Enzymatic Synthesis of Phosphatidylcholine with Fatty Acids, Isooctane, Carbon Dioxide, and Propane as Solvents

Magnus Härröd* and Inger Elfman

Department of Food Science, Chalmers University of Technology, Göteborg, Sweden

ABSTRACT: Phosphatidylcholine (PC) was synthesized from lyso-PC and long polyunsaturated fatty acids (PUFA) with phospholipase A_2. In previous investigations, performed in small glass tubes, the enzymatic synthesis reaction was optimized. This paper presents results from experiments performed in a high-pressure reactor filled with an immobilized enzyme (Im.E.). Fatty acids were used as the main solvent while isooctane, CO_2, or propane was used as an additional solvent. The water content was carefully controlled over wide ranges. The temperature was kept constant at 45°C for up to 50 h. The highest initial reaction rate was attained with pure fatty acids under relatively humid conditions (water = 35% of dry Im.E.). The reaction rates were more than three times as high in the high-pressure reactor than in previous experiments in glass tubes. In all solvent systems, the best yield was attained after long times under dry conditions (water <15% of dry Im.E.). Addition of CO_2 to the PUFA reduced the yield, while addition of isooctane or propane increased the yield. After 20 h at 45°C, the best yield (25%) was attained at a solvent composition of 91% PUFA and 9% propane.

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KEY WORDS: Carbon dioxide, enzymatic synthesis, isooctane, near-critical fluids, phosphatidylcholine, phospholipase A_2, polyunsaturated fatty acids, propane, reaction rate, yield.

Phospholipids are functional compounds in many biological tissues, e.g., cell membranes, and phospholipids have many important nutritional and medical applications. For example, it is believed that fatty acids (FAs) could be more easily digested in the body as phospholipids than as triglycerides or ethyl esters, especially when the FA is in position 2 of the phospholipids (1–3). Enzymatic synthesis with phospholipase A_2 (PLA_2) is probably the best way to tailor-make phosphatidylcholine (PC) with a particular FA in position 2 of PC (Fig. 1).

In the first experiments with PLA_2, a microemulsion system was used to obtain good solubility of lyso-PC. However, the yield was low, below 7% (1). The yield was restricted by the high water activity in the microemulsion system. By using immobilized enzyme (Im.E.) in small glass tubes with isooctane as solvent, the synthesis reaction was optimized at different water activities, temperatures, times, and substrate concentrations (4–6). The best yield (22%) was attained at a low water content (25% of dry Im.E.), long reaction times (9 d), and high concentrations of polyunsaturated fatty acids (PUFA) (93%) (6). The temperature was maintained in these experiments at 45°C, owing to observed oxidation of PUFA and nonenzymatic synthesis reactions at high temperatures (above 60°C) and long reaction times (5). The reaction rates were limited by poor contact between the enzyme and the substrates (6).

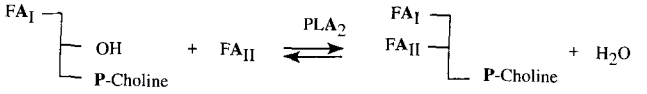

FIG. 1. Enzymatic synthesis of phosphatidylcholine (PC) from lyso-PC and fatty acids (FAs) with phospholipase A_2 (PLA_2). P-Choline, phosphate-choline.

Propane effectively dissolves different lipids (7), including phospholipids (8) and monoglycerides (9). Pure CO_2 does not dissolve phospholipids. Propane greatly reduces the viscosity of phospholipid/triglyceride mixtures, while CO_2 reduces the viscosity of glycerides to a much lower extent (10). These data suggest that propane could be a good solvent for phospholipid synthesis.

Therefore, propane was used as a solvent in this investigation, while isooctane and CO_2 were included as reference solvents. This paper presents the results in detail. A brief overview of the results has been presented elsewhere (11).

