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Synthesis of Mono- and Dioleylglycerols Using an Immobilized Lipase

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ABSTRACT

Transesterification between ethyl oleate and glycerol, or esterification between oleic acid and glycerine by the immobilized lipase preparation SP 435 from *Candida antarctica*, was investigated. Different temperatures and reactants ratios were tried. In all cases, transesterification yielded better results. When the reaction was carried out in *n*-heptane the addition of 3% water gave highest yields to mono and diolein; the formation of diolein can reach levels comparable to those of monoolein. However, when the reaction was carried out in one-liquid-phase only (in the presence of acetonitrile or acetone), the reaction was much more selective to monoolein.

Index Entries: Monoglycerides and diglycerides; lipase B; *Candida antarctica*; immobilized lipase; bioconversions; transesterification; esterification; organic solvents; food additives.

INTRODUCTION

Mono and diglycerides of fatty acids are good emulsifiers and stabilizers (E. E. C. code: E471). The use of enzymes for their preparation presents many advantages as compared to chemical procedures. Lipases (EC 3.1.1.3) comprise a group of enzymes whose biological function is to catalyze the hydrolysis and synthesis of triacylglycerols (*1*). They can be used for preparation of mixtures of mono- and diglycerides, either by the synthetic

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route or by the hydrolytic one. Since the demonstration that enzymes can catalyze reactions in organic solvents (2), lipases have received growing attention owing to their selectivity. When using lipases (and other hydrolases) as synthetic catalysts, it is absolutely necessary to decrease water concentration in the reaction mixture by the inclusion of suitable organic solvents (3). As regards their ability to hydrolyze triacylglycerols (4), lipases have been classified in unspecific, 1,3-specific, and fatty-acid-specific. These various specificities are being exploited in the synthesis of acylglycerols in order to determine if the specificity of action is the same for hydrolysis and synthesis. The presence of organic solvents hinders the enzyme-independent acyl migration in mono- and diglycerides commonly detected in water.

Previous investigations showing that C18:0 glycerides do not increase the serum levels of cholesterol, since the stearic acid liberated on hydrolysis is immediately oxidized by a desaturase system to form oleic acid, have been recently reviewed (5). However, glycerides of C12:0 to C16:0 increase these levels, whereas fatty acids of chain-length shorter than C12 do not (5). Consequently, our first objective has been the preparation of mixtures of mono- and diolein that could be employed as food additives.

While studying the preparation of C4:0 mono- and diglycerides by reaction between glycerol and ethyl butyrate in the presence of several lipases, we found that only monobutyrin was formed, with negligible amounts of dibutyrin (6). Therefore, in the present work we studied the preparation of mono + diolein by transesterification and esterification using an 1,3 (immobilized) lipase (from *Candida antarctica*), probing the effect of polar and apolar solvents.

MATERIALS

The immobilized enzyme SP 435-L (*Candida antarctica* lipase B insolubilized on a macroporous acrylic resin) was kindly provided by Novo Nordisk (Baegsvaerd, Denmark) (7). Glycerol (G) and dioxane were purchased from Merck (Darmstadt, Germany); oleic acid (OA) and ethyl oleate (EO), 1-monooleyl-*rac*-glycerol (Catalog No. M7765), 1,3-diolein (D3627) and triolein (T7140), from Sigma (St. Louis, MO); *n*-heptane, from Carlo Erba (Milano, Italy); acetonitrile (HPLC), from Scharlau (Barcelona, Spain); 2-pentanone, from Aldrich (Milwaukee, WI); and acetone (HPLC), from Panreac (Barcelona, Spain).

METHODS

Synthetic reactions were carried out as follows: To 55.2 mg (0.6 mmol) of glycerol and 62.2 mg (0.2 mmol) of ethyl oleate (EO) or 56.4 mg

(0.2 mmol) of oleic acid (OA) were successively added approx 2 mL of organic solvent, a variable amount of 0.1*M* phosphate buffer, pH 7.2, and 20–100 mg of immobilized lipase. The reaction mixture was maintained at constant temperature (30–80°C) with magnetic stirring at 250 rpm. The reactions were carried out using a refrigerated condenser on top of the reactor (a 10-mL round-bottom flask) to avoid loss of volatile compounds. In some experiments the molar ratio glycerol/EO or glycerol/OA was increased up to 3/9. When running the reaction in the esterification mode, 3A molecular sieves were added.

