Lipase-Catalyzed Transesterification of Phosphatidylcholine at Controlled Water Activity

Ingemar Svensson*, Patrick Adlercreutz and **Bo Mattiasson**
Department of Biotechnology, Chemical Center, University of Lund, S-221 00 Lund, Sweden

The incorporation of a free fatty acid into the *sn*-1 position of phosphatidylcholine by lipase-catalyzed transesterification was investigated. The thermodynamic water activity of both the enzyme preparation and the substrate solution was adjusted to the same value prior to the reaction. The reaction rate increased with increasing water activity but the yield of modified phosphatidylcholine decreased due to hydrolysis. By using a large excess of the free fatty acid (heptadecanoic acid), the hydrolysis reaction was slowed down, so a higher yield was obtained at a given degree of incorporation. The best results were obtained with *Rhizopus arrhizus* lipase immobilized by adsorption on a polypropylene support. With this preparation, a yield of 60% and nearly 50% incorporation of heptadecanoic acid (100% incorporation in the *sn*-1 position) was obtained at a water activity of 0.064. The enzyme preparation had good operational stability and position specificity. Little incorporation (<1%) was observed in the *sn*-2 position, when almost all the fatty acid in the *sn*-1 position was exchanged.

KEY WORDS: Lipase, phosphatidylcholine, transesterification, water activity.

There are several reasons why lipases and phospholipases are useful for modifying the acyl group composition in natural phospholipids. The mild reaction conditions and the high enzymatic regiospecificity are strong advantages, especially if the lipids are to be used in human food or for medical purposes. By exchanging fatty acids asymmetrically in the phospholipid molecule, new physical properties can be achieved. These modified lipids can be used in lipid/membrane research or as emulsifiers for food, cosmetics or medical substances. A special application of the enzymatic transesterification is the position-specific labelling of phospholipids with radioactive or photoactive acyl groups. Furthermore, biologically active polyunsaturated fatty acids that are chemically unstable can be incorporated under mild conditions.

The normal hydrolytic action of lipases and phospholipases has been used for preparing phospholipids with different fatty acids in the two carboxyl ester bond positions. After hydrolysis, the lysophospholipid formed can be nonenzymatically esterified with the desired fatty acid. To achieve acylation in a specific position not only must the lipase be position-specific but spontaneous acyl migration must also be considered (1). By reversing the lipase action to esterification or transesterification, it is possible to use the regioselectivity of the lipases in the acylation step and thereby reduce the problem of acyl migration.

A report of lipase-catalyzed regiospecific 1-position (1-pos) transesterification of fatty acid in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was made by Brockerhoff *et al.* (2). They used *Rhizopus delemar* lipase in buffer with phosphatidylcholine and oleic acid as substrates. Yoshimoto *et al.* (3) used polyethyleneglycol-modified *Candida cylindracea* lipase dissolved in benzene to incorporate polyunsaturated fatty acids into phosphatidylcholine. A two-phase water/oil system was used by Yagi *et al.* (4) for transesterification of PC and PE with different fatty acids. Yoichiro and Setsuko (5) also used a water/oil system but used sardine oil as source of polyunsaturated fatty acids for incorporation into soy phospholipid. We used a system with immobilized lipase in toluene for incorporation of heptadecanoic acid in egg phosphatidylcholine (6). In all systems the predominant problems were the hydrolysis reactions and low yields. Our system is practical because of the easy recovery of enzyme and the possibility to keep the water content low to avoid undesired hydrolysis.

Transesterification of the fatty acid in the *sn*-2 position should be possible with phospholipase A_2, but so far no practical method for doing this has been presented. It has been easier to carry out esterification between lysophosphatidylcholine (LPC) and free fatty acid, but the yield of PC formed was only in the range of 6–7% (7,8).

In this work the previous reaction system (6) has been improved. Both the yield of product PC and the amount of new fatty acid incorporated into the *sn*-1 position of egg PC have been increased.

