Elucidation of Acyl Migration During Lipase-Catalyzed Production of Structured Phospholipids

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ABSTRACT: Elucidation of acyl migration was carried out in the Lipozyme RM IM (*Rhizomucor miehei*)-catalyzed transesterification between soybean phosphatidylcholine (PC) and caprylic acid in solvent-free media. A five-factor response surface design was used to evaluate the influence of five major factors and their relationships. The five factors—enzyme dosage, reaction temperature, water addition, reaction time, and substrate ratio—were varied on three levels together with two star points. Enzyme dosage, reaction temperature, and reaction time showed increased effect on the acyl migration into the *sn*-2 position of PC, whereas increased water addition and substrate ratio had no significant effect in the ranges tested. The best-fitting quadratic response surface model was determined by regression and backward elimination. The coefficient of determination (R^2) was 0.84, which indicates that the fitted quadratic model has acceptable qualities in expressing acyl migration for the enzymatic transesterification. Correlation was observed between acyl donor in the *sn*-2 position of PC and incorporation of acyl donor into the intermediate lysophosphatidylcholine. Furthermore, acyl migration into the *sn*-2 position of PC was confirmed by TLC-FID, as PC with caprylic acid was observed on both positions. Under certain conditions, up to 18% incorporation could be observed in the *sn*-2 position during the lipase-catalyzed transesterification.

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KEY WORDS: Acyl migration, lipase-catalyzed acidolysis reaction, response surface methodology, Rhizomucor miehei, solvent-free system, structured phospholipids.

Production of structured phospholipids (PL) by lipase-catalyzed transesterification has attracted increased attention during the last couple of decades, and several publications have recently appeared on the subject (1–4). Unfortunately, many of these publications do not consider the problem of acyl migration, which in a practical reaction system cannot be simply avoided. No comprehensive reports have to our knowledge been published on acyl migration into the *sn*-2 position of PL during the lipase-catalyzed production of structured PL. Most commonly, the overall incorporation of novel FA has been measured, which gives no information about the location in the PL molecule. It is generally agreed that *sn*-1,3-specific lipases are specific for the *sn*-1 position of PL. However, during the transesterification reaction between PC and acyl donor, the formation of glycerophosphorylcholine (GPC) and the presence of the acyl donor in lysophosphatidylcholine (LPC) are usually observed, which are consequences of migration of the acyl group from the *sn*-2 position to the *sn*-1 position (5). The main reason for acyl migration is the existence of LPC, which is the intermediate produced during the lipase-catalyzed transesterification. The mechanism proposed for the acyl migration suggests that LPC goes through a cyclic ortho ester intermediate (6). Acyl migration from the *sn*-2 position to the *sn*-1 position or vice versa continues until a dynamic balance is reached.

Plückthun and Dennis (6) investigated acyl migration in LPC and reported that about 90% of thermodynamically stable 1-acyl LPC and 10% 2-acyl LPC were present in mixture at equilibrium under the conditions of their investigation. During the lipase-catalyzed acidolysis reaction between PC and EPA under solvent-free conditions, 85% of the FA in LPC were on the *sn*-1 position at equilibrium (5). Similar findings were reported for lipase-catalyzed acidolysis between PC and oleic acid where 90% of the FA were found on the *sn*-1 position in LPC at equilibrium (7).

Several factors possibly influence acyl migration. Increasing solvent polarity or addition of water to nonpolar solvents has been reported to cause lower rates of acyl migration (8). To prevent losses due to acyl migration, the acidolysis reaction should be carried out at high water activity (8). Increased water content in the reaction system, however, influences LPC formation and thus results in lower yields. In many cases excessive amounts of acyl donor have been applied to push the main reaction toward product formation. However, with increased substrate ratios, there would be a higher content of FFA, which have been reported to cause acyl migration on partial acylglycerols (8). Reaction temperature also influences the equilibrium of acyl migration. Higher temperatures decrease the overall incorporation of acyl donor into PC in a solvent-free system, but not into LPC (9). This indicates that the reaction rate for the acidolysis reaction becomes slower than acyl migration rates at elevated reaction temperatures. Certain supports for the immobilization of enzymes may cause increased acyl migration in the reaction system as well (10).

Response surface methodology (RSM) enables the evaluation of effects of multiple parameters, alone or in combination, on response variables. The objective of this study was to examine the relationship between five factors (enzyme dosage, reaction temperature, water addition, reaction time, and substrate ratio) and their effects on acyl migration into the *sn*-2 position of PC during lipase-catalyzed acidolysis. These five factors have been shown previously to have an effect on either the

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overall incorporation of acyl donor or the recovery of PC during acidolysis reaction (9).

