5 mL), crosslinker (0.2 mL), and poly(VP-co-HEMA) hydrogel (0.3 g) were placed, respectively, in different vials containing the substrate solutions and native lipase (0.02–0.05 mg [protein equivalent in 0.3 g of immobilized lipase]). The vials were then incubated in a horizontal water bath shaker at 150 rpm at 37°C for 5 h. The residual activities were expressed as a percentage of the activity of the untreated lipase.

Leaching Study by Washing with Hexane

Poly(VP-co-HEMA)-immobilized lipase (0.3 g) was placed in sealed vials with hexane (4.0 mL). The mixtures were shaken in a horizontal water bath shaker at 30°C for 30 min. The immobilized lipase was isolated from the organic solvent by filtration through Whatman no. 1 filter paper. This procedure was repeated up to four times. Then the residual activities were determined, which were expressed as a percentage of the activity of the untreated immobilized lipase.

Thermostability of Immobilized Lipase

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

Stability in Organic Solvent

The immobilized enzymes were incubated in hexane for between 1 and 12 d at room temperature. After incubation, their residual activities were determined at 37°C. The residual activities were expressed as a percentage of the activity of the immobilized lipase at d 1.

Storage Stability of Immobilized Lipase

The immobilized enzymes were stored at room temperature, 4°C, 0°C, and –80°C for 30 d in sealed vials. After leaving the enzymes at room temperature, the residual activities were determined. The residual activities were expressed as a percentage of the activity of the untreated immobilized lipase.

Operational Stability of Immobilized Lipase

The operational stability of the immobilized enzyme was investigated as follows. Immobilized lipases were placed in the reaction vials with the substrates. The reaction mixture was incubated with continuous shaking as already described. The enzyme was removed from the reaction mixture by

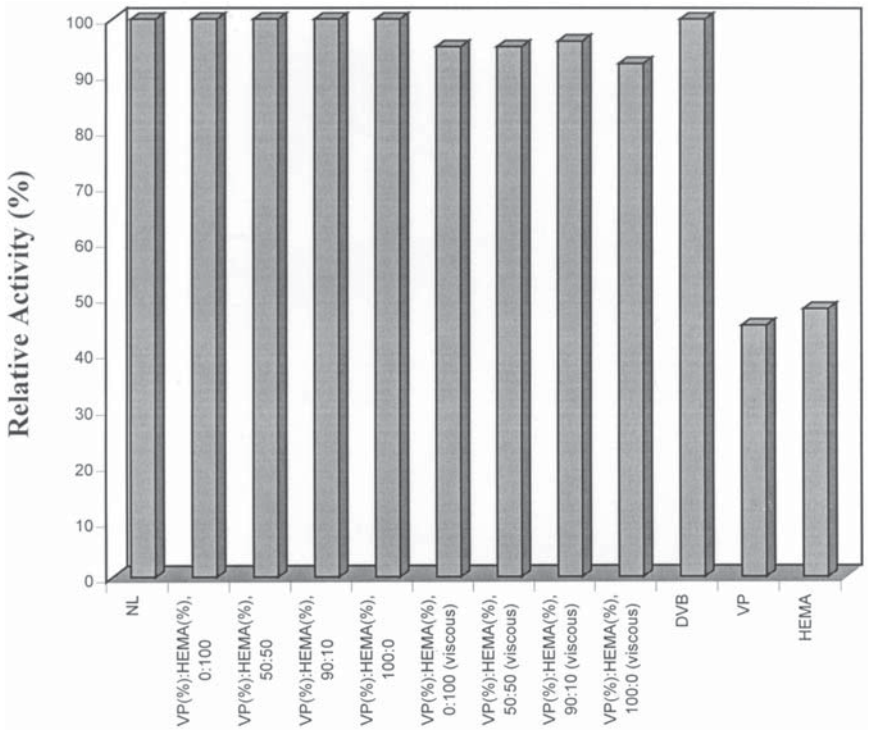

Fig. 1. Effect of monomers, crosslinker, viscous monomer mixtures, and poly(VP-co-HEMA) hydrogel on the activity of lipase. NL: native lipase; VP(%)/HEMA(%) 0:100, 50:50, 90:10, and 100:0: hydrogels with the respective compositions; VP(%)/HEMA(%) 0:100, 50:50, 90:10, and 100:0 (viscous): viscous monomer mixtures (50°C) with the respective compositions; DVB: divinylbenzene; VP: *N*-vinyl-2-pyrrolidone; HEMA: 2-hydroxyethyl methacrylate. % = wt% of monomer in total weight of (VP + HEMA).

