The Effect of Immobilization on the Hydrolytic/Interesterification Activity of Lipase from *Rhizopus niveus*

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ABSTRACT: Lipase from *Rhizopus niveus* was immobilized by physical adsorption on Celite 545 and glass beads. The results showed that the highest immobilization efficiency and specific hydrolytic activity of 96% and 9.2 meq/mg protein/min, respectively, were obtained with Celite as the carrier. However, the specific hydrolytic activity of lipase adsorbed on glass beads by acetone precipitation was similar to that obtained by the Celite carrier, although the protein loading capacity was relatively low. The results showed that lipase immobilized on glass beads exhibited similar activity profiles with respect to reaction time, different enzyme concentrations, and water content, using trimyristin and tripalmitin as substrates, to those obtained with the free enzyme. In contrast, the immobilized lipase on Celite exhibited a considerably lower hydrolytic activity. However, the results also showed that the lipase activities of the free enzyme and the immobilized Celite enzyme were similar when the more hydrophilic triolein was used as the substrate. The interesterification of a mixture of tripalmitin and trimyristin or triolein was carried out using both the free and immobilized enzymes. The results indicated that the hydrolytic activity of lipase was similar in both cases for the first 24 h, after which it decreased dramatically. These findings suggest that at this late stage an equilibrium between the hydrolytic and interesterification reactions was reached.

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Lipases (EC 3.1.1.3) are glycerol ester hydrolases that are most active at oil-water interfaces (1). Lipase-catalyzed reactions of triacylglycerols include hydrolysis, in which the fatty acid moieties are replaced by hydroxyl groups, and interesterification, in which positional interchanges among fatty acid groups on separate triacylglycerol molecules occur (2). Selectivity of interesterification over hydrolysis occurs by limiting the amount of water in the reaction mixture (3). The use of specific lipases to catalyze the interesterification reaction has received considerable attention lately; different lipases have shown preferences for both the nature of the acyl group and the positional distribution of fatty acids on the triacylglycerol molecules (4). Hayes *et al*. (5) reported that the interchange of palmitic or myristic acid with oleic acid at the *sn*-2 position of the glycerol molecule suppressed the cholesterol-raising potential of milk fat. Commercial lipase N, obtained from *Rhizopus niveus*, showed an interesting specificity in changing the positional distribution of selected fatty acids ($C_{18:1}$ and $C_{16:0}$) within the triacylglycerol molecules of butter fat resulting in an increased proportion of oleic acid at the *sn*-2 position (6). Lipases from different microbial sources such as *Pseudomonas fluorescens* (7), *Aspergillus niger* (8), *Candida cylindracea* (9), *C. deformans* (3), and *Mucor miehei* (8,10,11) have also been investigated for the interesterification of butter fat. Kennedy (12) reported that the use of microbial lipases of selected positional specificity resulted in the production of interesterified mixtures of triacylglycerols that could not be produced by simple chemical interesterification.

The immobilization of enzymes is of importance for the development of continuous large-scale processing (13). The choice of the support depends upon characteristics including mechanical and chemical resistance, durability, hydrophobicity/hydrophilicity, chemical functionality, toxicity, cost, and mass transfer. Malcata *et al*. (14) reported numerous methods for the immobilization of lipase on different carriers, each involving a different degree of complexity and efficiency. Immobilization by physical adsorption on a carrier such as glass beads or Celite presents certain advantages including low cost, simplicity, and preservation of substrate specificity (14). The major disadvantage of physical adsorption is the weak bond between the carrier and enzyme, which could result in leakage of the enzyme (15). However, the use of immobilized enzymes in organic solvents where enzyme solubility is limited overcomes this problem as the enzymes remain associated with the support. Martinek *et al*. (16) reported that the use of a micelle-forming surfactant, solubilized in organic solvents, enhanced the stability and catalytic activity of certain free lipases.

This study is a part of ongoing research (6,17–20) with the aim of optimizing the interesterification of selected fatty acids in butter fat by lipase activity in organic solvent media. The

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objective of this work was to compare selected methods of immobilization in terms of their efficiency and stability to carry out the hydrolysis/interesterification of selected fatty acid acylglycerols based on their nutritional implications, including trimyristin, tripalmitin, and triolein, by the lipase activity from *R. niveus*.

MATERIALS AND METHODS

Lipase N was obtained from the Amano Pharmaceutical Company (Nagoya, Japan). It was produced by a unique fermentation process of a selected strain of *R. niveus*.

Immobilization by adsorption of lipase on different carriers. Three supports were investigated, including Celite-545, glass beads, and aluminum oxide. The immobilization of lipase on Celite was achieved according to the method described by Triantafyllou *et al*. (21); lipase (1 g) was suspended in water (8 mL), followed by the addition of Celite (4 g) and stirring. The suspension was then lyophilized and stored at – 20°C.

The adsorption of lipase onto glass beads and aluminum oxide was performed according to the modified method described by Cao *et al.* (22). Lipase (1 g) was suspended in 10 mL of sodium phosphate buffer solution (0.1 M, pH 7.0) and 4 g of glass beads or aluminum oxide was added. After 30 min of gentle stirring at 4°C, the above suspensions were filtered, lyophilized, and stored at –20°C.

