Preparation of Phospholipids Highly Enriched with n-3 Polyunsaturated Fatty Acids by Lipase

Gudmundur G. Haraldsson* and Atli Thorarensen

Science Institute, University of Iceland, 107 Reykjavik, Iceland

ABSTRACT: The immobilized 1,3-regiospecific *Rhizomucor miehei* lipase (Lipozyme™) was employed to catalyze the transesterification reaction (acidolysis) of 1,2-diacyl-*sn*-glycero-3-phosphatidylcholine with n-3 polyunsaturated fatty acids under nonaqueous solvent-free conditions. With a concentrate of 55% eicosapentaenoic acid (EPA) and 30% docosahexaenoic acid (DHA) and pure phosphatidylcholine from egg yolk, phospholipids of 32% EPA and 16% DHA content were obtained, presumably as a mixture of phosphatidylcholine and lysophosphatidylcholine. ^{31}P nuclear magnetic resonance (NMR) analysis turned out to be a valuable technique to study the details of the reactions involved. It revealed that when 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine was transesterified with 98% pure EPA, a substantial amount of hydrolysis side reaction took place (39%), leading to a product mixture of 39% phosphatidylcholine, 44% lysophosphatidylcholine, and 17% *sn*-glycerol-3-phosphatidylcholine. The lysophosphatidylcholine constituent comprised 70% EPA, whereas the phosphatidylcholine component contained 58% EPA. The ^{31}P NMR technique provided valid information about the mechanism of the reaction. It became evident that a high dosage of lipase containing 5% water afforded optimal conditions for the optimal extent of EPA incorporation into the phospholipids, under which the extent of hydrolysis side reaction remained relatively high.

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KEY WORDS: Acidolysis, acyl migration, DHA, EPA, hydrolysis side reaction, lysophosphatidylcholine, n-3 fatty acids, ^{31}P NMR, phosphatidylcholine, *Rhizomucor miehei* lipase.

Phospholipids (PL) are major constituents of cell membranes and play crucial roles in the biochemistry and physiology of the cell (1). They have been widely used in food, pharmaceutical, and cosmetic products as highly proficient emulsifiers. Of these, diacyl-*sn*-glycero-3-phosphatidylcholine (PC) is the most ubiquitous. Long-chain polyunsaturated n-3 type fatty acids are characteristic of marine fat and occur pervasively in the PL of fish and marine species (2). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are by far the most common and important of the n-3 fatty acids and commonly account for up to 50% of the fatty acid constituents of marine PL (3). The presence of the long-chain polyunsaturated and low-melting fatty acids is believed to add to membrane fluidity and mobility and thus adjust membrane integrity and function at lower ambient temperatures. The positive influence of n-3 polyunsaturated fatty acids on human health is now well established (4), and there is a growing demand for them by the pharmaceutical industry in the natural triacylglycerol form as well as in the PL form. One might believe that such PL enriched with n-3 polyunsaturated fatty acids would be readily available from fish, but rather laborious extraction and separation procedures are required. Therefore, we decided to focus on the generation of such PL highly enriched with EPA and DHA from inexpensive and commercially available plant or animal lecithins using biotechnology methods involving enzymes.

Lipases have become among the most commonly employed and versatile biocatalysts in organic synthesis, and their scope of applicability extends far beyond their natural glyceryl ester substrates (5). They are ideally suited as catalysts for transformations involving the highly labile long-chain n-3 polyunsaturated fatty acids (6). They have previously been employed in the preparation of both triacylglycerols (7,8) and glyceryl ether lipids (9) highly enriched in EPA and DHA and homogeneous with EPA or DHA (i.e., lipids containing either 100% EPA or 100% DHA). In the recent literature are several successful reports of employing lipases on PL as substrates in both hydrolysis (10–12) and various transesterification reactions (13,14). Attempts to prepare PL enriched with n-3 polyunsaturated fatty acids by various esterification reactions involving lipases (15,16) and phospholipases (16,17) have so far usually resulted in low yields as a result of predominant hydrolysis side reactions, and the incorporation of the n-3 polyunsaturated fatty acids did not reach any elevated levels.

