An Enzymatic Method for the Synthesis of Mixed-Acid Phosphatidylcholine

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ABSTRACT: The enzymatic synthesis of PC with decanoic acid in the *sn*-1 and hexanoic acid in the *sn*-2 position is described. The procedure comprises the following enzymatic steps: (i) treatment of egg yolk with phospholipase A_2 (PLA_2) to hydrolyze egg yolk PC to 1-acyl lysophosphatidylcholine (LPC); (ii) esterification of 1-acyl LPC with hexanoic acid catalyzed by PLA_2 to yield PC with hexanoic acid in the *sn*-2 position; (iii) removal of the FA in the *sn*-1 position by lipase-catalyzed ethanolysis to yield 2-hexanoyl LPC; and finally (iv) introduction of decanoic acid in this position by lipase-catalyzed esterification of 2-hexanoyl LPC to yield 1-decanoyl-2-hexanoyl-PC. Two egg yolks with a weight of 16 g were required to obtain 160 mg of the desired product. The chemical purity of the PC product and the positional purity of the FA were around 99%. The method is applicable for the synthesis of other mixed-acid PC species as well.

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KEY WORDS: Acidolysis, alcoholysis, enzymatic phospholipid modification, esterification, hydrolysis, phospholipase A_2, *Rhizomucor miehei, Rhizopus oryzae.*

Mixed-acid phospholipids are high-priced fine chemicals required in membrane and lipoprotein research, in liposome technology, and in the cosmetic industry (1–4). The synthesis of mixed-acid phospholipids is very complex (for a review, see Paltauf and Hermetter, Ref. 5) and involves both toxic chemicals and harsh reaction conditions, which reduce the attractiveness of the products for medical and biological applications. Enzymes, in contrast, catalyze chemical reactions under mild conditions, providing a promising alternative to the chemical approach (6–8). 1,3-Specific lipases (EC 3.1.1.3) and phospholipase A_2 (PLA_2) (EC 3.1.1.4) have been used to specifically modify PC in the *sn*-1 and *sn*-2 positions, respectively. The most important of these modification reactions is the PLA_2-catalyzed hydrolysis of PC to produce 1-acyl lysophosphatidylcholine (LPC) (9,10), an effective bio-emulsifier and important intermediate in the synthesis of PC with a defined FA composition. PLA_2 also has been used in organic media to synthesize PC with defined FA in the *sn*-2 position from 1-acyl LPC and FA (11–13). 1,3-Specific lipases have been used mainly to exchange the FA in the *sn*-1 position of PC by acidolysis (14–17) and to synthesize 2-acyl LPC (18).

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Most studies concerning enzymatic phospholipid modification have focused on a single reaction. In the present work, four enzymatic steps were combined to convert egg yolk PC (containing various FA; see Ref. 19) to PC containing decanoic acid in the *sn*-1 position and hexanoic acid in the *sn*-2 position (Fig. 1). The hydrolysis (ethanolysis)/re-esterification approach for FA introduction was chosen instead of the acidolysis approach to obtain a product of higher purity (14). PC containing decanoic acid in the *sn*-1 position and hexanoic acid in the *sn*-2 position is, in contrast to egg PC, water-soluble and therefore attractive in certain applications. Water-soluble PC species have, for instance, been used as substrates in enzymatic studies in which the use of organic co-solvents or detergents for phospholipid solubilization was undesirable (20). Water-soluble PC species also have been used for membrane solubilization and for the solubilization and reconstitution of integral membrane proteins (21).

Both lipases and PLA_2 accept a wide range of FA as substrates, which makes this enzymatic method applicable for the preparation of other mixed-acid PC species as well.