After a certain reaction time, the enzyme was separated by filtration. Since the organic solvents used in these studies—with the exception of acetonitrile—cause interferences in the high pressure liquid chromatography (HPLC) analysis, the rest of the reaction mixture was heated at 100°C until complete solvent evaporation. After that, the remaining mixture was dissolved in 2 mL of chloroform.

The reaction products were analyzed by HPLC using a Spectra Physics isocratic pump, a refraction index detector (Shodex, Tokyo, Japan) connected to a Spherisorb ODS-2 column, and a Spectra Physics integrator SP 4290. The separation of products was achieved using (1:1) (v/v) acetonitrile/acetone as mobile phase, with a flow of 2 mL/min. The column temperature was kept constant at 40°C. Calibration analyses were obtained with pure grade OA, EO, 1-monoolein (MO), 1,3-diolein (DO), and triolein (TO). Retention times were: 1.5 min for MO, 1.7 min for OA, 2.1 min for EO, 3.3 min for DO, and 14.7 min for TO.

RESULTS

Previous experiments of glycerine acylation using several lipases (results not shown) showed that the most promising one was SP 435, and therefore this investigation was carried out with it.

Influence of the Water Content

In a previous study we found (*6*) that the addition of 5% buffer to the reaction mixture formed by glycerol and ethyl butyrate—no solvent added—yielded the optimal conversion to monobutyrin. The amount of water in the reaction mixture has been found to have a considerable effect on the relative amounts of hydrolysis products (mono- and diglycerides and fatty acids) formed in the enzymatic interesterification of butter fat (*8*). Therefore, we checked—using heptane as a solvent—the influence of the aqueous content in the formation of mono- and diolein. Figure 1 presents the transesterification results obtained when the amount of buffer (sodium phosphate buffer 0.1*M*, pH 7.2) added was varied from 1 to 9% (v/v); the experimental values are given in percent conversion or yield (referred to the starting concentration of glycerol) as a function of

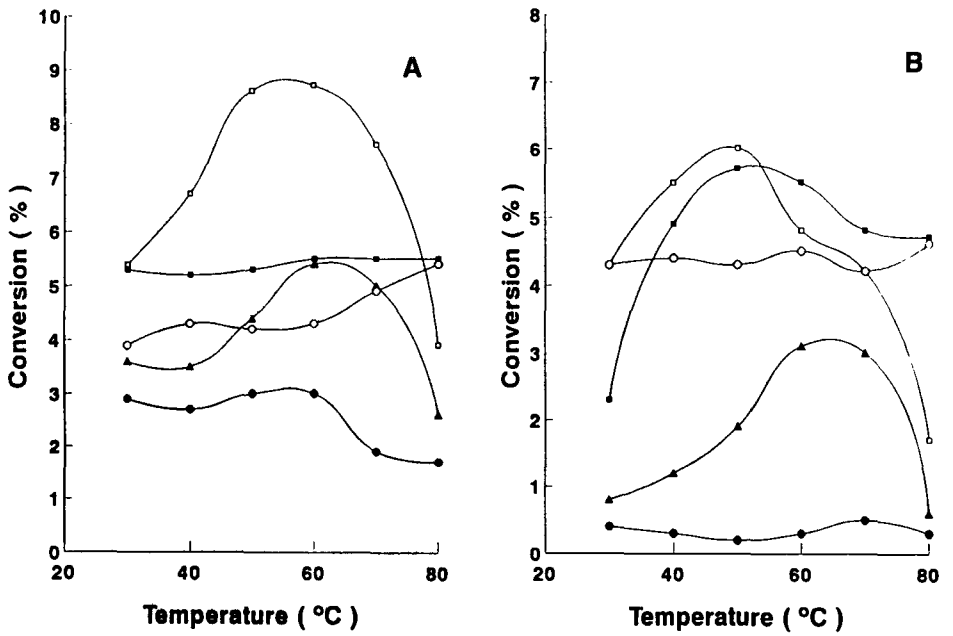

Fig. 1. Formation of MO (**A**) and DO (**B**) at different temperatures as a function of the amount of buffer added: (○) 1%; (□) 3%, (■) 5%; (▲) 7%; (●) 9%. Reaction conditions: G/EO = 3/1; 20 mg lipase; heptane as solvent; 30 min reaction time.

