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Poly(Methyl Methacrylate) as a Matrix for Immobilization of Lipase

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ABSTRACT

Poly(methyl methacrylate) (PMMA) was found to be suitable for the immobilization of lipase from *Candida rugosa*. The best result based on hydrolytic activity was obtained by adsorption of the purified unbuffered enzyme solution onto PMMA beads without any modification of the beads. Prolonged exposure of the protein to the beads increased its adsorption but the expressed activity decreased after 1 h of exposure. The magnitude of the immobilized activity also varied with the size of the beads. Immobilization of the lipase shifted its optimal reaction temperature from 37 to 45°C. The immobilized enzyme is also more stable than the free enzyme in solution. The operational half-life of the immobilized lipase packed in a column and assayed in a closed system is 40 d.

Index Entries: Immobilization; lipase; *Candida rugosa;* poly(methyl methacrylate); hydrolysis.

INTRODUCTION

Lipases (glycerol ester hydrolase, EC. 3.1.1.3) are a group of enzymes that are useful as catalysts for the preparation of some industrially important chemicals. The use of lipases for lipid hydrolysis provides an alternative reaction to the conventional physicochemical splitting of fat.

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Enzymatic hydrolysis of lipids allows the reaction to be carried out under moderate conditions and does not produce any undesirable materials that may contaminate the products (*1*). Other industrially important chemical reactions include the synthesis of esters (*2,3*), biomodification of fats (*4*), and resolution of chirals compounds (*5,6*). The use of immobilized lipase in the above reactions has been extensively carried out. Although many supports have been used in the immobilization of lipase (*7*), the expressed activities of most of the immobilized lipase preparations were low compared to those obtained with other immobilized enzymes (*8*). Hydrolysis of oil by a lipase is an interfacial reaction that requires that the enzymes be adsorbed onto the surface of the oil droplet (*9*). Consequently, in order for the immobilized lipase to show a high expressed activity, the bound enzyme must easily be approached by the substrate.

MATERIALS AND METHODS

Materials

Lipase from *Candida rugosa*, Amberlite XAD-4 and 7 and Poly(methyl methacrylate) (PMMA) was obtained from BDH. Natural olive oil, 100% pure (Bertolli Luca, Italy) was obtained from the local distributor. All other reagents were of analytical grade.

Methods

Activity Assay

Lipolytic activity of the free enzyme was determined as described by Samad et al. (*10*). The substrate used was a water emulsion containing 50% (v/v) of olive oil emulsion with 1% of poly(vinyl alcohol). The reaction mixture comprised 2.5 mL of substrate, 0.02 mL of 0.02*M* $CaCl_2$. The mixture was incubated at 28°C for 30 min with continuous shaking of 150 rev/min. The reaction was terminated by the addition of an equivolume of acetone-ethanol (50% v/v). Free fatty acid liberated was then titrated with 0.05*M* NaOH using a Radiometer ABU90 autotitrator. One unit of activity is equivalent to 1 μmole of free fatty acid liberated/min. To determine the activity of the immobilized enzyme, the free enzyme in the above procedure was replaced by 0.3 g (wet wt) of immobilized lipase. To determine the optimal reaction temperature, reactions were carried out at 28, 37, 45, 50, and 60°C. In the stability study, the free and immobilized enzymes were initially exposed to the chosen temperatures for 30 min and the residual activity was then assayed at standard conditions.

Immobilization of Enzyme

Purified lipase (140 U/mg) was used in all the procedures. Purification was carried out as described by Basri et al. (*11*). Protein concentration was determined by the Lowry method (*12*).

Table 1
Amount of Lipase Adsorbed
on Various Polymers and Their Specific Activities

Polymer	Amount of protein adsorbed, mg/g	Specific activity, U/min/g
PMMA	0.35	23.86
PMMA[a]	0.39	9.69
PMMA[b]	0.38	18.83
Amberlite XAD-7	0.50	9.90
Amberlite XAD-4	0.32	0.29

[a]With hydrazide groups.
[b]With carboxylic acid groups.

The immobilization of lipase was performed by shaking continuously (80 rev/min) 1 g of PMMA with 10 mL of purified enzyme (1 mg lipase in water) for 30 min. The immobilized enzyme was then separated by suction filtration and washed with 100 mL of distilled water and air dried before use.

Polymer Modification

Modification of PMMA was carried out by two methods. The attachment of hydrazide groups to the polymer was carried out by refluxing the polymer (10 g) in 100 mL of 25% hydrazine solution in methanol-water (50%, v/v) at 50°C for 4 h. Conversion of some of the ester groups into carboxylic groups was performed by hydrolysis of the polymer (10 g) with 100 mL of 10*M* sodium hydroxide solution at 40°C for 16 h. Both products were thoroughly washed with distilled water before they were used for enzyme immobilization.