The immobilization of lipase by adsorption onto glass beads using chilled acetone as a precipitant was also investigated. Lipase (1 g) was suspended in 10 mL of sodium phosphate buffer solution (0.1 M, pH 7.0), followed by the addition of glass beads (4 g). Ten mL of cold acetone (–20°C) was slowly added to the enzyme suspension under gentle agitation in an ice bath. The suspension was filtered after 30 min and the carrier was recovered. The immobilized enzyme was washed three times with cold acetone and the residual acetone was evaporated under a gentle stream of nitrogen. The enzyme preparation was lyophilized, and stored at –20°C.

Measurement of immobilization effectiveness. To evaluate effectiveness of enzyme adsorption to the carrier, the immobilized enzyme was washed three times with 10 mL of water or cosurfactant-free organic solvent medium (a mixture of sorbitan monostearate "Span-60" and polyoxyethylene sorbitan monostearate "Tween-60" solution in hexane) with a hydrophilic-lipophilic balance (HLB) value of 10. The protein content of the wash solutions was then determined using bovine serum albumin as standard (23).

Lipase hydrolytic assay in aqueous medium. Hydrolytic activity of lipase was assayed in aqueous medium according to the modified method described by Kermasha *et al.* (24). The reaction medium was prepared by mixing 1 mL of sodium chloride (5 mM), 0.1 mL of potassium chloride (0.5 M), 0.3 mL of Tween-20, and 0.1 mL of triacetin with 5 mL phosphate buffer solution (0.1 M, pH 7.0). The final volume of 10 mL was obtained by the addition of deionized water and pre-incubated to 37°C before the addition of the enzyme suspension. The reaction was carried out with 55 mg of immobilized enzyme or 4 mL of wash solution. The reaction mixtures were incubated in Erlenmeyer flasks (50 mL) for 15 min in a reciprocal shaking water bath. The reaction was stopped by adding 20 mL of a mixture of ethanol and acetone (50:50, vol/vol). The liberated free acids were titrated with a 0.005 M solution of sodium hydroxide. The enzymatic assays were performed in triplicate (5% RSD≤, where RSD is relative standard deviation).

Lipase hydrolytic assay in microemulsion system. The microemulsion system was prepared according to the procedure described by Kermasha *et al*. (20). Stock solutions of Span-60 (100 mM) and Tween-60 (3.4 mM) in hexane were prepared and combined (48:52, vol/vol) to produce a cosurfactant-free organic solvent medium with an HLB value of 10. The microemulsion was prepared by suspending substrate (trimyristin, tripalmitin, and/or triolein) in 2 mL of hexane to obtain a concentration of 50 mM and adding 500 µL of the cosurfactant-free organic solvent medium. The appropriate volume of sodium phosphate buffer solution (0.1 M, pH 7.0) was then added, and the final volume of 10 mL was obtained using hexane containing sodium xylenesulfonate (20 mg). In enzymatic assays that used the free enzyme, the enzyme suspension was previously dissolved in the buffer solution; otherwise, the immobilized enzyme was added after the formation of the microemulsion.

The hydrolytic assay was optimized using the microemulsion system described above. The reaction media was incubated for times ranging from 15 to 90 min to determine the period for optimal lipase activity; the water, substrate, and enzyme concentration of the microemulsion was selected to be 1% buffer (vol/vol) (20), 10 mM (25), and 11 mg, respectively. When the optimal reaction time for lipase activity was determined, the enzyme concentration was then varied to investigate the effect of mass transfer on the specific activity. The effect of water content in the reaction was also investigated and optimized. The hydrolytic assay using the microemulsion system was stopped by the addition of 20 mL of a mixture of ethanol and acetone (50:50 vol/vol) and the liberated free acids were titrated with a sodium hydroxide solution (0.005 M).

Lipase-catalyzed interesterification reaction. The interesterification was carried out according to the procedure described previously by Kermasha *et al.* (20). Stock solutions (50 mM) of trimyristin, tripalmitin, and triolein were prepared using the microemulsion system. Tripalmitin was mixed in equimolar concentrations (5 mM) with either trimyristin or triolein to obtain a final concentration of 10 mM in the reaction medium.

The 50 mL flasks containing the microemulsion system (tripalmitin with equimolar concentrations of trimyristin or triolein), as well as the free or immobilized enzyme, were incubated at 37°C and 160 rpm under vacuum. A control containing all the reaction mixture components, minus the enzyme, was run in tandem. Samples (3 mL) were withdrawn from the reaction medium and a mixture (6 mL) of ethanol and acetone (50:50, vol/vol) was added to stop the reaction.

Samples were centrifuged (5 min, 3000 rpm) and the organic phase, containing the fatty acid acylglycerols, was separated and any residual aqueous phase was removed using anhydrous sodium sulfate. The organic solvent was then evaporated under a gentle stream of nitrogen and stored at – 20°C for further analysis.

