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Stability of Hydrophobic Lipase Derivatives Immobilized on Organic Polymer Beads

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ABSTRACT

Lipase from *Candida rugosa* was immobilized by attaching various hydrophobic groups to the enzyme molecule and adsorbing these hydrophobic lipase derivatives on several organic polymer beads. The immobilized enzymes were more thermostable in organic solvents compared to the native and modified lipases. Thermostability was highest with XAD2 beads, followed by XAD7 and RCOOH. Initially modifying the enzyme with hydrophobic modifiers did not have any effect on the enzyme thermostability. The best conditions for storing these enzyme preparations were at very low temperature in the lyophilized form and in a solution containing the reaction substrate. Interestingly, PEG-lipase immobilized on XAD7 beads showed increased operational stability when used in a stirred-tank reactor. The operational stability was further increased by a mild glutaraldehyde treatment of the enzyme preparation.

Index Entries: Hydrophobic lipase; immobilization; stability.

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INTRODUCTION

Enzymes have great potential as catalysts for use in synthetic organic chemistry (*1*). Applications of enzymes in synthesis have so far been limited to a relatively small number of processes used in industry and to small-scale syntheses of materials used in analytical procedures and in research. Immobilization of enzymes makes these catalysts practical for wider use in large-scale synthetic organic chemistry (*2*). There are several advantages in the use of immobilized enzymes in industry. Among them are the possibility of continuous enzymatic reactions and their ease of recovery from the reaction mixture (*2*). Their use as industrial catalysts, however, will only be feasible if they can withstand the high temperatures and organic solvents that normally are used in industrial processes.

We reported recently a novel method of immobilizing lipase (*3*), which consists of attaching various hydrophobic groups to the enzyme molecule (*4–6*) and adsorbing these hydrophobic lipase derivatives onto several organic polymers. Our results showed that by attaching various hydrophobic groups to the enzyme molecule before adsorbing onto solid supports, the lipase derivatives adsorbed more strongly onto organic polymer beads. Furthermore, more active immobilized enzymes were produced since modification of enzymes with hydrophobic groups increased the activity of the enzyme (*4–6*). Our next step was to study other catalytic properties of the lipase immobilized in this manner.

This paper describes the stability of these immobilized enzymes when exposed to high temperatures and organic solvents. In addition, we discuss their storage and operational stability.

MATERIALS AND METHODS

Materials

Lipase from *Candida rugosa* (Type IV), Amberlite XAD2 (polystyrene), and XAD7 (polyacrylate) were obtained from Sigma Chemical Co. (St. Louis, MO). Polycarboxylic acid (RCOOH) with 5% divinyl benzene (DVB) crosslinking was synthesized in our laboratory. All other reagents were of analytical grade.

METHODS

Purification of Lipase

Commercial lipase from *Candida rugosa* was purified by water extraction and gel filtration through Superose 6 column on Fast Performance Liquid Chromatography systems (Pharmacia, Uppsala, Sweden). About 11–12-fold purification and an overall yield of 60% were obtained.

Modification of Lipase

Lipase was modified with monoethoxypolyethylene glycol (PEG) of mol wt 1900 (PL1900) as described by Basri et al. (*4*). Reductive alkylation of lipase with dodecyldehyde (DL) was as described by Ampon et al. (*5*). Amidination with methyl 4-phenylbutyrimidate (IL(VI)) was according to the method of Wofsy and Singer (*7*). The amount of excess modifier added to the enzyme was adjusted so that the derivatized enzyme was modified to a similar extent (40–50% modification).

Immobilization of Lipase

The lipase solutions and polymer beads (1 g) were mixed at room temperature by shaking at 100 rpm in a sealed vial for 1 h. The beads were separated from the supernatant by filtration through Whatman no. 1 filter paper. They were then washed three times each with buffered 1*M* KCl, pH 7 and 50% ethyleneglycol and once with 20 mL distilled water. The beads were lyophilized in the cold, kept in sealed vials, and stored at 0°C prior to use. The polymer beads used for immobilization of the enzyme were: Amberlite XAD2 (nonpolar), Amberlite XAD7 (medium polar), and polycarboxylic acid, RCOOH (polar).

Protein Assay

The amount of protein was determined by trinitrobenzene sulfonate (TNBS) titration of the amino acids produced following the hydrolysis of the enzyme or its derivatives (*8*). The extent of the protein modification was determined by comparing the number of amino acid groups that reacted with TNBS in the modified and unmodified protein (*9*).