filtering with Whatman no. 1 filter paper and washing three times with 3.0 mL of acetone:ethanol (1:1 [v/v]). The amount of FFA remaining in the reaction mixture was determined by titration with 0.05 *M* NaOH. Fresh substrate was added to the enzyme for the next cycle. Relative activities were expressed as the percentages of their residual activities at different cycles compared to the activity at the first cycle.

Results and Discussion

Effect of Monomers, Crosslinker, Viscous Monomer Mixtures, and poly(VP-co-HEMA) Hydrogel on Lipase Activity

Figure 1 presents the effect of monomers and crosslinker and the poly(VP-co-HEMA) hydrogel on the esterification reaction of lipases. Hydrogel of various compositions did not affect the activities of lipase, as shown by the relative activities of the enzyme, whereas the existence of monomers, VP, and HEMA, in the solution form, decreased the activity of lipase to <50%. By contrast, adding the crosslinking agent DVB showed

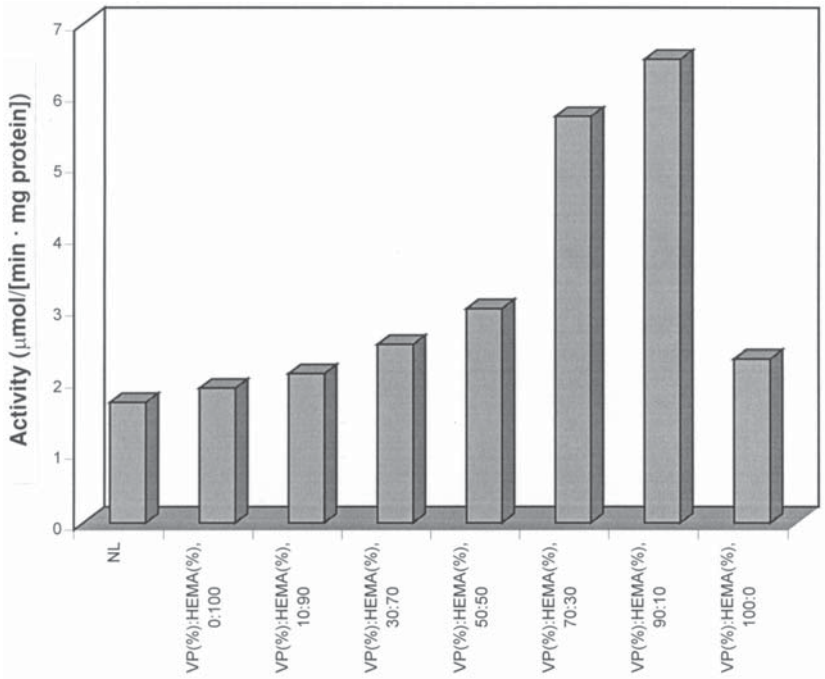

Fig. 2. Esterification activities of poly(VP-co-HEMA)-immobilized lipases. NL: native lipase; VP(%)/HEMA(%) 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0: hydrogels with the respective compositions.

100% of the residual esterification activities in this experiment. The effect of viscous monomer mixtures at 50°C was studied as the enzyme was introduced to the monomer mixtures at this condition. The result showed that the enzyme retained more than 90% of the relative activity. Thus, the immobilization procedure was carried out at this condition. The reactive monomers may have an inhibiting effect on the active site of the enzyme, thus decreasing the activity of the enzyme. However, when all the monomers were completely used up in the polymerization process, the hydrogel formed had no effect on the activity of the lipase.

