Solvent Engineering Applied to Lipase-Catalyzed Glycerolysis of Triolein

X. Rendón, A. López-Munguía, and E. Castillo*

Departamento de Bioingeniería, Instituto de Biotecnología, UNAM, Cuernavaca, Morelos 62271, México

ABSTRACT: Solvent engineering was applied to lipase-catalyzed glycerolysis of triolein for the selective synthesis of monoolein and diolein. The effect of different binary mixtures of *n*-hexane and 2-methyl-2-butanol (2M2B) on the selective production of mono- or diacylglyceride was established. Conditions for high selectivity toward monoolein synthesis were enhanced from 10.6 mol% in pure *n*-hexane to 64 mol% in 2M2B. On the contrary, the highest production of diolein, corresponding to 62 mol%, was achieved in *n*-hexane. Concerning triolein conversion, the best results were obtained in 100% 2M2B, with a conversion of 75%. The effect of the *n*-hexane/2M2B ratio on diolein regioisomer production during triolein glycerolysis was also evaluated. Two different profiles of diolein regioisomers were observed as a function of solvent composition: Although the production of the 1,2-diolein isomer was favored as the proportion of 2M2B in *n*-hexane was increased, the 1,3-isomer was preferentially synthesized in reactions where *n*-hexane was the predominant solvent. When 100% *n*-hexane was used as a solvent, 1,3-diolein comprised 72 mol% of the total diolein population (58 mM). On the contrary, when the reaction was carried out in 100% 2M2B, the total concentration of diolein was lower (21 mM) but the 1,2-diolein regioisomer was preferentially formed (89%). These results were explained as a consequence of the different extents of hydrolysis–synthesis reactions involved in the glycerolysis process, which are strongly dependent on solvent mixtures and water concentration. Finally, some advantages of the use of binary mixtures of solvents compared with other strategies applied to glycerolysis reactions are discussed.

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KEY WORDS: Diglyceride, glycerolysis, lipase, monoglyceride, organic solvents, solvent engineering, solvent-free system, triolein.

Surfactants are an important class of chemicals used widely in almost every sector of modern industry. They constitute a growing market, estimated at U.S. $4 billion in 1998; their worldwide production, exceeding 3×10^6 tons per annum in that same year, has been expected to rise to over 4×10^6 tons by year 2000 (1). Among all categories of surfactants, acylglycerides represent a substantial part of the market, having a world production estimated between 0.25 and 0.50 million tons per annum (2). Acylglycerols and their numerous derivatives are used in a great variety of industrial applications. They constitute the most important food-grade emulsifiers, and they have been used as surface-active agents in many consumer and industrial cleaning products such as detergents, shampoos, lotions, and toothpastes or as raw materials for the synthesis of chemical compounds such as alkyd resins (3).

*To whom correspondence should be addressed at Instituto de Biotecnología, UNAM, Apartado Postal 510-3, Cuernavaca, Morelos 62271, México. E-mail: edmundo@ibt.unam.mx

In particular, monoglycerides are the major nonionic food emulsifiers emerging from oleochemistry. They are mainly produced by glycerolysis, a process of reacting fats and glycerol in the presence of metallic catalysts under high temperature (220–260°C) and inert gas atmospheres (nitrogen), especially if unsaturated fats are used (4). In these extreme conditions, complex mixtures of products and by-products are formed, requiring molecular distillation for the production of high-purity monoglycerides (5). The use of lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) as biocatalysts for glycerolysis is an attractive alternative for improving process conditions and avoiding expensive purification steps, due to their high selectivity (6,7). Indeed, enzymatic transformations with lipases, such as hydrolysis, esterification, alcoholysis, and interesterification in nonconventional media under mild conditions have been widely reported for enzymatic modification of fats and other lipids (8,9). Enzymatic glycerolysis is one of the most investigated approaches for the lipase-catalyzed synthesis of monoglycerides. Different reaction systems have been proposed, using organic solvents in monophasic, biphasic (10–12), or solvent-free systems (13–15). In general, the main drawback in enzymatic glycerolysis is the low equilibrium conversion for triglycerides. An additional limitation is the low selectivity of the process for a specific degree of esterification, so in general, these types of products consist of complex mixtures of mono-, di-, and triglycerides (16).