Operational Stability of Immobilized Enzyme

The operational stability was studied using 10 g of the immobilized enzyme, packed in a glass column (id, 1.5 cm) of a closed system (*13*). The flow rate of the substrate was 0.2 mL/min. The substrate (50% olive oil emulsion in water) was continuously recycled through the column for 24 h before being replaced.

RESULTS AND DISCUSSION

PMMA was supplied in the bead form without any crosslinking. Treatments of this polymer with the hydrazine and sodium hydroxide solutions introduced hydrazide and carboxylic acid groups, respectively, onto the polymer. Results of the lipase adsorption study using the various polymers are given in Table 1. It clearly shows that PMMA without any modification is the best support for the enzyme immobilization. The amount of

protein adsorbed is 0.35 mg/g polymer with a specific activity of 23.86 U/g polymer, which is equivalent to 17% that of free lipase. The percentage of expressed activity of the immobilized enzyme is higher compared to those obtained on the supports reported by Shaw et al. (*8*).

Introduction of hydrazide and carboxylic groups increased slightly the amount of protein adsorbed but the activity that was expressed on the polymer was reduced. The loss of activity is probably owing to the increase in the hydrophilicity of the support surface, since the two functional groups introduced are polar. The reduction of lipase activity when the enzyme is immobilized onto hydrophilic supports is normally attributed to several factors. These include a change in the lipase comformation that deactivates its active sites, reduction of the amount of the nonpolar substrate exposed to the enzyme immobilized on the polymer surface, and steric hindrance of the support on the freedom of immobilized protein to catalyze the reaction.

For comparison, the lipase was also immobilized on Amberlite XAD-4 and 7. Amberlite XAD-4 and 7 are macroporous crosslinked polymers made from poly(styrene) and poly(acrylic ester), respectively (*14*). The use of Amberlite XAD-7 as a support for immobilized lipase had been reported (*15,16*). The results (Table 1) shows that although the amount of protein adsorbed onto Amberlite XAD-7 is high (0.50 mg/g polymer) its specific activity is less than 50% of that immobilized on PMMA. The higher activity expressed by lipase immobilized onto PMMA suggests that the surface of PMMA is a more appropriate environment in the hydrolysis of olive oil. Increasing the hydrophobicity of the support (using Amberlite XAD-4) not only reduced the amount of enzyme adsorbed but also drastically deactivated the enzyme. Amberlite XAD-7 has a lower surface area (about 50%) than XAD-4, though XAD-7 has doubled the pore size of XAD-4. It is tempting to conclude that the larger pore size may be contributory to the high expressed activity of the immobilized enzyme. However, the amount of protein adsorbed indicated that the surface property of XAD-7 was more suitable to protein adsorption. No data on the physical parameters of PMMA is available, but the chemical structure of PMMA may be similar to XAD-7 and may contribute to better expressed activity.

Further studies on a few immobilization parameters of lipase on PMMA were investigated. The effect of coupling period on the retention activity is shown in Fig. 1. Increasing the exposure period from 30 min to 1 h improved the hydrolysis activity by about 20%. However, a further increase in the shaking period decreased the expressed activity of the immobilized enzyme that was produced. The amount of protein adsorbed onto the polymer was slightly increased with increasing of exposure period. This suggests that there is a limit in the amount of protein that can be adsorbed on the polymer to express maximum activity. Increasing beyond this amount would reduce the specific activity. This reduction is

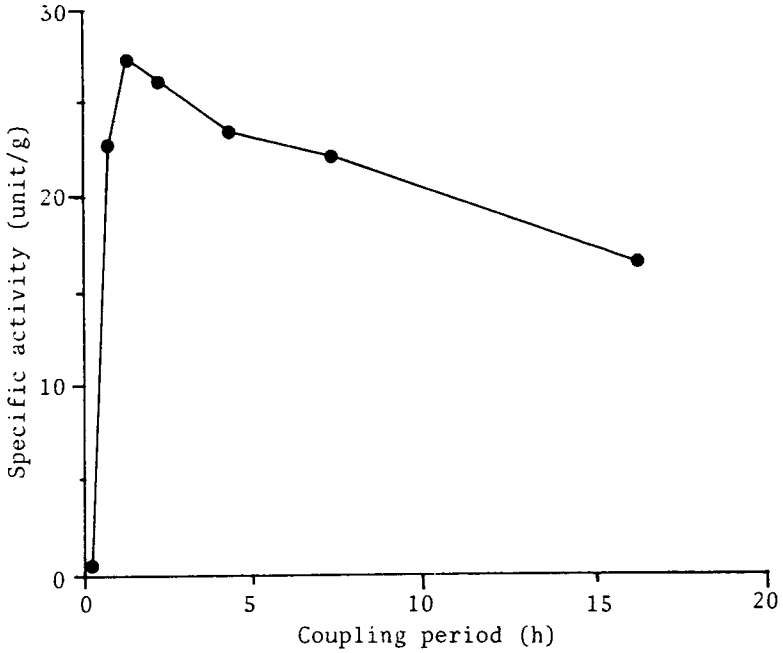

Fig. 1. Effect of coupling period on retention activity.