EXPERIMENTAL PROCEDURES

Materials. PLA_2 (EC 3.1.1.4), from porcine pancreatic glands (Novo Nordisk A/S, Bagsvaerd, Denmark), had an activity of 9900 μmoles min^{-1} mL^{-1}. The polymer carrier (Deloxan®, 0.2–0.4 mm) was a gift from Degussa (Hanau, Germany). The salts used ($BaCl_2$, KCl, K_2SO_4, NaCl, and P_2O_5) were of pro analysis grade, except the $BaCl_2$, which was of Suprapur® grade (Merck, Darmstadt, Germany). Isooctane (analytical grade; Merck), CO_2, or propane (standard gas and purity 2.0,

*To whom correspondence should be addressed at Department of Food Science, Chalmers University of Technology, P.O. Box 5401, S-402 29 Göteborg, Sweden.

respectively; AGA Gas AB, Lidingö, Sweden) was used as an additional solvent in the reactions.

The PUFA were of fish oil origin (Pronova Biocare A/S, Sandefjord, Norway), and the composition, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been presented in a previous paper (1). PC from soybeans (purity 95%; Avanti Polar-Lipids Inc., Alabaster, AL) and PC from egg yolk (Phospholipon 90; Nattermann Phospholipid GmbH, Köln, Germany) were hydrolyzed to lyso-PC with PLA_2 in a microemulsion of isooctane (analytical grade; Merck) and *bis*-(2-ethylhexyl)sulfosuccinate sodium salt (AOT) (Sigma Chemical Co., St. Louis, MO). The reaction mixture was separated on silica gel (silica gel 60, 230–400 mesh; Sigma Chemical Co.) with chloroform and methanol (analytical grade; Merck). The method used is described below.

The chemicals for the high-performance liquid chromatography (HPLC) analyses were of HPLC grade (Merck) except the triethylamine and the acetic acid, which were of synthesis grade (Merck) and analytical grade (PROLABO, Paris, France). Standards of PC and lyso-PC (purity >99%; Avanti Polar-Lipids Inc.) were used for calibration of the HPLC system.

Reaction unit. The reaction unit (Fig. 2) was constructed together with New Ways of Analytics (Lörrach, Germany). Its major parts were the tubular reactor and the circulation pump. The Im.E. was put in the reactor and kept in it by two filters, at the inlet (10 μm) and at the outlet (2 μm). An additional filter (15 μm) protected the pump from particles. The pump was an air-driven piston pump, and it could raise the pressure by 300 bar. Each stroke was about 1 mL, and the flow rate could be estimated by counting the strokes. With a pressure gauge and the valves, the static pressure and the pressure drops over the different filters could be measured. The temperature in the tubular reactor was controlled by an electric heating device.

Liquid substrates were filled into the reaction unit by the following procedure. The unit was connected to water suction, and a partial vacuum was attained. Lyso-PC was dissolved in the PUFA before the liquid mixture was sucked into the inlet tank. The inlet tank was pressurized with gaseous CO_2 or propane. The gas and the liquid were allowed to separate before the liquid was injected into the reaction loop by the pressure in the gas phase.

In experiments with isooctane, excess liquid reaction mixture for the reaction loop was filled into the inlet tank. Gaseous CO_2 in the inlet tank was used to fill the loop with the liquid reaction mixture. The surplus of the liquid reaction mixture remained at the bottom of the inlet tank and prevented the CO_2 from entering the reaction loop. In experiments with CO_2 and propane, just as much liquid reaction mixture as was needed was filled into the inlet tank and injected into the reaction loop. Finally, liquid CO_2 or propane was used to fill the reaction loop.

Between experiments, the reaction unit was carefully rinsed with isopropanol. Isopropanol was added to the reaction unit by the procedure described above for liquid substrates. Before the start of a new experiment, isopropanol was completely removed from the reaction unit by several rinsing procedures with CO_2.

