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Monitoring of Monooctanoylphosphatidylcholine Synthesis by Enzymatic Acidolysis between Soybean Phosphatidylcholine and Caprylic Acid by Thin-Layer Chromatography with a Flame Ionization Detector

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Thin-layer chromatography with a flame ionization detector (TLC-FID) was used for monitoring the production of structured phospholipids (ML type: L, long-chain fatty acids; M, medium-chain fatty acids) by enzyme-catalyzed acidolysis between soybean phosphatidylcholine (PC) and caprylic acid. It was found that the structured PC fractionated into two to three distinct bands on both plate thin-layer chromatography (TLC) and Chromarod TLC. These three bands represented PC of the LL type, ML type, and MM type, respectively. The TLC-FID method was applied in the present study to examine the influence of enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio (caprylic acid/PC, mol/mol) on formation of ML-type PC in a batch reactor with *Thermomyces lanuginosa* lipase as the catalyst. The formation of ML-type PC was dependent on all parameters examined except for the substrate ratio. The ML-type PC content increased with increasing enzyme dosage, reaction temperature, solvent amount, and reaction time. The substrate ratio had no significant effect on the formation of ML-type PC within the tested range (3−15 mol/mol). The formation of MM-type PC was observed in some experiments, indicating that acyl migration is taking place during reaction since the lipase is claimed to be 1,3-specific. The TLC-FID method offers a simple and cheap technique for elucidation of product and byproduct formation during enzyme-catalyzed reactions for production of phospholipids containing mixtures of long- and medium-chain fatty acids.

KEYWORDS: ***Thermomyces lanuginosa*** **lipase; acidolysis; response surface methodology; structured phospholipids; phosphatidylcholine; TLC−FID**

INTRODUCTION

Phospholipids containing medium-chain fatty acids have received increased attention (*1*, *2*). Phospholipids with medium-chain fatty acids are more water soluble than natural phospholipid and have better heat stability. In the native form soybean phospholipids contain more than 70% mono- or polyunsaturated fatty acids. For some applications, particular those involving very long shelf lives, more saturated grades of phospholipids may be desired.

In the area of liposome formulation it has been reported that the release of drug is very fast when small amounts of phospholipids containing medium-chain fatty acids are incorporated into the carrier liposome due to instantaneous activation of phospholipase A_2 (PLA_2) (*3*). The more rapidly PLA_2 is activated, the faster the drug release and the larger the drug absorption during the time which the carrier spends near the target. Elevated PLA_2 activity is often seen in inflamed and cancerous tissue. Furthermore, it has been observed in disorders such as epilepsy, bipolar disorders, and some types of pain and migraine associated with inflammatory processes (*4*, *5*).

In recent years there has also been an increasing interest in the synthesis of phospholipids containing drug molecules. Compounds comprising the anticonvulsant valproic acid bonded to the phospholipid moiety at the sn-2 position by chemical synthesis have been produced (*6*). These compounds were found to be effective at much lower equivalent molar doses compared to the doses currently used for valproic acid. The reduced therapeutic doses in turn reduce the toxicological risk, accompanying side effects, and the risk of undesirable interactions with other drugs. Depending on the fatty acid located at the sn-1 position of these phospholipid derivatives, different pharmacokinetic profiles were observed (*6*). The length of the alkyl moiety esterified at the sn-1 position of the phospholipid may determine the lipophilicity of the phospholipid derivatives, and thus also transport across the cellular membrane. Other drug molecules may be inserted into the phospholipids, and therefore, there will be a demand to have phospholipids with varying fatty

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acids, giving the opportunity to change pharmacokinetic properties for individual pseudo-phospholipids.

