The Role of Silica Gel in Lipase-Catalyzed Esterification Reactions of High-Polar Substrates

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ABSTRACT: The crucial role of silica gel in lipase-catalyzed esterification reactions using adsorbed high-polar substrates was established. It was found that in these kinds of reactions, the presence of silica gel-adsorbed substrates did not alter the kinetic mechanisms of the synthesis of acylglycerides but improved significantly the conversion yields. An explanation for the critical role of silica gel in these kinds of reactions was proposed whereby the silica gel behaves as a "polar substrate reservoir" and plays a protective role for the immobilized enzyme avoiding its blockage. In this way it was shown that the use of silica gel facilitates reactions at high glycerol concentrations without alteration of reaction rates or conversion yields. It also was demonstrated that highly hydrophilic polyols enhanced blockage of the enzyme. The importance of the presence of an organic solvent in the reaction medium was shown to have a critical role in the conversion yields of the reactions tested. Finally, the influence of different supports used for adsorption of the substrates was compared with respect to their efficiency in protecting the enzymatic activity.
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KEY WORDS: Diolein, glycerol, *n*-hexane, lipase-catalyzed esterification, lipase specificity, monoolein, silica gel, solvent-free systems, triolein, tailor-made synthesis.

The use of mono- and diacylglycerides as nonionic emulsifiers in the food and pharmaceutical industries as well as their utilization as synthetic intermediates in the chemical industry has been growing in recent years (1,2). In the food industry, for example, they have important potential applications in altering the melting range of food formulations and as a texturizing agent in different products (3). Generally, from an industrial point of view, they are chemically synthesized at high temperatures (200–250°C) under controlled atmospheres (nitrogen) and using several metallic catalysts (1,4). The substrates utilized in these reactions are generally glycerol and saturated fatty acids (5,6). In some papers, sugars such as sucrose and fructose also have been reported to be useful substrates for the synthesis of these kinds of emulsifiers (7). However, the extreme conditions and the nonspecificity of these chemical reactions lead to important problems for industrial synthesis of surfactants. In some cases, these result in the formation of free radicals from unsaturated free fatty acids and consequently the generation of toxic by-products.

To solve the above-mentioned problem, several alternative methods have been proposed. For example, the enzymatic hydrolysis and alcoholysis of oils and fats were reported in microemulsion systems (8,9). Since the early 1980s the use of enzymes in nonaqueous media, as a tool in organic synthesis, has been extensively studied (10–12). It has been reported, for example, that hydrolases in a nearanhydrous organic medium are able to catalyze reverse reactions in order to esterify alcohols and fatty acids (13,14). The low optimal temperatures at which these reactions were carried out (40–50°C), as well as the high regio- and stereoselectivity of the enzymes, render these enzymatic esterification reactions a very attractive way to synthesize acylglycerides. Hence, the synthesis of food emulsifiers with a desired hydrophilic-lipophilic balance (HLB) value can be accomplished as a result of the high specificity of enzymes and their capacity to reverse hydrolytic reactions. In recent years, many research groups have attempted to develop an enzymatic method for the synthesis of tailor-made pure acylglycerides with very specific properties (15–18).

Other researchers have been engaged in carrying out enzymatic reactions through direct condensation of glycerol or sugars with fatty acids in different reaction media. For example, the use of aqueous-organic two-phase systems or microemulsions has been reported (19,20) as well as the use of nearanhydrous organic systems or solvent-free reactions (21,22). In almost all reports, there are two main problems. First is the difficulty or even the impossibility of dissolving the polar substrates in an organic solvent, and second is the increased risk of coating the powdered or immobilized lipase when using high concentrations of oil-insoluble glycerol in a hydrophobic medium (9). To solve the solubilization problem, the use of 1,2-*O*-isopropylidene-*rac*-glycerol as a solubilization agent of glycerol for the production of monoacylglycerides has been suggested (6,16,17). However, as reported by Akoh (17), one of the main drawbacks of this approach in the industrial synthesis of biosurfactants is the extra step of making and recovering the isopropylidene-glyc-

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erol molecule. In 1972 Ferrier (23) reported an alternative method for solubilization of polyols using acylboronic acids, which form soluble complexes in organic solvents. Such systems have been reported as allowing the enzymatic esterification of mono- and disaccharides with fatty acids as well as acylglyceride production using lipases as catalysts (18, 24–27). Nevertheless, such methods will become industrially interesting only when an efficient method is developed for recovering the complexed substrates used and products formed.

In 1992, Berger *et al.* (15,28,29) reported a novel method for making enzymatic catalysis possible in a nonconventional medium using high-polar molecules as substrates. In their work, they adsorbed solvent-immiscible substrates, such as ethylene glycol and glycerol, onto a solid support of silica gel. Next, the silica gel-adsorbed glycerol was enzymatically esterified with various free fatty acids in the presence of immobilized Lipozyme™ as catalyst. This lipase-catalyzed esterification was found to be very efficient in producing mono- and diacylglycerols using *n*-hexane, diethyl ether or tBuOMe as solvents. To explain the role of silica gel in these reactions, they suggested the formation of an interfacial liquid-liquid interface generally assumed to be involved in lipase-catalyzed transformation of glycerides. Berger's method was also successfully tested by Castillo *et al.* (18) by performing enzymatic esterification of glycerol and fructose in supercritical carbon dioxide ($SCCO_2$). $SCCO_2$ was used as a hydrophobic solvent and because of its postreactional qualities for separation, esterification reactions were performed. In this way, high conversions of both polar substrates and oleic acid were obtained in $SCCO_2$ and no substrate limitations were found in the concentration range of the polar substrates used. In 1995, Charlemagne and Legoy (30) reported the enzymatic synthesis of polyglycerol-fatty acid esters in a solvent-free system using silica gel as a support for different polar polyglycerols. Using silica gel-adsorbed polyglycerols, they found that the time to reach the thermodynamic equilibrium and the reaction yields were clearly improved when compared to reactions carried out with nonadsorbed polyglycerols. Rather than the existence of an interface phenomenon, they proposed that the hydroxyl groups at the surface of the silica gel allow the formation of hydrogen bonds with the –OH groups of polyols, which lowered the polarity of adsorbed molecules and increased the miscibility with the lipid phase. They suggested that this increase in miscibility of polyols in organic solvents allows almost total conversion of the substrate. More recently, Kwon *et al.* (31), using the same principle, suggested a method to simplify the immobilization step of glycerol by direct addition of a solid support into the reaction medium containing the polar substrate. Presumably, this modification of the original method avoided the previously required immobilization step of the substrate. Similarly to Berger *et al.* (15,28) the use of polar substrates immobilized onto silica gel was shown to cause a significant increase in fatty acid conversion. They proposed that an optimal increase in this interfacial surface between hydrophilic glycerol and hydrophobic organic solvent largely improved the fatty acid conversions. Furthermore, they also proposed that the improvement in the conversion rates was favored by the silica gel adsorption of water produced during the esterification reactions. Finally, Stevenson *et al.* (9) took advantage of the method proposed by Berger *et al.* (28,29) to recover produced glycerol from alcoholysis reactions.