MATERIALS AND METHODS

Materials. Hens' eggs were purchased from a local supermarket. PLA_2 from pig pancreas (*Sus scrofa*) (Lecitase 10L, 12,000 IU mL^{-1}) was a gift from Novo Industri A/S (Bagsvaerd, Denmark), and lipase from *Rhizomucor miehei* (Lipozyme™) was purchased from Novo Industri A/S. Lipase 80.000 from *Rhizopus oryzae* (formerly *R. arrhizus)* was donated by Gist-Brocades S.A., now belonging to DSM (Delft, The Netherlands). PLA_2 was immobilized on DAX-8 from Supelco (Bellefonte, PA) (11), and lipase from *R. oryzae* was immobilized on an EP-100 polypropylene support from Akzo (Oldenburg, Germany) (14). Hexanoic and decanoic acids were from Sigma (St. Louis, MO), and toluene (LiChrosolv®) and diethyl ether (SeccoSolv®) were purchased from Merck (Darmstadt, Germany). Ethanol (95%) was from Kemetyl (Haninge, Sweden).

Preparation of 1-acyl LPC. 1-Acyl LPC was prepared as outlined in Figure 2. The yolks of 12 fresh eggs (206 g) were mixed with an electric stirrer, and the PLA_2 solution (12,000 U) was added to initiate phospholipid hydrolysis. The reaction temperature was 25°C. Samples of known weight were withdrawn for HPLC analysis (19) to follow the hydrolysis of PC to 1-acyl LPC. After 2 h, ethanol (0.9 L, 95%) was added to extract the LPC. The ethanol extraction was repeated twice.

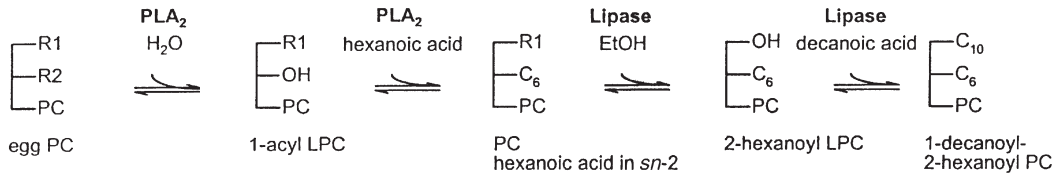

FIG. 1. Modification of egg yolk PC in four enzymatic steps: (i) phospholipase A_2 (PLA_2)-catalyzed hydrolysis of PC to 1-acyl lysophosphatidylcholine (LPC); (ii) PLA_2-catalyzed re-esterfication of 1-acyl LPC with hexanoic acid; (iii) lipase-catalyzed ethanolysis to yield 2-hexanoyl LPC; and (iv) lipase-catalyzed re-esterification of 2-hexanoyl LPC to yield 1-decanoyl-2-hexanoyl PC.

Extracts 1 and 2 were pooled, and the ethanol was removed in a rotary evaporator. The remaining solid was then extracted twice with diethyl ether (each 0.5 L) to remove FFA, and dried under vacuum to give a solid residue (12.1 g). Two grams of this residue was then suspended in 12 mL eluent (cyclohexane/isopropanol/water = 35:57:8 by vol). Insoluble material was removed by centrifugation, and the supernatant was loaded onto a silica column (2.5 × 17 cm) equilibrated with the eluent. The column was then eluted with 2.0 L eluent. Fractions of 50 mL were collected and analyzed by HPLC (19).

Introduction of hexanoic acid into the sn-2 *position.* PC containing hexanoic acid in the *sn*-2 position was synthesized by PLA_2-catalyzed esterification of 1-acyl LPC with hexanoic acid in toluene. A water-activity gradient and two different temperatures were applied for optimal exploitation of the enzyme (11). The 1-acyl LPC (860 mg) obtained after column chromatography was dissolved in 100 mL of a 1.2-M solution of hexanoic acid in toluene. The solution was then transferred to the reaction vessel, which was placed in a desiccator, together with a beaker containing a saturated solution of K_2CO_3 for water-activity adjustment (a_w 0.43) and another beaker containing toluene for saturation of the atmosphere with solvent