To enhance the selective synthesis of monoglycerides, different strategies have been reported in the literature: a stoichiometric approach, consisting of reacting glycerol and triglycerides at high concentration ratios. In theory, a stoichiometric ratio of glycerol/triglycerides equal to 2 should result in the exclusive production of monoglycerides, but for several reasons this is not the case in practice, among others, the selectivity for a specific degree of esterification and hydrolysis. In fact, the higher the glycerol/triglyceride ratio, the higher the triglyceride conversion to monoglycerides. Therefore, in almost all reports concerning glycerolysis, high ratios are employed and excessive quantities of nonreacted glycerol are found at equilibrium (15,16). A second alternative consists of avoiding the thermodynamic equilibrium by precipitating or extracting monoglycerides. This is generally accomplished by cooling the reaction mixture until monoglycerides are crystallized (11,17). Although this thermodynamic approach seems a good alternative, low temperatures in enzymatic processes are expensive in terms of energy demand, and they result in a decrease in the activities of enzymes. The optimal crystallization temperatures reported for monoglycerides production are generally between 30 and 45°C for animal fats and between 5 and 10°C for vegetable oils (16,18).

An additional alternative to carrying out the tailor-made

synthesis of acylglycerides is to use solvent engineering strategies. Several papers have pointed out that solvent polarity is the main factor influencing selectivity of lipase-catalyzed reactions (19,20). Kuo and Parkin (19) associated the selective production of mono- and diesters of glycerol and 1,3-propanediol to the differential solvation and extraction properties of solvents. On the other hand, Torres *et al.* (20) observed that solvent polarity had a great influence on the product distribution of malic acid monoesters. These results were explained by the authors as the result of the higher solubility of the monoester relative to malic acid in hydrophobic solvents, inducing a rapid esterification to produce the corresponding diester.

The synthesis of polyol esters, including a selective degree of esterification, has been predicted by considering the thermodynamic properties (21–24). Janssen *et al.* (21) reported the effect of organic solvents (polarity and structural nature) on the equilibrium position of enzymatic acylglycerol synthesis. Using a UNIFAC (Universal Function Activity Coefficient) group contribution method to predict activity coefficients, these authors showed how solvent polarity influenced monoglyceride yields in aqueous-organic two-phase systems. Bellot *et at.* (24) also applied the UNIFAC method in near-anhydrous monophasic media and proposed a thermodynamic model to predict the equilibrium concentrations in the lipase-catalyzed tailor-made synthesis of mono- and diacylglycerols in various solvent mixtures. Their results demonstrated that thermodynamic activities (reactivities) of the chemical species involved in the reaction are influenced by the nature of the reaction medium. Finally, Adachi *et al.* (23) also proposed a thermodynamic approach to studying the influence of the solvent on the selective synthesis of erythritol esters. However, no thermodynamic equilibrium data were provided.

The aim of this work is to apply solvent engineering strategies to the lipase-catalyzed glycerolysis of triolein. In particular, the effect of different binary mixtures of solvents on the selective production of mono- or diacylglycerides was studied in search of optimal conditions for high-selectivity monoolein synthesis. Some advantages in using solvents as reaction media over solvent-free media were also established.

EXPERIMENTAL PROCEDURES

Materials. Immobilized lipase from *Rhizomucor miehei,* Lipozyme IM-20, was kindly provided by Novo Nordisk Industry A/S (Bagsvaard, Denmark). Glycerol, oleic acid, (*cis*-9-octadecanoic acid), 1(3)-monooleoyl-*rac*-glycerol, 1,3-dioleoyl glycerol, 1,2-dioleoyl glycerol, trioleoyl glycerol, and silica gel 60 (70–230 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest purity available. All high-purity solvents and the salt used were purchased from J.T.Baker Co. (Phillipsburg, NJ). All solvents were dried extensively with 4 Å molecular sieves (Aldrich Chemical Co. Inc., Milwaukee, WI). Other chemicals were of analytical grade.