High-performance liquid chromatography (HPLC) analysis. HPLC analyses of acylglycerols were performed according to the procedure described by Kermasha *et al.* (26). The acylglycerols were separated using a reverse-phase HPLC system (Beckman Model 126, Beckman Instruments, Inc., San Ramon, CA) with an evaporative light-scattering detector ELSD IIA (Varex Corporation, Burtonsville, MD) and computerized integration and data handling. The analysis was performed on two Spherisorb-ODS-2 columns (150 × 4.6 mm i.d., 5 μm) (Alltech Associates, Inc., Deerfield, IL) using a linear gradient elution system of chloroform/acetonitrile (20 to 50% chloroform) at a flow rate of 1 mL/min for 45 min. Detector temperature was set to 120°C and the nitrogen flow rate was 30 mL min^{-1} under a pressure of 22 psi. The injection volume used was 20 μL. The interesterification rate was defined as:

$$R_{inter} = 100 \times ([OOP]_t + [PPO]_t + [OP.]_t)/([OOO]_o + [PPP]_o) \quad [1]$$

where $[OOO]_o$ and $[PPP]_o$ are the initial concentrations of triolein and tripalmitin, respectively, while $[OOP]_t$ and $[PPO]_t$ are the concentrations of transesterified triacylglycerols and $[OP.]_t$ is the concentration of oleoyl palmitoyl glycerol, at a given time. The hydrolytic rate was defined as the molar percentage of ester bonds hydrolyzed:

$$R_{hydro} = 100 \times (2\,[O..]_t + 2[P..]_t + [OO.]_t + [PP.]_t + [OP.]_t)/([OOO]_o + [PPP]_o) \quad [2]$$

where $[OOO]_o$ and $[PPP]_o$ are the initial concentrations of triolein and tripalmitin, respectively, while $[O..]_t$ and $[P..]_t$ are the concentrations of monoacylglycerols, and $[OO.]_t$, $[PP.]_t$ and $[OP.]_t$ are the concentrations of diacylglycerols at a given time.

RESULTS AND DISCUSSION

Immobilization of Lipase N. Table 1 shows the immobilization efficiency of Celite 545, glass beads, and aluminum oxide. These supports were selected on the basis of their inertness and nontoxicity as well as their use by numerous investigators for interesterification (27). The results show that 96% of the protein content was immobilized on Celite and showed a similar specific hydrolytic activity to that of the free enzyme. The results also indicate that the immobilization of lipase on glass beads by acetone precipitation retained 70% of the protein content, of which 96% exhibited a similar hydrolytic activity to that of the free enzyme. Similar results were reported by Wisdom *et al.* (28), who showed that immobilization of lipase from *R. arrhizus* on Hyflo-Supercel retained only 50% of the protein content and recovered almost 100% of the free enzyme solution.

However, Table 1 also shows that the adsorption of lipase onto glass beads and aluminum oxide retained only 47 and 42% of the protein content, respectively, with a 44% recovery of the specific hydrolytic activity compared to that of the free enzyme. The comparison of glass bead immobilization, with and without the use of acetone, showed that the former fixed higher amounts of protein onto the support. These results suggest that the addition of chilled acetone into the free enzyme suspension forces its adsorption onto the carrier without adverse effects on its activity. The overall results indicate that immobilization by physical adsorption was more effective using Celite or glass beads combined with acetone precipitation, whereas that obtained with glass beads without acetone precipitation and aluminum oxide immobilization exhibited a relative low percentage of adsorbed enzyme.

Table 2 shows that more than 90% of the lipase immobi-

TABLE 1
Immobilization of Lipase N on Different Carriers

Carrier	Protein[a]	Hydrolytic activity[b]	Immobilization efficiency[c]
Free enzyme	0.380	9.5	—
Glass beads			
Support	0.170	4.0	45
Filtrate	0.200	4.8	
Glass beads with acetone precipitation			
Support	0.360	9.0	70
Filtrate	0.009	0.4	
Celite 545			
Support	0.380	9.2	96
Aluminum oxide			
Support	0.150	3.9	40
Filtrate	0.220	5.3	

[a]Amount of mg protein per mg free enzyme or mg immobilized enzyme.
[b]Hydrolytic activity was defined as meq acid per mg protein per min.
[c]Immobilization efficiency was determined as the relative percentage of adsorbed enzymatic protein with respect to that present in the free enzyme.

TABLE 2
Effect of Washing on the Release of Immobilized Enzymes

Carrier/wash medium	Protein[a]	Hydrolytic activity[b]	Immobilization efficiency[c]
Glass beads			
Aqueous medium			
Support	nd[d]	nd[d]	5
Filtrate	0.366	8.9	
Microemulsion medium[e]			
Support	0.360	8.9	100
Filtrate	nd[d]	nd[d]	
Celite 545			
Aqueous medium			
Support	0.038	0.7	10
Filtrate	0.345	8.6	
Microemulsion medium[e]			
Support	0.378	8.8	100
Filtrate	nd[d]	nd[d]	