In consideration of all the hypotheses to explain the role of silica gel in the enzymatic synthesis of acylglycerides, no complete mechanism has been proposed to understand the availability of the polar substrates for these esterification reactions. In this paper, lipase-catalyzed reactions of silica gel-adsorbed substrates and oleic acid in *n*-hexane are presented as a reaction model. We specifically focused attention on the mechanisms involved in the utilization of silica gel as pseudo-solubilizing agent together with the principal phenomena associated with this process. Finally, we present the reaction conditions under which the use of silica gel is advantageous.

MATERIALS AND METHODS

Materials. Free and immobilized lipases from *Mucor miehei,* Lipozyme™ 10,000 L, and Lipozyme™ IM-46 (IME), respectively, were kindly offered by Novo Nordisk Industry A/S (Bagsvaard, Denmark). In reactions carried out with free Lipozyme™, the commercial enzyme preparation was diluted in 25 mM potassium phosphate buffer at pH 7.0 and lyophilized before utilization. Glycerol, oleic acid (*cis*-9-octadecanoic acid), 1(3)-monoolein, 1,3-diolein, 1,2-diolein, triolein, 1,3-propanediol, and silica gel (mesh 70–230) were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest available purity. All high-purity solvents used were purchased from Prolabo (Paris, France), the enzymatic-UV kit for the analysis of soluble glycerol in *n*-hexane was purchased from Boehringer Mannheim France S.A (Meylan, France) and the resin Duolite™ A568 was purchased from Rohm and Haas (Paris, France).

Bioconversions. Substrates were adsorbed as described by Berger et al. (28) and Castillo *et al.* (18): 1 g of glycerol or 1,3-propanediol and 1 g of silica gel were carefully mixed until a homogeneous powder was obtained. In the case of the use of Duolite™, 1 g of powder was mixed with the glycerol and prepared similarly to silica gel-adsorbed substrates. In typical reactions, 1 g of silica gel-adsorbed substrate and 100 mg of Lipozyme™ were mixed in an assay tube containing a 50-mM solution of oleic acid in *n*-hexane (10 mL). In the case of nonadsorbed substrates, 0.5 g of glycerol was used. The reaction mixtures were shaken at 40°C and several samples of the reaction (50 μL) were withdrawn and analyzed by high-performance liquid chromatography (HPLC). For the reactions in a solvent-free medium the same procedure was followed without the use of *n*-hexane.

HPLC analysis. The *n*-hexane from the reaction samples was exhaustively evaporated in a thermostated bath at 80°C and resuspended in an equal volume of HPLC solvent. In the case of esterification of glycerol, analysis of recovered samples were performed using an integrated system HP 1050 se-

ries II (Hewlett Packard, Atlanta, GA) with a C_{18} reverse phase analytical column Spherisorb ODS-2 (5µ, 250 × 4.6 mm) thermostated at 45°C. Products and reactants were quantified at a wavelength of 210 nm with a detector UV/VIS HP 1050 series II. The eluent employed was acetone:acetonitrile (50:50, vol/vol) mixture, isocratically run. Elution of products was made with a flow gradient: 0.5 mL/min for 7 min, then increased in 3 min up to 3 mL/min and held for 6 min before decreasing to 0.5 mL/min in 4 min as previously described by Ergan and André (32). In the case of the esterification of 1,3-propanediol, samples were also analyzed using a HP 1050 series II (Hewlett Packard), with the same C_{18} column and a HP 1047A refractive index detector (Hewlett Packard). Elution of products was made at 50°C with methanol/acetic acid (99.7:0.3, vol/vol) and a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Effect of the use of silica gel in glycerol esterification reactions. In order to show the effect of silica gel on this kind of reaction, a model lipase-catalyzed reaction between the glycerol and oleic acid was chosen, and the feasibility of an enzymatic esterification reaction of silica gel-adsorbed glycerol and fatty acids in organic solvents was tested. These preliminary reactions were carried out as described for typical reactions and monitored for 30 h. The results obtained were compared with a control reaction that was carried out with nonadsorbed glycerol. Figure 1 shows the progress of the reaction using silica gel-adsorbed glycerol and the control reaction.

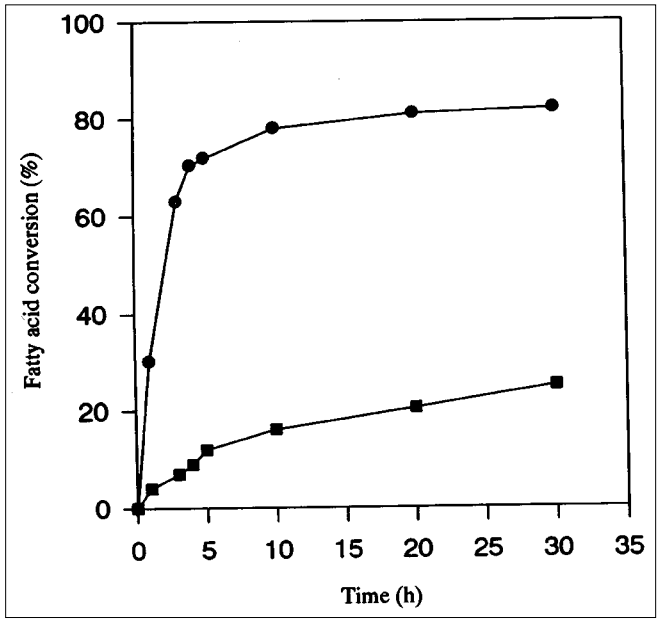


FIG. 1. Effect of silica gel on the esterification of glycerol with oleic acid, catalyzed by immobilized Lipozyme™. The reactions were carried out in a 10-mL assay tube containing 100 mg immobilized Lipozyme™ and 50 mM oleic acid solution in *n*-hexane. In the reaction with silica gel (●) and in the reaction without silica gel (■), 1 g silica gel-adsorbed glycerol and 0.5 g free glycerol were, respectively, used as substrates.