MATERIALS AND METHODS

Materials. Soybean PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The FA composition of the PC (mol%) was 16:0 (12.8%), 18:0 (3.9%), 18:1 (9.4%), 18:2 (65.8%), and18:3 (8.1%). Caprylic acid (purity 97%) was from Riedel-de-Haen (Seelze, Germany). *sn*-1,3-Specific lipase from *Rhizomucor miehei* immobilized on macroporous ion resin (Lipozyme RM IM) and phospholipase A_1 (PLA_1) from *Fusarium oxysporum* (Lecitase Novo) were donated by Novozymes A/S (Bagsværd, Denmark). All solvents and reagents for analyses were of analytical grade.

Experimental design. A three-level with two star points and partial five-factor fractional factorial design according to the principle of RSM was used in this study. The five factors and their levels were enzyme dosage (e_d, 10–50 wt% based on substrate), reaction temperature (t_e, 40–60°C), water addition (w_a, 0–4 wt% based on substrate), reaction time (t_i, 10–90 h), and substrate ratio (s_r, 3–15 mol/mol caprylic acid/PC). The design generated 29 experimental settings as determined by the use of the software Modde 6.0 (Umetri, Umeå, Sweden) (Table 1).

Transesterification. The transesterification (acidolysis) between soybean PC and caprylic acid was carried out in a system previously described (9). PC and caprylic acid (10 g reaction mixture) were mixed in a brown flask with tight screw cap. Reactions were started by the addition of lipase (wt% based on total substrate). Reactions were conducted in a water bath with magnetic stirring at 300 rpm. After reaction, the samples were centrifuged at $2879 \times g$ for 5 min, and the supernatants were collected. All samples were stored at −20°C prior to analysis.

TLC. Analytical separations of PC, LPC, and FFA were performed on Silica Gel 60 thin-layer plates (20 × 20 cm; Merck, Darmstadt, Germany). The solvent system used for the separations consisted of: chloroform/methanol/water (65:35:5, by vol). Lipid bands on TLC plates were visualized by spraying with 0.2% 2,7-dichlorofluorescein in ethanol. Lipid bands were scraped off and methylated for FA analysis.

FA position analysis of PC. Caprylic acid-enriched PC was separated from LPC and FFA on Silica Gel 60 thin-layer plates as described above. PC was extracted from the silica gel with 4 × 10 mL chloroform/methanol/water (65:35:5, by vol). After drying in a rotary evaporator, the PC was hydrolyzed to LPC with Lecitase Novo to remove the FA in the *sn*-1 position. A 2.5 mg portion of PC was dissolved in diethyl ether (2 mL) and incubated with 10 μL Lecitase Novo dissolved in 100 μL water. After shaking vigorously for 5 min, solvent was evaporated under nitrogen. Hydrolyzed PC was redissolved in chloroform and applied to the TLC plate for separation. The LPC band was scraped off and methylated for GC analysis.

Methylation and GC analysis. Methylation and GC analysis were performed on PC, LPC, and the PLA_1-catalyzed hydrolysis product of PC. Methyl esters were prepared by treating scrapings from TLC with 0.5 M NaOH in methanol, followed by 20% boron trifluoride treatment, and analyzed by HP6890 series GC with FID (Hewlett-Packard) using a fused-silica capillary column (SUPELCOWAX, 60 m × 0.25 mm i.d., 0.20 mm film thickness; Supelco Inc., Bellefonte, PA) as described before (9).

Analysis of phospholipid profile by TLC–FID. Diluted sample (1 μL) was spotted to Chromarod SIII (Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (42:22:3 by vol). After the development, Chromarods were dried at 120°C for 5 min, and PL species were analyzed by TLC–FID (Iatroscan MK6s: Iatron Laboratories). Flow rates of 2 L/min and 160 mL/min were used during analysis for air and hydrogen, respectively. Peaks were identified by external standards. With TLC–FID, it is possible to monitor the replacement of long (L)-chain FA with medium (M)-chain FA during the lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid as previously described (11). PC can split into three peaks: the LL-type, the ML-type (LM-type), and the MM-type. Overall incorporation of caprylic acid into PC can thus be calculated by Equation 1:

$$\text{Inc (mol\%)} = 0.5\ \{[\text{ML}](\text{mol\%})\} + \{[\text{MM}](\text{mol\%})\} \qquad [1]$$

Statistical analysis. Data were analyzed by means of RSM with Modde 6.0. Second-order coefficients were generated by regression analysis with backward elimination. Responses were fitted for the factors by multiple regression. The fit of the model was evaluated by the coefficients of determination (R^2) and ANOVA. Insignificant coefficients ($P > 0.05$) were eliminated after examining the coefficients, and the model was finally refined. Linear regression analysis was performed with assistance of Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA). All samples were analyzed in duplicate, and mean values are reported.