Activity of Immobilized Lipases

Figure 2 presents the results of the esterification reactions with the poly(VP-co-HEMA)-immobilized lipases. Immobilization of lipase onto hydrogels showed increased esterification activity compared to native lipase. Increasing the percentage of VP from 0 to 90, which corresponds to an increase in the hydrophilicity of the hydrogel and thus the equilibrium water content (EWC) of the hydrogel *(4)*, seemed to increase the activity of the immobilized enzyme. Lipase that was entrapped on VP(%):HEMA(%) 90:10 was the best for the esterification reaction because it exhibited the highest activity in the experiment. This finding may be due to the water surrounding the enzyme in the hydrogel, which is needed to maintain the

three-dimensional active conformation of the enzyme. It may also be due to the ability of the hydrophilic support (90% VP) to absorb the water that was produced in the esterification reaction and thus push the equilibrium of the reaction forward, resulting in an increased formation of ester. Our work using poly(VP-co-HEMA) but using ethylene glycol dimethacrylate (EDMA) as crosslinker showed a similar result *(5)*. Similarly, Kosugi and Suzuki *(6)* reported that the activity of the entrapped lipase depended on the high concentration of water that surrounds the catalytic surface of lipase and the high aquaphilicity of the support.

The low activity observed when lipase was immobilized on VP(%): HEMA(%) 0:100 may be owing to the decreased EWC in the polymer-HEMA, which corresponds with the decrease in water, which is essential for enzyme catalysis. The activity of immobilized lipase on VP(%): HEMA(%) 100:0 (polymer-HEMA) was also low. This finding may be owing to the partial solubility of the hydrogel in water, which is accompanied by a decrease in the level of crosslinking, resulting in more lipases being diffused out of the hydrogel. The composition of the monomers in the hydrogel formation seemed to be an important factor in determining the activity of the enzyme.

Leaching Study by Washing with Hexane

Figure 3 presents the effect of the washing process on lipase activity. Lipases immobilized on VP(%):HEMA(%) 0:100 hydrogel retained their activities after washing four times with hexane. Lipases immobilized on VP(%):HEMA(%) 10:90, 30:70, and 50:50 retained their activities after washing three times but subsequently lost some activities after washing four times with hexane. With VP(%):HEMA(%) 70:30 and 90:70, the decrease in activity started after washing twice with hexane, whereas lipase immobilized on VP(%):HEMA(%) 100:0 showed the lowest retention stability in this experiment. Increasing the hydrophobicity of the hydrogel (increasing the percentage of HEMA) seemed to decrease the leaching effect with hexane. The physical crosslinking contributed by hydrophobic bonding of HEMA and DVB may prevent the enzyme from leaching out from the matrix. At high hydrophilicity of the hydrogel (VP[%]:HEMA[%] 100:0), the absence of physical crosslinking contributed by HEMA may lower the entrapment stability.

Thermostability of Immobilized Lipase

Immobilization of enzymes onto hydrogel seemed to increase thermal stability compared to free lipase after 1 h of incubation (Fig. 4). Immobilized lipases were more thermostable over the temperature range of 40–70°C than native lipase. The hydrogel may be acting as a heat sink, thus protecting the enzyme from denaturation. The relative activity of the immobilized lipase decreased starting at 50°C with a further decrease at 70°C. As temperature is raised, presumably the EWC of the hydrogel gradually decreases, which is accompanied by the shrinkage of the gel matrix. The