temperature (30–80°C). As can be seen, conversion after 30 min to MO (Fig. 1A) and DO (Fig. 1B) increased up to 3% buffer, and then decreased (in the absence of buffer, the yield to MO and DO was negligible). Therefore, 3% was chosen as the optimum volume of the aqueous phase when using apolar solvents; this selection was also in agreement with the results obtained in experiments similar to those in Fig. 1, but using OA instead of EO (not shown). However, the values of conversion found (maximum of 9% to MO and 6% to DO, at 50°C) were too small, and therefore we tested the reaction in the presence of several polar solvents. The results obtained using acetonitrile, acetone, dioxane, and 2-pentanone as the reaction media for the transesterification between glycerol and ethyl oleate at 50°C are shown in Table 1 (reaction time, 30 min). No triolein was found. The best results were obtained with acetonitrile. When buffer (3%) was present in the reaction mixture, the conversions to MO and DO decreased drastically; therefore, in further experiments no buffer was added when the organic solvent was polar.

Influence of the Temperature

Next, we studied the influence of temperature on the reaction rate in polar solvents (acetonitrile and acetone). Figure 2 shows the yields obtained in acetonitrile for esterification with OA (Fig. 2A) and for trans-

Table 1
Transesterification Yields in Polar Solvents[a]

Solvent/ percent buffer	Yield, %	
	MO	DO
Dioxane/0	8	1.7
Dioxane/3	1.2	0
Acetonitrile/0	34	1.2
Acetonitrile/3	13	3.3
Acetone/0	20	0.8
Acetone/3	4.5	0
2-Pentanone/0	14	0.5
2-Pentanone/3	3.6	0.1

[a]Reaction conditions: G/EO = 3/1 (G concentration, approx 0.3*M*); 50°C; 60 mg lipase; 30 min reaction time. Each solvent was tried in the absence and presence (3%) of buffer.

