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Synthesis of structured phospholipids by immobilized phospholipase A2 catalyzed acidolysis

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Running title: PLA2 catalyzed synthesis of structured phospholipids

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Abstract

Acyl modification of the sn-2 position in phospholipids (PLs) was conducted by acidolysis reaction using immobilized phospholipase A₂ (PLA₂) as the catalyst. In the first stage we screened different carriers for their ability to immobilize PLA₂. Several carriers were able to fix the enzyme and maintain catalytic activity; however the final choice of carrier for the continued work was a non-ionic weakly polar macroreticular resin. Response surface methodology was applied to evaluate the influence of substrate ratio, reaction temperature and water addition during acidolysis reaction between caprylic acid and soybean phosphatidylcholine (PC). Reaction temperature and water addition had significant effect on acidolysis reaction, however no effect was observed for substrate ratio (mol caprylic acid/mol PC) in range tested. In general an inverse relationship between incorporation of caprylic acid and PC recovery was observed.

Highest incorporation obtained during acidolysis reactions was 36%. Such incorporation could be obtained under reaction temperature, 45°C; substrate ratio, 9 mol/mol caprylic acid/PC; and water addition of 2%; 30 wt % immobilized enzyme; and reaction time, 48h. The yield under these conditions was however only 29%. Lysophosphatidylcholine (LPC) was the major by-product formed during the reaction. Incorporation of acyl donor into LPC was very low (<4%), which indicates that acyl migration is only a minor problem for PLA₂ catalyzed synthesis reaction. Conjugated linoleic acid and docosahexaenoic acid were also tested as acyl donors, and were able to be incorporated into PC with 30 and 20%, respectively.

Keywords: Immobilization; PLA₂ catalyzed synthesis; response surface methodology; solvent-free system; structured phospholipids.
1. Introduction

Different enzymes can be used to tailor phospholipids (PLs) with defined fatty acid composition at the \textit{sn}-1 and \textit{sn}-2 positions. Using enzymatic acyl exchange it would be possible to acquire PLs for specific application requirements in food, pharmaceuticals and cosmetics by altering the technical or physiological properties of the natural compounds. Most of the work in this direction focuses on incorporation of saturated fatty acids (including both medium chain and long chain) or polyunsaturated fatty acids into PLs (Hossen et al., 2005; Lyberg et al., 2005; Reddy et al., 2005; Vikbjerg et al., 2005). The interest in the incorporation of saturated fatty acids is mainly to improve the heat stability, emulsifying properties and oxidation stability of the PLs (Chmiel et al., 1999; Pedersen, 2001), while the incorporation of polyunsaturated fatty acids is due to the claimed health promoting effects (Takahashi and Hosokawa, 2001).

Compared to enzymatic acyl exchange at the \textit{sn}-1 position of PLs, the enzymatic acyl exchange in the \textit{sn}-2 position has received less attention. Porcine pancreatic phospholipase A\textsubscript{2} (PLA\textsubscript{2}), which is the most commonly used enzyme for modification of PLs at the \textit{sn}-2 position, is considerably more difficult for synthesis in comparison with lipases from microbial sources commonly used for modification of the \textit{sn}-1 position of PLs. Pancreatic PLA\textsubscript{2} has requirement of calcium ions and a water activity above 0.2 to be catalytically active, which means that low yields can be expected compared to lipase-catalyzed reactions that can function in nearly anhydrous reaction systems without the presence of calcium ions (Pernas et al., 1990, Adlercreutz et al, 2003).
Despite these problems there remains a great interest in using PLA$_2$ for PL synthesis as fatty acids resided in the secondary position of PLs may have particular important influence on nutritional and medical functions (Takahashi and Hosokawa, 2001).

Commercial product of PLA$_2$ has so far only been provided in the free form (liquid solution), but some attempts have previously been made to immobilize the enzyme (Aura et al., 1995; Doig and Diks, 2003; Härrod and Elfman, 1995; Hossen et al. 2005; Lyberg et al. 2005). Main reason to use immobilized enzymes is the ability to isolate the biocatalyst from reaction mixture as well as to improve the stability. Some of the carriers selected in these previous studies would however not be suitable if having larger-scale production in mind. Enzymes immobilized on celite and certain other porous or powder inert materials have good initial activity, but are often difficult to handle or have insufficient enzymatic and physical stability in industrial processes (Eigtved, 1992). Dust formation, displacement of the enzyme from the carrier, and high pressure drops in packed bed columns are some of the problems that can occur using these types of carriers. Polymer or resin based carriers have been described, which offers strong adsorption, high activity, and stability of enzymes, which would accommodate enzymes and transport lipid substrate without major diffusion problems (Eigtved, 1992).