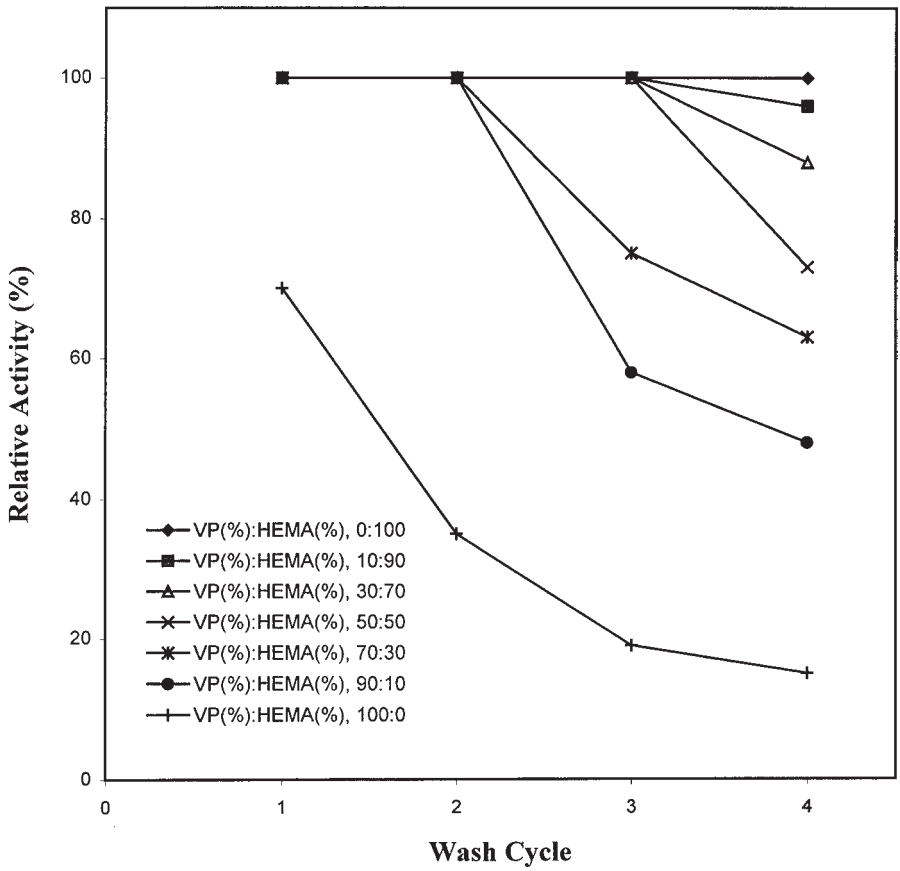

Fig. 3. Leaching study of poly(VP-co-HEMA)-immobilized lipases by washing with hexane. NL: native lipase; VP(%)/HEMA(%) 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0: hydrogels with the respective compositions.

reduced pore size in the hydrogel results in a decrease in the diffusional rate of substrate *(7)*, thus resulting in a reduction in the residual esterification activities. In another study in which we immobilized lipase on poly(VP-co-HEMA) hydrogel but used DVB as crosslinker, a similar trend was seen, but the relative stability of the immobilized enzyme at higher temperature was higher *(5)*. This was expected because the more hydrophobic crosslinker (DVB) seemed to increase the stability of the enzyme compared to the more hydrophilic crosslinker (EDMA).

The lipase that was entrapped on VP(%):HEMA(%) 50:50 exhibited the highest esterification activities. This presence of balanced hydrophobic and hydrophilic moieties in the hydrogel seemed to stabilize the enzyme from heat-induced denaturation. The hydrophilic moiety (VP) offers the advantages of high water content and softness whereas the hydrophobic moiety (HEMA) gives rigidity and toughness to the hydrogel. In addition, the balance structure in this hydrogel may give the correct amount of water for the lipase to function efficiently.