Activity Assay

The reaction system consisted of solvent (0.5 mL), alcohol (2.67 mmol), fatty acid (0.35 mmol), and immobilized lipase (0.3 g). The mixture was incubated at 28°C for 24 h with continuous shaking at 150 rpm. The reaction was terminated by dilution with 3.5 mL of ethanol:acetone (1:1 v/v) and the remaining free fatty acid in the reaction mixture was determined by titration with 0.05*M* NaOH using an automatic titrator (ABU 90, Radiometer, Copenhagen) to an endpoint of pH 9.5.

Thermostability

To investigate the effect of temperature on the stability of the enzyme structure, enzymes were incubated in benzene at various temperatures (20, 30, 40, 50, 60, 70°C) for 1 h in sealed vials. After the incubation, the enzyme mixtures were cooled to room temperature and the residual esterification activities determined. The activities were expressed as percentages of the residual activities at different temperatures relative to the activity of the untreated enzyme (kept at room temperature).

Stability in Organic Solvent

The enzyme preparations were incubated in benzene, without shaking, for 1–10 d at room temperature. After the incubation, their residual activities were determined at 28°C. The relative activities of the enzymes were expressed as percentages of their activities at the different time intervals compared to the activity at day one.

Storage Stability

This experiment was conducted to investigate the stability of the enzyme preparations in benzene at different storage temperature, in the lyophilized form and in the presence of its substrate. The enzymes were kept in the manner indicated for 60 d. The residual activities were then determined and the activities were expressed as percentages of their residual activities at different times compared to the initial activity (day one).

Operational Stability

The operational stability of the immobilized enzyme was investigated as follows. Lipase modified with PEG1900 (PL1900) and immobilized onto XAD7 beads under optimum conditions was placed in a stirred-tank reactor (4.5 × 5.5 cm) equipped with a Teflon magnetic stirring bar suspended from the top. An outlet tubing attached to the bottom of the reactor was used to drain out the reaction mixture after each cycle. The size of the reaction mixture used was a 10-fold scale up of the reaction described in the activity determination. The reaction mixture was incubated with continuous shaking at room temperature. The substrate was replaced after every 24 h. The amount of free fatty acid remaining in the reaction mixture was determined by titration with 0.05 NaOH. Relative activities were expressed as the percentages of their residual activities at different cycles compared to the activity at the first cycle. XAD7 was chosen as the support in this experiment because of the higher immobilized activity compared to XAD2.

Glutaraldehyde Crosslinking

The effect of glutaraldehyde crosslinking on the operational stability of the immobilized enzyme was investigated as follows: Immobilized enzymes (10 g) were added to a solution (100 mL) containing different concentrations of glutaraldehyde (1–6%). The mixture was shaken (100 rpm) for 2 h at 28°C. The beads were filtered through Whatman no. 1 filter paper. They were washed three times with 20 mL of distilled water, lyophilized in the cold, kept in sealed vials, and stored at −20°C prior to use.

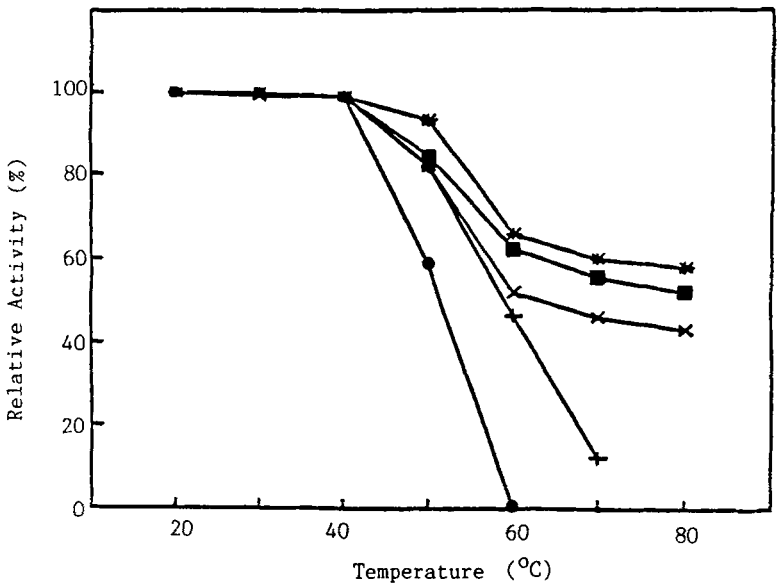

Fig. 1. Thermostability of PEG-lipase immobilized onto several types of polymer beads incubated for 1 h in benzene. Symbols: ● = NL (native lipase); + = PL (PEG1900 lipase); * = XAD2PL; ■ = XAD7PL; × = RCOOHPL.