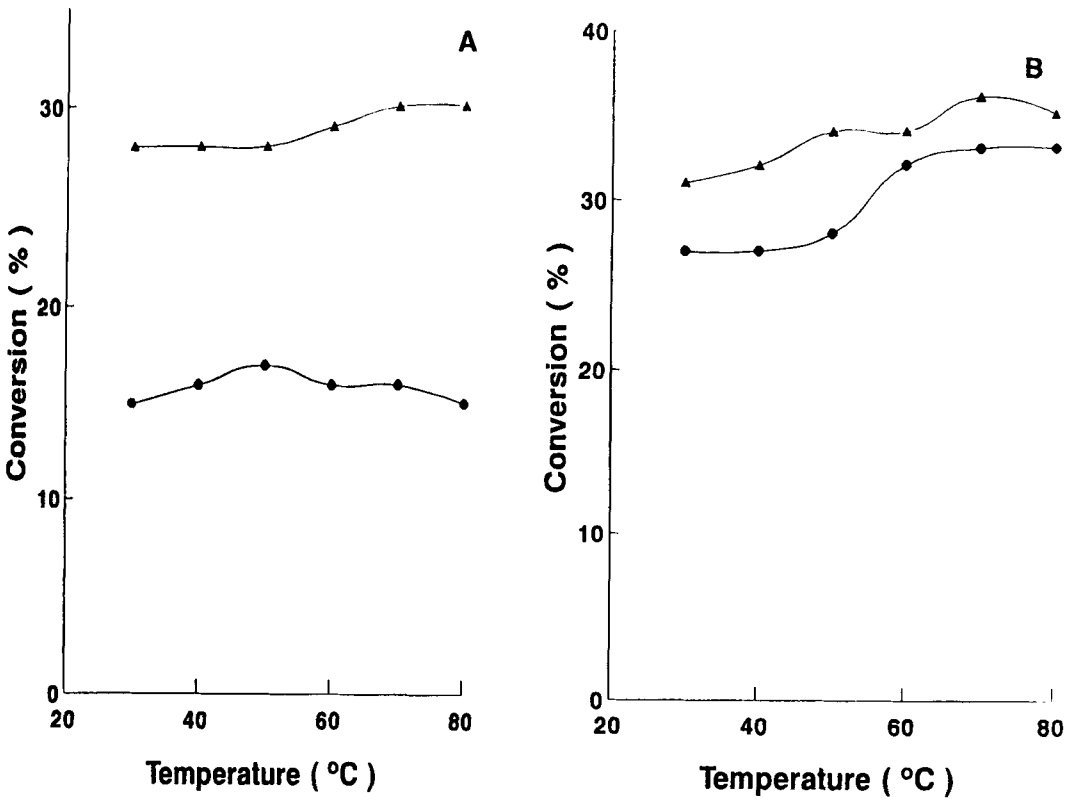

Fig. 2. Formation of MO in esterification (**A**) and transesterification (**B**) experiments in acetonitrile using two concentrations of lipase. Reaction conditions: G/Oleyl (OA or EO) = 3/1; 30 min reaction; (●) 20 mg, (▲) 60 mg.

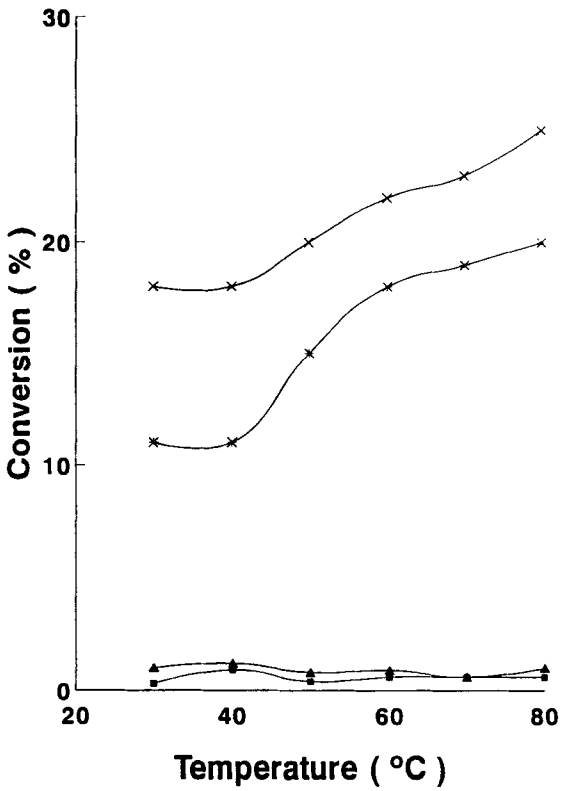

Fig. 3. Transesterification conversion in acetone to MO and DO as a function of temperature, using two concentrations of lipase. G/EO = 3/1; 30 min reaction; (*) MO, 20 mg; (■) DO, 20 mg; (×) MO, 60 mg; (▲) DO, 60 mg.

esterification with EO (Fig. 2B). Figure 3 shows the results when the transesterification was done in acetone. Best results were obtained by transesterification at 70°C in acetonitrile with 60 mg of lipase. (The influence of temperature on reaction rate in heptane was included in Fig. 1.)

Effect of the Reactants Ratio

The above described experiments were carried out at a ratio G/oleyl = 3/1. In order to enhance the yield to mono + diolein, the molar ratio was then increased up to 3/9. Figure 4 displays the esterification (Fig. 4A) and transesterification (Fig. 4B) results in acetonitrile at the optimum temperature (70°C). As can be seen, the highest conversion with OA to MO + DO (61% = 42% MO + 19% DO) was obtained at 3/6 ratio, whereas with EO the highest conversion was 94% (= 63% + 31%) at 3/8 ratio; the same yields were obtained at a ratio of 3/9. Similarly, Fig. 5 shows the transesterification yield obtained in acetone at 50°C. In this solvent the highest conversion found was 58% (51% MO and 7% DO) at 3/6 ratio.