Structured phospholipids with a defined fatty acid profile can be manufactured by enzyme-catalyzed synthesis reactions. The most commonly used enzymes for these purposes have been sn-1,3-specific lipases and PLA_2 for exchange of fatty acids at the sn-1 position and the sn-2 position, respectively (*7*−*10*). In many studies the overall incorporation of novel fatty acids into phospholipids has been determined during reactions, which unfortunately does not give any information concerning the distribution of the novel fatty acids. The sn-2 position on the phospholipid may be involved in the lipase-catalyzed acidolysis reaction, which could lead to the false assumption that higher incorporation at sn-1 has occurred since these enzymes are stated to be specific for the sn-1 position. This is based on observations of higher incorporation of novel fatty acids than theoretically possible (*11*). Incorporation of novel fatty acids has also been determined for the whole reaction mixture including both phospholipids and lysophospholipids, without any fractionation into individual compounds (*1*). During acidolysis it has been reported that the intermediate lysophosphatidylcholine (LPC) may have high incorporation of novel fatty acids as a result of acyl migration.

Chromarod thin-layer chromatography with an Iatroscan flame ionization detector (TLC-FID) has become more accepted as standard and is being used routinely for lipid analysis in several fields, including food, medical, environmental, toxicological, and ecological studies. TLC is a fast, easy, and cost-saving method for the qualitative determination of most compounds. It can also be used quantitatively to determine the purity of a sample, after reaction to determine recovery and purity, and has in several cases been used to evaluate the lipase-catalyzed hydrolysis and esterification reactions of phospholipids (*12*−*14*).

We observed that phosphatidylcholine (PC) splits into two to three bands by TLC corresponding to the differences in the fatty acid composition for samples taken during lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid. These three bands represented PC of the LL type, ML type, and MM type (L, long-chain fatty acids; M, medium-chain fatty acids). This observation was made on both Chromarod TLC and Kieselgel silica plate TLC. LPC was also observed to separate into two bands depending on the fatty acid composition. Fractionation of triacylglycerols into several bands on TLC has also been reported. This separation is based on the difference in fatty acid chain length of triglycerides as well as a result of stereospecific distribution of fatty acids within the triglycerides (*15*).

In this paper we describe a TLC-FID method for examining the fatty acid distribution in PC during the lipase-catalyzed acidolysis between soybean PC and medium-chain fatty acid. Mixtures of the products of the reaction could be spotted directly on the Chromarods without any preparation. With the aid of response surface methodology (RSM) the developed analysis method was applied for the evaluation of parameter effects during acidolysis reactions.

MATERIALS AND METHODS

Materials. Granulated soybean PC (purity 95%) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The fatty acid composition (mol %) of soybean PC was C16:0, 13.7; C18:0, 3.6; C18:1, 9.5; C18:2, 66.0; C18:3, 7.2. 1,2-Octanoyl-*sn*-glycero-3-phosphatidylcholine (purity, 99%), soybean LPC (purity 98%), and glycerophosphorylcholine (GPC; purity 99%) were purchased from Larodan Fine Chemicals (Malmoe, Sweden). Caprylic acid (C8:0; purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Lipozyme TL IM, silica granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S (Bagsvaerd, Denmark). All solvents and chemicals used were of analytical grade.

Table 1. Experimental Setup for Five-Factor, Three-Level Surface Response Design and the Responses[a]

experiment no.	factors					concn of ML-type PC (mol %)
	E_d	T_e	S_a	T_i	S_r	
1	15	40	10	20	12	4.6
2	25	40	10	20	6	23.1
3	15	50	10	20	6	16.3
4	25	50	10	20	12	32.9
5	15	40	20	20	12	15.7
6	25	40	20	20	6	33.3
7	15	50	20	20	6	32.7
8	25	50	20	20	12	42.0
9	15	40	10	40	12	18.9
10	25	40	10	40	6	31.4
11	15	50	10	40	6	17.7
12	25	50	10	40	12	48.2
13	15	40	20	40	12	25.9
14	25	40	20	40	6	41.9
15	15	50	20	40	6	26.6
16	25	50	20	40	12	63.2
17	10	45	15	30	9	14.5
18	30	45	15	30	9	61.6
19	20	35	15	30	9	21.1
20	20	55	15	30	9	45.3
21	20	45	5	30	9	6.5
22	20	45	25	30	9	48.2
23	20	45	15	10	9	11.9
24	20	45	15	50	9	54.3
25	20	45	15	30	3	27.2
26	20	45	15	30	15	55.0
27	20	45	15	30	9	38.1
28	20	45	15	30	9	41.5
29	20	45	15	30	9	35.0