RESULTS AND DISCUSSION

Phospholipase hydrolysis of PL. Determination of positional distribution of FA in PL is usually done by enzymatic hydrolysis (12). Different enzymes have been suggested in the literature for specific acyl hydrolysis of PC. Phospholipase A_2 from snake venoms and porcine pancreases has been used to hydrolyze the ester bond in the *sn*-2 position of PL, releasing the FA in this position (10,12). The FFA products from the *sn*- 2 position and the LPC-containing FA in the *sn*-1 position can be isolated for analysis so the distribution of FA in both positions of the glycerol moiety is determined. Alternatively, enzymes specific for the *sn*-1 position can be used to hydrolyze FA in the *sn*-1 position, and hydrolysis products can be examined in a similar way. Recently Vijeeta *et al.* (13) proposed a method for determining positional distribution of PC by using phospholipase A_1 (Lecitase Novo). Hydrolysis reactions are performed over 6 h in tertiary alcohol. Acyl migration rates are usually lower in alcoholic solutions compared with nonpolar organic solvents (8); however, the reaction time is considered

TABLE 1
Set Factor Levels and Observed Responses in Response Surface Methodology Experiments for Acyl Migration into *sn*-2 Position of PC During Lipase Catalyzed Acidolysis Reactions Between Soybean PC and Caprylic Acid

	Factors[a]					Migration to
Exp. no.	e_d	t_e	w_a	t_i	s_r	*sn*-2 position (%)
1	20	45	1	30	12	3.8
2	40	45	1	30	6	6.0
3	20	55	1	30	6	3.8
4	40	55	1	30	12	8.2
5	20	45	3	30	6	4.6
6	40	45	3	30	12	4.3
7	20	55	3	30	12	2.0
8	40	55	3	30	6	11.9
9	20	45	1	70	6	14.0
10	40	45	1	70	12	12.5
11	20	55	1	70	12	8.8
12	40	55	1	70	6	18.0
13	20	45	3	70	12	12.2
14	40	45	3	70	6	5.9
15	20	55	3	70	6	9.9
16	40	55	3	70	12	12.9
17	10	50	2	50	9	3.7
18	50	50	2	50	9	9.4
19	30	40	2	50	9	5.1
20	30	60	2	50	9	12.2
21	30	50	0	50	9	12.3
22	30	50	4	50	9	5.9
23	30	50	2	10	9	0.9
24	30	50	2	90	9	14.5
25	30	50	2	50	3	6.0
26	30	50	2	50	15	8.8
27	30	50	2	50	9	13.3
28	30	50	2	50	9	16.1
29	30	50	2	50	9	16.9

[a]Abbreviations: e_d, enzyme dosage (wt% based on substrate); t_e, reaction temperature (°C); w_a, water addition (wt% based on total substrate); t_i, reaction time (h); s_r, substrate ratio (mol/mol caprylic acid/PC).

very long from a practical point of view. The 2-acyl LPC formed is a thermodynamically unstable molecule that, over time, will convert to the more stable 1-acyl LPC, which can be further hydrolyzed to GPC by the lipase. We previously examined the regioselectivity of the Lipozyme RM IM-catalyzed incorporation of caprylic acid into PC (9). Structured PC was hydrolyzed with PLA_1 and snake venom to verify the FA composition in the *sn*-1 and *sn*-2 positions, respectively. The accuracy of the hydrolysis procedures were checked by summing the results for the concentration of each FA in *sn*-1 and *sn*-2 positions, dividing by two, and comparing this quantity with the analysis for each component in the original PC. These two results agreed well, showing that snake venom and PLA_1 are suitable for determining the positional distribution of structured PC containing a mixture of long- and medium-chain FA.