RESULTS AND DISCUSSION

Thermostability

The stability of the various lipase preparations incubated in benzene at different temperatures is shown in Fig. 1. Immobilization of the modified lipase on XAD2, XAD7, and RCOOH beads increased their thermal stability. The thermostability of lipase or other enzymes had been known to improve after immobilization (*10–12*). An immobilized enzyme has a considerably reduced conformational flexibility but is still quite capable of the movement required for efficient catalysis. Heat-induced unfolding (denaturation) of enzymes that require ample conformational mobility (*13*) is prevented by attachment to the inflexible solid supports. Substrate and product molecules, being of much smaller molecular mass, can freely diffuse in and out of the polymer matrix.

XAD2-bound PL1900 had higher thermal stability compared to those immobilized on XAD7 and RCOOH beads. Hydrophobic interaction is considerably enhanced at high temperature, and the increase in thermostability of the immobilized enzyme preparation using the relatively nonpolar supports probably is a consequence of this increased interaction (*14*). The improvement in the enzyme stability thus would be an advantage for reaction at high temperature.

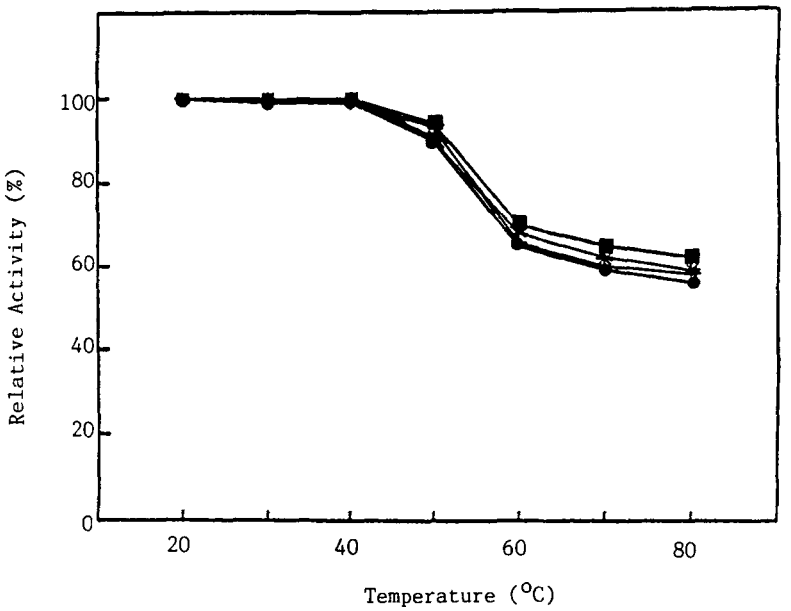

Fig. 2. Thermostability of XAD2 immobilized, modified lipases incubated for 1 h in benzene. Symbols: ● = XAD2NL; + = XAD2PL; * = XAD2IL(VI); ■ = XAD2DL.

In an attempt to investigate the effect of different modifiers on the thermostability of immobilized lipase, XAD2 beads were used as the support, as immobilized enzymes prepared from them had shown the highest thermal stability. Very little change in the relative thermostability of the immobilized enzymes was observed (Fig. 2). In contrast, lipase immobilized via PEG spacer on to silica is more stable than lipase bound directly onto silica (*15*).

Stability in Organic Solvent

The immobilized lipases showed increased stability in benzene over a period of 10 d (Figs. 3 and 4). All the immobilized lipases retained their activities for the first 3 d but lost about 50% of them toward the 10th d. Lipases are known to lose activity as time elapses (*16*).

Not much difference was observed in the stability of the modified lipase immobilized onto different supports in benzene. The stability of the native and modified lipases immobilized onto XAD2 was also similar (Fig. 4). This is as expected, since hydrophobic interaction between the enzyme and relatively nonpolar support is not increased at this temperature.

