Synthesis of Phosphatidylcholine with (n-3) Fatty Acids by Phospholipase A 2 in Microemulsion

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Phosphatidylcholine containing a long chain polyunsaturated acyl group at the 2-position has been prepared by phospholipase A 2 catalyzed esterification of lysophosphatidylcholine with polyunsaturated fatty acids EPA (C20:5) or DHA (C22:6). Preliminary studies showed that the other fatty acids, such as lauric acid (C12), palmitic acid (C16), stearic acid (C18) and linoleic acid (C18:2), were also incorporated. To our knowledge, phospholipase A 2 catalyzed condensation reactions have not been reported in the literature before. The reactions were performed in sodium *bis*(2-ethylhexyl)-sulfosuccinate-based microemulsions containing small amounts of water. Synthesis of the same phospholipid by transesterification of phosphatidylcholine with the polyunsaturated acids in microemulsion failed; however, enzymatic hydrolysis to lysophosphatidylcholine was facile, quantitative conversion from phosphatidylcholine being attained after 16 hr reaction time.

An additional observation was that, unlike enzymatic hydrolysis of phospholipids, the condensation reaction catalyzed by phospholipase A 2 was totally independent of the presence of calcium.

KEY WORDS: AOT, DHA, EPA, microemulsion, phosphatidylcholine, phospholipase A 2, synthesis.

The effect of n-3 fatty acids on the metabolism of prostaglandins and on serum lipid, cholesterol, phospholipids, triglycerides, platelet aggregation, as well as on immune regulation, have been demonstrated. The roles of n-3 fatty acids in health promotion and disease prevention, especially in the treatment of cardiovascular disease, are under extensive investigations (1-6). It is believed that phosphatidylcholine (PC) with eicosapentaenoic acid (EPA C20:5) or docosahexaenoic acid (DHA C22:6) at the 2-position could more easily be digested in the body and might be of value in nutritional and medical applications (7).

It has recently been shown that lipase catalyzed transesterification is an efficient way to prepare triglycerides with unusual fatty acid composition. The reactions are conveniently carried out in microemulsions of low water content and both saturated (8) and unsaturated (9) acyl groups can be incorporated. Sodium *bis* (2-ethylhexyl)-sulfosuccinate (AOT) gives a large isotropic phase (L 2 region) in the oil-rich region of the water-surfactant-hydrocarbon phase diagram and has been found to be the surfactant of choice in many instances.

The aim of this work was to investigate conditions for the incorporation of polyunsaturated fatty acids (PUFAs) into the 2-position of phosphatidylcholine by a phospholipase A 2 catalyzed process in microemulsion. Attempts were made to prepare the modified PC (mod-PC) either by enzymatic transesterification of PC or by enzymatic esterification of lysophosphatidylcholine (lyso-PC). Since it was believed that several compositional and environmental factors affect these reactions, statistical experimental designs were applied.

EXPERIMENTAL PROCEDURES

Materials. Phospholipase A 2 (from porcine pancreatic glands, 11.000 U/mL) was purchased from Novo, Denmark, AOT and TLC-plates were from Merck, Ltd. (West Germany). PUFAs were obtained from Jahres Fabrikker (Norway), and were of fish oil origin. PC (from egg yolk, purity 99%), lyso-PC (99%), standard fatty acids, sodium arsenite, ceric sulphate, silica gel 60 (230-400 mesh) and boron trifluoride-methanol (14%) were from Sigma Chemical Co. (St. Louis, MO). Isooctane, chloroform, methanol and other chemicals were analytical grades.

Hydrolysis of phosphatidylcholine. Aqueous buffer, AOT, PC, isooctane and phospholipase A 2 were mixed in sealed tubes and kept at 30°C under N_2 in a shaker for 16 hr. After completed reaction, 10 g reaction mixture was charged on a silica gel column (2.5 × 8.0 cm). Chloroform/methanol (9:1, v/v) was used as eluent to remove free fatty acid and AOT. Lyso-PC was eluted with pure methanol. The eluent fractions containing lyso-PC were collected and evaporated to dryness at 60°C under vacuum. The yield of lyso-PC was calculated according to the equation:

$$\text{yield (\%)} = \text{lyso-PC/PC} \cdot 100 \text{ (w/w)} \qquad [1]$$

Synthesis of phosphatidylcholine containing a polyunsaturated acyl group (mod-PC). Aqueous buffer, AOT, lyso-PC, PUFAs, isooctane and enzyme were mixed in sealed tubes and the reactions were carried out under the same conditions as above. Mod-PC was eluted from the silica gel column by chloroform/methanol/water (80:20:0.2) after removal of fatty acids and AOT. The unreacted lyso-PC was recovered by methanol. The eluent fractions containing mod-PC were collected and evaporated to dryness. The yield of mod-PC was calculated according to the equation:

$$\text{yield (\%)} = \text{mod-PC/lyso-PC} \cdot 100 \text{ (w/w)} \qquad [2]$$

Quantitative analysis of mod-PC. A standard quantity (10 μL) of reaction mixture was spotted on a TLC plate and eluated with a solvent system consisting of chloroform/methanol/water (75:20:4, v/v/v). After drying, the plate was developed in iodine vapor for 5 min and sprayed with glacial acetic acid. The round brown area of mod-PC (R_f=0.32) was scraped off and transferred into an Eppendorf centrifuge tube (1.5 mL). Sulphuric acid (1.0 mL, 1.8 M) was added to extract the iodine

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and mod-PC was analyzed quantitatively by measuring the amount of iodine released (10). A standard curve was constructed by subjecting known quantities of PC to the same procedure.

Quantitative analysis of fatty acids. Methyl esters of fatty acids were prepared according to the method of Morrison and Smith (11), and their amount determined by gas chromatography. Commercial fatty acid methyl esters were used as standards.

Statistical design. In the synthesis experiments, a fractional factorial (12) with 16 experimental points and a central composite design with 29 experimental points were combined to give a summary estimate of the effect of the different experimental conditions. A total of 123 experiments were performed.

The fraction factorial was a 2^{7-3} design and had the following experimental settings: Water, 0.5 and 1%; AOT, 2 and 4%; lyso-PC, 1 and 3%; PUFA, 2 and 8%; enzyme, $2 \cdot 5 \times 10^3$ and $2 \cdot 5 \times 10^4$ units/g of lyso-PC; calcium, 0 and 10 nM; and reaction time, 16 and 24 hr.

The central composite design had the following experimental conditions: Water, 0.25, 0.5, 1, 1.5, 2.5%; AOT, 3, 4, 5, 6, 7%; lyso-PC, 2, 3, 4, 5, 6%; PUFA, 2, 3, 4, 5, 6%; enzyme, 5×10^3, 1×10^4; 1.5×10^4, 2.0×10^4, 2.5×10^4, units/g of lyso-PC. The minimum, maximum and centerpoint of these variables represents the "star" part of the design, and the remaining the factorial part. The estimated regression model (13,14) is shown below in equation [3].

$$Mod\text{-}PC = 1.236 + 0.150\ A - 0.546\ A^2 + 0.213\ B$$
$$- 0.041\ B^2 - 0.096\ C - 0.025\ C^2$$
$$+ 0.190\ D + 0.113\ AC - 0.024\ BC$$
$$- 0.041\ E \qquad [3]$$

$$n = 45,\ R^2, = 0.81,\ S^2 = 0.044$$

where, A, water content -1; B, lyso-PC concentration -3; C, AOT concentration -4; D, (enzyme concentration $-1.25 \cdot 10^4)/2.5 \cdot 10^4$; E, PUFAs concentration -4; n, the number of experiments; R^2, coefficient of determination; and S^2, the estimated variance in the residuals.

RESULTS AND DISCUSSION

Attempted transesterification of phosphatidylcholine with polyunsaturated fatty acids. In analogy with lipase catalyzed transesterfication of triglycerides in microemulsions, addition of phospholipase A 2 to a properly formulated microemulsion containing PC and PUFA should lead to incorporation of a polyunsaturated acyl group in the 2-position of the phospholipid. A series of microemulsions based on AOT, PC, PUFA, isooctane and an aqueous buffer were prepared and the enzymatic reaction investigated. A number of reaction parameters, such as time, temperature, pH, Ca^{2+} and Na$^+$ concentrations, water to surfactant ratio and amount of enzyme, were varied. Analysis of the phospholipid fraction after completed reaction invariably showed a negligible content of polyunsaturated acyl groups, indicating that little or no reaction had taken place. Evidently, the phospholipase A 2 catalyzed the transesterification of a phospholipid is

not as facile as the lipase catalyzed triglyceride conversion.