Adsorption of glycerol onto silica gel. Glycerol was adsorbed onto silica gel as described by Berger *et al.* (25): 1 g of glycerol and 1 g of silica gel were carefully mixed until a fluid and homogeneous powder were obtained. When higher quantities of glycerol/silica gel were prepared (>5 g), the mixtures were fluidized with the aid of a mechanical homogenizer. As previously reported (26), enzymatic assays for adsorbed glycerol content on silica-gel preparations showed a glycerol content of 0.5 g of glycerol per gram of this mixture. The adsorbed glycerol was stored under anhydrous conditions prior to use.

Substrates and enzyme conditioning. Enzyme preparations and substrates were equilibrated over a saturated salt solution at 25°C to obtain a defined initial water activity (a_w). A saturated salt solution of CH_3COOK ($a_w = 0.23$) was used in this study to minimize hydrolysis of substrates. Equilibration of enzyme and substrates at $a_w = 0.23$ was performed for at least 72 h. To confirm that a_w equilibrium was attained, the water content in the reaction mixture was determined by Karl Fisher titration on a 758/B10 Metrohm KF Titrator (Herisau, Switzerland). Samples were regularly withdrawn and analyzed without further treatment.

Glycerolysis reaction. Unless otherwise stated, typical glycerolysis experiments were carried out in closed, screw-capped reaction vessels containing 0.1 g of Lipozyme IM20 pre-equilibrated at $a_w = 0.23$, 0.25 mmol of triolein, 0.092 g of silica-gel-adsorbed glycerol (equivalent to 0.5 mmol of glycerol), and 5 mL of solvent or solvent mixture. The reaction was incubated in a thermostated water bath at 40°C and vigorously agitated at around 200 rpm with magnetic stirring. Samples of 100 µL were withdrawn at intervals and centrifuged to separate the liquid phase from the enzyme and the residual silica gel. In order to monitor the reaction progress, samples were either diluted 1:10 (vol/vol) in *n*-hexane and analyzed by thin-layer chromatography (TLC) or evaporated in a thermostated water bath at 60°C under vacuum; the oily residual phase was resuspended in acetone for analysis by high-performance liquid chromatography (HPLC).

TLC analysis. Samples of 100 µL were withdrawn from reaction vessels, diluted 1:10 (vol/vol) in *n*-hexane and applied to TLC plates (Alugram Kiesegel 60; Macherey-Nagel, Düren, Germany). Substrates and products were eluted using *n*-hexane/ethyl ether/acetic acid (75:25:1). For visualization of products, the plates were dried and sprayed with a saturated solution of copper acetate diluted 50:50 (vol/vol) with 85% orthophosphoric acid and heated to 200°C for 10 min.

HPLC analysis and quantification of acylglycerides. The extent of reaction was measured by using an HPLC system equipped with a Waters 600E multisolvent delivery controller, a Waters 486 series detector Ultraviolet (UV)/Visible at 210 nm (Waters Corp., Milford, MA), and a C_{18} reversed-phase analytical Spherisorb ODS-2 column (5 µm, 250 × 4.6 mm) (Macherey-Nagel, Düren, Germany) thermostated at 45°C. The column was eluted with both eluant and flow gradients run concurrently. The eluant gradient consisted of an initial mixture of acetone/acetonitrile (50:50, vol/vol) held for 2 min and then changed to 40:60 (vol/vol) in 3 min, held for 13 min, and then reversed to the initial conditions in 5 min. The flow gradient consisted of an initial flow rate of 0.5 mL/min held for 12 min, increased to 3.0 mL/min for 2 min, held for 4 min, and finally reversed to the initial conditions in 4 min. The substrate and product concentrations were calculated using response factors derived from calibration curves for oleic acid, mono-, di-, and triolein.

RESULTS

Comparative reactions in solvent and solvent-free media. In order to select the more suitable reaction system and to define reference parameters for the process, three reaction systems were investigated under similar conditions: silica-gel-adsorbed glycerol in *n*-hexane, free glycerol in *n*-hexane, and free glycerol in a solvent-free system (0.1 g of enzyme, 0.25 mmol of triolein, and 0.5 mmol of glycerol at 40°C).