In this report, the application of lipase to generate such PL highly enriched with n-3 polyunsaturated fatty acids by acidolysis reaction is described. High levels of EPA and DHA incorporation into the PL, which were afforded in moderate yields, were accomplished although the hydrolysis side reaction level was rather high.

EXPERIMENTAL PROCEDURES

Solvents and materials. PC was isolated from egg yolk according to a procedure described by Singleton *et al.* (18) and

*To whom correspondence should be addressed at Science Institute, University of Iceland, Dunhaga 3, 107 Reykjavik, Iceland.
E-mail: gghar@raunvis.hi.is

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was purified by high-performance liquid chromatography (HPLC) according to the method of Patel and Sparrow (19). Pure *sn*-glycerol-3-phosphatidylcholine (GPC) was obtained by hydrolyzing the purified PC by the method of Brockerhoff and Yurkowski (20). Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine and 1-palmitoyl-*sn*-glycero-3-lysophosphatidylcholine (2-LPC) were obtained 99% pure from Sigma Chemicals (St. Louis, MO), as well as 14% BF_3 in methanol. Concentrates of 55% EPA and 30% DHA, as well as 98% pure EPA, were supplied as a gift from Norsk Hydro A/S (Porsgrunn, Norway). Free fatty acid (FFA) concentrates of essentially identical composition were obtained by alkaline hydrolysis according to previously described procedures (7). Immobilized *Rhizomucor miehei* lipase (Lipozyme™) of 2.5, 5.0, 7.5, 10, and 20% water content (by weight) was a gift of Novo Nordisk A/S (Bagsvaerd, Denmark). Solvents (chloroform, methanol, and acetic acid) and other chemicals used (NaOH, NaCl, and anhydrous $MgSO_4$) were obtained from Merck (Darmstadt, Germany) and were of analytical grade and used without any further purification. Rhodamine 6G was obtained from Merck.

Transesterification reactions. In a typical procedure, PC (80 mg, 0.11 mmol) was placed into a 3-mL round-bottomed flask together with EPA as FFA (240 mg, 0.79 mmol) and Lipozyme (30 mg). The mixture was gently stirred on a magnetic-stirrer hot-plate under argon at 60–65°C on an oil bath for 72 h. The fat was separated from the lipase by adding chloroform (1 mL) to the mixture and filtering through a cotton wool plug. This was repeated with methanol (1 mL) and, finally, deuterated chloroform (0.5 mL) was added to the resulting solution, which then was subjected to ^{31}P nuclear magnetic resonance (NMR) analysis.

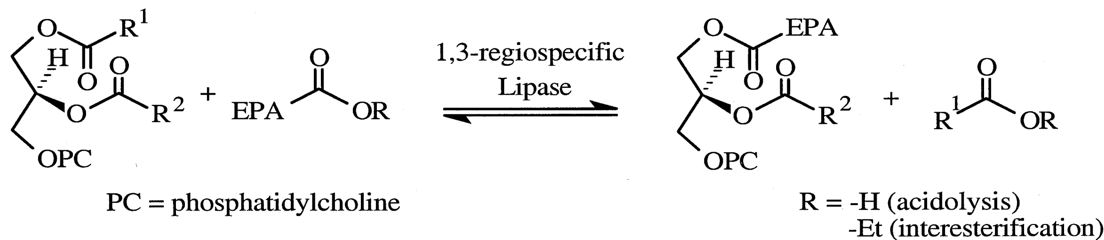
^{31}P NMR analysis. ^{31}P NMR analysis was performed on a Bruker AC 250 NMR spectrometer (Karlsruhe, Germany) in the chloroform d_6/chloroform/methanol (0.5:1:1) mixture at 101.26 MHz. Typical chemical shift values obtained (relative to H_3PO_4 as an external reference) were as follows: δ0.51 (GPC), 0.27 (2-LPC), 0.17 (1-LPC), and −0.42 ppm (PC). The relative integrated intensity (I) of each peak was used to calculate the composition of the product mixture (as mol%). The percentage hydrolysis was calculated according to the following equation: % hydrolysis = $(2 \cdot I_{GPC} + I_{2\text{-LPC}} + I_{1\text{-LPC}})/2 \cdot (I_{GPC} + I_{1\text{-LPC}} + I_{2\text{-LPC}} + I_{PC})$ and was based on the total number of moles of ester equivalents present at the initial PC.