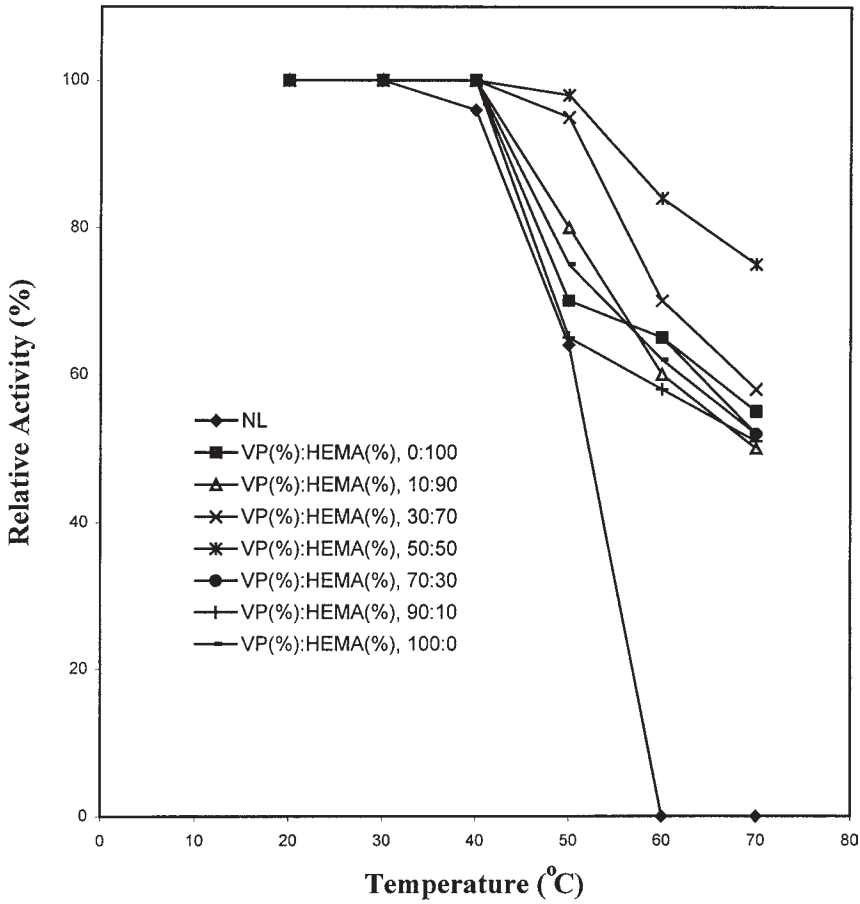

Fig. 4. Thermostability of poly(VP-co-HEMA)-immobilized lipases incubated for 1 h in hexane. NL: native lipase; VP(%)/HEMA(%) 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0: hydrogels with the respective compositions.

Hydrogels with a percentage of composition less or greater than VP(%):HEMA(%) 50:50 showed lower stability compared to the VP(%): HEMA(%) 50:50. This finding is presumably owing to the decrease in the balance between the hydrophilicity and hydrophobicity of the hydrogel. The decrease in activity of immobilized lipase with respect to temperature may also be owing to the inhomogeneous crosslinking of the polymer network that was noted particularly at high contents of VP.

Stability in Organic Solvent

The stability of the immobilized lipase in hexane was investigated (Fig. 5). All immobilized lipases exhibited higher solvent stability than native lipase. Lipases immobilized on VP(%):HEMA(%) 30:70, 50:50, 70:30, 90:10, and 100:0 retained their activities for the first 3 d, whereas the immobilized lipase on VP(%):HEMA(%) 50:50 showed the highest stability