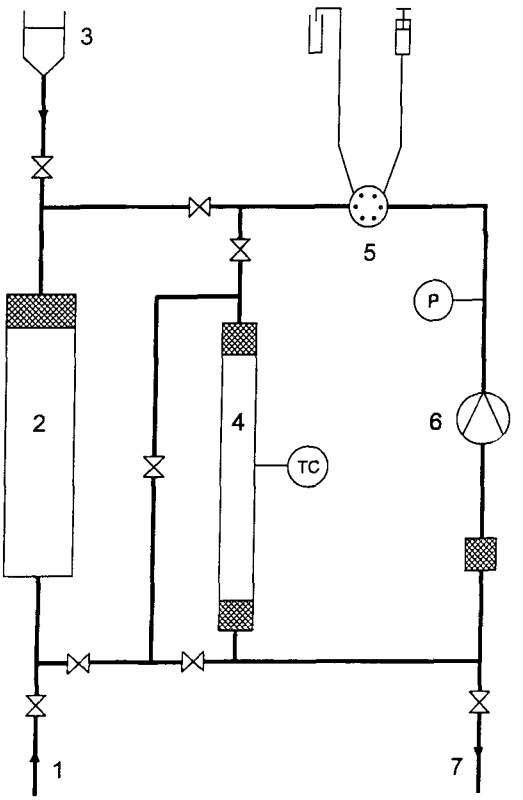

FIG. 2. Schematic view of the high-pressure reaction unit: 1, inlet CO_2/propane; 2, inlet tank (100 mL); 3, inlet liquids; 4, tubular reactor (5 mL); 5, sample outlet; 6, circulation pump; 7, outlet or vacuum; P, pressure gauge; TC, temperature control; double triangles, valve; thatched box, filter.

Samples of the reaction mixture were drawn with an ordinary HPLC injection valve. The sample was collected into an HPLC vial by switching the valve and pressing air through the sample loop with an HPLC syringe. The procedure was repeated until enough material for HPLC analysis was collected.

Experimental design and modelling. PUFA was used as the main solvent in three experimental series. In these series, isooctane, CO_2, or propane was used as additional solvent. The levels of three variables (additional solvent concentration, water content, and time) were selected according to a central composite design (2^3, 16 experiments) (12). The levels of these variables are given in Table 1. The following variables were kept constant through all experiments: lyso-PC (7.75 mg mL^{-1}), temperature (45°C), Im.E. (2175 mg), system pressure (isooctane 60 bar; CO_2 120 bar; propane 80 bar), reaction loop volume (51 mL), and flow rate (5 mL min^{-1}).

The experimental data were evaluated with models that included most terms up to the third degree for the independent variables (see above), with yield as the dependent variable. The least significant terms were successively excluded until

TABLE 1
The Levels of the Variables Systematically Changed in the Experiments[a]

Variables	Levels				
	Very low	Low	Median	High	Very high
Solvent (% of reaction mixture)	0	0	10	20	28
Water content (% of dry Im.E.)	1	15	35	55	70
Time (h)	0.5	1.3	5	18.9	50

[a]Im.E., immobilized enzyme.

TABLE 2
Water Content of the Im.E. Equilibrated over P_2O_5 and Four Different Salt Solutions[a]

Storage condition	Water activity (Ref. 13) (at 5°C)	Water content (% of dry Im.E.)
P_2O_5		0
NaCl solution	0.76	17
KCl solution	0.88	43
$BaCl_2$ solution	0.93	68
K_2SO_4 solution	0.98	79

[a]See Table 1 for abbreviation.

the smallest standard error of estimate (SEE) was attained. The estimations and the graphic presentation of the models were made with computer programs (SYGRAPH; SYSTAT Inc., Evanston, IL).

Preparation of lyso-PC. PC was hydrolyzed in a mixture that contained aqueous buffer, AOT, PLA_2, and isooctane (1). The mixture was kept at 37°C in a shaker for 16 h. The product, lyso-PC, was separated from the rest of the reaction mixture on a silica gel column (30 × 300 mm, 50 g silica gel). Two mixtures of chloroform and methanol (9:1 and 75:25, vol/vol) were used to remove FAs, AOT, and unreacted PC. The lyso-PC was eluated with pure methanol. The fractions containing lyso-PC were evaporated to dryness at 30°C under vacuum. The lyso-PC was freeze-dried and kept below 0°C over silica gel with an indicator (blue gel) until use. The water content of the lyso-PC was then 7.0% for the lyso-PC from soybeans and 3.0% for the lyso-PC from egg yolk. Lyso-PC from soybeans was used in the experimental series with isooctane and CO_2, while lyso-PC from egg yolk was used in the experimental series with propane.

Preparation of different water levels in the reaction mixtures. Lyso-PC and Im.E. contributed to the total water content of the reaction mixture. In most mixtures, the largest share of water came from Im.E. Therefore, the water concentrations of Im.E. were controlled, while lyso-PC was kept as dry as possible. Isooctane was dried with $Na_4P_2O_7$ before use, and its contribution to the water content was disregarded. CO_2 and propane were regarded as completely dry.