Thin-layer chromatography (TLC). Preparative silica gel TLC plates from Merck (Kieselgel, Art. 5721, 0.25 mm) were used after washing them overnight in chloroform/methanol (1:1) and activation in an oven at 120°C for 30 min prior to use. Samples (5–10 mg) of the product mixture were introduced on the plates, and chloroform/methanol/acetic acid/water (75:40:8:3) used as an eluent. Visualization was achieved with an aqueous 0.12% Rhodamine 6G solution, followed by inspection under a short-wavelength ultraviolet light. Obtained R_f values were 1.0 for FFA, 0.25 for PC, and 0.12 for LPC. The PL bands were scraped off, methylated, and analyzed by gas chromatography (GC) (see below).

GC analysis. GC was performed on a Perkin Elmer 8140 gas chromatograph using a 30-m capillary column, DB-225 30N, 0.25 mm (J&W Scientific, Folsom, CA). Hydrogen was used as a carrier gas at a flow rate of 1.6 mL/min. Detection was with flame-ionization detection and areal quantitation was made with an auxiliary automatic integrator. A temperature of 180°C was used at the beginning, rising 3°C/min to a final temperature of 210°C, which was maintained for 12 min. Injector and detector were kept at 265°C. Methyl esters were prepared according to our previously described procedure (21) by treating the scrapings from TLC with 0.5 M NaOH in methanol, followed by 14% BF_3 in methanol treatment.

RESULTS

Preliminary studies. Preliminary studies were conducted on pure (approx. 99%) PC isolated from egg yolk (18) and purified by HPLC (19). The predominant fatty acids in the PC were palmitic ($C_{16:0}$; 33.1%), stearic ($C_{18:0}$; 11.3%), oleic ($C_{18:1}$; 32.2%), and linoleic ($C_{18:2}$; 9.2%) acids. Concentrates of 55% EPA and 30% DHA content were used as free acids and ethyl esters in two types of lipase-catalyzed transesterification reactions, acidolysis, and interesterification, respectively, according to Scheme 1 (simplified and only shown for EPA). Reaction conditions almost identical to those previously described for cod liver oil triglycerides (8,21) and shark liver oil ether lipids (9) were applied. Based on the weight of substrates, a 10% dosage of the immobilized 1,3-regiospecific *R. miehei* lipase, containing 10% water, was employed under solvent-free conditions at 60–65°C with a 3.5-fold molar excess of the concentrates, as based on the number of moles of ester equivalents present at the glyceryl backbone of the phospholipids.



SCHEME 1

The progress of the reactions was monitored by collecting samples of the product mixture after allowing the reactions to proceed for a chosen length of time, usually 72 h, unless otherwise stated. They were introduced on preparative TLC to aid separation of the FFA or ethyl esters and the PL mixture, followed by GC analysis of the fatty acid composition of the PL. In these initial studies the PL were not fractionated into individual PC, LPC, and GPC, the latter adducts being presumed products of hydrolysis side reactions. This means that the extent of the possible hydrolysis side reactions under the initial study conditions was not known.

As might be expected for lipase, the rate of the reactions involving the PL, possessing the zwitterionic head groups, was much lower when compared to their native triglycerides. As before (8,21), EPA was a considerably better substrate than DHA. The interesterification reaction was found to proceed at a comparable rate to the acidolysis reaction, and similar incorporation levels of EPA and DHA were obtained at an equilibrium. We decided, however, to look further into the acidolysis reaction, since the water-associated hydrolysis side reactions were believed to be suppressed by the large excess of FFA present in that case.

The acidolysis reaction was observed to be very dependent on lipase dosage. This is clearly evident from Figure 1, which is a graphical presentation of how the EPA and DHA incorporation levels into the total PL were affected after a 72-h reaction time when varying the amount of lipase. High incorporation levels of EPA and DHA were obtained with 70% lipase dosage, but the highest levels of incorporation were obtained when employing 100% dosage of lipase, when PL containing 32% EPA and 16% DHA were accomplished after 72 h reaction time.