Acyl migration into the sn-2 position of PC. The effect of different parameters on the overall incorporation and distribution of PL has been examined during the lipase-catalyzed acidolysis reaction (9). Several compromises have to be made in order to have high product yield since parameters favoring acyl incorporation also result in increased by-product formation in

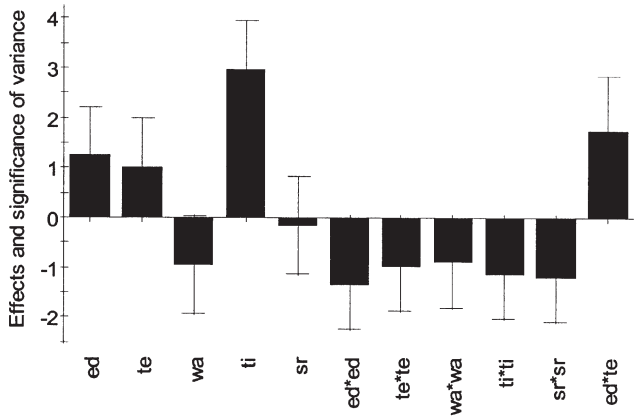

FIG. 1. Effect and significance plot of parameters and interactions on acyl migration into *sn*-2 position of PC during lipase-catalyzed acidolysis reaction between PC and caprylic acid. Abbreviations: e_d, enzyme dosage (wt% based on substrate); t_e, reaction temperature (°C); w_a, water addition (wt% based on total substrate); t_i, reaction time (h); s_r, substrate ratio (mol/mol caprylic acid/PC).

the reaction system. Under optimal conditions (based on overall incorporation), 20% of the FA were found in the *sn*-2 position. Owing to the complexity of the acidolysis reaction it is difficult to predict the influence of different parameters on the acyl migration into the *sn*-2 position of PC. A statistical experimental design was therefore set up with the assistance of RSM to evaluate the influence of the individual parameters mentioned above, as well as their interactions, on acyl migration into the *sn*-2 position. The practical experimental settings are given in Table 1 including responses from the experiments. The structured PC produced were hydrolyzed with PLA_1 so as to have a direct measure of the migration into the *sn*-2 position.

Partial least-squares regression was used to fit the responses. Insignificant variables were refined in steps of backward elimination. The coefficient of determination was 0.84 for acyl migration into the *sn*-2 position of PC, and according to the ANOVA there was no lack of fit. The effect of each parameter can be seen from the plot of the main effects (Fig. 1). The

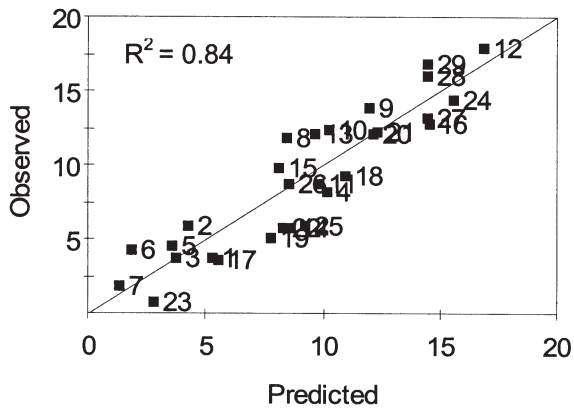

FIG. 2. Linear relationship between observed responses and those predicted for acyl migration into *sn*-2 position of PC. Numbers in figure are experimental setting number.

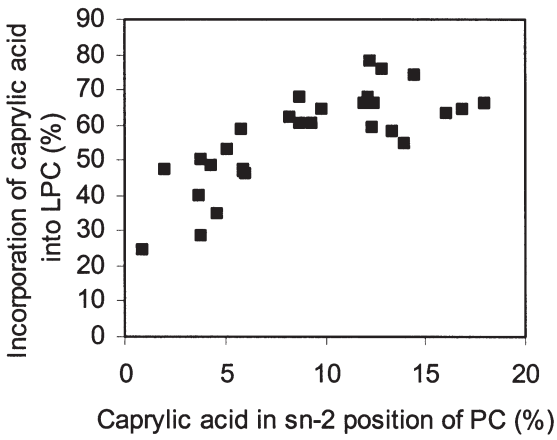

FIG. 3. Correlation between the caprylic acid in the *sn*-2 position of PC and the incorporation of caprylic acid into lysophosphatidylcholine (LPC).

model for the migration to the *sn*-2 position was generally satisfactory for the evaluation of such a system, as the observed and predicted results were well correlated (Fig. 2). According to regression analysis the relationship between observed and predicted results was significant ($P < 0.01$).