[a]Amount of mg protein per mg free enzyme or mg immobilized enzyme.
[b]Hydrolytic activity was defined as meq acid per mg protein per min.
[c]The immobilization efficiency was determined as the relative percentage of adsorbed enzymatic protein with respect to that present in the free enzyme.
[d]Not detected.
[e]The microemulsion medium consisted of a mixture of sorbitan monostearate and polyoxyethylene sorbitan monostearate solution in hexane with a hydrophilic-lipophilic balance value of 10.

lized on Celite or glass beads was released when the carriers were washed with deionized water or phosphate buffer solution; the removal of lipase from the glass bead and Celite carriers was indicated by the high protein content and residual hydrolytic activity in the filtrate solutions. These findings suggest that the interaction between the support carrier and the enzyme was relatively weak. The adsorption of an enzyme onto a carrier is dependent on experimental variables such as pH, the nature of solvent, ionic strength, concentration of enzyme and adsorbent, and temperature. A close control of these variables is required for optimal adsorption and retention of activity, owing to the relatively weak binding forces between protein and adsorbent (29).

The results (Table 2) also suggest that the enzyme was not denatured by the immobilization process, as the hydrolytic activity of the free enzyme (9.5 meq acid/mg protein/min) was close to that obtained in the residual wash solutions from glass beads (8.9 meq acid/mg protein/min) and Celite (8.6 meq/mg protein/min). In the case of physical adsorption, the forces responsible for the immobilization include hydrogen bonding, van der Waals forces, and hydrophobic and ionic interactions; since no reactive species are involved, there are little or no conformational changes in the enzyme on immobilization (30).

Table 2 shows that almost all of the enzyme was retained on the glass bead and Celite supports, as indicated by the relatively high protein content and hydrolytic activity, when the immobilized enzyme was washed with the cosurfactant-free organic solvent medium (a mixture of Span-60 and Tween-60 in hexane with an HLB value of 10). These findings suggest that the enzyme remained associated with the support after adsorption, thereby indicating that the enzyme was unable to dissolve in a nonaqueous organic solvent for which it has little affinity.

Determination of specific activity. Figure 1 shows that the specific hydrolytic activity of free and glass bead or Celite-immobilized Lipase N reached a maximum at 15 min using trimyristin, tripalmitin, or triolein as substrates. In addition, the results (Figs. 1A and 1B) show that the free and glass bead-immobilized enzymes exhibited similar hydrolytic activities using trimyristin and tripalmitin, while the Celite- immobilized enzyme exhibited considerably lower activity. The results (Fig. 1C) also show that the free and Celite-immobilized lipases showed similar activities toward the longer-chain fatty acid triolein, while the glass bead-immobilized lipase exhibited lower activity in the first 20 min of reaction. These findings suggest that the hydrophobic glass beads seem to be a more efficient support for the hydrolysis of the two saturated triacylglycerols, trimyristin and tripalmitin, while the hydrophilic Celite carrier is more likely to attract the relatively more polar triolein.

These overall findings suggest that the kinetic behavior of lipase from *R. niveus* adsorbed to a charged or hydrophobic support may differ from that observed for the free enzyme. This could be due to the fact that the concentration of the substrates, products, and hydrogen and other ions in the environment of the immobilized enzyme could be different from that in the outer solution, owing to the electrostatic interactions with the fixed charges on the support, which produce a partitioning effect (29).

Effect of enzyme concentration. Figures 2A and 2B show that the specific hydrolytic activity of the free and glass bead-immobilized lipases at different enzyme concentrations were similar using trimyristin and tripalmitin as substrates, while

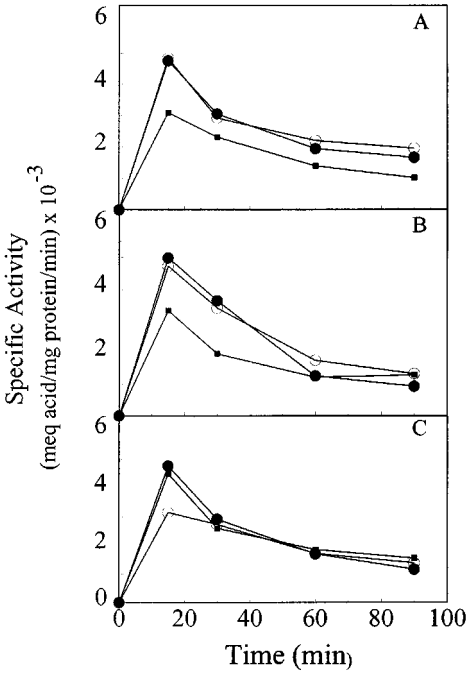

FIG. 1. Effect of time on the specific hydrolytic activity of the free (●) and immobilized *Rhizopus niveus* lipase on glass beads (○) and Celite (■), using trimyristin (A), tripalmitin (B), and triolein (C) as substrates.