When relatively low dosages of the lipase were employed, relatively minor changes seemed to take place in the fatty acid composition of the *sn*-2 position. On the other hand, when large amounts of lipase were used and high conversion achieved, more profound changes occurred, indicating that the *sn*-2 position of PC was participating in the reaction. This is based on the presumption that $C_{18:1}$ and $C_{18:2}$ fatty acids are exclusively confined to the *sn*-2 position of the egg-yolk PL (22).

The reaction was observed to be very medium dependent. It proceeded by far the fastest when conducted without a solvent, but it was also found to be very solvent-dependent and the rate was inversely proportional to the solvent polarity, fastest in hexane, slower in toluene, but slowest in ethyl acetate.

More comprehensive studies. More detailed information about the nature of the acidolysis reaction was obtained through ^{31}P NMR spectroscopy analysis. In order to enable the application of that technique the reaction system had to be redesigned and simplified in terms of substrates as a result of line-broadening due to the great variety of fatty acids present in the substrates. This was brought about by employing pure dipalmitoyl-*sn*-glycero-3-phosphatidylcholine and 98% pure EPA as free acid. In addition to that, the PL were further fractionated by preparative TLC (after each reaction), and the resulting PC and LPC fractions (as a mixture of 1-LPC and 2-LPC) analyzed by GC to reveal their fatty acid composition.

The results confirmed that the hydrolysis side reactions were much more extensive in the interesterification reaction than the acidolysis reaction, approximately threefold more when lipase containing 10% water was employed. They also revealed that optimal results were obtained at 60–65°C. Further studies on varying the water content of the lipase revealed that optimal results in terms of activity were obtained with lipase containing 5% rather than 10% water, as had been employed in the previous experiments. This is graphically demonstrated in Figure 2 when using a 10% dosage of lipase after 72 h. It is evident from

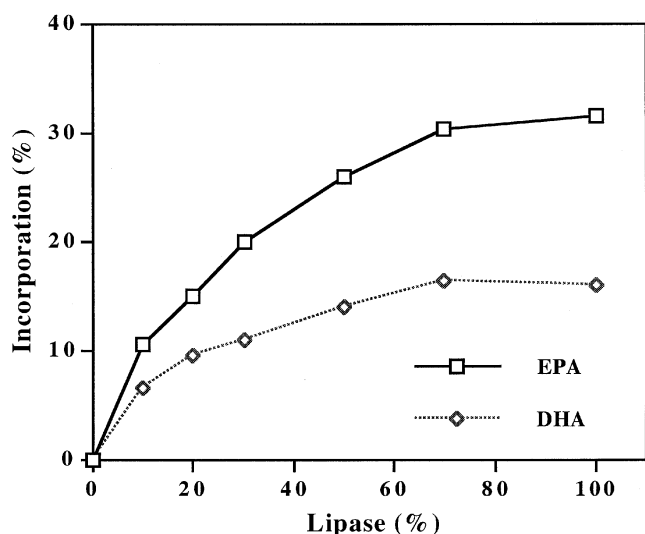


FIG. 1. A graphical presentation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) incorporation into the total phospholipids after 72 h when varying the lipase dosage, expressed as a percentage based on the combined substrate weight.

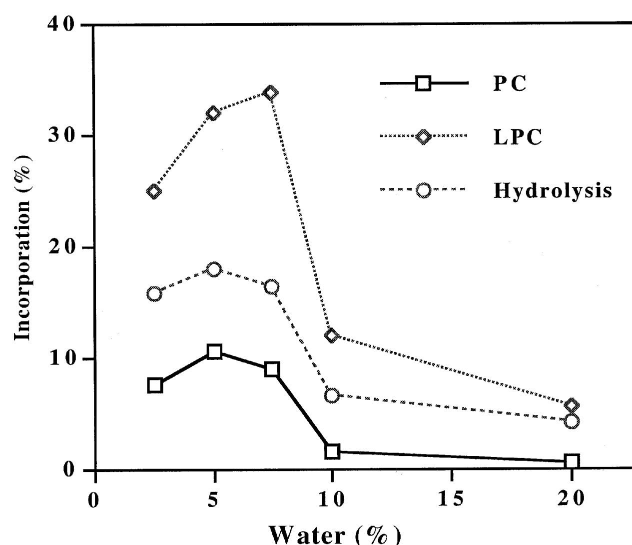


FIG. 2. Extent of hydrolysis side reaction and incorporation of EPA into phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) after 72 h when varying the water content of the immobilized *Rhizomucor miehei* lipase using 10 % dosage of lipase. For abbreviation see Figure 1.