[a] Abbreviations: E_d, enzyme dosage (wt %, based on the amount of substrates); T_e, reaction temperature (°C); S_a, solvent amount (mL of hexane); T_i, reaction time (h); S_r, substrate ratio (caprylic acid/PC, mol/mol).

Acidolysis. Reactions between soybean PC and caprylic acid were carried out using a 3 g reaction mixture in varying amounts of hexane in a brown flask with a tight screw cap. Reactions were conducted in a water bath with magnetic stirring at 300 rpm, and reaction was started by the addition of lipase (wt % based on total substrates). After reaction the samples were centrifuged at 4000 rpm for 5 min. All samples were stored at −20 °C prior to analysis.

Experimental Design. A fractional factorial design based on the principle of RSM was used in this work with the assistance of the commercial software Modde 6.0 (Umetri, Umeå, Sweden). The five factors chosen were enzyme dosage (E_d, wt % based on substrates), reaction temperature (T_e, °C), solvent amount (S_a, mL of hexane), reaction time, (T_i, h), and substrate ratio (S_r, caprylic acid/PC, mol/mol). The variables and their levels are presented in **Table 1**. The mole percent of ML-type PC of the total PC was used as the response.

Analysis of the Phospholipid Profile by TLC-FID. A 1 μL aliquot of diluted sample was spotted onto Chromarod SIII (Iatron Laboratories Inc., Tokyo, Japan), which was developed in a mixture of chloroform/methanol/water (42:22:3, v/v/v). After the development, the Chromarods were dried at 120 °C for 5 min, and phospholipid species PC, LPC, and GPC were analyzed by TLC-FID (Iatroscan MK6s, Iatron Laboratories, Tokyo, Japan). Flow rates of 2 L/min and 160 mL/min were used during the analysis for air and hydrogen, respectively. Peaks were identified by external standards.

Plate Thin-Layer Chromatography. Analytical separations were also performed on silica gel 60 thin-layer plates (20 cm × 20 cm; Merck, Darmstadt, Germany). Double determinations were performed for each sample. After development in chloroform−methanol−water (65:35:5, v/v), the plate was sprayed with 0.2% 2,7-dichloroflourescein in ethanol (96%), making the lipid bands visible under UV light. The

following bands were observed: LPC of the M type ($R_f = 0.07$), LPC of the L type ($R_f = 0.15$), PC of the MM type ($R_f = 0.24$), PC of the ML type ($R_f = 0.30$), and PC of the LL type ($R_f = 0.35$), where L refers to long-chain fatty acids and M refers to medium-chain fatty acids (caprylic acid), and fatty acids ($R_f = 0.78$). The lipid bands were scraped off, methylated, and analyzed by GC.

Methylation of Phospholipid Species. The scrapings from TLC were transferred to test tubes with tight screw caps. A 1 mL sample of 0.5 M NaOH in methanol was added to each tube, and the tubes were kept at 80 °C for 5 min. Then 1 mL of 20% BF_3 in methanol and 0.5 mL of 0.5% hydroquinone in methanol were added, and the tubes were kept at 80 °C for 2 min. A 2 mL sample of 0.73% NaCl solution was added and subsequently 1 mL of heptane. The upper phase was transferred to a new tube. A 1 mL sample of a saturated salt solution was added to the new tube, and the upper phase was taken for GC analysis.

GC Analysis of the Fatty Acid Composition. The methyl esters were analyzed on an HP6890 series gas−liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with an FID, as described elsewhere (*1*).