Table 2
Effect of Bead Size on Specific Activity of Lipase Immobilized on PMMA

Bead size, μm	Specific activity, U/min/g
75-300	67.44
300-850	18.93
850-1700	14.01

probably owing to the increase of hydrophilicity of the polymer surface as more protein is adsorbed.

Primary investigation on the effect of pH on the coupling process was carried out. A relatively high activity was obtained when the coupling was done at pH 6 and 7 (81 and 94% of maximum activity). The best result (100% activity), however, was obtained when the coupling was done in distilled water (pH 6.2). This suggests that ionic concentration may suppress the adsorption of lipase onto the beads. However, to test this hypothesis, a more systematic investigation is required.

Table 2 shows the effect of PMMA bead size on the specific activity of the immediate lipase. The dependency of the activity on the bead sizes

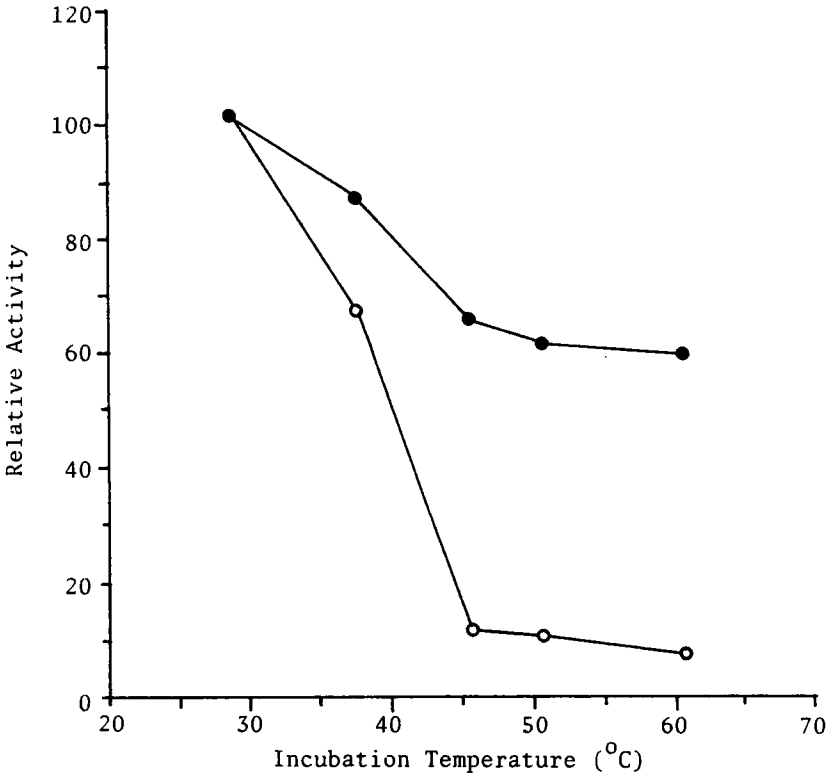

Fig. 2. Stability of free (○) and immobilized lipase (●) to different temperatures.

suggests that the polymer is not sufficiently porous to allow for all of the enzyme trapped within the pores to be fully effective and functional. The immobilization of pancreatic lipase on Biogel P-2 and stainless steel powder as reported by Lieberman and Ollis (*17*), suggested that the low immobilized activity that was obtained was owing to the difficulties of the oil droplets to penetrate the pores of the supports. As stated earlier, other physical specifications for PMMA are not available for further analysis.