Hexane was selected as a solvent owing to the high solubility of all types of triglycerides and oils in this medium. Although solvent-free reactions were not the aim of this work, these systems were tested to demonstrate the advantages of solvents for tailor-made synthesis of acylglycerides. Substrate conversions and product distributions at equilibrium in the systems described are presented in Table 1.

From Table 1, we observe that at equilibrium, triolein conversion was similar in all systems investigated. In reactions 1 and 2, in which *n*-hexane was used as the solvent, the distribution of products at equilibrium was almost the same. Both conditions showed a preferential accumulation of hydrophobic species (diolein and triolein) at equilibrium. In reaction 3, when *n*-hexane was absent, a higher synthesis of monoolein was observed compared to reactions 1 and 2. These values were in accordance with other reports of glycerolysis reactions in solvent-free media (14,27,28). In fact, the final mixture of glycerol and acylglycerides presumably increased the polarity of the medium. In agreement with previous reports on the direct synthesis of acylglycerides (22), we found that more polar environments enhanced the selective synthesis of monoolein. Although the solvent-free system was advantageous for the synthesis of acylglycerides in terms of productivity, the thermodynamic properties of these systems were not easily manipulated to enhance the selectivity of the reaction.

Furthermore, reactions carried out in *n*-hexane with adsorbed glycerol (reaction 1), showed faster transformation rates of triolein than other reaction systems evaluated, as in these conditions the thermodynamic equilibrium was reached in 10 h. On the contrary, reactions in which nonadsorbed glycerol was used required 48 to 72 h to reach equilibrium (reactions 2 and 3, respectively). In both systems the low reaction rates were probably the result of mass-transfer limitations owing to a blockage of the immobilized enzyme. Indeed, it has been well documented that adsorption of glycerol onto silica gel has a crucial effect in lipase-catalyzed esterifications involving polar substrates (2,16,25,29). In addition to mass transfer limitations, we observed that in a solvent-free system, difficulties in mechanical mixing owing to the high viscosity of substrate mixtures were present. This problem may become critical if solid fats are intended to be transformed at industrial scale (30).

TABLE 1
Product Distribution at Equilibrium in Lipase-Catalyzed Glycerolysis of Triolein Using Three Different Reaction Systems

Reaction system	Triolein (mol%)	Diolein (mol%)	Monoolein (mol%)	Free oleic acid (mol%)
1. Silica gel-adsorbed glycerol in *n*-hexane	28.3	57.3	10.1	4.3
2. Free glycerol in *n*-hexane	29.7	53.3	12.4	4.6
3. Free glycerol in a solvent-free system	28.1	39.9	29.1	2.9

From these results, reaction with silica-gel-adsorbed glycerol in *n*-hexane was selected as the reference system to study the effect of different solvent engineering strategies in the glycerolysis of triolein.

Glycerolysis reactions in binary mixtures of solvents. Thermodynamic equilibrium of the overall glycerolysis reaction considering a total conversion of glycerol to monoglycerides may be described by the following equations:

$$[\text{triglyceride}] + 2[\text{glycerol}] \overset{K_{eq}}{\rightleftharpoons} 3[\text{monoglyceride}] \quad [1]$$

The final product concentration can also be described in terms of a more detailed mechanism:

$$[\text{triglyceride}] + [\text{glycerol}] \overset{K_{eq_1}}{\rightleftharpoons} [\text{monoglyceride}] + [\text{diglyceride}] \quad [2]$$

$$[\text{diglyceride}] + [\text{glycerol}] \overset{K_{eq_2}}{\rightleftharpoons} 2[\text{monoglyceride}] \quad [3]$$

Previous studies (22,24) have demonstrated that the influence of polarity on the specificity of the enzymes for the synthesis of mono- and diacylglycerides can be controlled through an appropriate mixture of solvents. In this report the same approach was extended to glycerolysis. As explained above, in hydrophilic solvents the thermodynamic activity of monoglycerides is the lowest, favoring the production of monoolein. In the same sense, in hydrophobic media triolein and diolein are the predominant species.