Statistical Analysis. Data were analyzed by means of response surface methodology using the commercial software Modde 6.0 from Umetri (Umeå, Sweden). Responses were fitted to the factors by multiple regression, and the fit of the model was evaluated by the coefficient of determination (R^2) and analysis of variance (ANOVA). R^2 above 0.8 indicates that the model has acceptable qualities. The significance of the results was established at $P \leq 0.05$. The response surface model was fitted to the following equation:

$$Y = \beta_o + \sum_{i=1}^{5}\beta_i X_i + \sum_{i=1}^{5}\beta_{ii} X_i^2 + \sum_{i=1}^{4}\sum_{j=i+1}^{5}\beta_{ij} X_i X_j \qquad (1)$$

where Y is the response variable of the sample (ML-type PC content), X_i the ith independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadric coefficient for variable i, and β_{ij} the model coefficient for the interaction between factors i and j. The insignificant coefficients were eliminated after the coefficients were examined, and the model was finally refined. For process factors the main effect plot displays the predicted changes in the response when the factor varies from its low to its high level, all other factors in the design being set at their averages.

RESULTS AND DISCUSSION

Acidolysis Reaction Between Soybean PC and Caprylic Acid. The 1,3-specific lipase was used for synthesis of PC with medium-chain fatty acids at the sn-1 position by acidolysis between soybean PC and caprylic acid. TLC-FID analysis of the acidolysis product (**Figure 1**) illustrates how the PC composition changed on Chromarods for a sample taken at different reaction times. Two to three peaks were observed on the chromatograms for samples taken during acidolysis reaction. The peaks might represent PC of the LL, ML, and MM types. A mixture of 1,2-octanoyl-PC (MM-type PC) and soybean PC (LL-type PC) was spotted on Chromarods, and it was observed that they were separated into two separate peaks, suggesting that the retention value of PC on TLC depends on the fatty acid composition (**Figure 2**).

To verify that the peaks observed represented PC of the LL, ML, and MM types, the sample taken at 72 h (reaction conditions described in **Figure 1**) were separated by plate TLC. Similarly, PC was observed to split into three bands. The fatty acid composition of each PC band was measured, after conversion to methyl esters (**Table 2**). These data confirm that the three bands represent PC of the LL type, ML type, and MM type, since the first band contains practically no caprylic acid and the second and third bands contain approximately 50% and 100% caprylic acid, respectively.

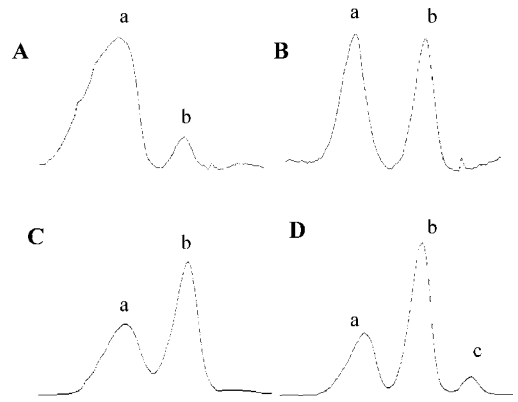

Figure 1. Separation of acidolysis products using TLC-FID. Observed changes in PC during lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid at different reaction times. Reaction conditions: enzyme dosage (E_d), 30%; substrate ratio (S_r), 6 mol/mol; solvent amount (S_a), 20 mL of hexane; reaction temperature (T_e), 45 °C. Key: (**A**) 6 h, (**B**) 24 h, (**C**) 50 h, (**D**) 72 h. Peaks a, b, and c represent LL-type PC, ML-type PC, and MM-type PC.