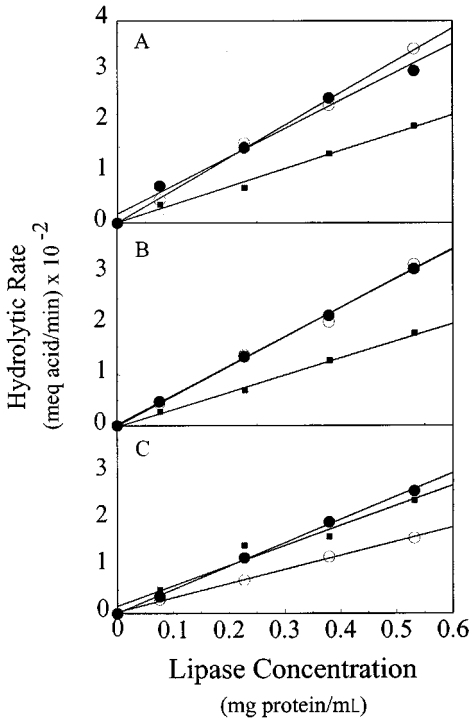

FIG. 2. Effect of enzyme concentration on the specific hydrolytic activity of the free (●) and immobilized *R. niveus* lipase on glass beads (○) and Celite (■), using trimyristin (A), tripalmitin (B), and triolein (C) as substrates. For abbreviations see Figure 1.

that of the Celite-immobilized lipase decreased dramatically, by approximately 40%. In contrast, Figure 2C demonstrates that the specific hydrolytic activity of the glass bead-immobilized enzyme decreased using triolein as substrate, while that of the Celite-immobilized enzyme was similar to that obtained with the free enzyme.

The overall results indicate that the hydrolytic rate increased with increasing enzyme concentration, approaching a saturation value asymptotically at higher enzyme concentrations, with a following decrease in specific activity. These results are similar to those reported for kinetically controlled reactions (31,32), thereby suggesting that the mass transfer rate of the substrates was not a limiting factor in the enzymatic activity. A major influence on the activity of lipase adsorbed to a solid support is the enzyme concentration exposed to the unit surface of carrier during the immobilization process. When the diffusion rate of substrate is slower than its rate of transformation by the immobilized enzyme, the observed reaction rate is lower than that of a given amount of free enzyme, since not all enzyme molecules are in contact with the substrate at a concentration similar to that of the bulk solution. However, the results suggest that the external mass transfer between the bulk phase of the reaction mixture and the surface of the support may have been increased by the vigorous 150 rpm agitation during the reaction, as well as by the addition of surfactants, resulting in an increase in the interfacial area between the enzyme and the substrates. These findings show that immobilization of lipase using glass beads and Celite as carriers was efficient with increasing enzyme concentrations (29).

Effect of water content. Figure 3 shows that the specific hydrolytic activity of lipase was largely altered by the presence of different concentrations of water in the microemulsion. The results (Figs. 3A and 3B) show that the free and glass bead-immobilized lipases exhibited similar activities using trimyristin and tripalmitin as substrates, while that of Celite demonstrated an overall lower specific activity. In addition, lipase activity increased with increasing water concentrations, thereby suggesting that water is involved as a co-substrate in the hydrolytic reaction. The results (Fig. 3C) also indicate that the free and Celite-immobilized enzymes showed similar activities with triolein as substrate at water contents up to 4 mmol, while that of the glass bead-immobilized lipase showed approximately 40% less activity. These results suggest that the decrease in the specific hydrolytic activity for the glass bead- and Celite-immobilized lipases at water concentrations of 5.5 mmol and above could be due to the limited interaction of the substrate with the enzyme. Mass transfer effects, i.e., external diffusional resistances could arise when the substrate is transported from the bulk solution to the enzyme surface across a boundary of liquid and internal diffusional resistances inside the porous catalytic medium (29).

Miller *et al.* (31) reported that the water layer around the enzyme must be thin enough to permit substrate diffusion. At high water concentrations, the water layer may be too big to permit diffusion of hydrophobic substrates, thereby resulting in a decrease in activity such as that observed using the hydrophilic Celite support, which is more likely to attract water.

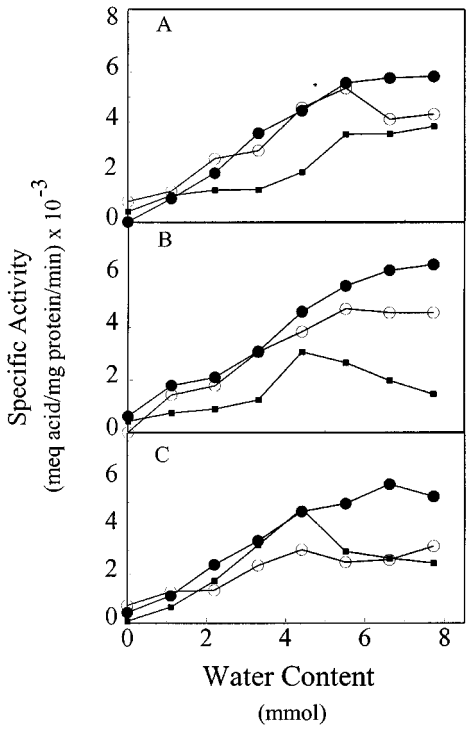

FIG. 3. Effect of water content on the specific hydrolytic activity of the free (●) and immobilized *R. niveus* lipase on glass beads (○) and Celite (■), using trimyristin (A), tripalmitin (B), and triolein (C) as substrates. For abbreviations see Figure 1.