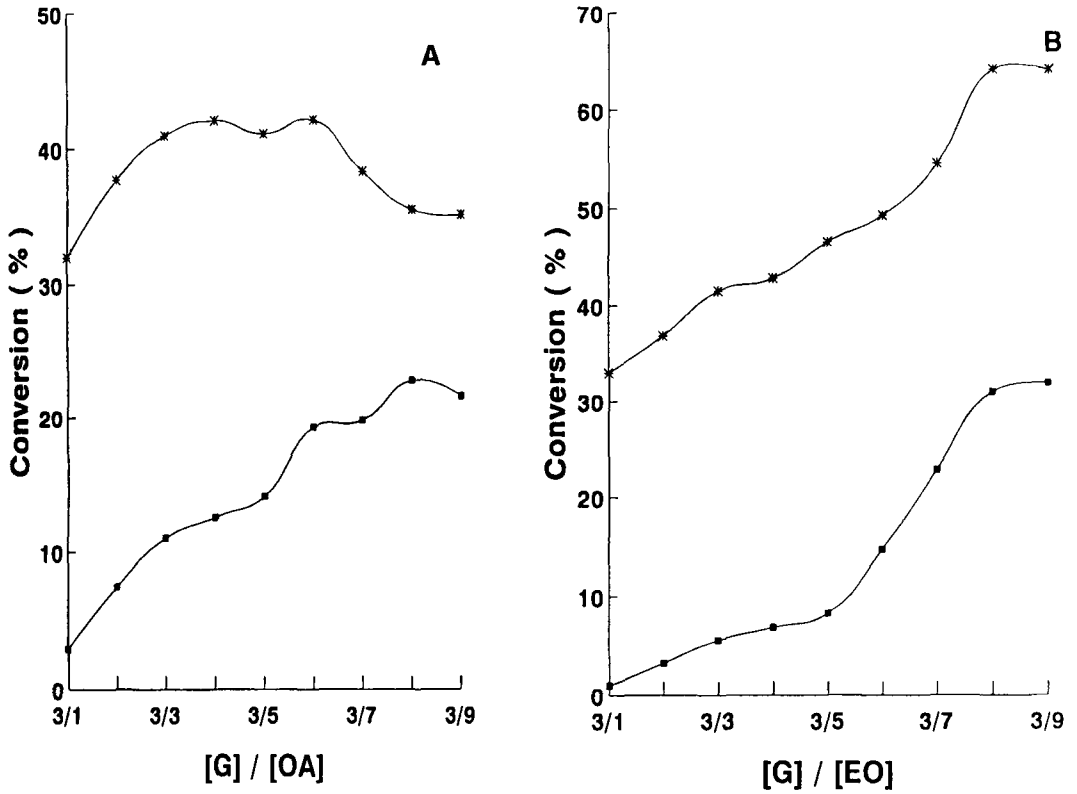

Fig. 4. Esterification (**A**) and transesterification (**B**) yields at 70°C in acetonitrile vs reactants ratio. Conditions: 60 mg lipase; 30 min reaction; (*) MO; (■) DO.

Transesterification at Long Reaction Times

The reaction time in the above experiments was in all cases 30 min. Transesterification at 50°C in heptane at a ratio G/EO = 3/9 was followed up to 6 d (*see* Fig. 6). In this case TO is present in the reaction products, reaching 19% conversion after 6 d. The highest conversion to acylglycerols was observed after 3 d (46% MO + 37% DO + 10% TO).

Figure 7 shows the conversion to glycerides in heptane at a ratio G/EO = 3/9 as a function of the amount of lipase. Total conversion (47% MO + 36% DO + 16% TO) was measured with 100 mg lipase.