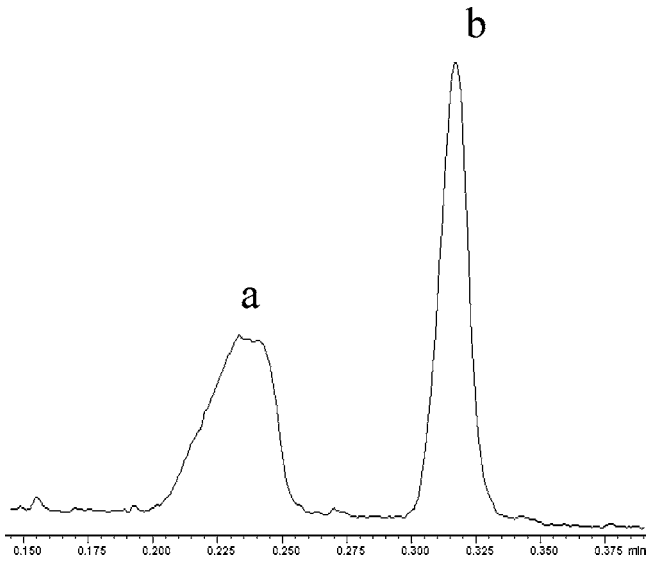

Figure 2. Separation of soybean PC and 1,2-dioctanoyl-PC. Peaks a and b represent soybean PC and 1,2-dioctanoyl-PC.

Table 2. Fatty Acid Distribution (mol %) in Structured Phosphatidylcholine for a Sample Taken at 72 h[a] Measured by GC

fatty acid	band 1	band 2	band 3	$[PC]_{total}$[b]
C8:0	1.3	49.0	92.1	38.7
C16:0	9.7	2.7	1.7	4.8
C18:0	4.8	0.6	0.8	1.8
C18:1	11.1	5.8	1.3	6.9
C18:2	66.5	38.3	3.6	43.5
C18:3	6.6	3.7	0.5	4.3

[a] For reaction conditions see **Figure 1**. [b] Mole percent of caprylic acid incorporated into PC when all PC bands were methylated together (bands 1–3).

The content of ML-type PC increased with reaction time, whereas that of LL-type PC decreased as expected. However, after 72 h MM-type PC was produced (**Figure 1D**). This is an undesirable byproduct formed during acidolysis reaction. Acyl migration is a serious problem with these types of reactions, leading to lower yields and formation of byproducts as illustrated in **Figure 3** (*2*, *11*). Acyl migration is a problem often

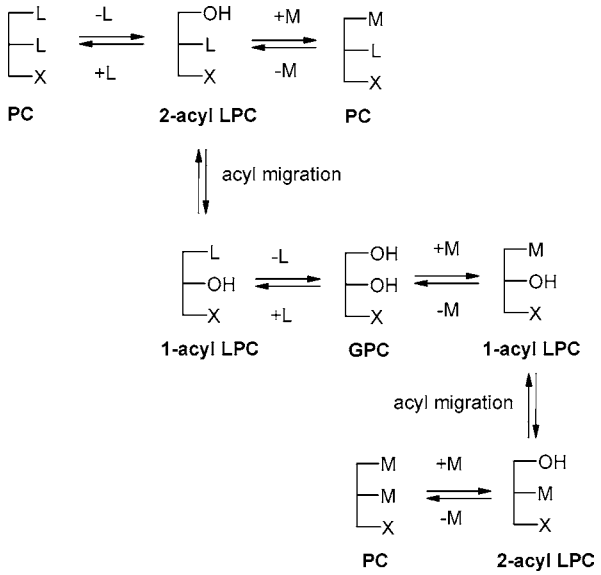

Figure 3. Diagram of the reaction and principle of the lipase-catalyzed acidolysis and side reactions for the production of specific structured PC (L, long-chain fatty acids; M, medium-chain fatty acids).

encountered in selective synthesis of regiospecific glycerophospholipids, i.e., intramolecular transfer of one fatty acid moiety from one hydroxyl group to an adjacent one. In the intermediate LPC, there is a free hydroxyl group, making the reaction possible. 2-Acyl-LPC is less stable than 1-acyl-LPC and converts into the more stable 1-acyl-LPC by acyl migration. Even though an sn-1,3-specific lipase is used for the production of the structured PC, caprylic acid on both positions may therefore occur. In addition, it was observed from GC analysis that acidolysis products contain LPC with caprylic acid incorporated, which further illustrates that acyl migration is taking place (data not shown). Acyl migration cannot be simply avoided in applied systems. Many factors possibly influence acyl migration. Often balancing acyl incorporation and migration is necessary to have optimal conditions since an important parameter for acyl incorporation may result in an increase in acyl migration as well.