EXPERIMENTAL PROCEDURES

Chemicals. L-α-Phosphatidylcholine (PC from egg, 98.5% purity, average molecular weight 762 g/mole) was a gift of Karlshamns AB, Division Lipid Teknik (Stockholm, Sweden). L-α-phosphatidylethanolamine (PE from egg yolk, 98%), L-α-phosphatidylinositol (PI from soybean, sodium salt, 99%), L-α-phosphatidic acid (PA from egg yolk, sodium salt, 98%), heptadecanoic acid (purity 99%) and phospholipase A_2 from porcine pancreas (Type II) were obtained from Sigma Chemicals (St. Louis, MO). Immobilized lipases from *Rhizomucor miehei* (Lipozyme IM 20 and IM 60) were gifts of Novo Industri A/S (Bagsvaerd, Denmark). Lipase from *Rhizopus arrhizus* (Lipase 80,000) was a gift of Gist-Brocades S.A. (Delft, The Netherlands). Polypropylene support (EP 100, 400–1000 μm) was donated by Akzo (Obernburg, Germany). Sodium methoxide was obtained from Merck (Darmstadt, Germany). Solvents used were of high-performance liquid chromatography (HPLC)-grade, and all other chemicals were of analytical grade.

Preparation of immobilized enzyme. Lipase from *Rhizopus arrhizus* (200 mg unless otherwise stated) was dissolved in 20.0 mL sodium phosphate buffer (20 mM, pH 6.0). The enzyme solution was mixed with 1.0 g polypropylene support (EP 100), which was prewet with ethanol (normally 0.5 mL). After incubation for 24 h in a shaking bath at 25°C, the preparation was filtered and washed two times with distilled water. The preparation was dried overnight under reduced pressure.

*To whom correspondence should be addressed at Department of Biotechnology, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden.

Transesterification reaction. To obtain a defined initial water activity of the enzyme preparation and the substrate solution, both were equilibrated over saturated salt solutions in closed vessels for 24 h at 25°C (9). Lipozyme was equilibrated with a solution of LiCl ($a_w = 0.113$) and *Rhizopus arrhizus* lipase on EP100 with a solution of K_2CO_3 ($a_w = 0.43$) unless otherwise indicated. Other salt solutions used were: LiBr ($a_w = 0.064$), KAc ($a_w = 0.22$), $MgCl_2$ ($a_w = 0.33$) and NaCl ($a_w = 0.75$). To start the reaction, the enzyme/support preparation (150 mg) and 1.0 mL of a solution of PC (10 mM) and heptadecanoic acid (400 mM) in toluene were mixed in Teflon-lined screw-cap vessels (4 mL). The vessels were then incubated in a shaking water bath at 40°C and 150 rpm.

Isolation of reaction products. The immobilized enzyme was removed from the product solution by filtration, and it was washed with 3 × 1 mL $CHCl_3$/MeOH (2:1) to extract phosphatidylcholine adsorbed on the enzyme preparation. These extracts were added to the product solution (in toluene) and the solvents were removed with a rotary evaporator. The dry residue was redissolved in 1.0 mL of toluene, and 50 μL was withdrawn for thin-layer chromatography to separate PC and LPC. The quantitation of PC and LPC was done by measuring the total fatty acid content in phospholipid fractions (see below).

Thin-layer chromatography. Samples (50 μL) from the reaction solutions were applied as bands on thin-layer chromatography (TLC) plates (Kieselgel 60, 0.2 mm, Merck). The solvent system used to separate phosphatidylcholine, lysophosphatidylcholine (LPC) and free fatty acid consisted of $CHCl_3/MeOH/H_2O$ (65:35:5). Spots on the TLC plates were visualized by spraying the plates with 0.1% 2,7-dichloro-fluorescein in ethanol and air drying. The PC spots ($R_f = 0.37$) were scraped off and stored in screw-cap reaction tubes at −20°C for fatty acid analysis.

Fatty acid analysis of phosphatidylcholine. Fatty acid methyl esters were formed by mixing 2.0 mL of 0.5 M sodium methoxide in methanol with the scrapings from the TLC plates, and 10 μL internal standard (129.5 nmol docosanoic acid methyl ester) in heptane was added. After incubation for 10 min in a water bath at 50°C, 400 μL of hexane and 4 mL of saturated NaCl solution were added to each tube. After vortexing (3 × 5 s) and centrifugation, 1 μL of the upper layer was withdrawn for gas chromatography (GC) analysis.

Gas chromatography analysis. Fatty acid methyl esters were analyzed on a Shimadzu gas chromatograph GC-14A with flame-ionization detector (Shimadzu, Kyoto, Japan). The column was a SP-2380 (30 m, 0.32 mm i.d., 0.20 μm film thickness, Supelco, Bellefonte, PA); the column temperature was programmed between 180–200°C. Experimental response factors for the different fatty acid methyl esters and internal standard were used, and corrections were made for the methylation yield.