these results that the 5% water content resulted not only in the highest incorporation levels of EPA into both PC and LPC, but also in the highest extent of hydrolysis side reaction. This demonstrates rather well the complex role played by water (23) in terms of compromising the lipase activity, hydrolysis side reactions, reaction rate, and extent of incorporation under the nonaqueous reaction conditions.

Further experiments were therefore conducted with lipase containing 5% water. Figure 3 shows how the level of EPA incorporation into both PC and LPC and the extent of hydrolysis side reaction were affected when varying the lipase dosage for lipase containing 5% water after 72 h. Maximal incorporation of EPA was obtained with 100% dosage of lipase, 58% into PC and 70% into LPC. Under that condition, the extent of hydrolysis reached 39%, as based on number of moles of ester equivalents present at the initial PC, with 39% PC, 44% LPC, and 17% GPC present in the product mixture, based on moles of PL, according to the ^{31}P NMR studies.

Agitation became problematic with that high dosage of lipase and additional experiments were conducted with 70% lipase dosage. Figure 4 shows how the incorporation of EPA into PC and LPC varied with time as the reaction was allowed to proceed. An equilibrium had been reached after 72 h reaction time, when 70% EPA had been incorporated into LPC and 44% into PC. This remained constant after 160 h reaction time. During the first 3 h of the reaction EPA incorporation into PC was higher than into LPC, but from there onward the reverse became true.

Additional results are exhibited in Figure 5, which clearly illustrates the power of the ^{31}P NMR technique in this field. The main advantage is that a true image of the product mixture is reflected without much disturbance or loss due to separation procedures. Figure 5A shows the extent of hydrolysis

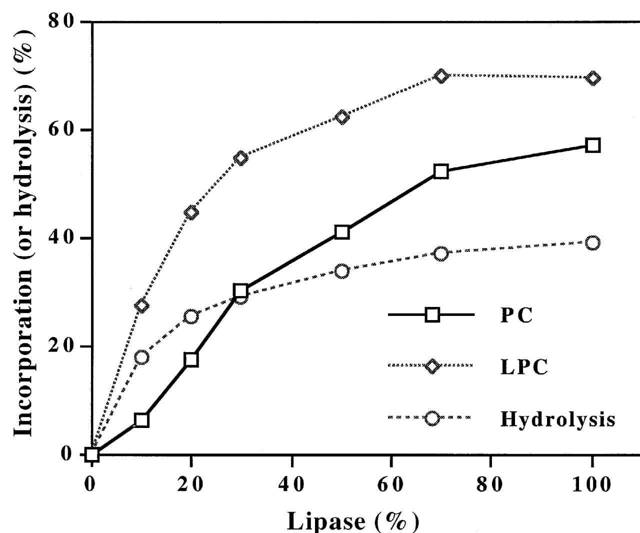


FIG. 3. Extent of hydrolysis side reaction and incorporation levels of EPA into PC and LPC after 72 h when varying the dosage of the immobilized *R. miehei* lipase containing 5% water expressed as percentages based on combined substrate weight. For abbreviations see Figures 1 and 2.

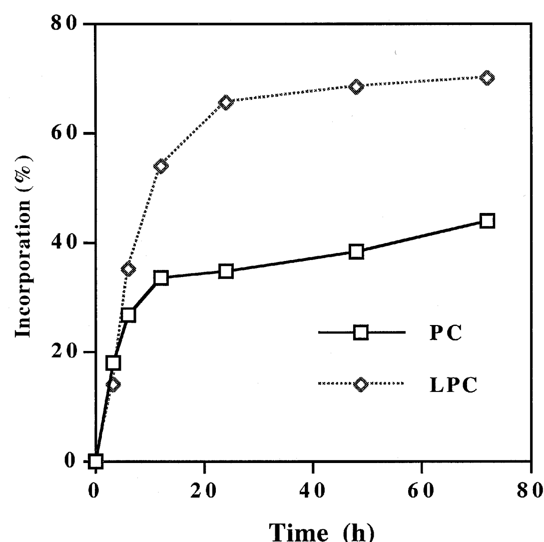


FIG. 4. Incorporation of EPA and DHA into PC and LPC during the progress of the acidolysis reaction with 70% lipase dosage. For abbreviations see Figures 1 and 2.