In the different mixtures of 2-methyl-2-butanol (2M2B)-amended *n*-hexane that were evaluated, as the amount of 2M2B was increased, the solvent mixture became more hydrophilic.

Figure 1 shows the influence of solvent composition on the global thermodynamic equilibrium. From this figure it is clear that as the proportion of 2M2B in *n*-hexane increased, the monoolein concentration increased to become the most abundant product in 100% 2M2B (64 mol%, equivalent to 60 mM). Nevertheless, owing to its high polarity, this product became less favored in *n*-hexane (10.6 mol%, equivalent to 9.3 mM). On the contrary, diolein concentration decreased from 58 mM (62 mol%) to 21 mM (22 mol%) when 2M2B was used instead of *n*-hexane. However, in this case the effect of solvent polarity was extreme, as the selectivity toward diolein dropped rapidly in the presence of 25% 2M2B. Finally, the proportion of nonconverted triolein followed a bell-shaped curve. The lowest conversions corresponding to a residual triolein 25 mM, was observed in the hydrophobic mixtures (25% 2M2B).

Although a usual strategy to favor monoglyceride production is to change the stoichiometric ratio (Eq. 1) (15,16,31), the same effect can be achieved at the minimum glycerol required by the stoichiometry (Eq. 1) by an appropriate mixture of solvents, avoiding the excess of glycerol. Hence, in terms of monoglyceride content at equilibrium, reactions carried out in solvents containing 2M2B reached a monoglyceride concentration of 40–65% without exceeding the stoichiometric ratio

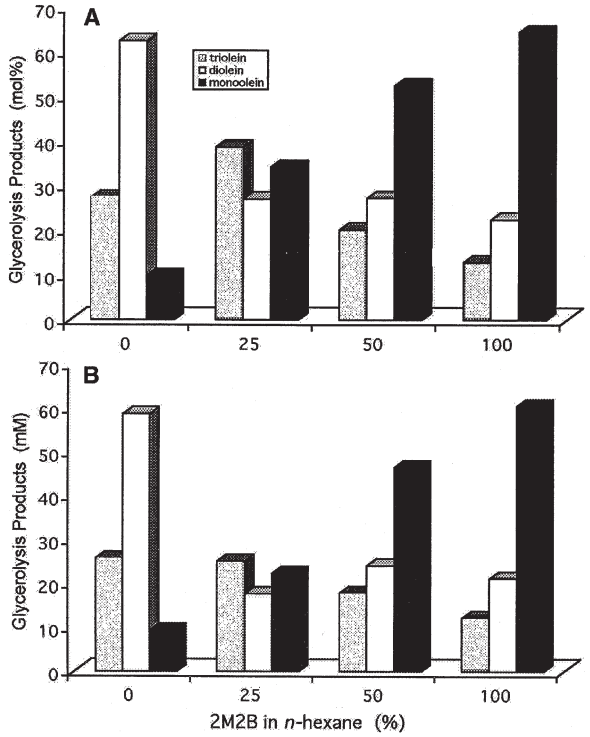

FIG. 1. The influence of solvent composition on product distribution at thermodynamic equilibrium. Initial conditions: 0.25 mmol of triolein, 0.1 g of enzyme, 0.092 g of silica-gel-adsorbed glycerol (0.5 mmol of glycerol), 40°C, and different percentages of 2-methyl-2-butanol (2M2B) in *n*-hexane (vol/vol); (A) proportional distribution of products; (B) final concentrations of products in the mixture.

for a glycerolysis system designed for monoolein production.

Effect of the n-*hexane/2M2B ratio on regioselectivity*. The effect of the *n*-hexane/2M2B ratio on regioselectivity of triolein glycerolysis was evaluated in terms of the production of diolein regioisomers. For this purpose, the profiles of 1,2-diolein and 1,3-diolein produced at equilibrium were measured for mixtures of 2M2B-amended *n*-hexane.