Fatty acid position analysis of phosphatidylcholine. Heptadecanoic acid-enriched phosphatidylcholine was purified from the reaction mixture on preparative thin-layer chromatography plates (Kieselgel 60 F254, 2.0 mm, Merck) developed in $CHCl_3/MeOH/H_2O$ (65:35:5). The silica containing the PC band was extracted with 6 × 10 mL of $CHCl_3/MeOH/H_2O$ (5:5:1). Phosphatidylcholine was hydrolyzed to LPC with lipase *(Rhizopus arrhizus)* to remove the fatty acid in the *sn*-1 position or with phospholipase A_2 for the *sn*-2 position. Phosphatidylcholine (3 mg) was dissolved in 1.0 mL buffer (10 mM *tris*-HCl buffer, pH 9.0, 10 mM sodium cholate and 5 mM $CaCl_2$ with phospholipase A_2 and 20 mM phosphate buffer pH 7.0, 10 mM sodium cholate with the lipase) and incubated with enzyme (7 U of phospholipase A_2 or 20 mg lipase). After rapid shaking at 40°C for 30 min, the LPC was extracted to the lower chloroform phase, that was formed by adding 4 mL $CHCl_3$/MeOH (2:1), and isolated by thin-layer chromatography. The fatty acids in LPC were analyzed in the same way as those in PC.

RESULTS AND DISCUSSION

The influence of the substrate concentration on the lipase-catalyzed transesterification of PC was investigated. When the concentration of the fatty acid to be incorporated (heptadecanoic acid) was varied, the degree of incorporation after 10 h of reaction varied only slightly (Fig. 1A). However, the amount of PC remaining increased with increasing fatty acid concentration. With low fatty acid concentrations, a large proportion of the PC was lost through hydrolysis. To obtain a good yield of modified PC,

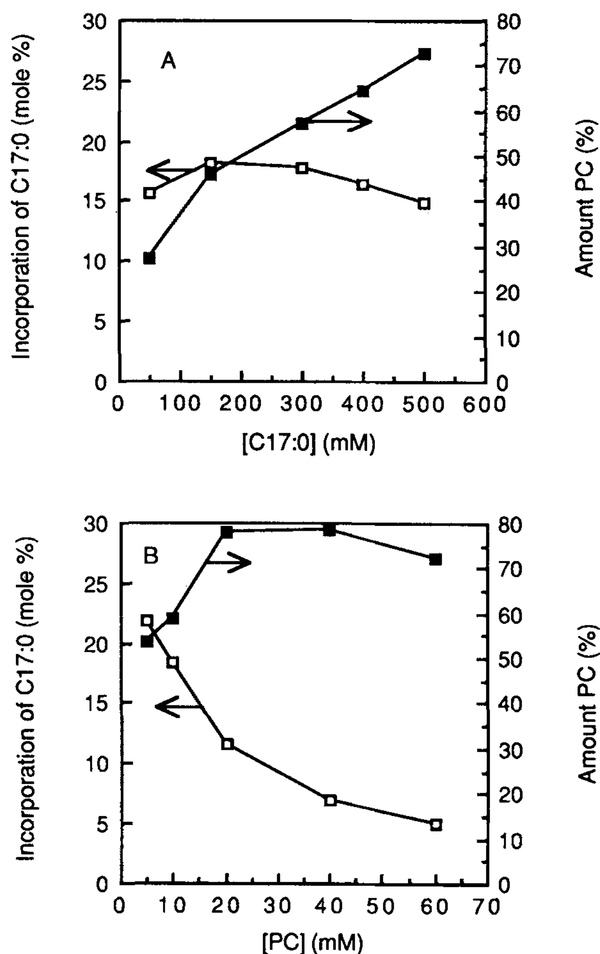

FIG. 1. Incorporation of heptadecanoic acid (□) in phosphatidylcholine (PC) and the amount of PC (■) remaining after 10 h of reaction as a function of (A) initial fatty acid concentration (10 mM PC) or (B) initial concentration of PC (400 mM C17:0). The enzyme was Lipozyme IM20 and initial water activity was 0.113.

it is thus important to have a high fatty acid concentration; a concentration of 400 mM was used in the rest of the experiments unless otherwise stated. These reactions were carried out in toluene. The reaction rate in hexane was slightly higher, but the solubility of fatty acids was considerably lower than in toluene, so the latter was considered the best solvent for this application. The degree of incorporation of heptadecanoic acid in 10 h decreased with increasing concentration of PC (Fig. 1B). This was expected since the enzyme/substrate ratio decreased with increasing PC concentration. The amount of PC remaining increased with increasing PC concentration up to 20 mM. Higher concentrations of PC do not seem suitable if a high yield of modified PC is to be achieved because the same degree of hydrolysis was then obtained and a lower degree of incorporation of the new fatty acid. In the rest of the experiments, a PC concentration of 10 mM was used, unless otherwise stated.