as well as changes of PC and LPC composition during the progress of the reaction. From these results, it is evident that major changes composition, in in terms of extent of hydrolysis and PC and LPC incorporation, occurred during the first 3 h of the reaction. More detailed insight into the progress of the reaction is provided in Figure 5B. At the beginning of the reaction, 1-LPC was the predominant hydrolysis product. After 3 h, 27% 1-LPC, 9% 2-LPC, 10% GPC, and 55% PC were present, as based on the number of moles produced. After 6 h, 1-LPC and 2-LPC had leveled out at 16% content, but after 48 h, an equilibrium was reached with 2-LPC now dominating in the hydrolysis product mixture at 36% content, with only 6% 1-LPC, 15% GPC, and 45% PC present in the reaction mixture.

DISCUSSION

An ideal lipase for undertaking work of the type described in this report would require a very low water content to maintain its optimal activity, but at the same time would tolerate both PL and n-3 polyunsaturated fatty acids well as substrates. This makes it possible to maintain the hydrolysis side reactions at a minimum but still offer a high level of incorporation of EPA and DHA into the PL and afford them in good yields. The immobilized *R. miehei* lipase was observed to satisfy both requirements rather well and the results offered in this report represent the highest extent of enrichment of n-3 polyunsaturated fatty acids into PL described so far in the literature—60–70% as compared to <20%.

In the current work, it was obviously of interest to obtain maximal incorporation of EPA (or DHA) into the PL. To enable that, lipase of optimal activity was required in relatively high quantities, 70–100% as based on combined substrate weight, to maintain a satisfactory lipase activity, mainly because the zwitterionic PL are inferior substrates to their nat-

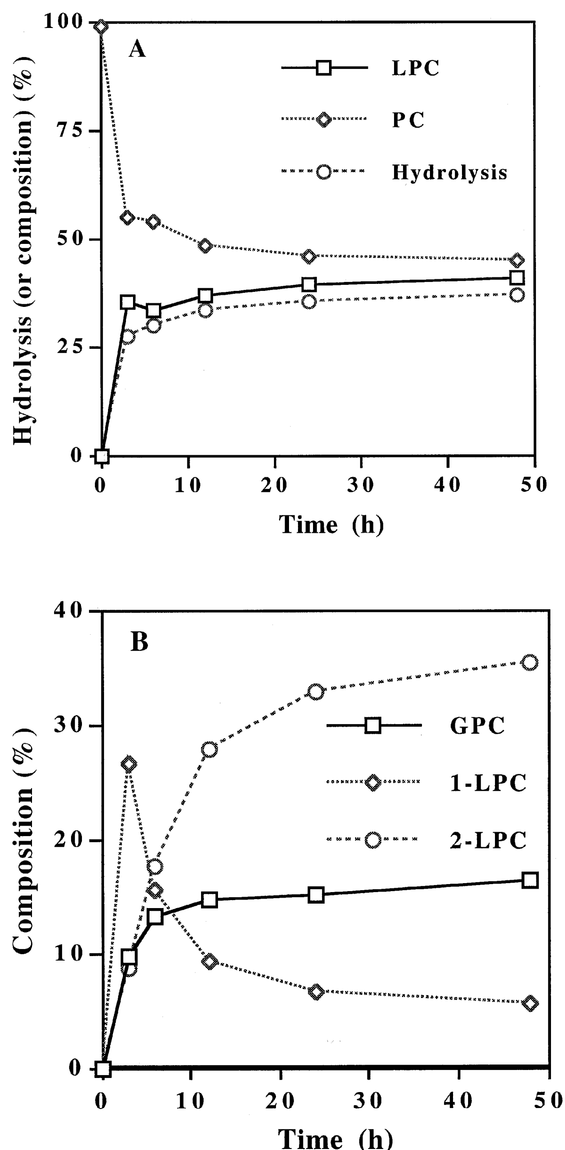


FIG. 5. (A) Extent of hydrolysis and changes in PC and LPC composition during the progress of the acidolysis reaction with 70% lipase dosage. (B) Composition of product mixtures in terms of various phospholipid intermediates during the progress of the acidolysis reaction with 70% lipase dosage. GPC, *sn*-glycerol-3-phosphatidylcholine; for other abbreviations see Figures 1 and 2.