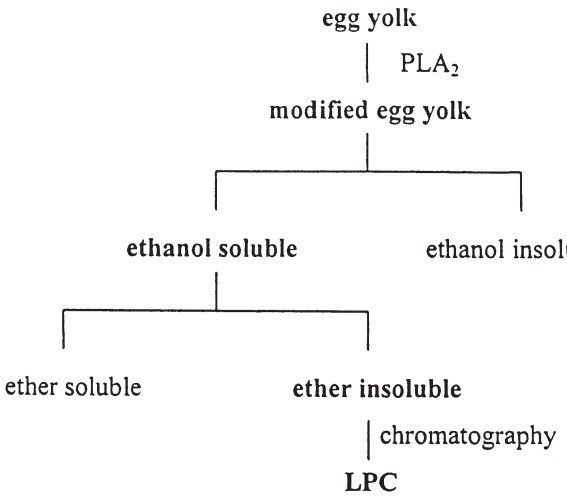

FIG. 2. Preparation of 1-acyl LPC from egg yolk. 1-Acyl LPC was synthesized by PLA_2-catalyzed hydrolysis of egg yolk PC and then purified by extraction with ethanol, precipitation with diethyl ether, and column chromatography. For abbreviations see Figure 1.

(22). The enzyme preparation (11 g immobilized PLA_2) was adjusted to the same water activity in a separate desiccator. The reaction was started after 20 h of water-activity equilibration by mixing the enzyme preparation and the reaction medium and conducting the reaction in the desiccator. The water activity in the system was then decreased by exchanging the K_2CO_3 with potassium acetate (a_w 0.22) at the start of the reaction and by exchanging the potassium acetate with LiCl (a_w 0.11) after 48 h. The initial reaction temperature was 25°C. After 48 h, the temperature was raised to 40°C. Conversion of 1-acyl LPC to PC was followed by HPLC (19). The water content of the medium was measured by Karl Fischer titration. After completion of the reaction, the enzyme preparation was filtered off, and the toluene was removed in a rotary evaporator. The remaining oil was loaded onto a silica column (28.5 × 3 cm) equilibrated with toluene. The column was eluted with approximately four column volumes of toluene to remove the FA and then with a mixture of cyclohexane/isopropanol/water (35:57:8 by vol) to elute first the PC and then the unconverted LPC. The fractions containing PC were pooled, the eluent was removed in a rotary evaporator, and the preparation was dried by lyophilization.

Introduction of decanoic acid into the sn-*1 position*. The PC obtained in the previous enzymatic step (370 mg) was dissolved in 15 mL ethanol, Lipozyme IM (1.5 g) was added, and the mixture was shaken vigorously at 25°C for 5 h. The enzyme preparation was then filtered off, and the ethanol was removed in a rotary evaporator. The remaining solid was lyophilized, extracted three times with 20 mL diethyl ether to remove FFA and FA ethyl esters, and then lyophilized once again. The lyophilized powder (263 mg) was then dissolved in 30 mL of an 800 mM solution of decanoic acid in toluene, and dry enzyme preparation (Lipase 80.000 on EP-100, 1.5 g) was added to start the reaction. The reaction vial was placed in a desiccator together with a saturated solution of LiCl (a_w 0.11). The reaction temperature was 25°C. After 49 h, the enzyme preparation was filtered off, and the PC product was purified on a silica column (2.5 × 17.0 cm) as described in the previous section.

FA position analysis of the PC product. The enzymatically produced PC was subjected to either Lipozyme-catalyzed ethanolysis in the *sn*-1 position or to PLA_2-catalyzed hydrolysis in the *sn*-2 position. The PC and LPC products were then analyzed for their FA composition. PC (6 mg) was dissolved in 500 μL of ethanol or 500 μL of a diethyl ether/ethanol mixture

(4:1 vol/vol), and Lipozyme (20 mg) or immobilized PLA_2 (10 mg) was added. The reactions were complete after 26 and 3 h, respectively. Fifty microliters of the reaction medium was withdrawn before addition of the enzyme, and 100 μL was withdrawn at the end of the reaction for FA analysis. The FA analysis was carried out as described by Svensson and coworkers (16).