It is clear that hydrolysis is the cause of the low yields of modified PC under some conditions. On the other hand, some hydrolysis must occur because the mechanism of the transesterification reaction involves LPC as an intermediate. In the first reaction step, a molecule of PC is hydrolyzed in the 1-position. The LPC can react with an acyl enzyme complex formed from the enzyme and the fatty acid to be incorporated. The extent of hydrolysis is to a large extent dependent on the amount of water on the biocatalyst. A good measure of the amount of water is the thermodynamic water activity. A series of experiments was carried out at fixed water activities; the water activity of the enzyme preparations and the substrate solutions were adjusted prior to the reaction by vapor phase equilibration with saturated salt solutions.

In these experiments another type of lipase catalyst was also tried for the PC transesterifications. Lipase from *Rhizopus arrhizus* was immobilized on the polypropylene support EP100 by adsorption. The adsorption process was effective; at least 99% of the hydrolytic lipase activity (measured by pH-stat method on emulsified tributyrin, pH 8.0 and 30°C) was removed from the solution. To obtain such high removal of lipase activity, it was necessary to use sufficient ethanol to prewet the polypropylene (3 mL/g EP100). The effect of the enzyme loading on the transesterification of PC is shown in Figure 2. A loading of 2000 U/g EP100 (200 mg lipase/g EP100) was chosen for the water activity study.

After 10 h of incubation with Lipozyme IM60 as catalyst, the incorporation of heptadecanoic acid varied between 33 and 45% (Fig. 3A). The highest incorporation was observed at $a_w = 0.43$. At low water activities, incorporation of heptadecanoic acid into LPC was also observed. The amount of remaining PC (Fig. 4A) decreased with increasing water activity, as expected. The highest amount of LPC was observed at the highest water activity used, but a large proportion of the phospholipid was further hydrolyzed to *sn*-glycero-3-phosphorylcholine, so that the sum of the yields of PC and LPC was around 40%

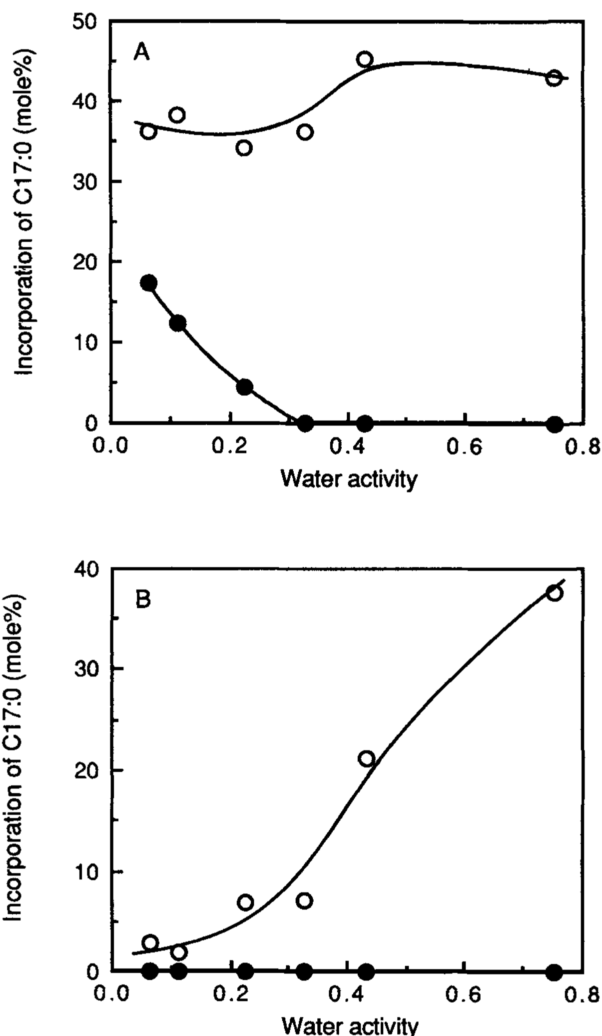

FIG. 3. Incorporation of heptadecanoic acid into phosphatidylcholine (○) and lysophosphatidylcholine (●) fractions as a function of initial water activity. The reaction was run for 10 h at conditions described in Experimental Procedures. Lipase preparation; (A) Lipozyme IM60, (B) *R. arrhizus*/EP100 prep (0.2 g/g support).