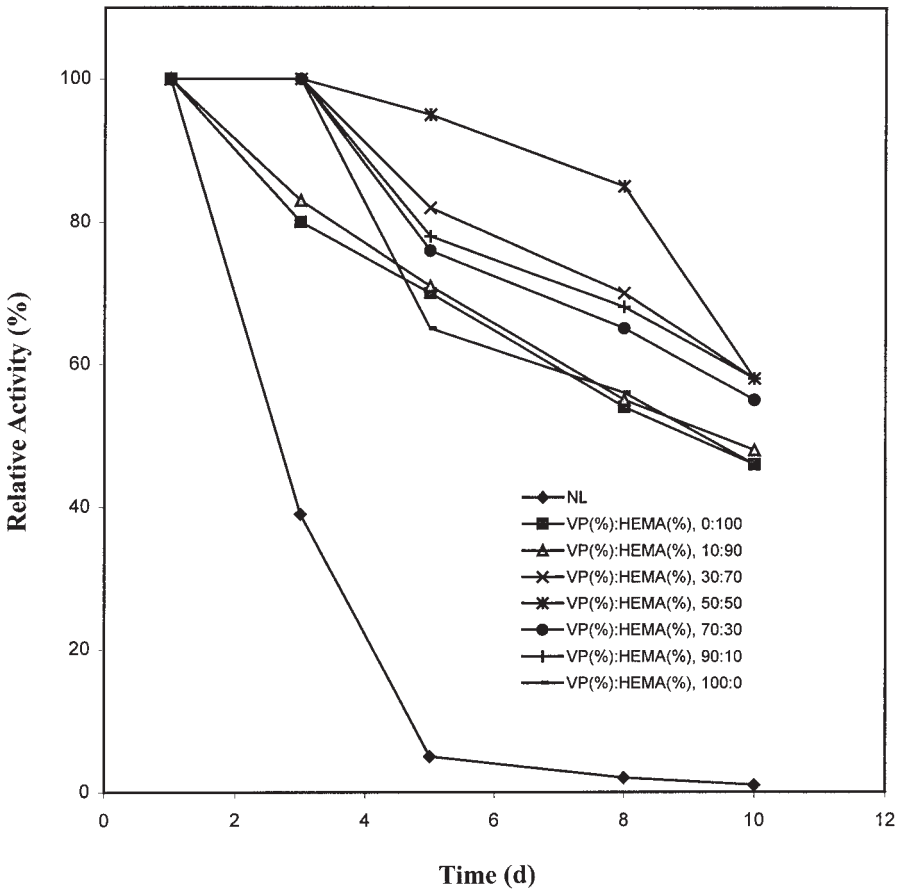

Fig. 5. Stability of poly(VP-co-HEMA)-immobilized lipases incubated in hexane for 12 d at room temperature. NL: native lipase; VP(%)/HEMA(%) 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0: hydrogels with the respective compositions.

in organic solvent. Lipase immobilized on VP(%):HEMA(%) 0:100 and 10:90 hydrogels exhibited relatively lower stability, whereas native lipase was the least stable in organic solvent. It seemed that the protein active structure was easily denatured by the organic solvent. However, immobilizing the enzyme increases the stability in organic solvent significantly. Hydrogels of high hydrophilicity seemed to be most effective in protecting the proteins against unfavorable direct contact with the organic solvent used (hexane), which is a relatively nonpolar solvent.

Storage Stability of Immobilized Lipase

Table 1 presents the stability of the various immobilized lipases incubated in hexane for 30 d at different storage conditions. All immobilized lipases and native lipase showed full catalytic activity after storing them at –80°C. Immobilized lipases retained their full catalytic activities when stored at 0°C, whereas the native lipase showed 67% of the residual esteri-

Table 1
Stability of Immobilized Lipase in Different Storage Conditions After 30 d

	Relative activity (%)[a]			
Immobilized lipase	Room temperature	4°C	0°C	–80°C
VP(%):HEMA(%) 0:100	52	94	100	100
VP(%):HEMA(%) 10:90	60	90	100	100
VP(%):HEMA(%) 30:70	69	100	100	100
VP(%):HEMA(%) 50:50	71	100	100	100
VP(%):HEMA(%) 70:30	44	76	100	100
VP(%):HEMA(%) 90:10	46	51	100	100
VP(%):HEMA(%) 100:0	47	53	100	100
Native lipase	34	50	67	100

[a]Activity is expressed as percentage of the lipase activity at d 1. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing butanol and oleic acid.

fication activity. At 4°C, the lipases immobilized on the more hydrophobic hydrogel exhibited an increase in storage stability compared to native lipase, with lipase immobilized on VP(%):HEMA(%) 30:70 and 50:50 showing the best stability. However, lipase immobilized on the more hydrophilic hydrogel and native lipase showed relatively lower stability. When stored at room temperature, the immobilized lipases expressed a relatively higher storage stability compared to native lipase. The decrease in the activity of the immobilized lipases at room temperature may be contributed to the presence of water in the immobilized lipases introduced during the immobilization procedure, which may cause proteolysis of the enzyme *(8)*.

Operational Stability of Immobilized Enzyme

Figure 6 presents the operational stability of the immobilized lipases. Lipases immobilized on the more hydrophobic hydrogels (VP[%]:HEMA[%] 0:100–50:50) showed a higher operational stability as compared to lipase immobilized on the more hydrophilic hydrogel, with VP(%):HEMA(%) 50:50 showing the highest stability. They retained their initial activity for three cycles. The activities decreased rapidly to about 60–45% of their initial values after 6 cycles, and the remaining activities were maintained with gradual loss to 12 cycles. For the lipase immobilized on the more hydrophilic hydrogel (VP[%]:HEMA[%] 70:30–100:0), the activities decreased rapidly until the ninth cycles and then gradually to about 30–15% to 12 cycles. Lipase immobilized on VP(%):HEMA(%) 100:0 showed the lowest stability.