Hydrolysis of phosphatidylcholine. Phospholipase A 2 catalyzed hydrolysis of PC was performed in AOT based microemulsions of varying water content. The reaction was sluggish in the absence of Ca^{2+}, but gave an almost quantitative yield (over 95%) of lyso-PC after 16 hr reaction time when Ca^{2+} was present in a concentration of at least 10 mM, as is seen in Figure 1. A Ca^{2+} dependence for phospholipase A 2 catalyzed hydrolysis has been reported earlier (15).

The enzymatic hydrolysis turned out to be extremely pH sensitive, as is shown in Figure 2. Optimum pH was around 7, and a suitable formulation for the conversion of PC into lyso-PC is PC, 3.0%; AOT, 7.0%; isooctane, 87%; 0.1 M borate-HCl buffer (pH 7), 3.0%; CaCl$_2$, 10 mM; and enzyme 3×10^3 units/g of PC.

Synthesis of phosphatidylcholine containing a polyunsaturated acyl group. A number of reaction parameters including the concentrations of water, AOT, lyso-PC, PUFA, enzyme and Ca^{2+}, as well as buffer pH and reaction time, were systematically tested.

In order to shift the equilibrium, lyso-PC + PUFA $\xrightarrow{enzyme}$ mod-PC + water, to the right the amount of water must be kept low. It was found, however, that reducing the amount of water to below about 1% led to a dramatically decreased production of mod-PC (Fig. 3A). Evidently, a certain amount of water is needed to hydrate the enzyme and enable it to attain its desired conformation. Analogous behavior has been reported in the lipase catalyzed synthesis of triglycerides (16).
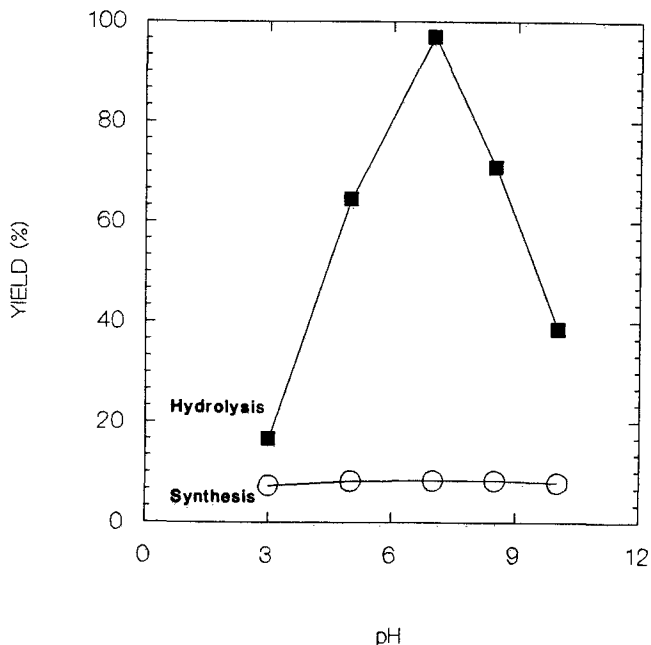

FIG. 1. Effect of calcium ion concentration on hydrolysis of PC and synthesis of mod-PC. Hydrolysis and synthesis reactions were carried out respectively at their preferred conditions (see Results and Discussion), except the calcium ion concentration.

The optimal water content was found to be about 1.2% (Fig. 3B).