The reaction time was the most significant factor in the acyl migration into the *sn*-2 position of PC. Increased reaction time also resulted in an overall higher incorporation, and acyl migration therefore seems difficult to avoid in the present reaction system. Other parameters having an effect on the acyl migration were enzyme dosage and reaction temperature. Water addition and substrate ratio had no individual effect on the acyl migration. The acyl migration rate of LPC has previously been shown to decrease in toluene solution in the presence of increased amounts of water (8). Svensson *et al.* (10) observed the incorporation of novel FA into LPC at low water activity. At higher water activity, incorporation into the LPC was very low. The highest incorporation into LPC was reported at 5% water addition (5). Higher water content resulted in lower incorporation into LPC, indicating lower acyl migration. We previously observed that the overall incorporation into PC and LPC was not influenced by the water content (1,9). With increased water in the system, the hydrolysis rate increases and GPC becomes a major reaction product. In considering the yield, a high water content in the system cannot be recommended.

Acids can catalyze acyl migration in LPC. Adlercreutz (8) examined the dependence of acyl migration from the *sn*-2 to the *sn*-1 position in LPC on the FA concentration in toluene. The acyl migration rate was observed to increase with increasing FA concentration. The highest rate was observed when LPC was dissolved directly in the FA in the absence of solvent. In the current study no significant difference in acyl migration into the *sn*-2 position was seen within the range of substrate ratios tested. An increased substrate ratio decreased the amount of LPC in the reaction mixture during acidolysis in a solvent-free system, but incorporation into PC also was decreased (9).

A higher conversion degree can usually be obtained by increasing the temperature in the solvent system (14). However, in a solvent-free system it was more beneficial to operate at low temperatures (9). Not only the yield decreased with increased temperature, but also the overall incorporation into PC. Temperature also was reported to have an effect on the acyl migration (8), as confirmed in the current study. With increase in temperature, the acyl migration in the reaction system increases. Enzyme dosage also had a significant effect on acyl migration into the *sn*-2 position. The anion exchange resin used for immobilization of the *R. miehei* lipase has been reported to cause the acyl migration (10). Acyl migration was also observed with the commercially available *Thermomyces lanuginosa* lipase immobilized on hydrophilic silica granules (11). For *Rhizopus oryzae* lipase immobilized on polypropylene, no acyl migration was observed into the *sn*-2 position of PC during PL transesterification (10); however this lipase cannot be obtained commercially in the immobilized form.

Some interaction was observed between the enzyme dosage and reaction temperature. High enzyme dosage together with high temperature resulted in increased acyl migration.

A correlation between incorporation into the *sn*-2 position of PC and incorporation into LPC was set up with varying parameters (Fig. 3). As expected, acyl migration into the *sn*-2 position seemed to increase with increased incorporation into LPC. When caprylic acid is incorporated into LPC, most FA will be in the *sn*-1 position; however, some will migrate to the *sn*-2 position until some balance is reached. With migration to the *sn*-2 position, the lipase has opportunity to incorporate caprylic acid into the *sn*-1 position, resulting in PC with caprylic acid on both positions. Haraldsson and Thorarensen (5) reported that the maximal incorporation into LPC was 70%. Even higher incorporation into LPC can be seen, depending on the reaction conditions (9). As for acyl migration to the *sn*-2 position of PC, the reaction time was the factor having the most significant effect on the incorporation of acyl donor into LPC (9).

PC molecular distribution. With TLC-FID, the distribution of different FA species in individual PC molecules can be followed. A typical chromatogram for PC molecular distribution is depicted in Figure 4.

The incorporation of caprylic acid into PC was calculated based on the distribution of individual PC species and compared with results obtained by GC (Fig. 5). The two different ways of analysis correlated fairly well ($R^2 = 0.82$). According to regression analysis, the intercept does not equal zero ($P < 0.01$). This indicates that incorporation of caprylic acid into PC should be above a certain level in order to be detected by TLC-FID. The *P*-value for slope was less than 0.01 showing that there is a significant relationship between the two data sets. Incorporation of caprylic acid into PC (determined by GC) was also correlated with the PC molecular distribution (Fig. 6). LL-type PC was observed to decrease with increase of incorporation of caprylic acid, primarily with formation of ML-type PC. However MM-type PC was also observed under certain reaction conditions, confirming acyl migration to the *sn*-2 position. In Table 1, experiment no. 12 resulted in the largest migration to the *sn*-2 position. TLC–FID analysis showed that the PC dis-