Figure 2 shows the relative activity of the enzyme after they were exposed for 30 min at different temperatures. It clearly showed that the immobilized enzyme was more stable than that in the free enzymes. Adherence of the enzyme on the solid support may protect the protein from unfolding. Figure 3 shows the effect of temperature on the lipolytic activity of the immobilized and free enzyme. The optimal reaction temperature shifts from 35°C for free lipase to 45°C for the immobilized lipase. Higher optimal temperature for immobilized enzyme has also been observed by Kimura et al. (*13*) and Shaw et al. (*8*). High temperature facilitates efficient diffusion of the substrate and products from the

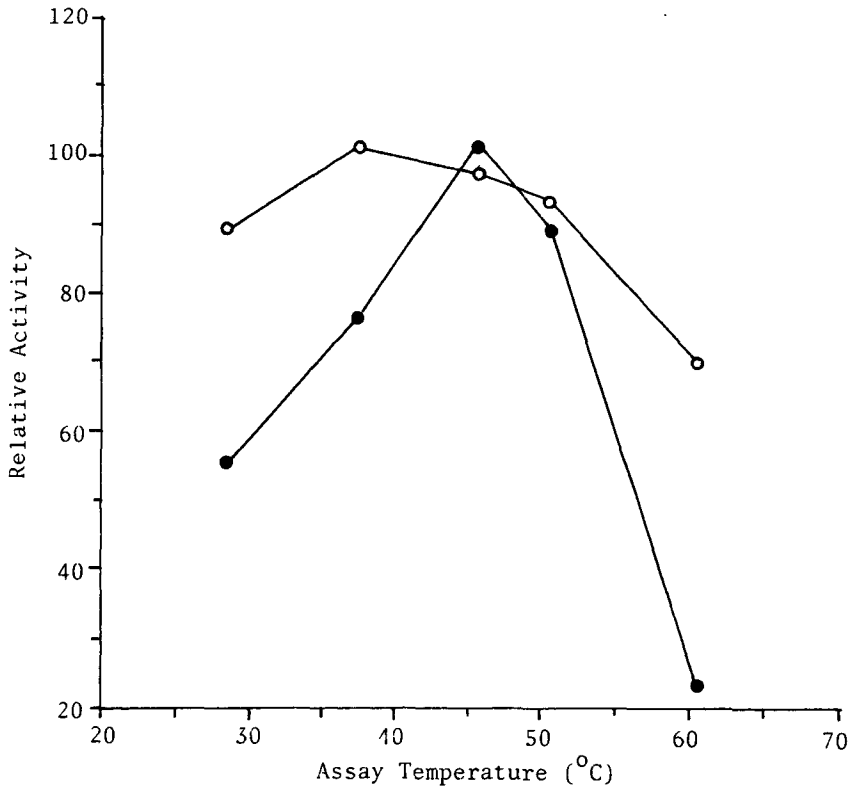

Fig. 3. Temperature profile of free (○) and immobilized lipase (●).

interfacial layer. However, further increase of temperature drastically reduced the lipolytic activity of the immobilized enzyme. Reduction of the activity is probably also owing to the reverse reaction beside deactivation of enzyme as the temperature increases.

The operating stability of the immobilized enzyme packed in a column of a closed system was also studied. Figure 4 shows that the half-life of the column is about 40 d. The half-life of the immobilized lipase system is similar to that reported by Pronk et al. (*18*) using lipase immobilized on membrane, and is much higher than those reported by Brady et al. (*1*), Ibrahim et al. (*16*), and Kimura et al. (*13*), albeit different immobilized lipase-reactor systems were used.

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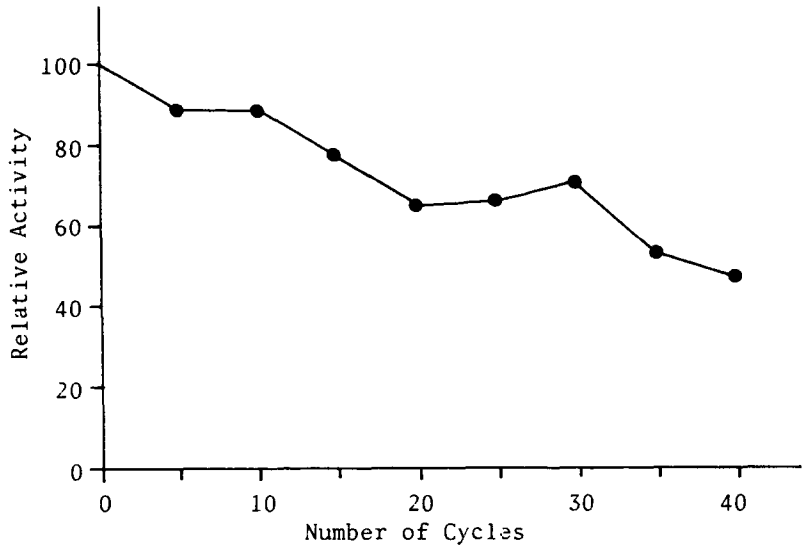

Fig. 4. Operational stability of immobilized lipase. The substrate is replaced every 24 h, but readings were recorded every 5 d.

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