ural triglyceride substrates. This is still a considerably lower amount than Svensson *et al.* (13) used in their interesting work describing the incorporation of heptadecanoic acid into the *sn*-1 position of PC under nonaqueous conditions using organic solvents. Unfortunately, this brings high quantities of water to the system, which results in a relatively high extent of hydrolysis side reactions, leading to formation of LPC and GPC, and thus lowering the yields of the desired PC. Under these conditions the extent of hydrolysis remained below 40%, with 40–45% of PC and a similar percentage of LPC present in the product mixture.

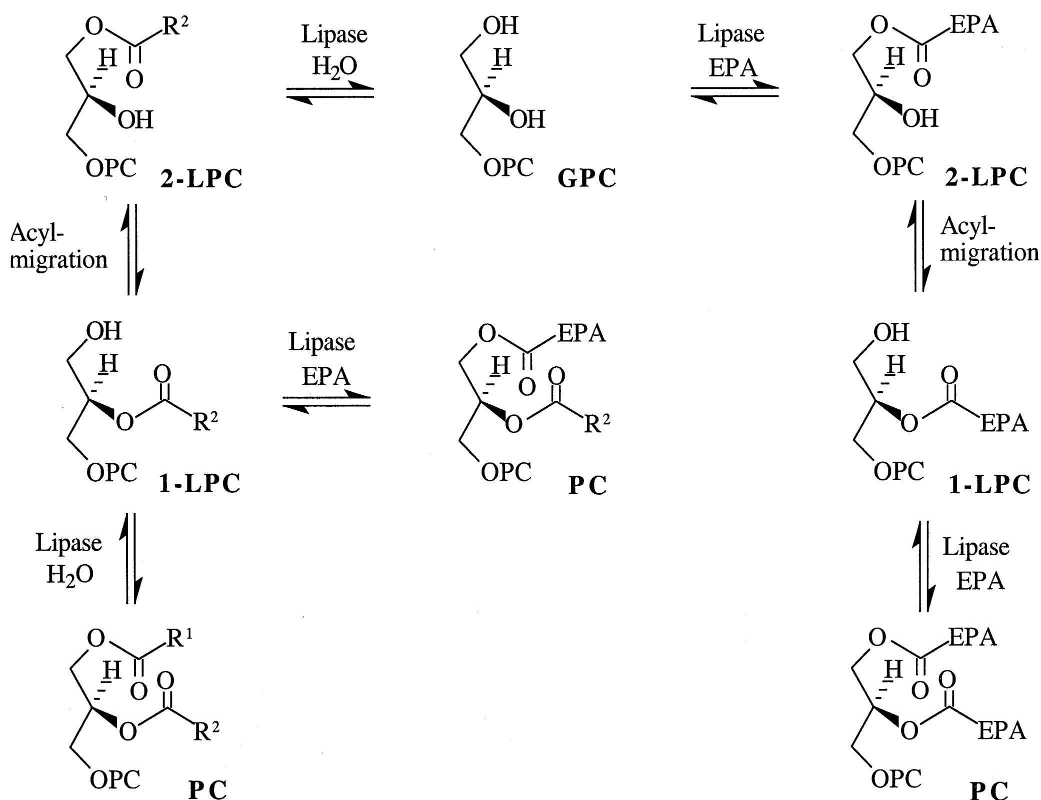
To suppress the extent of this hydrolysis side reaction Svensson *et al.* (13) employed a vast excess of FFA. They used up to a 20-fold excess as based on number of mol equivalents of acyl groups present in the PC. In the current work a constant 3.5-fold excess was consistently used, and it is most certain that the yield of PC as well as the extent of EPA and DHA incorporation can be increased and the extent of hydrolysis reduced by increasing the FFA/PC ratio.