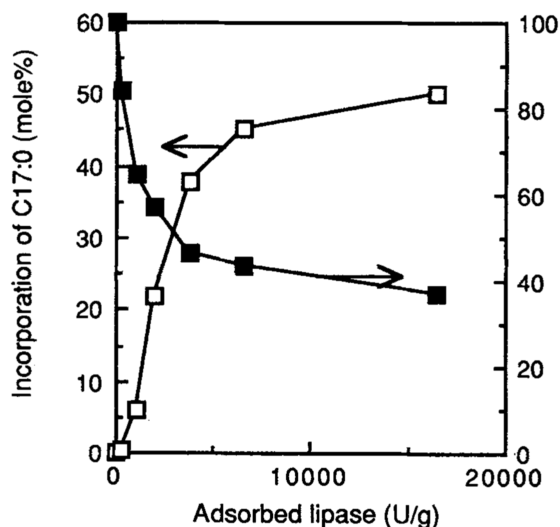

FIG. 2. Incorporation of heptadecanoic acid (□) and amount phosphatidylcholine (PC) remaining (■) after 4 h of reaction at different amounts of *R. arrhizus* lipase activity adsorbed onto EP100 support. Conditions described in Experimental Procedures.

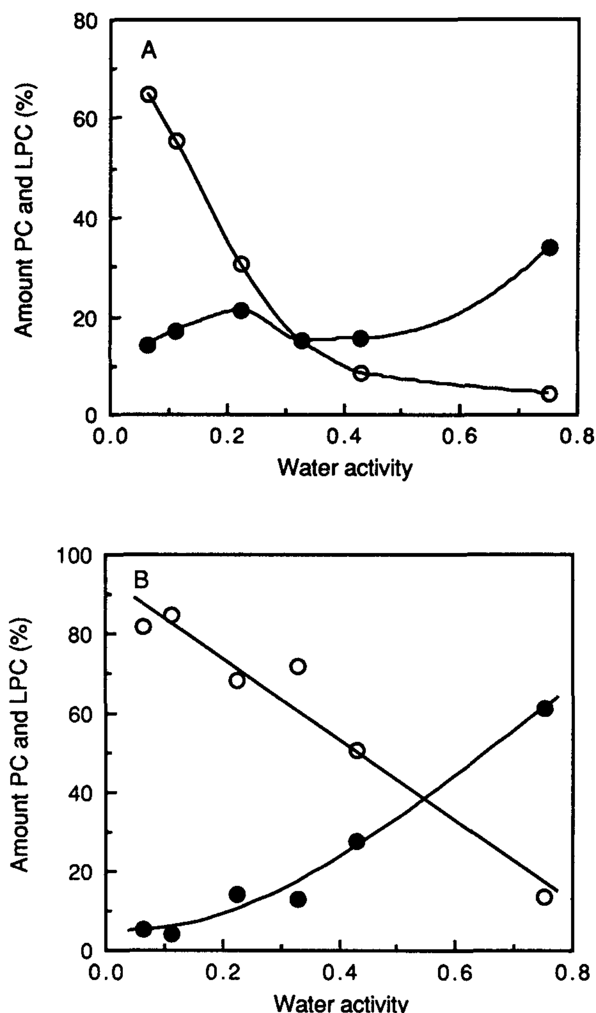

FIG. 4. Amount of remaining phosphatidylcholine (○) and formed lysophosphatidylcholine (●) after 10 h reaction as a function of initial water activity. Lipase preparation; (A) Lipozyme IM60, (B) *R. arrhizus*/EP100 prep (0.2 g/g support). Conditions as described in Experimental Procedures.

at high water activity. If the enzyme acts only in the 1-position, further hydrolysis of LPC or incorporation of heptadecanoic acid in LPC must be a consequence of acyl migration of the acyl group from the 2-position to the 1-position.

After 10 h of reaction with the *R. arrhizus* lipase preparation, the degree of incorporation of heptadecanoic acid in PC increased with increasing water activity; no incorporation into LPC was observed (Fig. 3B). On the other hand, the amount of remaining PC decreased with increasing water activity (Fig. 4B). Most of the rest of the phospholipid was in the form of LPC; further hydrolysis occurred only to a minor extent.