RESULTS AND DISCUSSION

From egg yolk to 1-acyl LPC. PLA_2 was used to hydrolyze PC to 1-acyl LPC and FA. The reaction was carried out directly in an egg yolk suspension, since the PC present in LDL particles in the egg yolk is readily hydrolyzed by PLA_2 without the addition of a detergent or solvent. Also, it was unnecessary to add water to the egg yolk suspension, as the natural water content of the egg yolk, about 50%, was sufficient for hydrolysis. The hydrolysis of PC to 1-acyl LPC reached a maximal conversion of 75% within 70 min. The progress of the reaction is shown in Figure 3.

The high solubility of LPC in ethanol and its insolubility in diethyl ether were then utilized to separate LPC from other egg yolk components. Ethanol extraction and ether precipitation yielded 12.1 g of a solid phospholipid mixture, consisting mainly of LPC, lysophosphatidylethanolamine (LPE), PC, and PE (Fig. 4A). A part of this phospholipid mixture (2 g) was then subjected to column chromatography for further purification (Fig. 4B). Fractions 18–40 contained about 95% of the LPC eluted, to give 860 mg of LPC (Fig. 4C). The regioisomeric purity of 1-acyl LPC was about 96%. Interestingly, there was no measurable rearrangement between the regioisomers of LPC during purification by column chromatography.

Incorporation of hexanoic acid into the sn-2 *position.* The progress of the PLA_2-catalyzed esterification of 1-acyl LPC with hexanoic acid is shown in Figure 5. The reaction proceeded stepwise, as was expected, when a water-activity gradient was applied (11). During the first step, when the water activity was decreased gradually from 0.43 to 0.22, a conversion of 27% was reached within 44 h. Thereafter, the reactions leveled off, indicating that the equilibrium position for that water activity was reached. When the water activity was decreased to 0.11 and the temperature was raised to 40°C, further conversion occurred until a new equilibrium value of 49% was reached. The increase in temperature during this second step led to a reaction rate that was almost as high as in the beginning of the reaction. At room temperature, the enzyme was nearly inactive at a water activity of 0.11 (11). However, the increase in temperature also led to acyl migration. The loss due to acyl migration (as seen in the decrease in the total concentration of PC and 1-acyl LPC) was around 7% during the second step, compared with practically zero when the reaction was carried out at room temperature.