We can observe on this graph that the oleic acid conversion rate is clearly improved when the reaction is carried out using silica gel as a support for glycerol. Conversion of 75% of oleic acid was obtained in the first 4 h of reaction and a maximal conversion of 84% was achieved after 30 h of reaction. In contrast, in the control reaction with nonadsorbed polar substrate, a very slow reaction rate and a very low final conversion of oleic acid of about 20% were observed. In the case of the reaction using silica gel-adsorbed glycerol the profiles of the synthesis of mono-, di-, and triolein and the consumption of oleic acid are presented in Figure 2.

A rapid production of 1(3)-monoolein and 1,3-diolein with a fast consumption of oleic acid in the first 5 h of reaction is observed. Moreover, in the first step of this reaction, no significant production of triolein is detected. Once 1-(3)-monoolein and 1,3-diolein achieve their maximal concentration (4.75 mM and 13.5 mM, respectively) and the consumption rate of oleic acid slows down, the concentration of triolein increases significantly consuming monoolein, diolein, and oleic acid at the same time. This production profile of triolein strongly agrees with the results published by Ergan *et al.* (21) and the kinetic model proposed by Lortie *et al.* (22) for the enzymatic synthesis of triolein in a solvent-free medium. These authors postulated that, in the esterification reactions using 1,3-specific enzymes, there is a slow acyl group migration from 1-(3)-monoolein and 1,3-diolein that allows the formation of 2-monoolein and 1,2-diolein, respectively. From these reports, we can deduce that when acylglycerides in position 2 are present in the reaction mixture, 1,3-specific enzymes are able to esterify this kind of molecule in free 1 or 3 positions leading to the triolein synthesis. However, it is obvious that the limiting step in triolein synthesis would be the

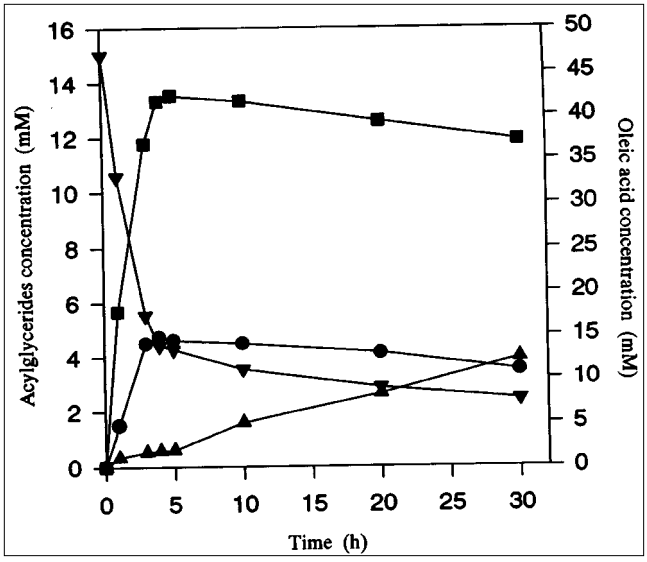


FIG. 2. Time course of triolein, diolein, and monoolein synthesis from oleic acid (50 mM) and adsorbed silica gel glycerol (1 g) using 100 mg immobilized Lipozyme™. (▼) Oleic acid, (●) monoolein, (■) diolein, (▲) triolein.

isomerization rates. The global mechanism involved in the enzymatic synthesis of acylglycerides is shown in Scheme 1.

Thus, in considering the partial reactions illustrated in Scheme 1 and the production profile of triolein shown in Figure 2, it is highly possible that the limiting step in the triolein production was also highly dependent on the isomerization steps (ki_1 and ki_2 in Scheme 1). Therefore, the low rate of disappearance of 1-(3)-monoolein, 1,3-diolein, and oleic acid observed at the end of our reaction might be directly associated with the increase in triolein formation as a function of isomerization rate of 1,3-products. In our previous paper (18), a similar profile of acylglycerides production was obtained in a similar reaction performed in $SCCO_2$. According to the results presented here and those obtained with $SCCO_2$, it is clear that the reaction mechanism proposed by Lortie *et al.* (22) for reactions performed in a solvent-free medium seems to be also valid for the reactions performed in *n*-hexane and $SCCO_2$. Indeed, in reactions where silica-gel adsorbed substrates are present, a similar kinetic mechanism was observed in the reaction. From these results, it is clear that the use of silica gel significantly improved the reaction rate and conversion yields, in a lipase-catalyzed esterification reaction of glycerol in different reaction media, without significantly changing the profile of synthesis of different products.

Direct contact between IME and glycerol adsorbed onto silica gel required for reaction. It has been shown that the rates of the esterification reaction involving glycerol are enhanced by the adsorption of this polar substrate on silica gel. In order to determine how the silica gel participates in this phenomenon, it would be necessary to understand the mechanisms involved.

Four different reaction schemes were carried out using the experimental system illustrated in Figure 3, to determine if direct contact was required between IME and glycerol for the reaction to proceed. In all four reactions (A to D), 100 mg immobilized Lipozyme™ were put in magnetically stirred reactor 1. For tests A and B, 1 g silica gel-adsorbed glycerol and 0.5 g free glycerol were, respectively, put in reactor 1 in contact with the IME. For tests C and D, 1 g silica gel-adsorbed glycerol and 0.5 g free glycerol were, respectively, put in magnetically stirred reactor 2 separated from the IME placed in the reactor 1. Then, 100 mL of a 50 mM oleic acid solution in *n*-hexane were circulated for 48 h through each reaction system, at a flow rate of 0.5 mL/min in a controlled temperature room at 40°C. Samples of the *n*-hexane phase were withdrawn at different reaction times and assayed with the commercial enzymatic kit for glycerol determination. Final oleic acid conversions obtained in these reactions are as follows: Reaction A, 71%; B, 16%; C, 0%; D, 0%.