Storage Stability

The residual activities of the various lipase preparations were determined after storing for 60 d under various conditions (Table 1). All lipase

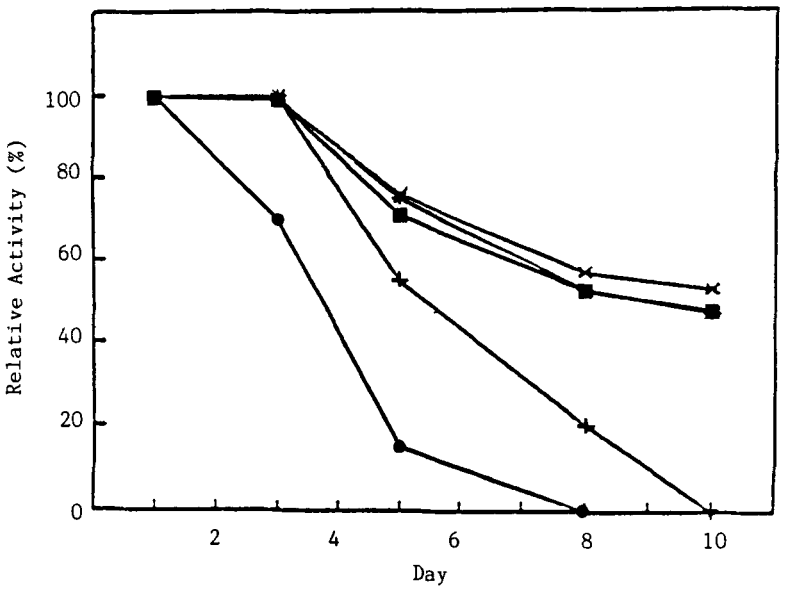

Fig. 3. Stability of immobilized PEG-lipases incubated in benzene for 10 d at room temperature. Symbols: ● = NL (native lipase); + = PL (PEG1900 lipase); × = XAD2PL; ■ = XAD7PL; * = RCOOHPL.

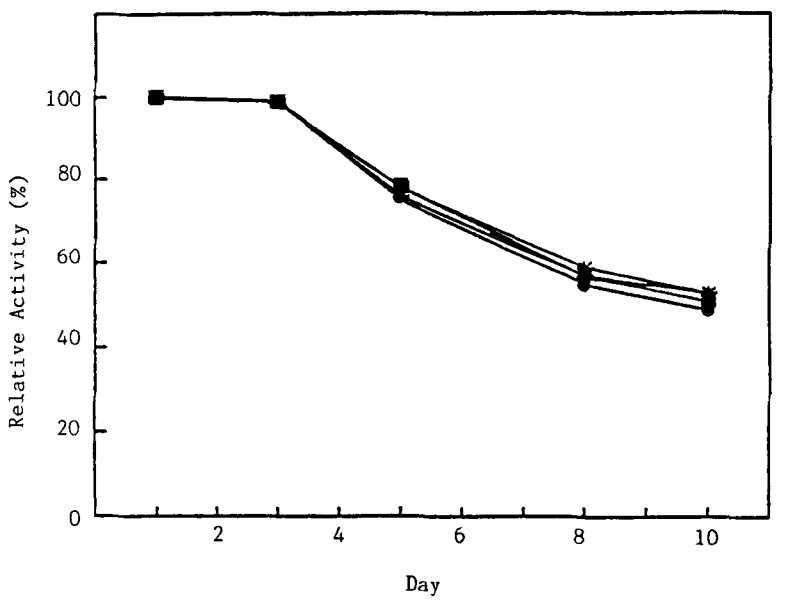

Fig. 4. Stability of XAD2 immobilized, modified, lipases incubated in benzene for 10 d at room temperature. Symbols: ● = XAD2NL; + = XAD2PL; * = XAD2IL(VI); ■ = XAD2DL.

Table 1
Stability of Lipase Preparations in Different Storage Conditions After 60 D

	Residual activity, %[a]			
	rt[b]	4°C	0°C	−20°C
NL				
In solution		19	80	100
Lyophilized		70	100	100
PL1900				
In solution		25	92	100
Lyophilized		80	100	100
XAD7NL				
Air-dried[c]	20	83	100	100
Lyophilized	78	95	100	100
Air-dried[d]	85	100		
Lyophilized[d]	85	100		
XAD7PL1900				
Air-dried[c]	20	85	100	100
Lyophilized	80	96	100	100
Air-dried[d]	86	100		
Lyophilized[e]	85	100		

[a]100% is based on the initial synthetic activity of lipases using propanol and oleic acid as substrates.
[b]Room temperature.
[c]The immobilized lipase was air-dried in a 37°C oven before storing.
[d]The air-dried immobilized lipase was stored in the presence of substrate mixture as described in [a].
[e]The lyophilized lipase was stored in the presence of substrate mixture as described in [a].

preparations exhibited full catalytic activity after storing them at −20°C. Immobilized lipases retained their full catalytic activity when stored at 0°C. At very low temperatures (enzyme in solid state), the lipase is probably locked in its native, catalytically active conformation (*17*). When stored at higher temperatures, immobilized lipases showed increase storage stability compared to native and modified lipases. At these temperatures, the stabilization may be owing to multipoint attachment of the enzyme to the supports, creating a more rigid enzyme molecule (*18*). Hence, disruption of the active center becomes less likely to occur. However, there was very little change in the relative storage stability between the immobilized native and modified lipases.