The enzyme, PLA_2, was immobilized as described previously (5), resulting in a powder with a high water content (about 65% of dry Im.E.). The immobilized enzyme was freeze-dried to low water content (below 0.1%). One part of the freeze-dried Im.E. was placed over P_2O_5 to keep it as dry as possible. The water content of the rest of the Im.E. was controlled by equilibration over different saturated salt solutions with defined water activity. The four chosen salt solutions and their water activities are shown in Table 2. Freeze-dried Im.E. (3–4 g) was spread in a thin layer in Petri bowls. They were equilibrated over salt solutions in vacuum chambers at 4°C for at least one week. Analyses of the water content of Im.E. were made before every experiment. The water content of the Im.E. over P_2O_5 and the four different salt solutions at equilibrium are shown in Table 2.

Analyses. The water content of lyso-PC and Im.E. was measured by drying samples for approximately two hours at 105°C until constant weight was attained.

The composition of the reaction mixture, PUFA, PC, and lyso-PC, was analyzed in an HPLC system (model SCL 6B; Shimadzu, Tokyo, Japan) equipped with a diol column (LiChrospher® 100 diol, 5 μm, 4 × 125 mm; Merck) and an evaporative light scattering detector (model 750/14; Applied Chromatography Systems Ltd., Macclesfield, Cheshire, England). The sample was eluated with hexane, 1-propanol, 2-propanol, water, acetic acid, and triethylamine at a temperature of 55°C. The composition of the mobile phase at the injection was 82:17:0:0:1.5:0.08 and was kept constant for 3 min. Then, the composition was linearly changed to 65.6:13.6:16.8:2.8:1.5:0.08 during 11 min, followed by a linear change to 0:0:84:14:1.5:0.08 during 9 min and finally kept constant for 10 min. The flow rate was 1.3 mL min^{-1}. A similar procedure has been presented elsewhere (14). An Integration System (Kontron Instruments, Milano, Italy) was used for data collection and integration. It was calibrated with known amounts of PC and lyso-PC standards. Samples from the reaction mixture were drawn after preparation and just after it entered the reactor. Double samples were drawn at the times determined by the experimental design (Table 1). The yield was calculated by Equation 1, where PC and lyso-PC were the analyzed amounts in each sample. The factor 1.53 was used to obtain a molar ratio.

$$\text{yield} = \text{PC}/(\text{PC} + 1.53 \times \text{lyso-PC}) \quad [1]$$

RESULTS AND DISCUSSION

General comments. In this study, PUFA was the main solvent, while isooctane, CO_2, or propane was used as an additional solvent. The results for each additional solvent were summarized by using estimated models. The yield was correlated to solvent composition, water content, and reaction time. The results are illustrated with contour plots (Figs. 3–5). In each figure, two variables are changed, while the third variable is kept constant, and the contour lines represent the yield.

The precision of the models is given by SEE. When the experimental series of isooctane, CO_2, and propane were estimated, SEE was 0.45, 0.73, and 0.25, respectively. One way to interpret SEE is that 95% of the experimental data are within ±1.96 × SEE. This means that changes less than 2 ×

SEE should be disregarded. Careful measurements of water content and composition of the reaction mixture were vital to obtain small SEE values.

Reaction rates and yields. For enzymatic synthesis of PC with PLA_2, the yield after 1 h was considered as a good measure of initial reaction rate. The initial rates are illustrated in Figure 3. In all three experimental series, the initial reaction rates showed basically the same levels and patterns (Fig. 3). The highest initial reaction rates were attained at relatively humid conditions (water = 35% of dry Im.E.). At low water activity (water = 15% of dry Im.E.), there was no reaction after 1 h (Fig. 3).

The yields after a long reaction time (20 h) at different water activities and concentrations of additional solvents are illustrated in Figure 4. The best yields were attained at low water activity (water <15% of dry Im.E.). This was valid for all three experimental series (Fig. 4). The reaction conditions that gave the best yield after 20 h for each solvent system are summarized in Table 3. The best yield for all experimental series was 26%, it was attained with propane as additional solvent (Table 3).