These results are shown in Figure 2, where two different profiles of diolein regioisomers are observed as a function of solvent composition: Although the synthesis of 1,2-diolein was favored as the proportion of 2M2B was increased, the 1,3-isomer was preferentially produced in reactions where *n*-hexane was predominant.

In order to explain this change in selectivity, apparently induced by the hydrophobicity of the reaction medium, glycerolysis reactions were carried out in 100% *n*-hexane and in 100% 2M2B, following the production kinetics of the diolein isomers. Lipozyme IM20 has been defined as a 1,3-regiospecific lipase; therefore, upon hydrolysis of triolein, the 1,2-diolein regioisomer is preferentially formed. In Figure 3A, we observe that when 100% *n*-hexane was used as a solvent, a rapid hydrolysis of triolein took place early in the reaction, as deduced from the free oleic acid released. In this first step of the reaction, the hydrolysis products 1,2-diolein, monoolein, and free oleic acid were obtained at stoichiometric concentrations. It may be concluded, therefore, that hydrolysis is the main reaction occurring during the first 10 min of the process, when water is available. Afterward, although the hydrolysis of triolein continues, as inferred from the additional formation of free oleic acid, monoolein, and 1,2-diolein, after 40 min the oleic acid and 1,2-diolein concentrations reached a maximum. 1,3-Diolein, which was absent during the first 10 min, was also formed, probably by esterification from free oleic acid and glycerol, both found in high concentrations, and by the low concentration of water, consumed during hydrolysis. After 40 min, when the consumption rate of triolein decreased considerably, a rapid decrease in oleic acid concentration with a simultaneous increase in 1,3-diolein concentration confirmed that this last product was mainly obtained by direct esterification between oleic acid and glycerol. Although 1,3-diolein may also have come from the spontaneous isomerization of 1,2-diglycerides (or from 2-monoglycerides followed by esterification), it seemed that under these reaction conditions isomerization played a minor role. In the last part of the process, 1,3-diolein was produced at a lower rate in a steady state between the hydrolysis of triolein and the consumption of oleic acid, with a almost constant concentration of 1,2-diolein and monoolein. Once the equilibrium was reached, the reaction mixture was incubated for 3 d without any observed change in 1,3-diolein and 1,2-diolein concentrations attributable to isomerization. According to different authors (32–34), in reactions carried out at temperatures lower than 40°C and solvents such as hexane, the rates of spontaneous acyl migration from 1,2-diglycerides and 2-monoglycerides are slower than the rates of lipase-catalyzed transformations. Therefore, in *n*-hexane, monoolein and 1,3-

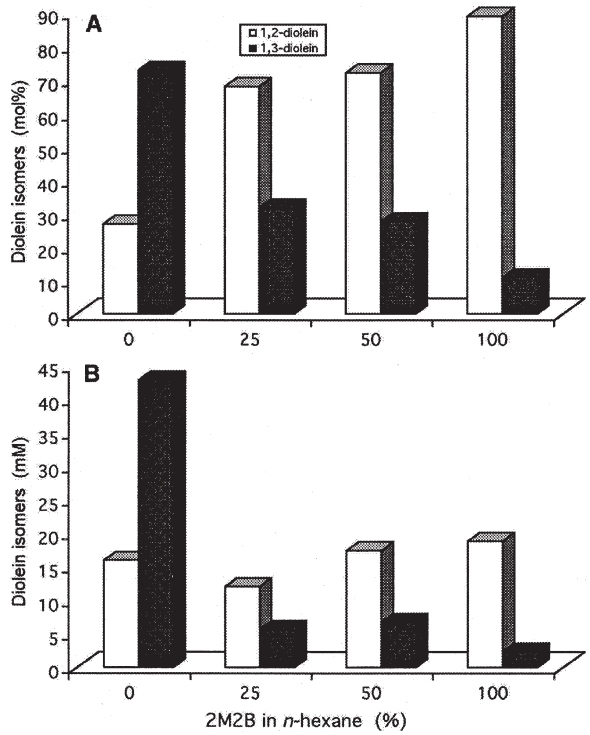

FIG. 2. The influence of solvent composition on diolein isomer distribution at thermodynamic equilibrium. Initial conditions: 0.25 mmol of triolein, 0.1 g of enzyme, 0.092 g of silica-gel-adsorbed glycerol (0.5 mmol of glycerol), 40°C, and different percentages of 2M2B in *n*-hexane (vol/vol); (A) proportional distribution of products; (B) final concentrations of isomers in the mixture. See Figure 1 for abbreviation.