Figures 3A and 4A show that decreasing the amount of AOT enhanced the production of mod-PC. However, it was discovered that the solubility of lyso-PC in the system was proportionally affected by AOT concentration. In order to completely dissolve lyso-PC, a molar ratio of AOT to lyso-PC of at least 1.0 (corresponding to a weight ratio of 0.85) was needed. In the region
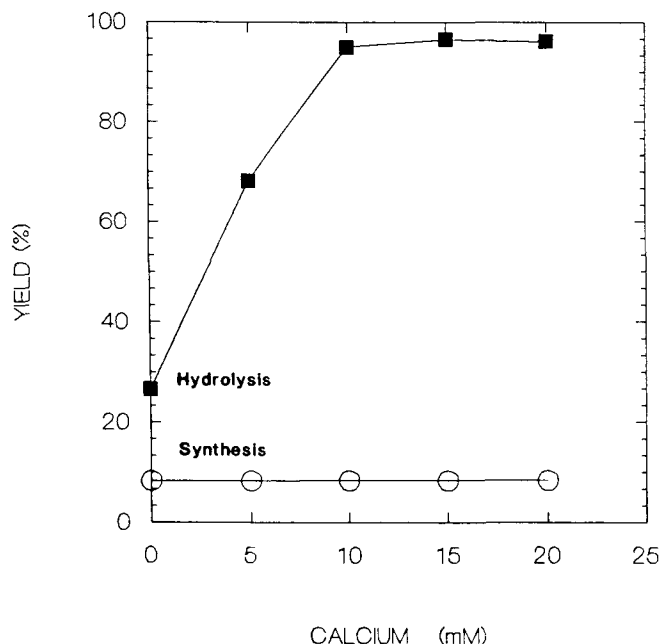

FIG. 2. Effect of buffer pH on hydrolysis of PC and on synthesis of mod-PC. Hydrolysis and synthesis reactions were carried out respectively at their preferred conditions, except the buffer pH.

with a ratio below 0.85, lyso-PC would partly precipitate (precipitation region in Fig. 4B) and lead to low production of mod-PC. In order to obtain an isotropic and thermodynamically stable microemulsion and high production of mod-PC, the preferred concentrations of lyso-PC and AOT were 4% and 3.4%, respectively (Fig. 4B).

The molar ratio (R) of water to AOT has previously been reported to be a critical parameter and governs most of the structural and physical properties of microemulsion. At about R = 10, the reactions catalyzed by hydrolases attain their maximum rates (17). In the present work, at optimal water content of 1.2% and AOT concentration of 3.4%, the R-value can be calculated to be 8.6.

Experimental results (data was not shown in this paper) indicated that increasing the amount of enzyme could effectively improve the production of mod-PC, but increasing it further was confined by low water content in the system. Under the experimental conditions, the allowed maximum of enzyme amount was 2.5 $\times 10^4$ units/g of lyso-PC.

Calcium is known to be essential for the enzymatic hydrolysis of phospholipids. However, we found no obvious effect of it in the reverse reaction. Evidently in the latter case, the enzyme-substrate complex formation can occur without calcium (Fig. 1).

Unlike the hydrolysis reaction, the yield of mod-PC is very sensitive to pH variations, the yield being approximately constant between pH 5 and 10 (Fig. 2). However, the difficulty in defining pH in the aqueous pools of reversed micellar systems is well-known (18).

The preferred formulation for the synthesis of mod-PC from lyso-PC and PUFAs by phospholipase A 2 is summarized as follows: lyso-PC, 4.0%; PUFA, 4.0%; AOT, 3.4%; isooctane, 87.4%; 0.1 M borate-HCl buffer (pH 8.2), 1.2%; and enzyme, 2.5 $\times 10^4$ units/g of lyso-PC.

A synthesis reaction (10 g of microemulsion) was carried out under the preferred conditions. The yield
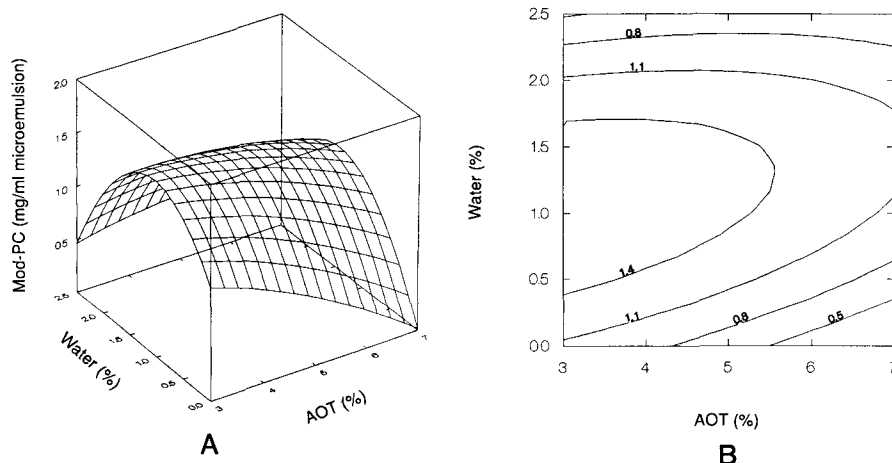