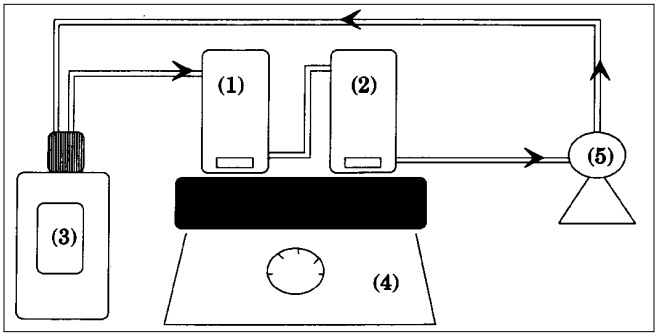
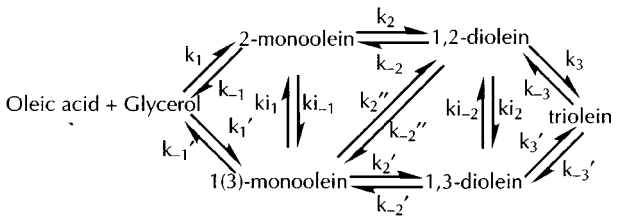


FIG. 3. Reaction system designed to test the solubility of glycerol in *n*-hexane phase during the lipase-catalyzed synthesis of acylglycerides from oleic acid and glycerol. (3) Oleic acid solution, (1 and 2) magnetically stirred reactors, (4) magnetic stirring plate, and (5) recirculation pump.

From these data we observe that only when glycerol is in direct contact with the IME does the enzymatic reaction take place (i.e,. reactions A and B). No soluble glycerol was detected in any of the samples from all reactions. From the results of the reactions A and B, it is confirmed that higher conversion rates were always obtained when silica gel-adsorbed glycerol was used (i.e., reaction A). In reaction C where glycerol was adsorbed onto silica gel but separated from IME during the reaction, no conversion of oleic acid was observed after 48 h of reaction time. So, the use of adsorbed glycerol in the reaction improves the accessibility of glycerol to the IME rather than changing the solubility of glycerol. Thus, an enhanced lipase-catalyzed reaction will be obtained only when adsorbed glycerol is in direct contact with the IME.

Factorial analysis of interactions of reaction components. In order to understand what is the specific role of silica gel in enhancing the reaction yields and also why the reaction with nonadsorbed glycerol shows a certain degree of conversion, additional experiments were carried out. The experimental design described in Table 1 was used to elucidate the more important parameters influencing the esterification reaction performed with adsorbed substrates and the combined effects resulting from these variations. Thus, all the combined effects of the components of the reaction medium of esterification of glycerol were tested. As it is assumed that a high ratio of glycerol/oleic acid is favorable to the formation of monoolein, arbitrary concentrations of immobilized glycerol (3 mmol) and oleic acid (0.25 mmol) were chosen to carry out the esterification reactions. Lyophilized free Lipozyme™ (30 mg) and free glycerol (3 mmol) were utilized for all reactions performed in the absence of enzymatic support. In all reactions the oleic acid and the IME concentrations were kept constant at 0.25 mmol and 50 mg, respectively.

In Table 1, the conversion yields were measured after 24 h of reaction. The reaction rates were not used to analyze these



SCHEME 1

TABLE 1
Effect of the Components of the Reaction on Conversion Yields After 24 h of Reaction[a]

Reaction	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Enzymatic support	+	−	+	+	+	−	−	−	+	+	+	+	−	−	−	−
Silica gel	+	+	−	+	+	−	+	+	−	−	+	−	+	−	−	−
n-Hexane	+	+	+	−	+	+	−	+	−	+	−	−	−	+	−	−
Water	+	+	+	+	−	+	+	−	+	−	−	−	−	−	+	−
Conversion (%)	46	50	28	67	48	9	69	52	70	28	66	73	74	7	11	12

[a]Immobilized enzyme = 50 mg; glycerol = 3 mmol; *n*-hexane = 10 mL; water = 2 μL; oleic acid = 0.25 mmol. + = added to the reaction. − = not added to the reaction.

results because some of the rate curves have not shown a Michaelis-Menten profile (data not shown, and explanation is given in Figure 5). In some cases, it is clear that the thermodynamic equilibrium was not reached after this time. All of these results will only be discussed in terms of conversion yields reached after 24 h.

As shown in Table 1, all reactions performed with adsorbed glycerol on silica gel (i.e., reactions 1, 2, 4, 5, 7, 8, 11, and 13) were positive, and the conversions were in general higher than 50%. The best conversion yields were achieved when enzyme was used in the presence of silica gel or enzymatic support and in the absence of *n*-hexane (i.e., reactions 4, 7, 9, 11, 12, and 13). The reactions where silica gel was not present but IME was used as catalyst (i.e., reactions 3, 9, 10, and 12) showed a peculiar behavior. For example, in reactions 3 and 10, tested with *n*-hexane as solvent and free glycerol, a low oleic acid conversion yield (28%) was observed. That corresponds to conversions previously shown on Figure 1 (25%) when nonadsorbed glycerol was used. In contrast, the same reactions but in the absence of *n*-hexane (i.e., reactions 9 and 12) showed a significant increase in conversion yield, in the range of 70%. It seems that the reactions carried out in *n*-hexane resulted in a large increase in the number of polar interactions between the glycerol and IME, compared with the results of the solvent-free reactions. From these results, it was observed that under certain conditions the use of silica gel may not be necessary to obtain high conversion yields (solvent-free esterification reactions), owing to the enzymatic support which is likely to substitute for the silica gel. In fact, in the presence of *n*-hexane, the increased number of polar interactions between glycerol and the IME could be expected to form a coating around it. In consequence, the reaction would be inhibited by limiting substrate and product diffusion. On the contrary, in solvent-free reactions, these forced numbers of interactions would be not large enough to coat the enzyme and to prevent the reaction. In these solvent-free systems a larger concentration range of glycerol in the reaction could be used before problems of blockage of the enzyme occurred.