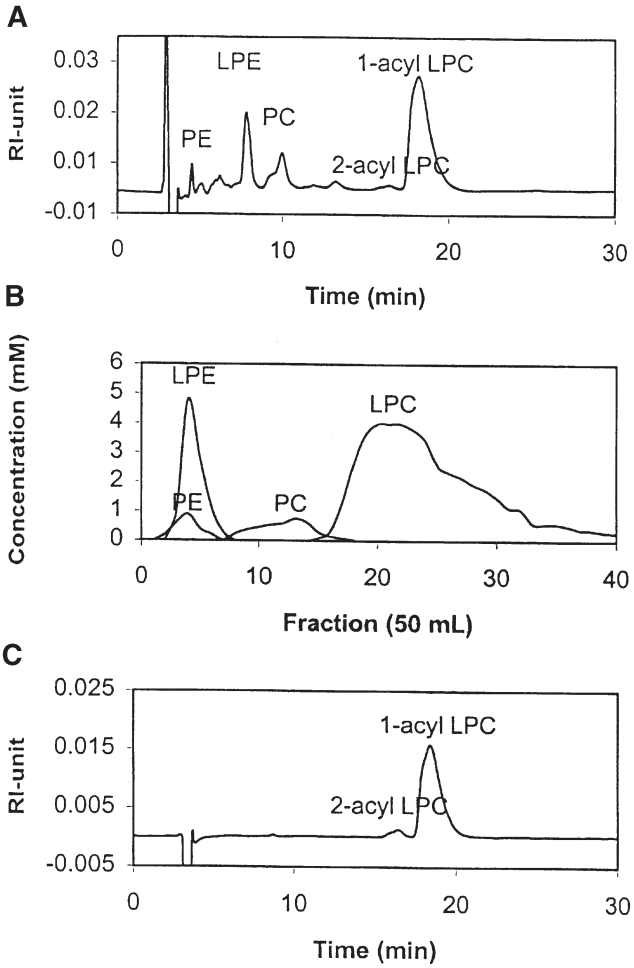

FIG. 4. Purification of LPC. (A) Ethanol extract, HPLC profile. (B) Separation of the substances present in the ethanol extract by silica column chromatography. (C) Purified LPC, HPLC profile. LPE, lysophosphatidylethanolamine; RI, refractive index; for other abbreviations see Figure 1.

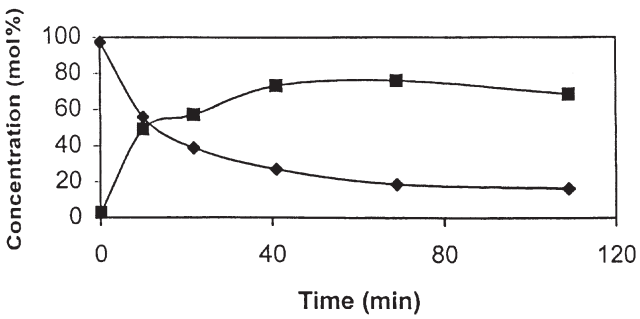

FIG. 3. Time course of the PLA_2-catalyzed hydrolysis of egg yolk. (◆) Concentration of PC; (■) concentration of 1-acyl LPC. For abbreviations see Figure 1.

The water content of the reaction medium decreased during the reaction from an initial 65 mg/mL^{-1} to 48 mg mL^{-1} after 44 h and finally to 25 mg mL^{-1} at the end of the reaction. These water contents are close to the ones expected for the given water activities (11). This indicates sufficient control of the water activity. However, it is possible that more efficient water removal might have speeded up the reaction.

The reactants were then separated by column chromatography. The FA eluted with toluene, and PC and LPC were eluted

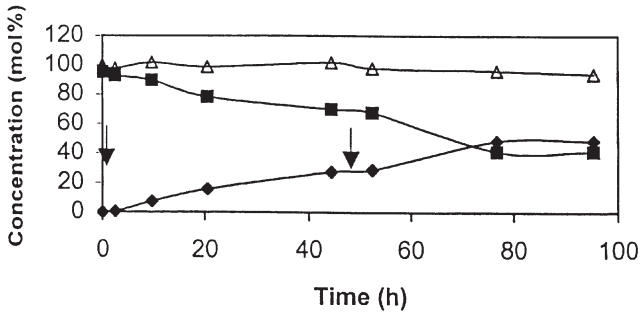

FIG. 5. PLA_2-catalyzed esterification of 1-acyl LPC with hexanoic acid. The reaction medium consisted of 860 mg LPC dissolved in a 1.2-M solution of hexanoic acid in toluene. The water activity of the reaction medium was decreased during the reaction from an initial 0.43 to 0.11 at the end of the reaction. Arrows indicate when the saturated salt solution, used for water-activity equilibration, was exchanged. (For more details see the Materials and Methods section.) The initial reaction temperature was 25°C, and after 48 h the temperature was raised to 40°C. (■) Amount 1-acyl LPC; (◆) amount PC; (△) total amount of PC and 1-acyl LPC. For abbreviations see Figure 1.