From the results presented, it is clear that during lipase-catalyzed transesterification of PC, net hydrolysis occurs concomitantly with the incorporation of the new fatty acid. An increase in the degree of incorporation can be achieved by an increase in reaction time, but then the yield is also reduced. When comparing results obtained under different conditions, both yield and incorporation must be considered. The best way to compare results obtained with different reaction parameters, such as different supports or different water activities, is to study the time courses under the different conditions. The amount of remaining PC can then be plotted *vs.* the incorporation of new fatty acid. Under the different conditions quite different curves were obtained. From the two curves obtained with Lipozyme, it was observed that the curve for the lower water activity was above the curve for the higher water activity in the entire range (Fig. 5). This means that regardless of which degree of incorporation is needed, the lower water activity gives a higher yield of the product. The curves obtained with *R. arrhizus* lipase on EP100 were much flatter than those for Lipozyme. This means that during the incorporation of the new fatty acid in PC, there was a long period (between 15 and 45% incorporation) when little net hydrolysis occurred. As a result, high yields of modified PC were achieved at high degrees of incorporation. One aspect not shown in Figure 5 is time; the reaction rate increases with increasing water activity. To obtain reasonable reaction times at low water activities, it was necessary to use a high loading of *R. arrhizus* lipase on EP100 (2 g/g). Experiments at a water activity of 0.43 were also carried out with a lower enzyme loading (0.2 g/g), and at a given degree of incorporation a higher yield of modified PC was obtained at this lower enzyme loading. This was probably due to the larger amount of water present in the preparation with high enzyme loading; some of this water may have participated in a hydrolysis reaction, thus decreasing the yield. However, at water activities below 0.4 it was not possible to achieve a high incorporation of the new fatty acid in a reasonable time, due to the low reaction rate with the low enzyme loading. With the high enzyme loading, the higher yields obtained by conducting the reaction at low water activity more than compensated for the loss by hydrolysis. The highest yields

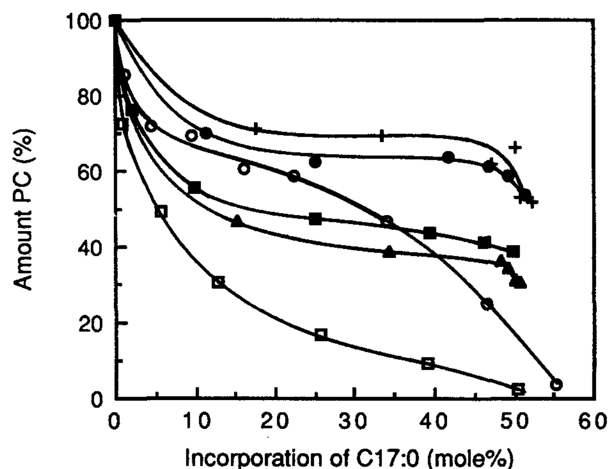

FIG. 5. Amount of remaining phosphatidylcholine as a function of incorporation of heptadecanoic acid with different enzymes and at different water activities. Reactions were carried out as described in Experimental Procedures with incubation times between 15 min and 120 h. Each point represents a separate reaction. Lipozyme IM 20 was used at water activities of 0.113 (○) and 0.43 (□) and *R. arrhizus* lipase on EP100 at water activities of 0.064 (+), 0.113 (●), and 0.43 (▲) with an enzyme loading of 2 g/g support. One series of experiments was done with a lower loading (0.2 g/g) of *R. arrhizus* lipase on EP100 at a water activity of 0.43 (■).

were obtained at the lowest water activity (0.064); a yield of 60% was obtained at 50% incorporation. This is considerably higher than what was obtained with Lipozyme as catalyst.

One important aspect in the use of enzymes for the preparation of chemicals is the operational stability of the catalyst. This was investigated by carrying out repeated batch reactions. After one batch, the catalyst was washed with toluene, excess solvent was evaporated and the water activity was again adjusted to the desired value by vapor phase equilibration with saturated salt solutions before new substrate solution was added. Hardly any decrease in reaction rate was observed for Lipozyme or *R. arrhizus* lipase when three consecutive batch reactions were carried out (Fig. 6). Most of the reaction products were removed from the biocatalyst in the toluene-washing step. However, to be sure of removing even strongly adsorbed reaction products, the biocatalysts were also washed with water after the third batch. Because of the strong adsorption of the enzyme on the support, no detectable loss of enzyme occurred in this operation. The fourth batch was then carried out at the same water activity as before. In the case of Lipozyme, about the same activity was observed after this extra washing as before. For *R. arrhizus* lipase, a slight increase in activity was observed after washing.