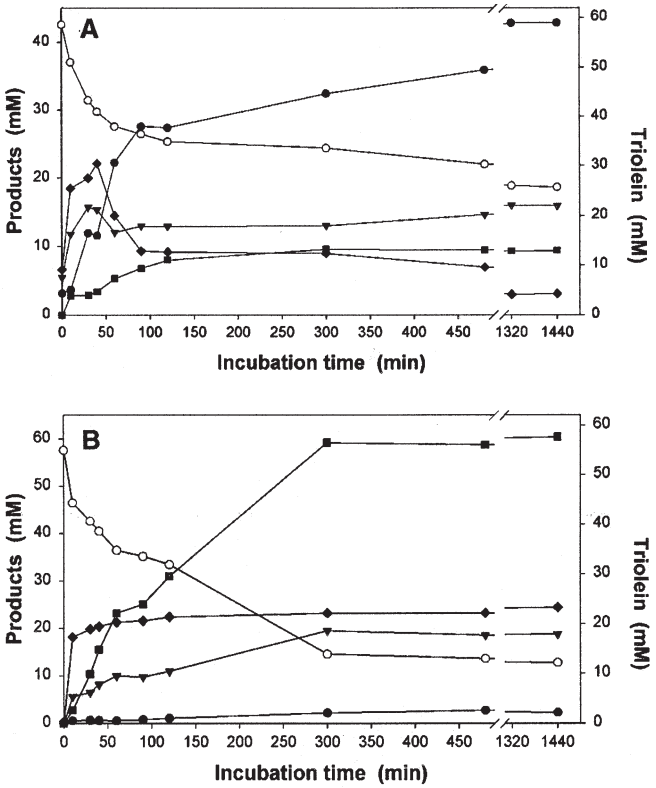

FIG. 3. Time course of oleylglyceride production in lipase-catalyzed glycerolysis of triolein. Initial conditions: 0.25 mmol of triolein, 0.1 g of enzyme, silica-gel-adsorbed glycerol 0.092 (0.5 mmol of glycerol), 40°C. Oleic acid (◆), monoolein (■), 1,2-diolein (▼), 1,3-diolein (●), and triolein (○); (A) kinetics in *n*-hexane ; (B) kinetics in 2M2B. See Figure 1 for abbreviation.

diolein were produced by hydrolysis and esterification reactions, with 1,2-diolein occurring only to a minor extent as the product of hydrolysis or a strongly limited glycerolysis.

When the reaction was carried out in 100% 2M2B, hydrolysis of triolein was also the first step of the process. However, in this solvent, 1,3-diolein, the esterification product, was not produced (Fig. 3B). As with *n*-hexane, the hydrolysis products 1,2-diolein, monoolein, and oleic acid were also detected in stoichiometric concentrations. After 10 min and until equilibrium was reached, the hydrolysis reaction was clearly reduced, and as no esterification was observed (absence of 1,3-diolein), it was concluded that the main product (monoolein) was obtained from glycerolysis of both triolein and 1,2-diolein. The small amount of 1,3-diolein obtained is an indication of inadequate conditions for direct esterification to occur. In fact, as has been previously reported (24), lipase-catalyzed esterification of glycerol and oleic acid carried out in 2M2B, resulted in very low yields of diolein and triolein as a consequence of unfavorable thermodynamic conditions.