Finally from Table 1, we can observe that the conversion yields obtained in reactions run without silica gel and without enzymatic support were strongly limited by the presence of glycerol. In fact, in reactions 6 and 14 the enzyme in suspension in *n*-hexane seems to interact with the insoluble glycerol creating glycerol–enzyme suspended particles that are difficult to separate. In these systems the glycerol will entirely coat the enzyme, preventing the substrate and product diffusion. In consequence, the conversions obtained in the case of reactions 6 and 14 without any support and in the presence of *n*-hexane as solvent are obviously the lowest. These conversions, compared to those obtained when only enzymatic support is present without silica gel (i.e., reactions 3 and 10), are approximately three times lower. In contrast, comparing with the reactions where silica gel is present without the enzymatic support and in the presence of *n*-hexane (i.e., reactions 2 and 8) we can observe a very significant difference in the conversion yields of about eight-fold. Indeed, under the same conditions as in reactions 6 and 14 but in the absence of solvent (i.e., reactions 15 and 16), the conversion yields are slightly improved owing to the absence of *n*-hexane. From these results, it is clear that in the last four reactions (i.e., reactions 6, 14, 15, and 16) the absence of any support regardless of the type—enzymatic support or silica gel—leads to a drastic fall in conversion yields.

In all reactions carried out in absence of any hydrophilic support, the strong limitation observed on the reaction rate suggests that a diffusional phenomenon is present. This shows that the thermodynamic equilibrium of these reactions is not reached after 24 h.

All these results suggest a simple mechanism of action of silica gel (Fig. 4). In this mechanism, the glycerol accumulated on the enzyme creates a polar barrier between the bulk media and the enzyme, limiting the substrate and product diffusion. The presence of *n*-hexane could force this polar barrier to form. These transport problems result in a critical fall in the conversion rate when there is no protective support for the enzyme. As shown in Fig. 4, the silica gel in the reaction behaves as a "reservoir" for the glycerol, preventing the enzyme from being blocked and allowing the reaction to take place. These results suggest that the transport of glycerol is possible by direct contact between the "reservoir" of glycerol and IME. Moreover, as reported by Stevenson *et al.* (9), the glycerol has a higher affinity for silica gel than for enzymatic support. Thus, the partition of glycerol will be in favor of silica gel. Therefore, it is highly probable that the silica gel, adsorbing insoluble polar substrates, will protect the enzyme from the blockage effect of these kinds of substrates.

Blockage level of IME with various polyols. As outlined above, immobilized Lipozyme™ has the capacity to adsorb

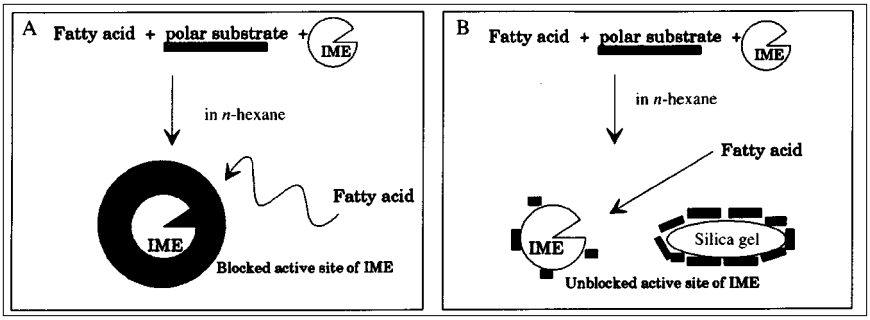


FIG. 4. Reaction mechanism proposed for the enzymatic esterification reactions: (A) nonadsorbed high-polar substrates; (B) high-polar substrates adsorbed on silica gel.

the glycerol present in the reaction. It also was shown that under certain conditions the use of silica gel is not necessary, owing to the presence of IME in the reaction. However, the maximal adsorption capacity of IME for different polyols while retaining its maximal activity must be determined. Similarly, the nature of different polyols influencing the blockage of immobilized Lipozyme™ must be established. Different concentrations of glycerol and 1,3-propanediol were used in the reaction while keeping the IME concentration constant. The use of 1,3-propanediol allowed the evaluation of results already obtained for glycerol compared to a different highly polar substrate.

Results obtained with glycerol and 1,3-propanediol as substrates are presented in Table 2. In this table we observed that the critical weight ratio of glycerol/IME prior to mass transfer limitations is lower (0.32) than the critical weight ratio obtained for the 1,3-propanediol (0.8). Weight ratios above these critical points resulted in decreased conversion rates, with negligible conversions at weight ratios of about 0.7 for the glycerol and 1.5 for 1,3-propanediol. Indeed, glycerol, having an additional hydroxyl group compared to the 1,3-propanediol, is a more polar molecule. Therefore, the affinity of glycerol for the polar enzymatic support will be stronger than that of 1,3-propanediol resulting in lower weight ratios of glycerol/IME and higher diffusion-limited reaction. In Table 2 we observed, with both polar substrates, that no mass transfer limitations were detected in all reactions performed in the presence of silica gel. Thus, under all conditions tested with adsorbed glycerol, high conversion yields were achieved. These results agree with the proposed hypothesis affirming that the saturation of the IME by these polyols was probably the main cause of a decrease in conversion rates of these reactions.

In Figure 5, concerning the use of nonadsorbed 1,3-propanediol as substrate, it is observed that the reactions carried out above the critical ratio of saturation of IME (i.e., weight ratio = 1.2) show a slow conversion rate at the beginning of the reaction. This slow initial step becomes more important as the 1,3-propanediol concentration in the reaction increased. In this initial step, the IME is considered saturated by the substrate, and the rate of conversion is controlled by the rate of transport of substrates and products between the bulk medium and the IME. Thus, as the reaction proceeded and the substrate was consumed, a significant increase in reaction rate was observed. Normal values of reaction rate were only achieved at the end of the reaction when concentrations of polar substrate were low. From Figure 5 we can conclude that the higher the concentration of 1,3-propanediol present in the reaction, the longer the step of mass transfer limitation on the kinetics. On the contrary, when silica gel-adsorbed substrates were used, no bimodal reaction behavior was observed (data not shown). Therefore, the use of nonadsorbed polar substrates in these kinds of reactions without loss of efficiency of enzyme will only be recommended at low substrate concentrations. Otherwise, if high substrate concentrations are needed for the reaction, the presence of silica gel will be recommended to achieve good final conversion yields.