Study of the Time Reaction Course at the Optimal [G]/[EO] Ratio

As seen in Table 1, the maximum theoretical yield of conversion (33.3% MO for G/EO = 3/1) was obtained in acetonitrile after 30 min of reaction. However, when acetone or heptane were used, the complete

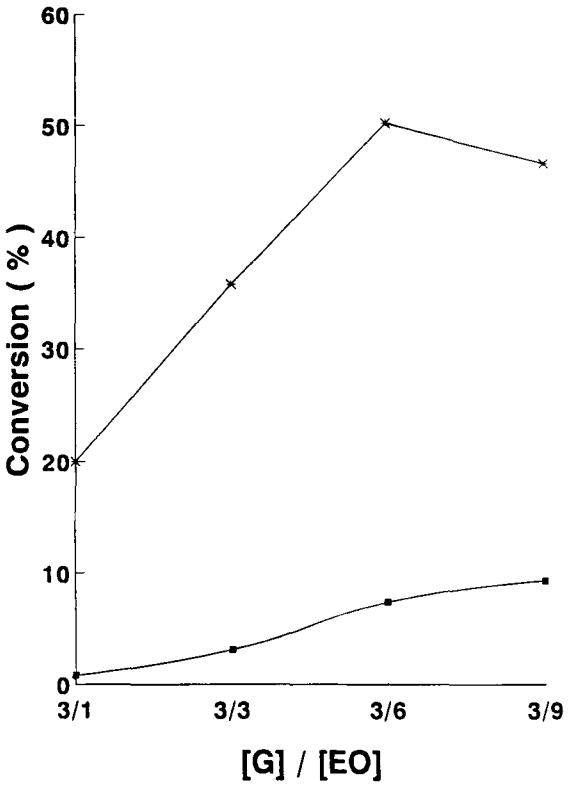

Fig. 5. Transesterification in acetone at 50°C vs reactants ratio. Other conditions as in Fig. 4.

transformation of glycerol to mono + diolein could not be obtained in 0.5 h. Hence, we studied the reaction course at different times and ratios. Table 2 lists the comprehensive results obtained with three solvents.

DISCUSSION

Lipases have been used in vitro to prepare mixtures of glycerides (*9–14*) for use in the food and other industries. Monoglycerides can be prepared by glycerolysis of triacylglycerols (*15–17*) or by esterification of glycerol and an acyl donor (*18–21*). The preparation of diglycerides has also been reported (*22,23*). Finally, the synthesis of triolein (*24*) and of other triacylglycerols (*25,26*) has recently been investigated. In addition, Akoh has just reported (*27*) the esterification of fatty acids in the absence of organic solvent or the transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols. Concerning the engineering of the reaction in the synthesis of glycerides in aprotic solvents, Schneider and collaborators (*21,23*) have reported the adsorption of glycerol onto a solid support to enhance its solubility. And Lang et al. (*28*) added phenylboronic acid to the reaction mixture.

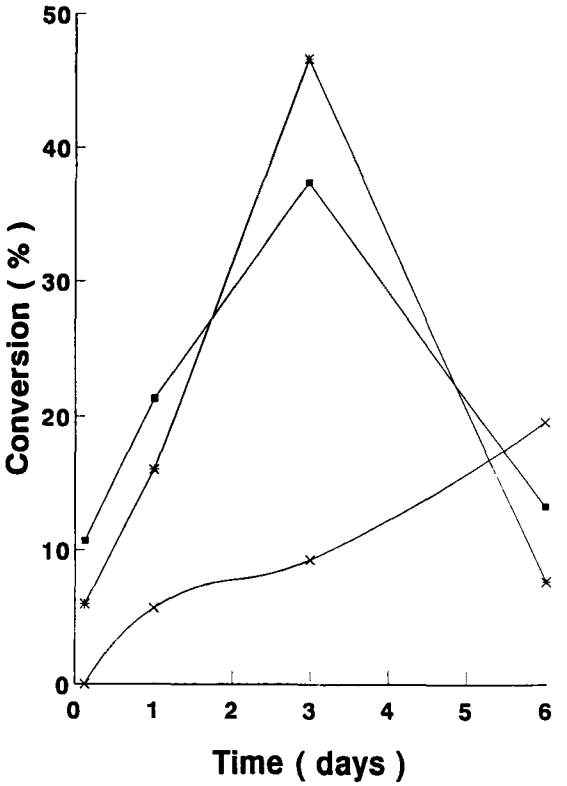

Fig. 6. Transesterification yield in heptane (+ 3% buffer) vs reaction time. Conditions: G/EO = 3/9; 100 mg lipase; 50°C; (*) MO; (■) DO; (×) TO.