When enzyme preparations are used for preparative purposes, it is important that the product obtained has a well-defined composition. To test the repeated use of a lipase preparation to produce modified PC, an experiment was carried out with *R. arrhizus* lipase on EP100. The reaction was continued to give almost complete exchange of the fatty acid in the 1-position (50% incorporation of heptadecanoic acid). A larger amount of enzyme was immobilized on the support to speed up the reaction (2 g lipase adsorbed on 1 g EP100). The variations in reaction rate between the different batches were small, and the incorporation of heptadecanoic acid reached close to 50% in 2 h for all 4 batches (Fig. 7). Position analysis of the fatty acids in this modified PC showed that the new fatty acid, heptadecanoic acid, was incorporated almost exclusively in the 1-position (Table 1). The degree of incorporation of heptadecanoic acid in the *sn*-1 position (97.7%) was close to what was expected considering the amounts of the two substrates: 10 mM PC and 400 mM fatty acid. This should give 97.6% incorporation, provided the free fatty acid and those in the *sn*-1 position were randomly distributed. The fatty acid composition in the *sn*-2 position was largely the same as in the starting material. The small but detectable amount of heptadecanoic acid in the *sn*-2 position was probably due to acyl migration in the LPC fraction during the reaction.

The applicability of the lipase-catalyzed transesterification reaction for different kinds of phospholipids was investigated with Lipozyme IM20 as catalyst. The reaction conditions were as described in Experimental Procedures, but the phospholipid concentrations were 7.6 mg/mL. It was possible to incorporate heptadecanoic acid in all phospholipids tried and the reaction rate varied as follows: phosphatidylcholine > phosphatidic acid > phosphatidylethanolamine > phosphatidylinositol (data not shown). Probably better conditions (concerning enzyme, substrate concentrations, *etc.*) can be found for these new substrates, and maybe these reactions can be made to proceed as rapidly as the one with phosphatidylcholine.

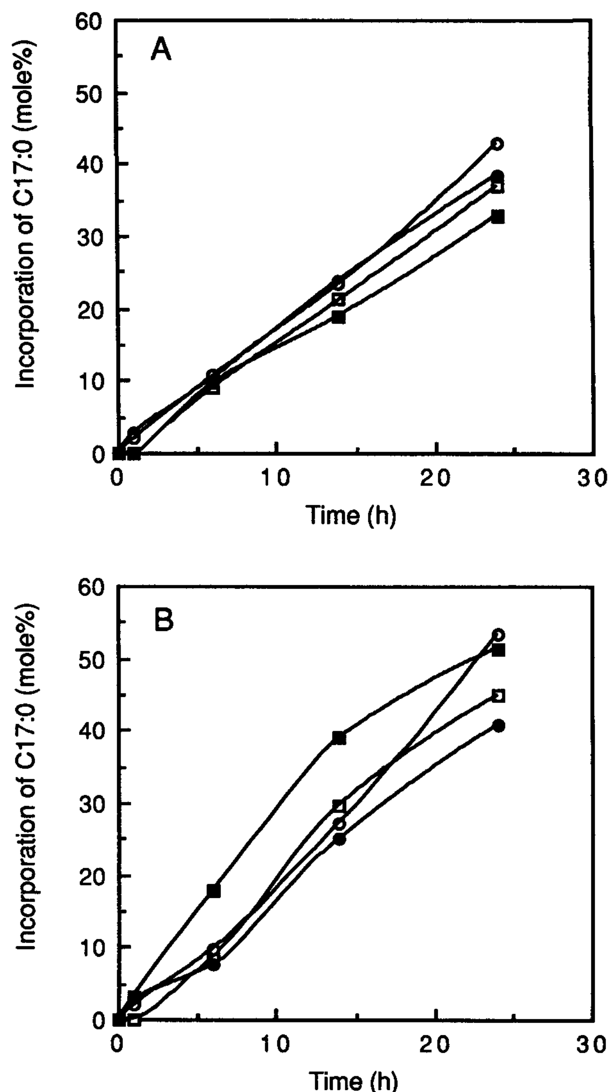

FIG. 6. Operational stability of lipases in the transesterification of phosphatidylcholine. Time courses of the incorporation of heptadecanoic acid into phosphatidylcholine for repeated batch reactions. Reaction conditions were as described in Experimental Procedures but the scale was 5 times as large. Samples (50 μL) were taken directly from the reaction mixture for analysis. After each batch the lipase preparations were washed with 4 × 3 mL toluene, dried at reduced pressure and equilibrated to a water activity of 0.43 before new substrate solution was added. After the third batch, the lipase preparations were washed three times with water after the toluene washing. Batch no. 1 □; 2 ●; 3 ○; 4 ■. Lipase preparations: (A) Lipozyme IM60, (B) *R. arrhizus*/EP100 prep (0.2 g/g support).