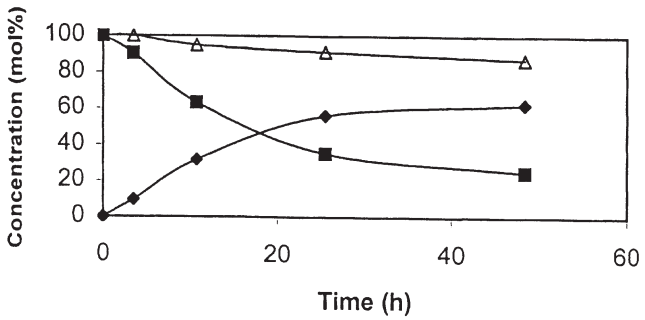

FIG. 6. Lipase-catalyzed esterification of 2-hexanoyl LPC with decanoic acid. The reaction medium (30 mL) consisted of 263 mg of 2-hexanoyl LPC dissolved in an 800-mM solution of hexanoic acid in toluene. The reaction temperature was 25°C, and the water activity was 0.11. (■) Amount 2-acyl LPC; (◆) amount PC; (△) total amount of PC and 2-acyl LPC. For abbreviations see Figure 1.

separately from each other. Unconverted substrates could thus easily be reused. The isolated PC yield was 370 mg, which corresponded to 36.0 mol% of egg yolk LPC (Table 1).

Introduction of decanoic acid into the sn-*1 position.* Decanoic acid was introduced into the *sn*-1 position in two steps. In the first step, the FA originally present in the egg yolk PC was removed by Lipozyme-catalyzed ethanolysis, and in the second step, lipase from *R. oryzae* was used to re-esterify the 2-acyl LPC with decanoic acid. Lipase from *R. oryzae* was chosen for the esterification, since in a similar reaction a slightly higher yield was obtained when using this enzyme instead of Lipozyme (16). On the other hand, the lipase from *R. oryzae* was inactive in the ethanolysis reaction.

The ethanolysis of PC by Lipozyme was complete within 5 h. Ether extraction and lyophilization yielded 263 mg of a white powder. This yield was a bit too high to account for only the 2-acyl LPC. Possibly, there were still small amounts of FA or ethyl ester present in the preparation.

The reaction progress for the second step, esterification of the 2-acyl LPC with decanoic acid, is shown in Figure 6. The reaction yielded 60% of the desired product within 50 h. The isolated yield was 160 mg, which corresponded to 17.0 mol% of egg yolk LPC. The PC product was chemically pure according to HPLC (19) and TLC analyses (silica; $CHCl_3$/MeOH/H_2O = 35:65:5 by vol). The positional purity of the FA was around 99% (Table 2).

TABLE 1
Enzymatic Synthesis of 1-Decanoyl-2-hexanoyl PC[a]

Substance	Yield (mg)	Yield (mol%)
LPC, from egg yolk	860	100
PC, hexanoic acid in the *sn*-2 position	370	36.0
LPC, 2-hexanoyl	263	—
PC, 1-decanoyl-2-hexanoyl	160	17.0

[a]Yield (mol%) = mol synthesized substance/mol lysophosphatidylcholine (LPC) purified from egg yolk × 100.

TABLE 2
FA Composition of the Enzymatically Modified PC and of Its Partial Hydrolysis Products, 1- and 2-Acyl LPC[a]

	FA composition (mol%)		
FA	PC	2-Acyl LPC	1-Acyl LPC
C 6:0	50.5	99.8	0.53
C10:0	49.3	0.2	98.9
Others	0.2		0.5

[a]For abbreviation see Table 1.

Additional remarks. Mixed-acid PC can be produced conveniently by enzymatic modification of natural PC. Egg yolk is a suitable starting material since egg yolk PC, being present in LDL particles, is readily hydrolyzed by PLA_2. PLA_2 then also catalyzes the re-esterification of 1-acyl LPC. The FA exchange in the *sn*-1 position is carried out advantageously in a two-step reaction when a product of high purity is desired. The enzymatic procedure provided a yield of 17%. The product had a chemical purity of >99% and a positional purity of the FA of around 99%.

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