In the present study our aim was the preparation of mixtures of mono- and diolein, useful as food additives. Directives of the World Health Organization require that these mixtures have at least 70% (w/w) of mono + diglyceride, a minimum of 30% monoacylglycerol, and a content of glycerol and triglyceride both below the 10% level. To achieve that aim we have tested and compared both the glycerol transesterification (with ethyl oleate) and esterification reactions.

First, we studied the influence of the water present in the reaction in apolar solvents (heptane). Smaller yields to MO and DO were obtained in the esterification compared with the transesterification. In the latter (Fig. 1), 3% buffer and 50°C were selected as the optimal parameters for further experiments. (Other authors (*19*), studying the lipase esterification with oleic and other fatty acids found the highest conversion—to mono and diglycerides—in the presence of 6% water.) However, the yield values found after 30 min reaction were too low (9% MO and 6% DO).

On the other hand, yields were good in plain polar solvents (no buffer added, *see* Table 1) of different hydrophobicity (logP values: dioxane, −1.1; acetonitrile, −0.33; acetone, −0.23; pentanone, 0.80). After 30 min reaction, total conversion (35%) was obtained in acetonitrile and 21% in acetone. Another important difference between the results in polar

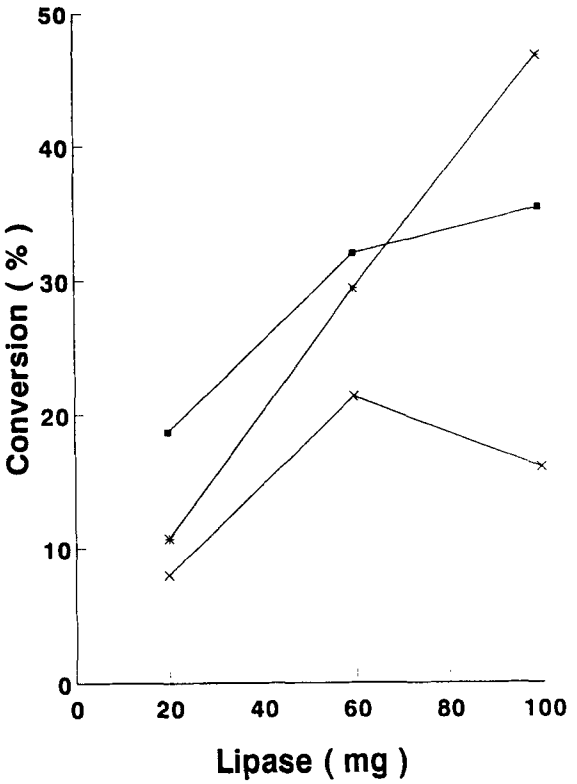

Fig. 7. Transesterification conversion in heptane (+3% buffer) using different amounts of lipase. Conditions: G/EO = 3/9; 50°C; 3 d reaction; (*) MO; (■) DO; (×) TO.

Table 2
Transesterification Yields of MO, DO, and TO at Different Times and Reactants Ratio[a]

Solvent	Reaction time	Percent Bu	mg Lipase	G/OE	Yield, percent MO	DO	TO
Acetonitrile	0.5 h	0	60	3/1	34	1.2	0
Acetone	0.5 h	0	60	3/1	20	0.8	0
	24 h	0	60	3/1	23	0.9	0
	3 d	0	60	3/1	26	2.5	0
	0.5 h	0	60	3/6	50	7.4	0
	5 h	0	60	3/6	55	11.2	0.2
Acetone	24 h	0	60	3/6	64	14.7	0.5
Acetonitrile	3 h	0	60	3/9	53	17.3	0.9
Acetone	2 h	0	60	3/9	68	14.6	2.0
Heptane	24 h	3	100	3/9	16	21.3	5.7
	3 d	3	100	3/9	46.6	37.3	9.3
	6 d	3	100	3/9	7.7	13.3	19.6