In conclusion, a practical method to prepare phosphatidylcholine with one single fatty acid in the 1-position is described. To further increase the yield of transesterified phospholipid, we believe that acyl migration must be avoided, and controlled removal of water at the end of the reaction may force the equilibrium between LPC and PC to yield more PC.

ACKNOWLEDGMENTS

This project was supported by the Nordic Industrial Foundation (NI) and the Swedish National Board for Technical Development (STU). The authors wish to thank Scott Bloomer for linguistic advice.

TABLE 1

Regioselectivity of *R. arrhizus*-Catalyzed Transesterification of Phosphatidylcholine (PC) and Heptadecanoic Acid[a]

Fatty acid	Position *sn*-1	Position *sn*-2	(position *sn*-1) + (position *sn*-2)[b] / 2	Direct analysis of modified PC
C16:0	1.6	1.0	1.3	1.9
C16:1	0.0	0.9	0.5	0.3
C17:0	97.7	0.9	49.3	49.9
C18:0	0.6	0.2	0.4	0.7
C18:1	0.1	64.9	32.5	31.3
C18:2	0.0	26.6	13.3	13.6
C20:4	0.0	5.6	2.8	2.3

[a]The fatty acid composition (mole %) in positions *sn*-1 and *sn*-2 of enzymatically modified phosphatidylcholine (reaction time 2 h) was determined after regiospecific enzymatic hydrolysis of product.

[b]Fatty acid composition of the enzymatically modified phosphatidylcholine based on the data from selective hydrolysis (the two columns to the left).

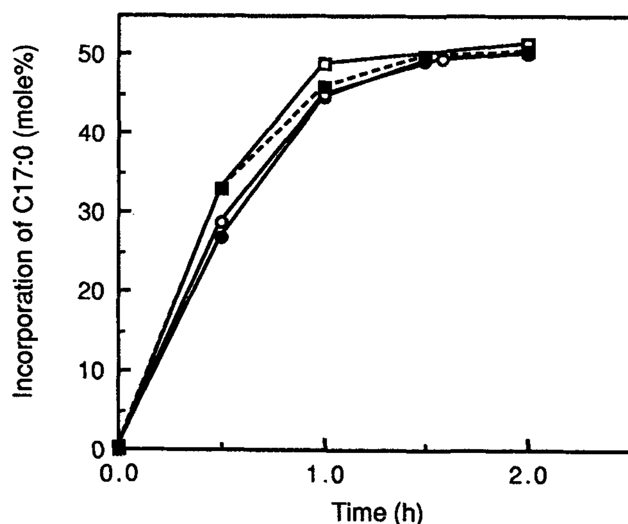

FIG. 7. Repeated use of a preparation with a high load of lipase (2 g *R. arrhizus*/g EP100). The experiment was performed as described in the legend of Figure 6 but each reaction was stopped after 2 h.

REFERENCES

1. Plückthun, A., and E.A. Dennis, *Biochemistry 21*:1743 (1982).
2. Brockerhoff, H., P.C. Schmidt, J.W. Fong and L.J. Tirri, *Lipids 11*:421 (1976).
3. Yoshimoto, T., M. Nakata, S. Yamaguchi, T. Funada, Y. Saito and Y. Inada, *Biotechnol. Lett. 8*:771 (1986).
4. Yagi, T., T. Nakanishi, Y. Yoshizawa and F. Fukui, *J. Ferment. Bioeng. 69*:23 (1990).
5. Yoichiro, T., and H. Setsuko, *J. Am. Oil Chem. Soc. 68*:848 (1991).
6. Svensson, I., P. Adlercreutz and B. Mattiasson, *Appl. Microbiol. Biotechnol. 33*:255 (1990).
7. Pernas, P., L.J. Olivier, M.D. Legoy and G. Bereziat, *Biochem. Biophys. Res. Commun. 168*:644 (1990).
8. Na, A., C. Eriksson, S.G. Eriksson, E. Österberg and K. Holmberg, *J. Am. Oil Chem. Soc. 67*:766 (1990).
9. Adlercreutz, P., *Eur. J. Biochem. 199*:609 (1991).

[Received April 10, 1992; accepted July 1, 1992]