When pure PUFA was used at low water activity, the yield was about 17%; the yield decreased at higher water activities (Fig. 4 at additional solvent = 0% and water from 15 to 45% of dry Im.E.). When CO_2 was the additional solvent, the yield decreased (Fig. 4b). However, when isooctane or propane was the additional solvent, the yield increased (Fig. 4a,c). This could be explained with the solubility of lyso-PC. It decreased when CO_2 was added, while it increased when propane or isooctane was added. A maximum yield was reached when the concentration of isooctane or propane was 9% (Fig. 4a,c). This might be explained by the reactivity of PUFA. When the concentration of PUFA was decreased below 91%, the reaction may have been restricted by low concentration of enzyme–PUFA complex. A more reactive FA donator (15) could perhaps improve the reaction rates, particularly when the concentration of the FA donor is lower than 91%.

When PUFA and isooctane were used as a solvent system in the high-pressure reaction unit, the initial reaction rate and the yield after 20 h were three times higher than the best of previous experiments in glass tubes (6). This was probably attributable to improved contact between the substrates and the enzyme. In the high-pressure unit, the contact was performed by forced circulation of substrates through the bed of Im.E. In previous experiments (6), the glass tubes were shaken, but Im.E. was free floating in the solvent/substrate solution, and contact depended on diffusion of substrates into Im.E.

During hydrolysis of a special egg yolk emulsion (water and bile acids were added) with PLA_2, the activity was declared as 9900 IU/mL (Product Sheet Lecitase™, B226e-GB; Novo Nordisk A/S, Bagsvaerd, Denmark). In this work, pure PC was hydrolyzed in a microemulsion. The initial activity corresponded to 50 IU/mL. According to the producer of the enzyme, this activity is normal when pure PC is the substrate. During the synthesis reactions in this paper, the highest initial reaction rates corresponded to an activity of 0.25 IU/mL.

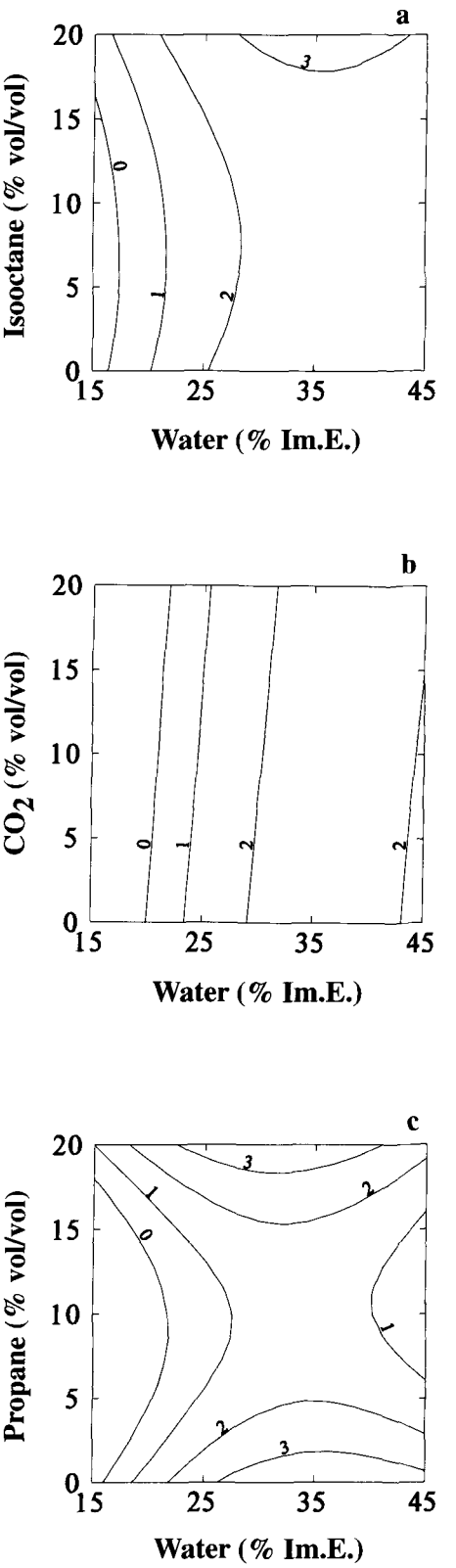

FIG. 3. Yields of phosphatidylcholine after 1 h (i.e., initial reaction rates) at different concentrations of water and additional solvents: (a) isooctane (SEE = 0.45), (b) CO_2 (SEE = 0.73), and (c) propane (SEE = 0.26). Im.E., immobilized enzyme; SEE, standard error of estimate.