The more hydrophobic hydrogel seemed to improve the operational stability for repeated use. The entrapment into these compositions of hydrogel would confine the enzyme within the matrix while allowing the transport of substrate and products in and out of the matrix. The loss of activity could be owing to leaching of the enzyme from the matrix, as was reported in the leaching study. It also may be caused by inactivation of the

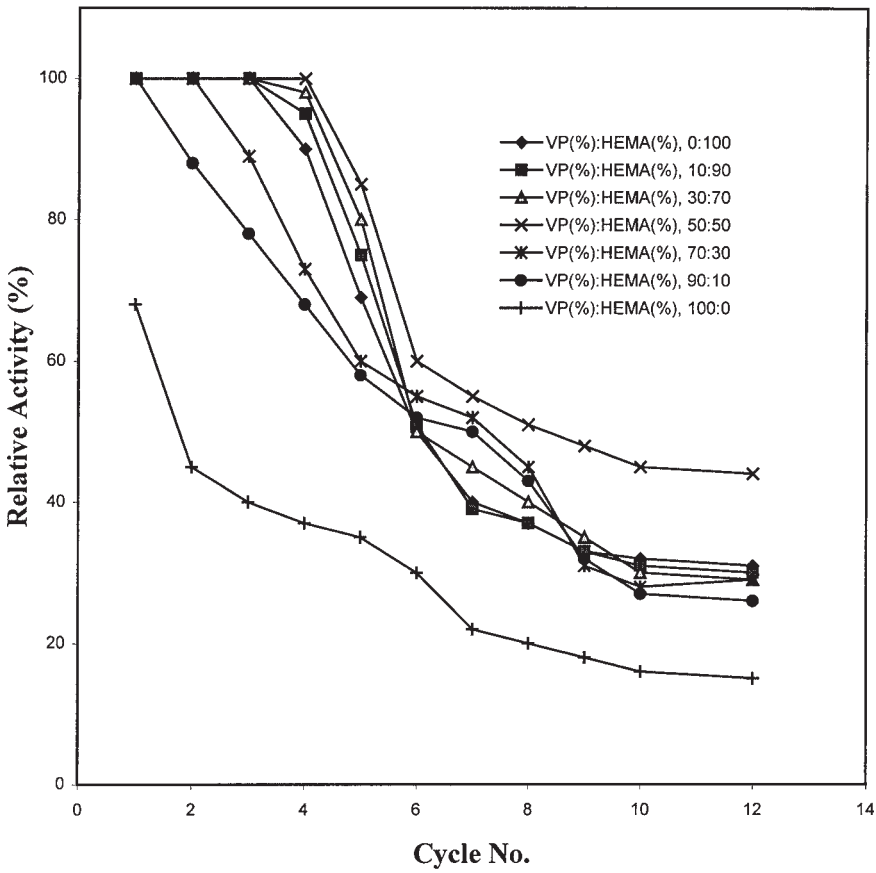

Fig. 6. Operational stability of poly(VP-co-HEMA)-immobilized lipases. VP(%)/HEMA(%) 0:100, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0: hydrogels with the respective compositions.

immobilized enzyme resulting from shearing produced during the shaking process. Earlier reports indicated that biologically active materials exposed to flow were subject to shear *(9,10)*, which altered their kinetics and caused inactivation, which is caused by the disruption of the tertiary structure when the enzyme molecule was oriented in the shear field *(11)*.

In conclusion, immobilization of lipase on hydrogels exhibited characteristics that may be suitable for applications in organic synthesis. The increased stability is quite favorable in commercial applications. The simplicity of the technique suggests that it will be applicable to a wide range of biologically active proteins and should stimulate their use in industrial processes.

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