Calibration. Calibration curves were prepared for soybean PC (LL-type PC) and 1,2-dioctanoyl-PC (MM-type PC). The response of the PC compounds was shown to depend very much on the fatty acid composition and concentration. The signal from the FID usually corresponds to the mass of each component. However, at a concentration below 2 mg/mL the responses of LL-type PC and MM-type PC were significantly different. When the concentrations were calculated into molar concentrations instead, the response was shown to be very similar for the two PC types. Two-way ANOVA showed that there was no significant difference in response between the soybean PC and 1,2-octanoyl-PC. The results illustrate that at low concentrations the signal from the FID does not follow the mass of the phospholipid components. The relationship between the peak area and the concentration of PC is shown in **Figure 4**. Calibration curves for these types of analysis are known to be nonlinear, and are usually represented by a power law equation, $y = ax^b$ (*16*).

Since the LL-type PC and MM-type PC had similar response factors based on molar concentration, it is expected that ML-type PC will as well. Therefore, the calibration curve would be suited for LL-, MM-, and ML-type PC.

From the analysis conducted by TLC-FID the distribution between the PC species was known, making it possible to

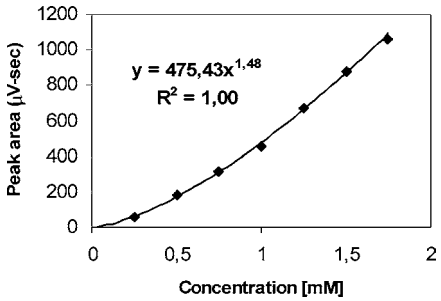

Figure 4. Standard calibration curve for PC. Lines were fitted to a power law equation. Calibration is based on soybean PC ($n = 3$) and 1,2-dioctanoyl-PC ($n = 3$).

Table 3. Multiple Linear Regression Coefficients Describing the Influence of Different Parameters on the Formation of ML-Type PC[a]

	ML-type PC formation			ML-type PC formation	
	regression coefficient	P[b]		regression coefficient	P[b]
constant	31.40	3.64×10^{-18}	S_a	7.15	2.43×10^{-5}
E_d	10.49	7.81×10^{-8}	T_i	6.59	6.78×10^{-5}
T_e	5.54	4.61×10^{-4}			

[a] For abbreviations see **Table 1** and the text. [b] The 95% confidence limit on each regression coefficient was ±2.81 (±2.60 for the constant).

calculate the overall incorporation of caprylic acid into the product by the following equation:

$$\text{Inc (mol \%)} = 0.5[[\text{LM}]\ (\text{mol \%})] + [\text{MM}]\ (\text{mol \%}) \quad (2)$$

where LM = LM-type PC and MM = MM-type PC. By applying eq 2, the incorporation of caprylic acid into PC for the 72 h sample (see **Figure 1** for details) was calculated as 38%. From GC analysis the same result was obtained when all PC bands from the TLC plate were methylated together (**Table 2**).

The TLC-FID method was applied in the present study to examine the influence of enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio on the formation of ML-type PC (mol %) during acidolysis reaction between soybean PC and caprylic acid with *T. lanuginosa* lipase as catalyst. RSM was used for evaluating the relationships of the parameters and predicting the results and behavior under the given reaction conditions.