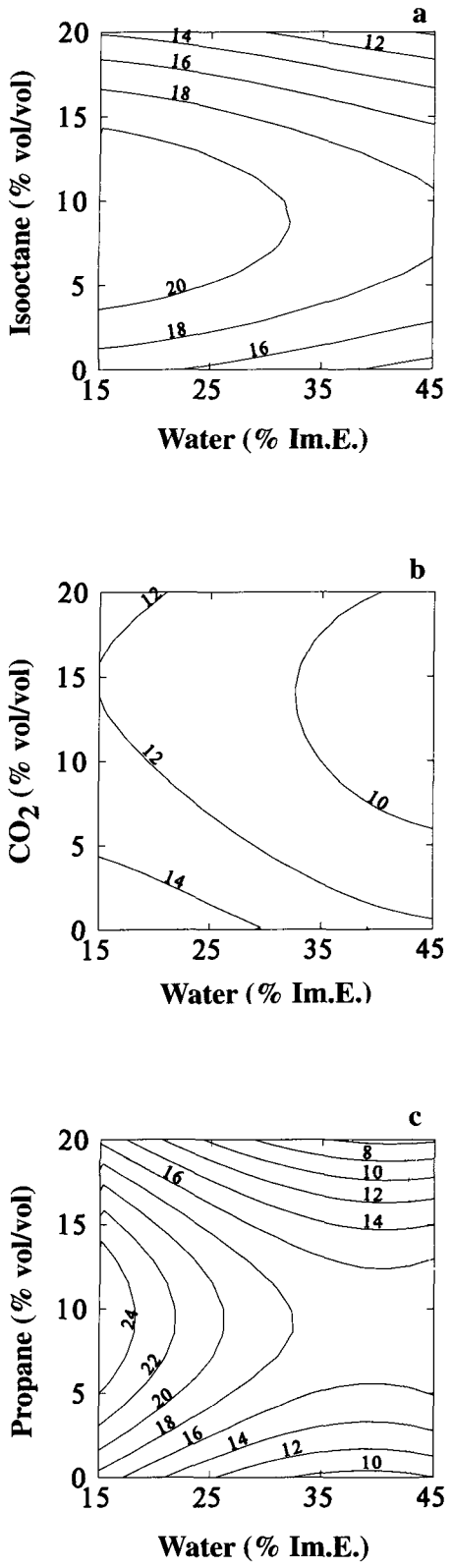

FIG. 4. Yields of phosphatidylcholine after a long reaction time (20 h) at different concentrations of water and additional solvents: (a) isooctane (SEE = 0.45), (b) CO_2 (SEE = 0.73), and (c) propane (SEE = 0.26). Abbreviations as in Figure 3.

TABLE 3
Maximum Yields of Phosphatidylcholine Attained in Different Solvent Systems After 20 h at 45°C and Different Reaction Conditions[a]

	Reaction conditions		
Solvent systems	PUFA + solvent (% vol/vol)	Water (% Im.E.)	Yields (%)
PUFA + isooctane	91 + 9	<15	21
PUFA + CO_2	100 + 0	<15	16
PUFA + propane	91 + 9	<15	26

[a]PUFA, polyunsaturated fatty acids; see Table 1 for other abbreviation.

The estimated models also can describe how the reaction proceeded at different reaction times and water contents when the concentration of additional solvent was kept at 9%, the most favorable level for isooctane and propane (Fig. 5). When CO_2 was the additional solvent, the reaction almost ceased after 10 h at high water content (water >35% of dry Im.E.), and the reaction proceeded slowly under dry conditions (Fig. 5b). When isooctane or propane was the additional solvent, the reaction rate also decreased with time but to a lower extent than with CO_2 (Fig. 5a,c vs. Fig. 5b). At the driest conditions (water <15% of dry Im.E.), the reaction rates were also considerable after 20 h (Fig. 5a,c). These results indicate that when isooctane or propane is used as the additional solvent, increased reaction time and decreased water content will increase the yield.