[a]Reaction conditions: 50°C; percent Bu, percent buffer added to the solvent.

solvents and those in heptane is the production of DO. In polar solvents high monoolein yield was obtained and the reaction was selective to that product. In contrast, in heptane (logP = 4) the yields of MO and DO were similar and very low. Therefore, from these results at short reaction times, it can be concluded that the transesterification (and also the esterification, results not shown) catalyzed by *C. antarctica* SP 435 lipase is faster and more selective to MO in these polar solvents than in *n*-heptane.

The effect of the temperature on the reaction rate during the first 30 min is also different in the polar and the apolar organic solvents studied. In acetonitrile and in acetone, without addition of water, the reaction rate increased with temperature in the range 30–80°C, both for the transesterification and the esterification (Figs. 2 and 3). It must be noted that the percent conversion of glycerol does not increase by more than 5% when the temperature is raised from 30 to 80°C. In contrast with this, when heptane is used as the reaction medium (containing 1–9% of buffer, Fig. 1) the temperature had a great influence. In heptane there are two behaviors:

1. For transesterification (Fig. 1) bell-shaped curves were normally found, from which 3% buffer and 50°C were obtained as optimum parameters. This seems to indicate that in 3% buffer the enzyme conformation is more active than in the presence of 1 or 0% buffer. The sharp decrease in conversion at 80°C points out that in the presence of larger proportions of buffer in the reaction mixture, the stability of the enzyme is drastically reduced.
2. For esterification (results not shown), conversion values increased up to 80°C for 1, 3, and 5% buffer addition, indicating that in this reaction mode the enzyme maintains its stability at higher temperatures. Nevertheless, transesterification was always more productive than esterification for the preparation of mono + diolein.

The high selectivity of the reaction in polar solvents is demonstrated in Figs. 2 and 3. For a G/EO molar ratio of 3/1, a nearly total conversion of glycerol to MO can be obtained in acetonitrile (about 33% yield) after 30 min. Figure 2 also shows that the transesterification is more interesting than direct esterification, since in the first case nearly the same conversion is obtained with a smaller amount of enzyme (20 mg).

The selectivity of the reaction after 30 min can be modified by changing the G/OA or G/EO molar ratio from 3/1 to 3/9 (Figs. 4 and 5). In acetonitrile, the selectivity of the reaction is the highest when the oleyl concentration is the lowest. However, maximum production of the monoglyceride was obtained with ratios higher than 3/1: 63% of MO in the transesterification in acetonitrile with 60 mg of enzyme and G/EO = 8 (Fig. 4B); conversion to DO was 31%. In acetone, the reaction is more selective (51% MO and 7% DO, *see* Fig. 5), although the productivity was smaller.

In the transesterification in heptane at long reaction times (*see* Fig. 6) TO was present in the reaction products. In heptane the reaction is always poorly selective. The yield of monoglyceride is higher than the corresponding one for diglyceride when OA or EO is in defect, and the opposite holds when the OA or EO is in excess (results not shown). Regarding conversion to glycerides, the optimal G/OE or G/OA ratio is 3/9. Transesterification using this ratio and 100 mg lipase gave best results after 3 d of reaction (cf. Fig. 6); at longer times overall conversion to glycerides decreased considerably, although TO amount increased. The relative proportions of MO, DO, and TO changed drastically when lower amounts of biocatalyst, 20 or 60 mg, were used (cf. Fig. 7). The study at different reaction times (Table 2) demonstrated that the best results for MO were obtained in acetone: 68% after 2 h with a G/EO = 3/9, or 64% after 1 d with a G/EO = 3/6. In both cases the conversion to DO was 15% and that to TO was 1%. Therefore these figures are above the minimum values of the directives of the World Health Organization concerning the composition of additive E471. These minimum values are also exceeded by transesterification in heptane after 3 d and using a G/EO molar ratio of 3/9.

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