It is clear that the polarity of polyol-substrate and the ratio polyol/IME play an important role in these reactions. However, the solvent also has an important influence on the reaction. To verify how the solvent influences the capacity of the enzymatic support to adsorb polyols, solvent-free esterification reactions of 1,3-propanediol were carried out. Indeed, if the solvent has a real influence on the capacity of the enzymatic support to adsorb a polyol, a change in the maximal weight ratio polyol/IME would be expected. Thus, different

TABLE 2
Conversion Yields (%) at Different Weight Ratios Glycerol/IME and 1,3-Propanediol/IME

Weight ratio glycerol/IME	0.17	0.24	0.32	0.4	0.7	1	2.0
Reaction with nonadsorbed glycerol	72	69	73	58	12	0	0
Reaction with silica gel-adsorbed glycerol	74	75	73	71	74	76	74
Weight ratio 1,3-propanediol/IME	0.47	0.81	1.21	1.5	2.4	2.8	3.8
Reaction with nonadsorbed 1,3-propanediol	68	78	78	20	4	0	0
Reaction with silica gel-adsorbed 1,3-propanediol	72	74	85	77	79	69	79

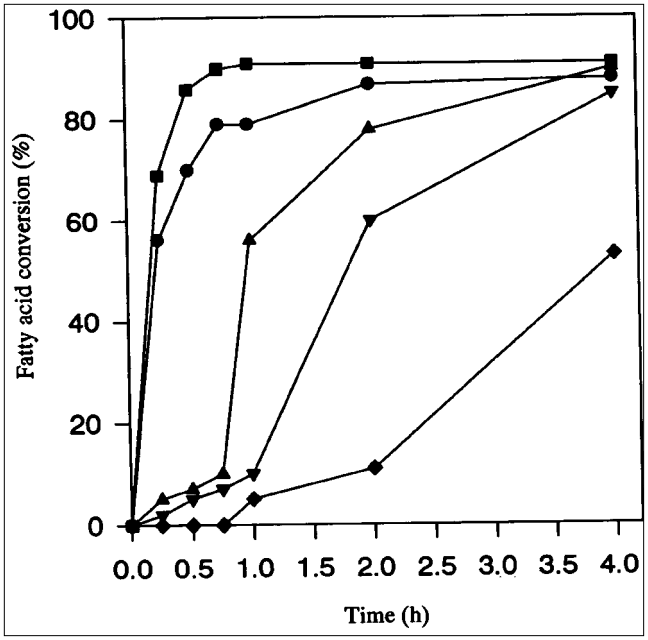


FIG. 5. Time course of the lipase-catalyzed esterification of free 1,3-propanediol with oleic acid at different weight ratios of 1,3-propanediol/immobilized enzyme. The reactions were carried out at different equivalent concentrations of oleic acid (1 mole of 1,3-propanediol/2 moles of oleic acid) in a 10-mL reaction volume with 10 mg of Lipozyme™. The different weight ratios tested were: (■) 0.47, (●) 0.81, (▲) 1.21, (▼) 1.5, (♦) 2.4.

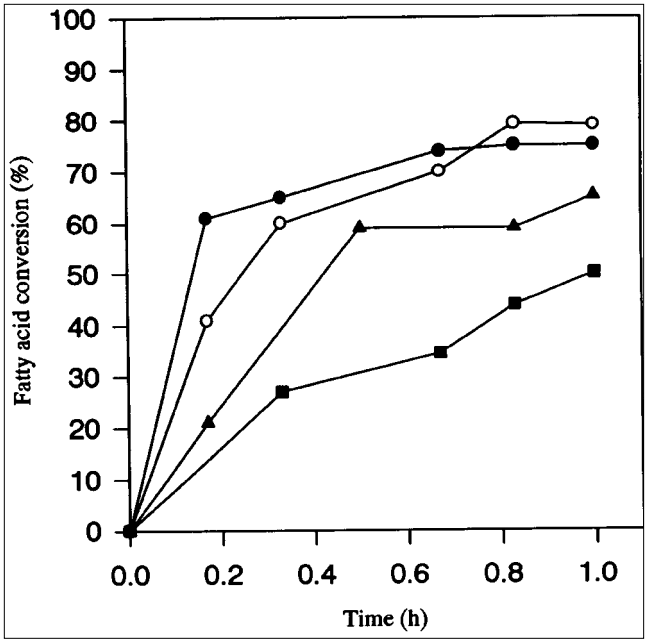


FIG. 6. Time course of the lipase-catalyzed esterification of free 1,3-propanediol with oleic acid at different weight ratios of 1,3-propanediol/immobilized enzyme. The reactions were carried out at different equivalent concentrations of oleic acid (1 mole of 1,3-propanediol/2 moles of oleic acid) with 10 mg of Lipozyme™ and in absence of *n*-hexane. The different weight ratios tested were: (●) 0.77, (○) 1.0, (▲) 1.9, (■) 4.1.

ratios of 1,3-propanediol/IME were used in the reactions keeping the IME concentration constant. The results reported in Figure 6 show that the weight ratio of 1,3-propanediol/IME increased compared with esterification reactions run in *n*-hexane. In fact, better conversions were obtained than those observed for reactions performed in *n*-hexane. With a weight ratio of 1,3-propanediol/IME of 4.1, the reaction occurred slower than the reactions at weight ratios of 0.77, 1.0, or 1.9 but these reaction rates are faster than those under the same reaction conditions performed in *n*-hexane. As suggested previously, the presence of *n*-hexane caused the increase in the number of polar interactions between the polyol and the enzymatic support causing a blockage of the IME. In contrast, in a solvent-free system, these interactions are weaker than in *n*-hexane, allowing the use of higher concentrations of polyol without mass transfer limitation. Even if the conversion rates in a solvent-free medium are improved, diffusional limitations also are present. This observation is made as a result of the decrease in the reaction rate as the substrate concentrations are increased. Obviously, in all these conditions the utilization of silica gel should avoid the mass transfer limitations and therefore no decrease in reaction rate should be observed.

Efficiency of Duolite™ as support for glycerol. The enzymatic support under certain conditions is able to protect efficiently the IME from coating by polyols. However, it also was shown that there is a limit to which the enzymatic support can protect this IME. It is suggested that the silica gel has a better protective effect on free and immobilized enzyme for the reactions with high-polar substrates. A final experiment was carried out to demonstrate that the silica gel has a preferential affinity for polar substrates over the enzymatic support. Thus, the silica gel was replaced by Duolite™ (enzymatic support) and this immobilized glycerol was used as a substrate for esterification reactions with oleic acid in *n*-hexane. The results of these experiments were compared with the results obtained with silica gel as a support for the glycerol.