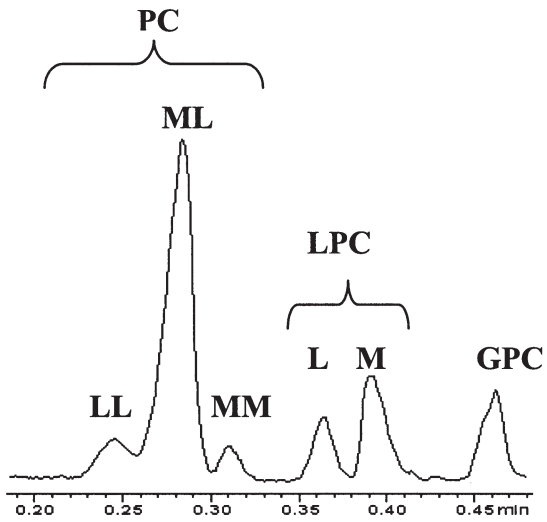

FIG. 4. TLC–FID chromatogram of structured PC. Structured PC produced with conditions given for Experiment 12 (enzyme dosage, 40%; reaction temperature, 55°C; water addition, 1%; reaction time, 70 h; substrate ratio, 6 mol/mol caprylic acid/PC). Abbreviations: L, long-chain FA; M, medium-chain FA. For other abbreviation see figure 3.

tribution of this product was 75% ML-type PC, 16% LL-type PC, and 9% MM-type PC. Combined, the GC and TLC results show that the majority of the caprylic acid is incorporated into the *sn*-1 position of PC during the lipase-catalyzed acidolysis reaction with the ML-type as the major product.

Acyl migration has been demonstrated to be a serious problem during lipase-catalyzed acyl exchange of PL. Lipase-catalyzed acyl exchanges of PL are normally conducted over several days; however, the present study has shown that reaction time is the factor having the most significant effect on acyl migration into the *sn*-2 position of PC. To increase the conversion rate, a higher enzyme load may be used; however, for the current reaction system this is not advisable as the enzyme concentration already is very large. With higher enzyme loads, mixing will be extremely difficult. Alternatively the reactions could be conducted in packed bed reactors. Packed bed reactors were demonstrated to be advantageous over stirred tank reactors during lipase-catalyzed production of structured lipid, since the former had a much lower level of acyl migration (15). For optimal conditions it is recommended that temperature, substrate ratio, and water addition should be low.

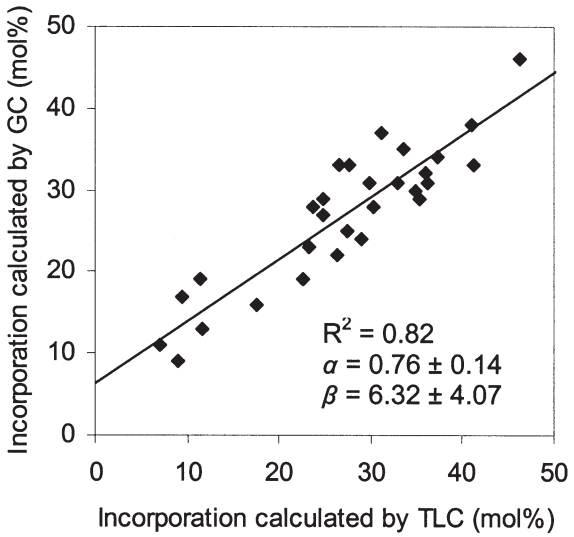

FIG. 5. Correlation between incorporation of caprylic determined by GC and TLC–FID. α and β represent the slope and intercept, respectively calculated from linear regression analysis.

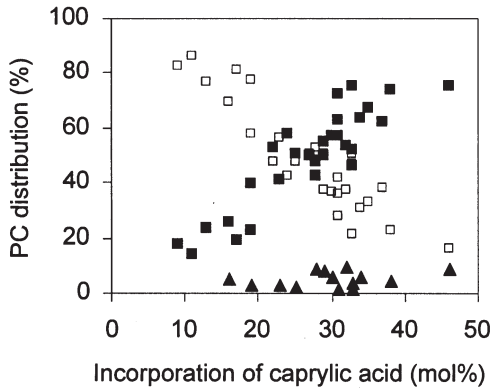

FIG. 6. Correlation between incorporation of caprylic acid into PC and PC molecular distribution. (□) LL-type PC, (■) ML-type PC, and (▲) MM-type PC. For abbreviations see Figure 4.

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