Model Fitting. A central composite rotatable design was selected with five factors: enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio. **Table 1** lists experimental parameter settings and the results based on the experimental design, which were obtained by the method developed above. The best model was determined by multiple regression and backward elimination. According to the model generated, ML-type PC formation was only affected by first-order variables. The model coefficients and *P* values for the regression variables are given in **Table 3**. All *P* values of the coefficient were below 0.05 after the model was refined. The coefficient of determination (R^2) of the model was 0.85 ($Q^2 = 0.77$). The observed and predicted values were sufficiently correlated as can be seen in **Figure 5**, except for no. 26, which was treated as an outlier. According to ANOVA, there was no lack of fit. This indicates that the model represents the actual relationship of the reaction parameters well within the ranges selected.

Main Effects of the Parameters. The effects of the parameters can be evaluated by the plots of the main effects

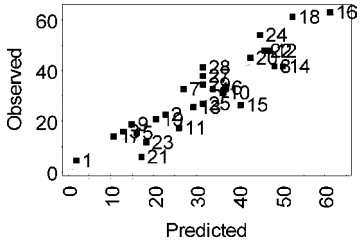

Figure 5. Relationship between the observed results and the data predicted by the models. The numbers inside the graph represent the experimental numbers. The solid line was obtained by regression.

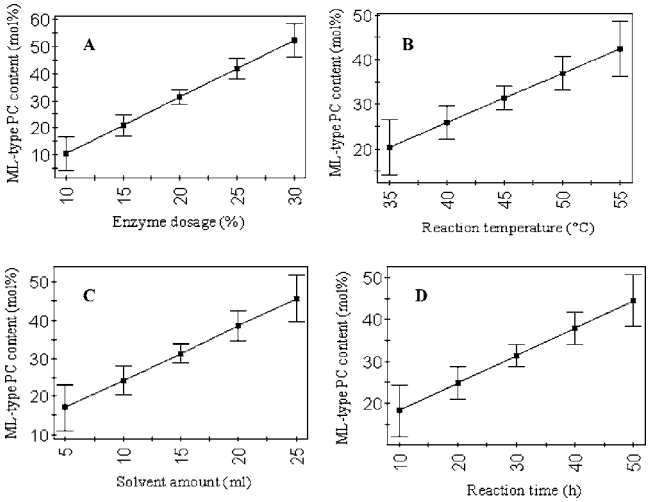

Figure 6. Main effects of the parameters on the ML-type PC content during Lipozyme TL IM-catalyzed acidolysis reaction between PC and caprylic acid: (**A**) enzyme dosage, (**B**) reaction temperature, (**C**) solvent amount, (**D**) reaction time.

(**Figure 6**). All parameters selected for the study except for the substrate ratio had a positive effect on the formation of ML-type PC. The enzyme dosage had the most significant effect followed by the solvent amount, reaction time, and reaction temperature. The substrate ratio showed no significant effect on the formation of ML-type PC within the tested range. Even higher settings for the other factors may increase ML-type PC formation since the studied effects increase over the entire range of values studied. According to the model the parameters should be on a high level to obtain the highest degree of conversion.

Typical contour plots between different parameters were generated as **Figure 7** for the ML-type PC content (mol %). All the plots in Figure 7 gave similar relationships with respect to the effects of the parameters. The higher the enzyme dosage, reaction temperature, solvent amount, and reaction time, the higher the incorporation obtained. These results are in agreement with the conclusions for the evaluation of the main effects. The generated model should be used with precaution since in certain cases MM-type PC is produced due to acyl migration. In the experimental design only in the sample from experiment 16 MM-type PC was detected. This sample also had the highest formation of ML-type PC.