Some comments on the equipment used. In preliminary experiments with the high-pressure reaction unit, there was no reaction. As reactions in glass tubes were obtained, this reaction was optimized (4–6). The high-pressure reaction unit was rinsed with large amounts of polar solvents, and finally a reaction was obtained in the high-pressure unit. This improvement was probably due to removal of metal ions from the system. There are two explanations in support of this statement: (i), Zn^{2+} is poisonous to the enzyme used (Product Sheet Lecitase™, B226e-GB; Novo Nordisk A/S) (ii) during the rinsing with polar solvents, grease was removed from the valves or the circulation pump. This grease might have contained metal ions. However, the grease can only enter the high-pressure unit during assembly. Once removed, the grease cannot enter the reaction loop again.

To control the water content in large amounts of Im.E., maximal vacuum in the vacuum chamber is strongly recommended. In this way, equilibrium will be reached fast and reliably.

Circulation pumps in small high-pressure units usually cause problems, e.g., leakage around the piston. These problems were solved by a proper choice of packing and filters. In the experiments, the pump was used for about 1500 h without problems.

The enzymatic synthesis of PC, starting from lyso-PC and PUFA, with PLA_2 has been optimized in different solvent systems: microemulsions (1), organic solvents (4–6, this study), and near-critical solvents (this study).

After 20 h, the best yield (26%) was attained at low water activity (water <15% of dry Im.E.) in a mixture of PUFA and

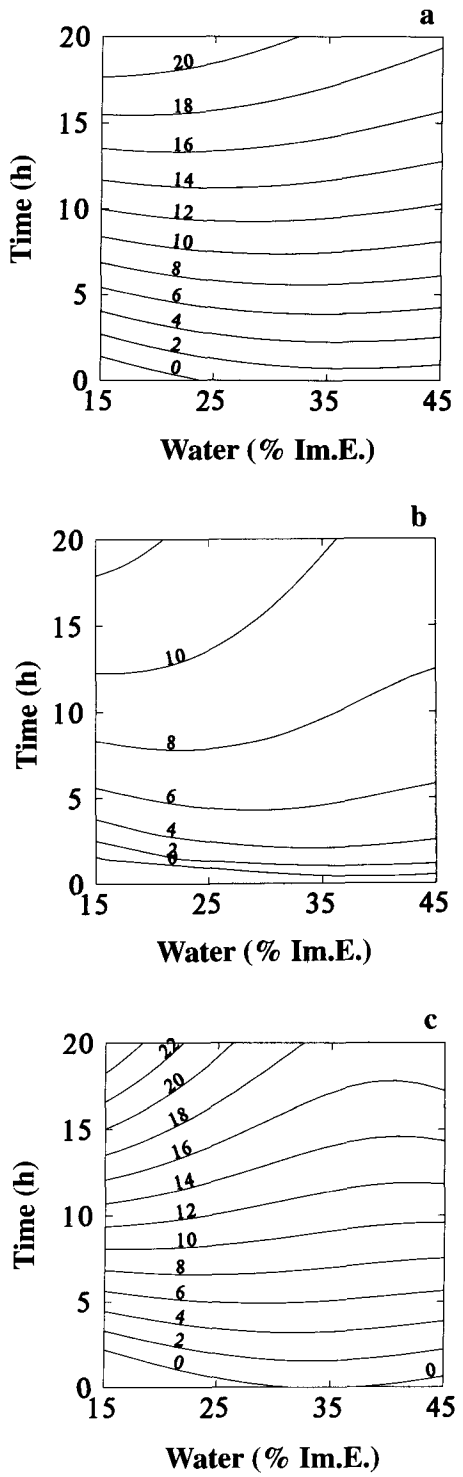

FIG. 5. Yields of phosphatidylcholine at different reaction times and water contents, when the concentration of the additional solvent was 9%: (a) isooctane (SEE = 0.45), (b) CO_2 (SEE = 0.73), and (c) propane (SEE = 0.26). Abbreviations as in Figure 3.

propane (near-critical) as solvent. The highest initial reaction rate was attained at higher water activity (water = 35% of dry Im.E.).

When a mixture of PUFA and isooctane was used as solvent, the reaction rate was three times faster in the high-pressure reactor than in glass tubes, because the contact between the substrates and the Im.E. was better in the high-pressure reactor.

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