For the reactions performed with Duolite™, equivalent weights of this support and glycerol were mixed in a similar way to that with silica gel. Then the Duolite™-adsorbed glycerol (1 g) was mixed with immobilized Lipozyme™ (50 mg) in an assay tube containing 10 mL of 50-mM oleic acid solution in *n*-hexane. The reaction mixture was incubated in a thermostated bath at 40°C, and samples were withdrawn during a 48 h period. The results of this reaction are shown in Figure 7. From this, and in agreement with Berger *et al.* (15) and Kwon *et al.* (31), a prior adsorption of glycerol onto a solid support is critical to the success of the esterification reaction. However, it is evident that the nature of the support for the polar substrate also will be very important. The two solid supports employed for glycerol clearly improved the esterification reaction rate, even though there are considerable differences in the respective final conversion yields. Thus, after 48 h of reaction, conversion yields of 73% and 52% were obtained, respectively, for reactions with silica gel and Duolite™ as support for glycerol. In contrast, the reaction carried out without any support shows very limited oleic acid

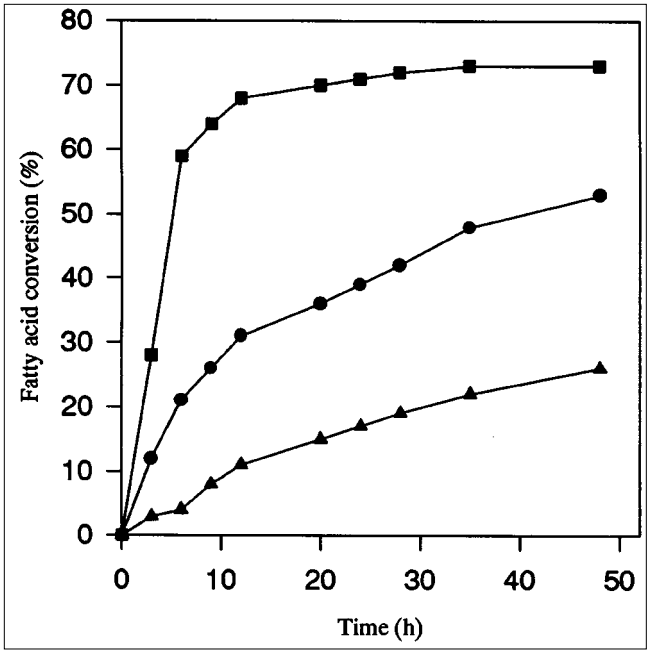


FIG. 7. Time course of the lipase-catalyzed esterification of free and adsorbed glycerol with oleic acid using different supports for the glycerol. The reactions were carried out with 50 mM of oleic acid solution in 10 mL of *n*-hexane and 50 mg of Lipozyme™. (▲) Nonadsorbed glycerol, (●) glycerol adsorbed on Duolite™, and (■) silica gel adsorbed glycerol.

conversions (26%). From Figure 7, we also can emphasize that only in the esterification reaction using silica gel-adsorbed glycerol were no mass transfer limitations observed. In fact, the two reactions where silica gel was not employed as a support for glycerol show important limitations in conversion rates. Presumably, under these conditions the reaction rate is highly limited by the substrate and product diffusion to and from the enzyme. This limitation, due to the coating effect of glycerol on the enzyme, is apparently not resolved to the same extent with the Duolite™-adsorbed glycerol. It is supposed that, in the presence of silica gel, mass transfer limitation problems in the reaction were prevented, allowing the enzyme to react more freely. Nevertheless, the presence of glycerol adsorbed onto Duolite™ in the reaction medium improved the conversion yield significantly when compared to reactions carried out without any support. In summary, contrary to glycerol adsorbed onto silica gel, it is clear that the use of glycerol adsorbed onto free Duolite™ was not effective in protecting the enzyme.

In this work, the lipase-catalyzed esterification of glycerol and 1,3-propanediol with oleic acid was examined. We have determined the crucial role of silica gel in these reactions. Unlike the hypothesis presented by Berger *et al.* (28), the formation of a liquid-liquid interface with the help of silica gel was not the only crucial step for promoting the reaction. It was demonstrated that the protective effect of silica gel against the blockage of enzyme with the high-polar substrates is even more critical. In fact, it was demonstrated that the silica gel adsorbs the polar substrates in the reaction, avoiding the blockage of the enzyme. Hence the silica gel, having a strong affinity for the glycerol, behaves as a "reservoir" controlling the transport of glycerol toward the enzyme. It also was shown that in the reactions carried out in the absence of silica gel but at low concentrations of glycerol the suggested liquid-liquid interface may already exist with the IME, as previously hypothesized by Charlemagne and Legoy (30). Therefore, reactions carried out under these conditions always will be possible as long as the concentration of polyol in the reaction is kept low. Reactions at high concentrations of polar substrate may be carried out by increasing substrate concentration while keeping the maximal weight ratio of polyol/IME constant. However, that is possible only if the increase in IME concentration is proportional to the increase of polyol concentration. In consequence this process will become very expensive owing to the requirement of high quantities of IME in the reactional medium. As an alternative approach the use of silica gel is recommended which allows the utilization of very high concentrations of polyol in the reaction without significantly increasing the enzyme concentration. Finally, we should emphasize that all the phenomena associated with the use of silica gel in esterification reactions will be strongly influenced by the nature of the substrate and the reaction medium. The effect of different enzymatic supports in the lipase-catalyzed esterification of different polyols with fatty acids is needed to fully understand this phenomena.