The Celite support is more porous than the glass bead support, so a smaller quantity of water would be required to close the opening of the pores to the substrate.

Monot *et al.* (33) reported that water plays several roles in enzyme structure and function: (i) action on enzyme structure by contribution to all noncovalent bonding, (ii) alteration of protein structure by disruption of hydrogen bonds, (iii) facilitation of reagent diffusion, and (iv) participation in the equilibrium constant where water is a substrate or product. The differences in the specific hydrolytic activity of the free and immobilized lipases could also be due to the distribution of water in the vicinity of the enzyme. Hydrophilic carriers such as Celite cause desorption of water from the enzyme, while hydrophobic carriers expel interfering water from the environment of the enzyme (34). The difference in the distribution of water could enhance the hydrolysis of hydrophobic or hydrophilic substrates, depending on the amount of water present in the microenvironment, and therefore an important layer of water around the lipase could produce a higher resistance as the hydrophobicity of the substrate increases.

Kinetic parameters. Table 3 shows the kinetic parameters, obtained from the Lineweaver-Burk plots, for the free and glass bead- or Celite-immobilized lipase. The results indicate that the glass bead-immobilized lipase demonstrated the highest affinity towards trimyristin, tripalmitin, and triolein, as indicated by its low K_m values of 4.2, 1.4, and 5.2 mM, respectively, as well as the highest efficiency, as indicated by its relatively high V_{max}/K_m values of 2.0, 3.6, and 1.4, respectively, in comparison to the free and Celite-immobilized enzymes. Miller *et al.* (31) reported K_m and V_{max} values of 7.8 mM and 4.4 μM mg protein^{-1} min^{-1}, respectively, for the lipase activity from *C. cylindracea* in the hydrolysis of trilaurin in cyclohexane. In addition, the lipase activity from *R. oryzae* (35) exhibited K_m and V_{max} values of 105 mM and 72 μM mg protein^{-1} min^{-1}, respectively, for the hydrolysis of triolein; the enzyme was immobilized on pore-controlled glass in a two-phase system containing high proportions of aqueous solvent.

The results (Table 3) also show that the Celite-immobilized enzyme exhibited a higher affinity toward trimyristin and tripalmitin as substrate in comparison to the free enzyme; however, the overall efficiency of the Celite-immobilized enzyme was similar to that of the free lipase. The findings indicate that the K_m values for the free and Celite-immobilized enzymes decreased dramatically as the carbon length of the acyl moieties increased, which is characteristic of lipases.

Table 3 also shows that the kinetic parameter of catalytic efficiency of the enzyme is more important for the hydrophobic glass bead support than the hydrophilic Celite. In addition, the results indicate that immobilization produced a greater change in the kinetic parameters when trimyristin and tripalmitin were used as substrates in comparison to that obtained with triolein. These variations could be attributed to an alteration in the conformation of the enzyme due to immobilization, thereby producing a change in the specificity and activity of the lipase (36). These modifications could also be due to the partitioning effects between the enzyme fixed to the support and the substrates; the K_m value of an immobilized enzyme could decrease if the charges on the support and substrates are opposite; this could produce an increase in the electrostatic attractive forces between the carrier and the substrate, resulting in an increase in the concentration of the substrate in the microenvironment of the immobilized enzyme (29).

TABLE 3
Kinetic Parameters of Immobilized Lipase N Using Different Supports

	Substrate					
	Trimyristin		Tripalmitin		Triolein	
Carrier	K_m[a]	V_{max}/K_m[b]	K_m[a]	V_{max}/K_m[b]	K_m[a]	V_{max}/K_m[b]
Free enzyme	47.8	0.6	32.7	0.6	7.1	1.1
Glass beads	4.2	2.0	1.4	3.6	5.2	1.4
Celite 545	12.2	0.9	9.1	0.6	7.9	0.9

[a]The K_m value is expressed as mM.
[b]The V_{max}/K_m value is expressed as the catalytic efficiency.

TABLE 4
Interesterification by Lipase Activity from *Rhizopus niveus* Using Trimyristin and Tripalmitin as Substrates