FIG. 3. Effect of water and AOT on the production of mod-PC from equation [3]. A shows the three dimensional graph and B shows the same relationship where each curved line in the graph represents one level of mod-PC production.
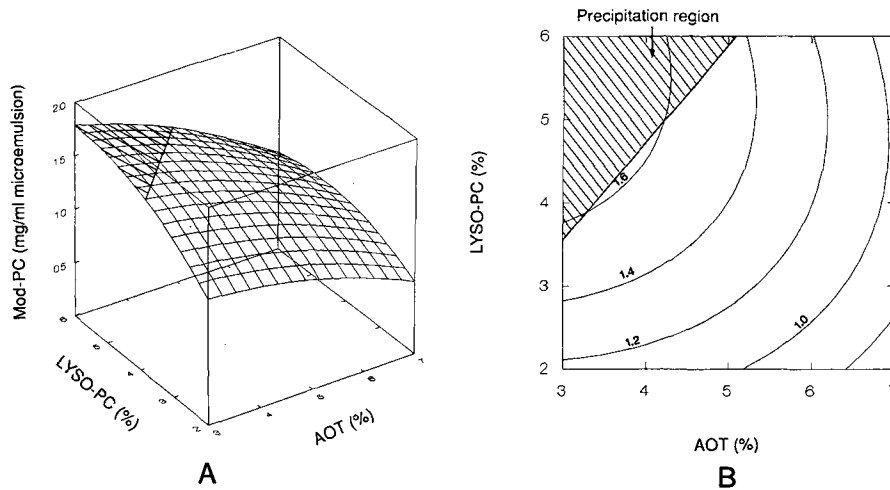
FIG. 4. Effect of lyso-PC and AOT on the productions of mod-PC from equation [3]. A shows the three dimensional graph and B shows the same relationship where each curved line in the graph represents one level of mod-PC production.

TABLE 1

Fatty Acid Composition of the Substrates and the Products Made from Them

Individual fatty acids	16:0	18:0	18:1	18:2	18:3	20:1	20:5	22:6
Expt 1.								
Substrate 1 (lyso-PC)	68.0	26.2	5.3	0.5	—	—	—	—
Substrate 2 (PUFA:s)	3.6	3.8	9.2	3.7	5.3	3.4	40.7	30.3
Product (mod-PC 1)	41.5	15.7	11.7	0.7	1.4	2.5	15.9	10.5
Expt 2.								
Substrate 1 (lyso-PC)	68.0	26.2	5.3	0.5	—	—	—	—
Substrate 2 (DHA)	3.9	6.1	—	—	—	—	—	90.0
Product (mod-PC 2)	34.6	19.8	8.4	0.9	—	—	—	34.6

of mod-PC was around 6% (method described in Experimental Procedures). After completed reaction the unreacted lyso-PC could be recovered from the column and reused in a new reaction. This procedure was repeated several times without considerable loss of yield. Thus, by a recycling process, theoretically all of lyso-PC could be transformed into the desired phospholipid containing a polyunsaturated acyl group at the 2-position. Using the same procedure, it was shown by TLC-analysis that C12, C16, C18 and C18:2 fatty acids also could be incorporated into lyso-PC.

Fatty acid composition of phosphatidylcholine containing a polyunsaturated acyl group. The synthesis of PC from lyso-PC has been carried out with two different fatty acids substrates. The first of them,

PUFAs, contains 71% of a mixture of EPA and DHA and the second, commercial DHA, contains 90% DHA, the balance in both products being standard C16-C22 fatty acids (Table 1).

Condensation of lyso-PC with PUFAs and DHA would, theoretically, give an EPA + DHA content of 35-36% and a DHA content of 45%, respectively. As can be seen from Table 1, the content of these acids in the phospholipid fraction is lower—26.4 and 34.6%, respectively.

The reduced degree of incorporation of the polyunsaturated, long chain fatty acids may reflect a slight discrimination of these acids in the formation of the acyl-enzyme intermediate (19). The effect also may be due to a migration of the 1-acyl group in the lyso-PC

to the 2-position followed by enzyme catalyzed hydrolysis. This side reaction, which is known to occur in the work-up of lysolecithins (20,21), will generate extra non-n-3 fatty acids available for condensation with lyso-PC.

ACKNOWLEDGMENTS

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