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REFERENCES

1. Sonntag, N.O.V., New Developments in the Fatty Acid Industry in America, *J. Am. Oil Chem. Soc. 61*:229–232 (1984).
2. Fiechter, A., Biosurfactants: Moving Towards Industrial Application, *Trends Biotechnol. 10*:208–217 (1992).
3. Van der Padt, A., J.T.F. Keurentjes, J.J.W. Sewalt, E.M. Van Dam, L.J. Van Dorp, and K. Van't Riet, Enzymatic Synthesis of Monoglycerides in a Membrane Bioreactor with an In-Line Adsorption Column, *J. Am. Oil Chem. Soc. 69*:748–754 (1992).
4. McNeill, G.P., S. Shimizu, and T. Yamane, Solid Phase Enzymatic Glycerolysis of Beef Tallow Resulting in a High Yield of Monoglyceride, *Ibid. 67*:779–783 (1990).
5. Akoh, C.C., C. Cooper, and C.V. Nwosu, Lipase G-Catalyzed Synthesis of Monoglycerides in Organic Solvent and Analysis by HPLC, *Ibid. 69*:257–260 (1992).
6. Bornscheuer, U.T., and T. Yamane, Activity and Stability of Lipase in the Solid-Phase Glycerolysis of Triolein, *Enzyme Microb. Technol. 16*:864–865 (1994).
7. Maag, H., Fatty Acid Derivatives: Important Surfactants for Household, Cosmetic and Industrial Puposes, *J. Am. Oil Chem. Soc. 1*:259–267 (1984).
8. Holmberg, K., and E. Osterberg, Enzymatic Preparation of Monoglycerides in Microemulsion, *Ibid. 65*:1544–1548 (1988).
9. Stevenson, D.E., R.A. Stanley, and R.H. Furneaux, Near-Quantitative Production of Fatty Acid Alkyl Esters by Lipase-Catalyzed Alcoholysis of Fats and Oils with Adsorption of Glyc-

erols by Silica Gel, *Enzyme Microb. Technol. 16*:478–484 (1994).
10. Zaks, A., and A.M. Klibanov, Enzyme-Catalyzed Processes in Organic Solvents, *Proc. Natl. Acad. Sci. USA 82*:3192–3196 (1985).
11. Deetz, J.S., and J.D. Rozzell, Enzyme-Catalyzed Reactions in Non Aqueous Media, *Trends Biotechnol. 6*:15–19 (1988).
12. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, *Enzyme Microb. Technol. 11*:194–210 (1989).
13. Chulalaksananukul, W., J.S. Condoret, P. Delorme, and R.M. Willemot, Kinetic Study of Esterification by Immobilized Lipase in *n*-Hexane, *FEBS. Lett. 276*:181–184 (1990).
14. Marty, A., W. Chulalaksananukul, J.S. Condoret, R.M. Willemot, and G. Durand,. Comparison of Lipase-Catalyzed Esterification in Supercritical Carbon Dioxide and in *n*-Hexane, *Biotechnol. Lett. 12*:11–16 (1990).
15. Berger, M., K. Laumen, and M.P. Schneider, Lipase Catalyzed Esterification of Hydrophilic Diols in Organic Solvents, *Ibid. 14*:553–558 (1992).
16. Pecnik, S., and Z. Knez, Enzymatic Fatty Acid Ester Synthesis, *J. Am. Oil Chem. Soc. 69*:261–265 (1992).
17. Akoh, C.C., Lipase-Catalyzed Synthesis of Partial Glyceride, *Biotechnol. Lett. 15*:949–954 (1993).
18. Castillo, R.E., A. Marty, D. Combes, and J.S. Condoret, Polar Substrates for Enzymatic Reactions in Supercritical CO_2: How to Overcome the Solubility Limitation, *Ibid. 16*:169–174 (1994).
19. Van der Padt, A., M.J. Edeme, J.J.W. Sewalt, and K. Van 't Riet, Enzymatic Acylglycerol Synthesis in a Membrane Bioreactor, *J. Am. Oil Chem. Soc. 67*:347–352 (1990).
20. Janssen, A.E.M., M. Hadini, N. Wessels Boer, R. Walinga, A. Van der Padt, H.M. Van Sonsbeek, and K. Van't Riet, The Effect of Organic Solvents on Enzymatic Esterification of Polyols, in *Biocatalysis in Non Conventional Media,* edited by J. Tramper, M.H. Vermüe, H.M. Beeftink, and U. Von Stockar*,* Elsevier Science Publishers B.V., Amsterdam, 1992, pp. 155–161.
21. Ergan, F., M. Trani, and G. André, Production of Glycerides from Glycerol and Fatty Acid Immobilized Lipases in Non-Aqueous Media, *Biotechnol. Bioeng. 35*:195–200 (1990).
22. Lortie, R., M. Trani, and F. Ergan, Kinetic Study of the Lipase-Catalyzed Synthesis of Triolein, *Ibid. 41*:1021–1026 (1993).
23. Ferrier, R.J., Applications of Phenylboronic Acid in Carbohydrate Chemistry, *Methods Carbohydr. Chem. 6*:419–426 (1972).
24. Oguntimein, G.B., H. Erdmann, and R.D. Schmid, Lipase Catalyzed Synthesis of Sugar Ester in Organic Solvents, *Biotechnol. Lett. 15*:175–180 (1993).
25. Steffen, B., A. Ziemann, S. Lang, and F. Wagner, Enzymatic Monoacylation of Trihydroxy Compounds, *Ibid. 14*:773–778 (1992).
26. Ikeda, I., and A.M. Klibanov, Lipase-Catalyzed Acylation of Sugars Solubilized in Hydrophobic Solvents by Complexation, *Biotechnol. Bioeng. 42*:788–791 (1993).
27. Scheckermann, C., A. Schlotterbeck, M. Schmidt, V. Wray, and S. Lang, Enzymatic Monoacylation of Fructose by Two Procedures, *Enzyme Microb. Technol. 17*:157–162 (1995).
28. Berger, M., K. Laumen, and M.P. Schneider, Enzymatic Esterification of Glycerol I. Lipase-Catalyzed Synthesis of Regioisomerically Pure 1,3-*sn*-Diacylglycerols, *J. Am. Oil Chem. Soc. 69*:955–960 (1992).
29. Berger, M., and M.P. Schneider, Enzymatic Esterification of Glycerol II. Lipase-Catalyzed Synthesis of Regioisomerically Pure 1(3)-*rac*-Monoacylglycerols, *Ibid. 69*:961–965 (1992).
30. Charlemagne, D., and M.D. Legoy, Enzymatic Synthesis of Polyglycerol-Fatty acid Esters in a Solvent-Free System, *Ibid. 72*:61–65 (1995).
31. Kwon, S.J., J.J. Han, and J.S. Rhee, Production and *in situ* Separation of Mono- or Diacylglycerol Catalysed by Lipases in *n*-Hexane, *Enzyme Microb. Technol. 17*:700–704 (1995).
32. Ergan, F., and G. André, Simple High Performance Liquid Chromatography Methods for Monitoring Lipase Reactions, *Lipids 24*:76–78 (1989).

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