Another possibility for suppressing the hydrolysis side reactions is to maintain strict control of water activity as Svensson *et al.* (13) demonstrated in their studies with PC and heptadecanoic acid. But even that did not appear to help very much in the *R. miehei* lipase case, since under their strict water content control with a high excess of FFA, they obtained even lower recovery of PC than reported in the current paper, although this worked better for a different lipase, which apparently requires less water. Despite the rather extensive hydrolysis reactions taking place in our experiments, and because of the large amount of lipase, the water content remained virtually constant throughout the reaction, roughly 2% as based on total weight of the reaction mixture when using 70% dosage of lipase.

A question arises as to whether and to what extent the *sn*-2 position of the PC was involved in these processes. There is little doubt that the lipase maintains its 1,3-regiospecificity and acts exclusively at the *sn*-1 position of the PC substrate. This has been demonstrated in several literature reports (13,14) and in fact, it appears that lipases in general may not act on acyl groups adjacent to a phosphoryl group in glycerophospholipids (24). From the results presented above there is unequivocal evidence for involvement of the *sn*-2 position in the reaction by an intramolecular acyl-migration isomerization process (24,25). The presence of excessive amounts of FFA is believed to enhance the acyl-migration process (7), as does the presence of the anionic-exchange resin support of the lipase (24).

Plückthun and Dennis (25) employed the ^{31}P NMR technique to investigate acyl migration in lysophospholipids. Their results revealed that about 90% of the thermodynamically more stable 2-LPC and 10% of 1-LPC were present in a mixture at equilibrium under their investigation conditions. The results reported in this paper are in good agreement with those of Plückthun and Dennis, 85% 2-LPC and 15% 1-LPC under our conditions. There was no evidence of any of the corresponding phosphoryl migration (25) from our results. It is obviously of interest to promote the acyl-migration process when one is interested in maximizing the EPA/DHA content of the phospholipids by using lipase, in order to involve both positions of the PL.

Based on these findings, the mechanism displayed in Scheme 2 is proposed for the lipase-catalysed acidolysis of PC with FFA. The proposed mechanism explains how PC of higher than 50% EPA content can be formed with both the *sn*-1 and *sn*-2 positions involved, as well as the presence of all the intermediates implied by the ^{31}P NMR spectroscopy analysis. According to that mechanism, the lipase utilizes water to bring about hydrolysis at the *sn*-1 position of the starting PC to produce 1-LPC. That intermediate can undergo



SCHEME 2

either reesterification to form EPA-enriched PC or a much slower acyl migration to form 2-LPC. Lipase can easily hydrolyze that intermediate to form GPC. Esterification at the *sn*-1 position of GPC with EPA by the aid of lipase, followed by acyl migration to form EPA-enriched 2-LPC and finally lipase-catalyzed esterification with EPA, leads to the formation of the highly EPA-enriched PC. Thus, the postulated mechanism can be used to rationalize the reaction system behavior: EPA-enriched PC and (presumably) EPA-deficient 1-LPC dominate in the reaction mixture during the early stages of the reaction, whereas at the later stage highly EPA-enriched 2-LPC and PC become dominant.

It should be emphasized that the process depicted in Scheme 2 is simplified and that at an equilibrium, all acyl constituents participating would be anticipated to have identical fatty acid compositions. That composition is determined by a weighted average of the initial fatty acid composition of the PL and the n-3 concentrates. When using a 3.5-fold excess of EPA with dipalmitoyl-*sn*-glycero-3-phosphatidylcholine it is easy to calculate the equilibrium composition, which is 78% in terms of EPA when both positions participate for all constituents including PC. When the *sn*-2 position is excluded from the equilibrium, PC should comprise pure palmitic acid in that position and 88% EPA at the *sn*-1 position, which means 44% EPA overall. The current results provide unequivocal evidence that the *sn*-2 position is indeed involved in the reaction, both in terms of fatty acid composition of PC, which is considerably higher than the calculated 44%, and the high levels of 2-LPC. Thus, an equilibrium in terms of PL constituents has been reached in the reactions displayed in this report, but an equilibrium in terms of fatty acid composition presumably requires longer reaction time. This is not surprising when the complicated process proposed in Scheme 2 is kept in mind.

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