Reaction time (h)	Interesterification		Hydrolytic			
	Rate (%)[a]	Trimyristin (%)[b]	Rate (%)[c]	Mono-[d]	Di-[d]	Mixed-[e]
				Acylglycerols		
	STD[f]	STD[f]	STD[f]	STD[f]	STDf	STD[f]
Free enzyme						
24	0.0 ± 0.0[c]	0.0 ± 0.0	30.4 ± 6.0	21.1 ± 1.8	11.2 ± 2.5	0.0 ± 0.0
36	39.0 ± 4.4	48.1 ± 0.3	14.6 ± 4.1	5.4 ± 1.0	22.6 ± 1.6	23.8 ± 2.5
48	52.7 ± 0.2	45.2 ± 0.2	15.1 ± 3.0	4.2 ± 1.7	21.3 ± 1.2	24.4 ± 0.5
60	55.9 ± 4.1	44.3 ± 1.5	20.3 ± 2.0	2.4 ± 1.2	20.9 ± 1.8	24.4 ± 0.7
Glass bead						
24	18.6 ± 1.8	50.0 ± 0.3	13.5 ± 1.1	10.4 ± 2.1	26.4 ± 4.6	18.6 ± 1.1
36	31.2 ± 3.2	48.8 ± 0.1	13.5 ± 1.5	9.2 ± 2.8	18.0 ± 1.7	21.5 ± 2.0
48	36.0 ± 1.2	45.8 ± 0.3	11.9 ± 3.7	6.2 ± 2.0	22.4 ± 1.5	20.9 ± 0.1
60	48.5 ± 3.0	45.3 ± 2.0	11.1 ± 2.7	1.6 ± 1.4	24.9 ± 0.7	20.4 ± 2.5
Celite 545						
24	13.6 ± 3.0	59.2 ± 1.9	6.5 ± 2.1	14.0 ± 4.3	12.8 ± 3.0	10.0 ± 2.8
36	37.0 ± 5.4	48.3 ± 1.8	9.0 ± 3.0	6.3 ± 2.0	1.6 ± 2.4	20.4 ± 2.6
48	45.1 ± 4.3	45.8 ± 3.0	23.0 ± 4.2	5.1 ± 3.2	7.5 ± 0.6	20.1 ± 0.3
60	59.6 ± 3.1	45.3 ± 3.4	20.1 ± 3.8	3.2 ± 2.1	23.1 ± 3.2	28.6 ± 4.4

[a]The interesterification rate was defined as: $R_{inter} = 100 \times ([MMP]_t + [PPM]_t + [MP]_t)/([MMM]_o + [PPP]_o)$, where $[MMM]_o$ and $[PPP]_o$ are the initial concentrations of trimyristin and tripalmitin, respectively, $[MMP]_t$ and $[PPM]_t$ are the concentrations of transesterified triacylglycerols, and $[MP]_t$ is the concentration of myristoyl, palmitoyl glycerol, at a given time.
[b]Percentage of dimyristoyl, palmitoyl glycerol divided by the total transesterified acylglycerols, multiplied by 100.
[c]The hydrolytic rate was defined as the molar percentage of ester bounds hydrolyzed: $R_{hydro} = 100 \times (2\ [M..]_t + 2[P..]_t + [MM.]_t + [PP.]_t + [MP]_t)/([MMM]_o + [PPP]_o)$, where $[MMM]_o$ and $[PPP]_o$ are the initial concentrations of trimyristin and tripalmitin, respectively, $[M..]_t$ and $[P..]_t$ are the concentrations of monoacylglycerols, and $[MM.]_t$, $[PP.]_t$, and $[MP.]_t$ are the concentrations of diacylglycerols at a given time.
[d]Percentage of mono- and diacylglycerols divided by total acyl glycerols at the beginning of the reaction, multiplied by 100.
[e]Percentage of palmitoyl, myristoyl glycerol divided by total transesterified acylglycerols, multiplied by 100.
[f]Standard deviation.

Interesterification reactions. Table 4 indicates that, during the first 24 h of the reaction, an increase in the specific hydrolytic activity of the free enzyme towards the mixture of trimyristin and tripalmitin was followed by a concomitant decrease and increase in the hydrolytic and interesterification rates, respectively. In addition, the results indicate that the percentage of monoacylglycerols was relatively high at the beginning of the reaction, when the rate of hydrolysis was predominant, and decreased progressively as the rate of interesterification increased. The results also show that the ratio of diacylglycerols decreased after 24 h of the reaction and became relatively stable after 48 h.

Safari and co-workers (19) reported similar results for the interesterification of butter. Macrae (3) suggests that the initial high hydrolytic activity rate may be due to the presence of water as a substrate in the reaction, resulting in the formation of a large amount of diacylglycerols and free fatty acids; once the ratio of diacylglycerols and free fatty acids reaches a certain concentration and water content is below a critical value, the interesterification reaction becomes more predominant than hydrolysis.

In contrast, Table 4 shows that the interesterification of trimyristin and tripalmitin by glass bead- and Celite-immobilized lipases occurred during the first 24 h. These findings suggest that immobilization of lipase produced an increase in the enzyme's affinity toward the substrates trimyristin and tripalmitin, resulting in a higher rate of hydrolysis which in turn produced a decrease in the water concentration in the vicinity of the enzyme so that the interesterification reaction became more predominant.

In comparison, the results (Table 5) indicate that the rate of hydrolysis of the mixture of tripalmitin and triolein by free and glass bead- or Celite-immobilized lipases was higher than that reported for the mixture of trimyristin and tripalmitin. The results show that the hydrolytic rate of the free enzyme decreased gradually during the 60 h of reaction, while that of the glass bead- and Celite-immobilized lipases decreased after 36 and 48 h, respectively. Table 5 also shows that the rate of interesterification of the mixture of tripalmitin and triolein was higher than that for the mixture of trimyristin and tripalmitin. These findings suggest that the increase in the rate of the interesterification reaction could be related to the associated hydrolytic activity, which may result in a concomitant decrease in the water concentration as well as an increase in the amount of free fatty acids.