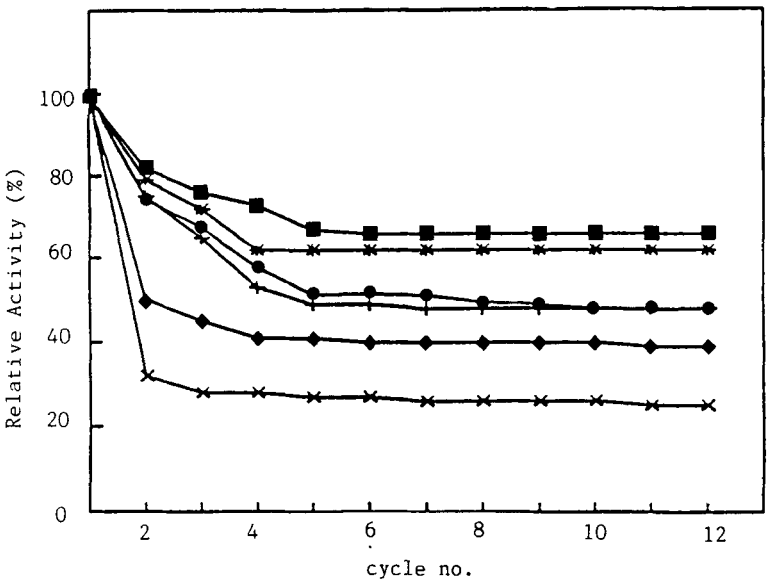

Fig. 5. Operational stability of immobilized lipases before and after crosslinking with different glutaraldehyde concentrations. Symbols: × = XAD7NL 0%; ◆ = XAD7PL 0%; ■ = XAD7PL 1%; * = XAD7PL 2%; + = XAD7PL 4%; ● = XAD7PL 6%.

Operational Stability

The activities of XAD7NL and XAD7PL in a stirred-tank reactor are shown in Fig. 5. The activities decreased rapidly to about 25 and 42% of their initial values, respectively, after the second cycle. Modifying the enzyme with PEG thus increases the operational stability of immobilized native lipase by about twofold. The remaining activity was maintained with almost no further loss after 12 cycles. Mild glutaraldehyde (1–6%) treatment of the XAD7-bound lipase further enhanced the operational stability of the preparation. The use of glutaraldehyde crosslinking was shown to improve the stability of immobilized enzyme for repeated use (*19*). The resultant crosslinking of beads would confine the enzyme within the matrix while allowing the transport of substrate and product in and out of the matrix (*20*). Crosslinking with 1% glutaraldehyde was found to give the best result. Ji (*21*) suggested that low concentration of glutaraldehyde is usually suitable for crosslinking.

There was also a gradual loss of activity for the first four cycles. This loss of activity in the reactor could be caused by inactivation of the immobilized enzyme resulting from shearing produced by the rotation of the suspended magnetic stirring bar. A number of reports indicated that biologically active materials exposed to flow were subject to shear (22,23),

which altered their kinetics and caused inactivation. Charm and Wong (22) suggested that the enzyme inactivation under this condition was caused by the disruption of the tertiary structure when the enzyme molecule was orientated in the shear field.

SUMMARY

Immobilization of native and modified lipase enhanced their stability toward high temperature and organic solvents. It appeared that the stability of the enzyme was affected more by the immobilization success rather than the modification procedure, as initially modifying the enzyme before adsorbing onto solid supports did not change the stability toward high temperature and organc solvents, but immobilization of the enzyme on the less polar organic polymer beads showed increased stability. By modifying the enzyme before adsorbing onto solid supports, however, the operational stability was enhanced significantly, and crosslinking the immobilized enzyme further increased the stability. The work presented here suggests that premodification of enzyme, selection of supports, and crosslinking of enzyme were important criteria to be considered in producing more stable immobilized enzymes. Immobilized lipases that are more versatile and adaptable to conditions in the oleochemical industry, thus, can be produced and the use of immobilized lipases can be expanded.

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