It should be kept in mind that the yield (recovery) of the total PC is also important for the reaction performance. The incorporation and the recovery of PC were examined for the reaction mixture with all parameters on a high level. The results show that with an increase in ML-type PC formation a decrease in the recovery of PC was observed (**Figure 8**). An explanation for the loss of product is the formation of byproducts with low

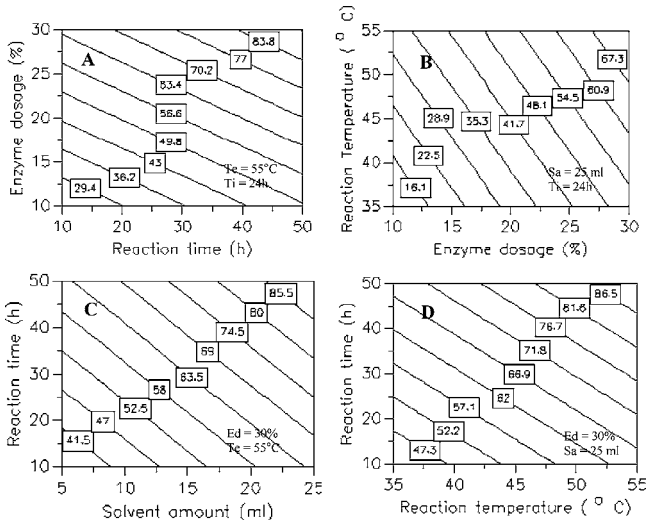

Figure 7. Contour plots of the ML-type PC content during Lipozyme TL IM-catalyzed acidolysis reaction between soybean PC and caprylic acid. The numbers inside the contour plots indicate the ML-type PC content (mol %). Key: (**A**) enzyme dosage vs solvent amount, (**B**) enzyme dosage vs reaction temperature, (**C**) solvent amount vs reaction time, (**D**) reaction temperature vs reaction time. For abbreviations see **Table 1**.

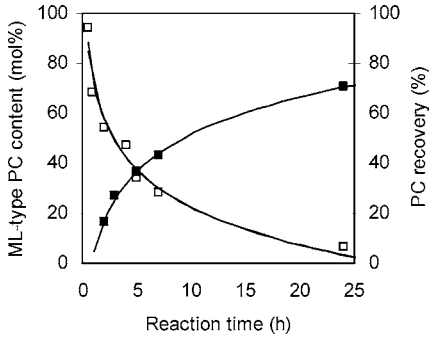

Figure 8. Time course for acidolysis reaction between PC and caprylic acid in hexane. Reaction conditions: enzyme dosage (E_d), 30%; substrate ratio (S_r), 15 mol/mol; solvent amount (S_a), 25 mL; reaction temperature, 55 °C. Key: (■) ML-type PC content (mol %), (□) PC recovery (%).

solubility in hexane, which are lost during removal of the enzyme. This was confirmed by extraction of the immobilized enzyme after the reaction with methanol−chloroform (50:50, v/v) and further analysis, which revealed that large amounts of GPC (totally deacylated PC) were produced. With all parameters on high levels during the acidolysis reaction, MM-type PC was not observed probably due to the rapid hydrolysis to GPC. According to the model having a reaction time of 48 h with other parameters on a high level, 90% of the PC would be of the ML type. However, with these conditions no PC could be observed in the reaction mixture. With a reaction time of 24 h the ML-type content should be 74% according to the generated model, which agrees well with the experimental value. The overall yield was however very low.

Optimal conditions for incorporation of novel fatty acids should be compromised with the consideration of recovery. Readers should thus make their own decisions concerning whether to have a high purity of ML-type PC or a compromise between the ML-type PC purity and recovery of PC.

In conclusion, the TLC-FID method developed has been shown to be suitable for analysis of enzymatic reactions for synthesis of structured phospholipids with mixtures of long- and

medium-chain fatty acids, since it is possible to follow the formation of both products and byproducts. The method was successfully used for the evaluation of reaction conditions assisted by RSM experimental design. The response model developed in this study satisfactorily expressed the formation of ML-type PC with regard to the selected parameters in the batch system. MM-type PC, an undesirable byproduct, is also formed during the lipase-catalyzed acidolysis reaction, due to acyl migration as seen from the developed method.

ABBREVIATIONS USED

FID, flame ionization detector; GC, gas chromatography; GPC, glycerophosphorylcholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; RSM, response surface methodology; TLC, thin-layer chromatography.

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