The results (Table 5) also show that the interesterification rate increased by 50% for the free lipase between 24 and 48 h, while a longer reaction time was needed to reach maximal activity for the glass bead- or Celite-immobilized enzyme. These findings suggest that the rate of interesterification was more important for the free enzyme than for the immobilized one. However, the results demonstrate that similar interesteri-

TABLE 5
Interesterification by Lipase Activity from *R. niveus* Using Tripalmitin and Triolein as Substrates

Reaction time (h)	Interesterification		Hydrolytic			
	Rate (%)[a]	Triolein (%)[b]	Rate (%)[c]	Mono-[d]	Di-[d]	Mixed-[e]
				Acylglycerols		
	STD[f]	STD[f]	STD[f]	STD[f]	STDf	STD[f]
Free enzyme						
24	36.3 ± 3.3	62.0 ± 3.5	36.8 ± 3.1	14.3 ± 3.7	11.2 ± 2.5	14.9 ± 3.0
36	43.5 ± 0.5	62.2 ± 5.0	30.6 ± 3.7	9.5 ± 2.3	22.6 ± 1.7	14.6 ± 2.2
48	47.0 ± 2.7	59.4 ± 1.1	29.9 ± 5.3	4.9 ± 1.3	21.3 ± 1.2	22.5 ± 3.3
60	47.5 ± 1.9	57.8 ± 2.1	28.9 ± 2.4	4.0 ± 2.1	20.9 ± 1.9	22.3 ± 1.4
Glass bead						
24	30.6 ± 0.5	62.2 ± 0.6	23.3 ± 0.6	13.9 ± 0.9	12.8 ± 3.0	18.7 ± 2.2
36	34.8 ± 0.9	59.9 ± 0.5	43.2 ± 0.2	10.5 ± 1.2	20.6 ± 2.5	15.3 ± 0.7
48	38.2 ± 0.4	59.3 ± 0.4	36.2 ± 1.7	5.1 ± 4.1	14.4 ± 0.6	18.7 ± 1.9
60	45.3 ± 2.5	59.6 ± 0.5	26.8 ± 0.8	4.2 ± 1.3	23.1 ± 3.3	23.8 ± 0.1
Celite 545						
24	32.5 ± 2.7	61.1 ± 0.3	33.7 ± 6.5	20.1 ± 7.4	26.5 ± 3.0	16.7 ± 6.3
36	40.0 ± 0.3	60.7 ± 2.1	30.7 ± 1.9	9.0 ± 4.2	18.0 ± 2.7	19.2 ± 4.4
48	41.4 ± 0.5	60.2 ± 1.2	39.5 ± 1.2	7.5 ± 2.7	22.5 ± 3.5	19.3 ± 2.2
60	45.0 ± 0.6	59.1 ± 0.8	31.2 ± 0.2	9.2 ± 0.6	24.9 ± 0.9	20.6 ± 0.7

[a]The interesterification rate was defined as: $R_{inter} = 100 \times ([OOP]_t + [PPO]_t + [OP.]_t)/([OOO]_o + [PPP]_o)$, where $[OOO]_o$ and $[PPP]_o$ are the initial concentrations of triolein and tripalmitin, respectively, $[OOP]_t$ and $[PPO]_t$ are the concentrations of transesterified triacylglycerols, and $[OP.]_t$ is the concentration of oleyl, palmitoyl glycerol, at a given time.
[b]Percentage of dioleoyl, palmitoyl glycerol divided by the total transesterified acylglycerols, multiplied by 100.
[c]The hydrolytic rate was defined as the molar percentage of ester bounds hydrolyzed: $R_{hydro} = 100 \times (2\ [O..]_t + 2[P..]_t + [OO.]_t + [PP.]_t + [OP]_t)/([OOO]_o + [PPP]_o)$, where $[OOO]_o$ and $[PPP]_o$ are the initial concentrations of triolein and tripalmitin, respectively, $[O..]_t$ and $[P..]t$ are the concentrations of monoacylglycerols, and $[OO.]_t$, $[PP.]_t$, and $[OP.]_t$ are the concentrations of diacylglycerols at a given time.
[d]Percentage of mono- and diacylglycerols divided by total acyl glycerols at the beginning of the reaction, multiplied by 100.
[e]Percentage of palmitoyl, oleoyl glycerol divided by total transesterified acylglycerols, multiplied by 100.
[f]Standard deviation.

fication rates were reached by free and glass bead- or Celite-immobilized enzymes after 60 h. These findings may indicate that the immobilization of the enzyme by physical adsorption resulted in little or no alteration in its conformation, thereby retaining its activity.

An enhancement of enzyme activity in organic solvents and its immobilization on specific carriers was reported for the lipases of *Penicillium expansum* (37), *R. delemar* (27), and *M. miehei* (38); this increase could be explained by an increase in the enzyme dispersion (37) in the surrounding aqueous layer, which is essential for its stabilization and activity as well as to the increase in its resistance against distortion (39). The selection of an immobilization process for a lipase depends on the nature of its protein in comparison to that of the other proteins present in the enzyme preparation.

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