These results suggest that the final product distribution obtained in the glycerolysis process may involve several reactions, which are strongly dependent on medium composition (mainly hydrophobicity and chemical nature of the solvent, as well as initial water activity). As a consequence, the product distribution profile at equilibrium may be completely different than the one observed early in the reaction. It has been established that in some conditions, lipase-catalyzed reactions of triolein with glycerol involve a first step of hydrolysis prior to direct esterification of fatty acids. Only a few reports in the literature deal with the eventual hydrolysis step previous to esterification (12,14,35). Actually, data from the initial product distribution in glycerolysis reactions (<1 h) are rarely monitored. In Figure 4, the alternative reactions occurring during the global glycerolysis process are summarized.

DISCUSSION

Solvent engineering strategies can be applied to lipase-catalyzed reactions for selective synthesis of mono- and diolein through glycerolysis. Selectivity for monoesters can be enhanced by changes in hydrophobicity, which in our case involved an increase in polar interactions between a tertiary alcohol such as 2M2B and glycerides (mono- and diesters). In fact, one of the advantages of solvent mixtures over pure solvents was that the use of solvent mixtures allowed for changes not only in the polarity of the reaction mixture but also in the structural interactions between solvents and reactants. By these means, the thermodynamic activities of reactants could be modified without drastic changes on the environment of the enzyme. The results presented in Figures 1 and 2 could not be explained by the common log P parameter. In fact, even if log P values reflected the overall lipophilicity of the medium, as demonstrated in the literature, this one-dimensional parameter contained limited information and became insufficient when topological or stereochemical features of molecules were analyzed in the context of intermolecular interactions and reactivities.

Thus, a glycerolysis process using solvent engineering strategies has been presented as a beneficial alternative to selectively produce biosurfactants. In addition, an attractive alternative to the selective synthesis of 1,3-diolein and 1,2-diolein has been presented. In effect, selectivity during the glycerolysis process was strongly influenced by the nature of the solvent, but hydrolysis was a determinant in this process; therefore, water activity became a critical parameter in controlling the overall process.

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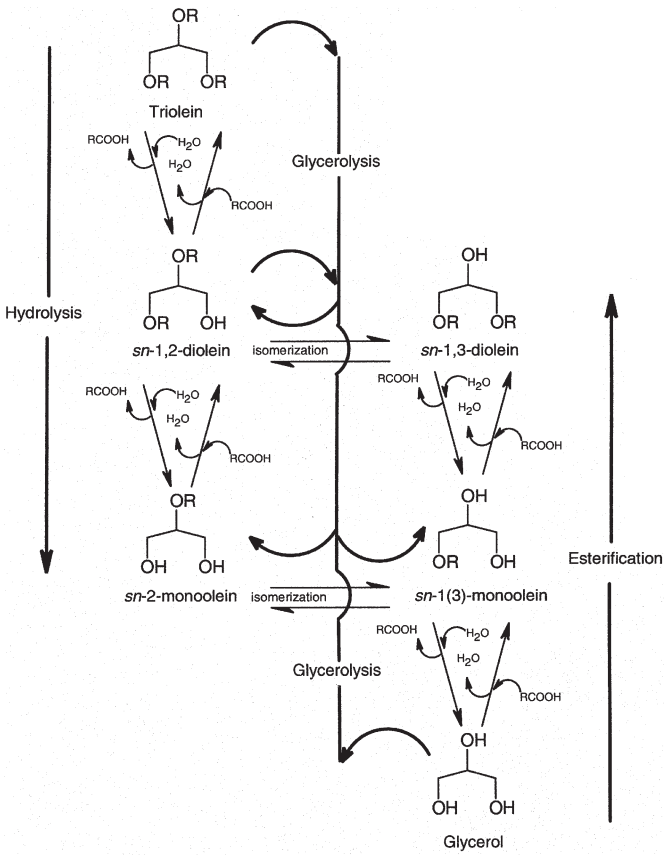

FIG. 4. Schematic representation of the reactions found in a lipase-catalyzed transformation using glycerol and triolein as initial substrates. 1- and 2-monoolein isomers are produced mainly by glycerolysis. The 1,3-diolein was predominantly produced by esterification, whereas 1,2-diolein was the product of both hydrolysis and glycerolysis. Isomerization was found as a minor reaction in this process. R, oleyl group.

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