Most work described for the PLA$_2$ catalyzed synthesis of structured PLs are based on esterification of lyso-PLs in organic solvent (Adlercreutz et al., 2003; Guo et al., 2005). In order to obtain lyso-PLs for this type of reaction it would require a hydrolysis step of the PL and subsequent purification step to remove free fatty acids. Direct transesterification (acidolysis) of PL with acyl donor would avoid these
additional steps as reaction can be performed in a single step. Some attempts have previously been made for transesterification; however in general the incorporation of fatty acids into the sn-2 position is rather low (<15%) (Aura et al., 1995; Hossen et al., 2005; Park et al., 2001).

In this study we screened different carriers for immobilization of PLA2. A promising carrier was selected and further experiments were performed to maximize catalytic activity of the immobilized enzyme. The immobilized PLA2 was subsequently used for synthesis of structured PLs under solvent-free conditions. The reaction scheme for PLA2-catalyzed acidolysis is depicted in Fig. 1. Different parameters were examined for their influence on incorporation and PL distribution during PLA2 catalyzed synthesis of structured PLs. Response surface methodology was used to assist the evaluation.

2. Materials and Methods

2.1. Materials

Epikuron 200 (PC, 93%) was purchased from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition (mol%) of PC can be seen in Table 1. Caprylic acid (C8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Conjugated linoleic acid (CLA, purity 80%) consisting of 38.8% 9c,11t isomer and 38.8% 10t,12c isomer was provided by Natural ASA (Hovdebygda, Norway). 4,7,10,13,16,19 all cis-Docosahexaenoic acid (DHA, purity 99+ %) was purchased from Loradan Fine Chemicals (Malmö, Sweden). Porcine pancreatic PLA2 (Lecitase 10L, 10,000 U/ml) was supplied by Novozymes A/S
(Bagsvaerd, Denmark). Carrier materials and their suppliers are listed in table 2. All solvent and chemicals were of analytical grade.

2.2. Immobilization of PLA2

Varying amounts of PLA2 solution was added to 5 ml buffer (10 mM Tris-HCl, 10 mM CaCl2, pH 8) followed by the addition of 250 mg carrier. The enzyme solutions containing the carrier were incubated overnight by end-over-end mixing at room temperature followed by centrifugation at 4000 rpm for 5 minutes. The fixation level was estimated subtracting the protein remaining in the supernatant after binding compared to the initial protein concentration. Protein was determined according to the method of Lowry et al. (1951) using Bovine Serum albumin (BSA) as the standard. Enzyme preparation was removed by filtration and subsequently dried overnight in fume hood. Immobilized PLA2 was stored at 5°C prior to use.

2.3. Hydrolytic activity of PLA2.

Evaluation of the catalytic activity was determined by hydrolysis of PC as described by Kim et al. (2001). Reactions were carried out in an ethanol-buffer (10 mM Tris-HCl, 10 mM CaCl2, pH 8.0) (ratio, 70:30) with 0.4 g PC/ ml. Capped flasks containing the PC solution were incubated in water bath with magnetic stirring (300 rpm) at 40°C. Hydrolysis reactions were initiated by the addition of PLA2. Samples were withdrawn during progress in reaction, and analyzed by TLC-FID. The activity
was defined as the amount of LPC produced per min, and specific activity was defined as the amount of LPC produced per min and mg protein.

2.4. Acidolysis reaction

Reactions between fatty acid and PC were carried out using a 1 g reaction mixture in 5 ml glass vials. Vials were incubated in a water bath with magnetic stirring (300 rpm) and reactions were initiated by the addition of 300 mg immobilized PLA$_2$ (carrier: Amberlite XAD7; 72 mg PLA$_2$/g carrier). After reactions, samples were withdrawn from the reaction mixture for analysis. A three-level three-factor fractional experiment with 2 star points (17 experiments) was carried out. The three factors chosen were: reaction temperature (°C), water addition (wt% based on total substrate), and substrate ratio (mol/mol caprylic acid/PC). The incorporation of caprylic acid into PC, and the PL distribution (PC, LPC and glycerophosphorylcholine (GPC)) were used as responses. In table 1 are listed the factors used, the parameter ranges applied, and the responses.

2.5. Analysis methods

Analytical separations PL species and fatty acids were performed on Silica Gel 60 thin-layer plates (20cm x 20cm, Merck, Darmstadt, Germany). After development in chloroform-methanol-water (65:35:5, v/v), the plate was sprayed with 0.2% of 2,7-dichloroflourescein in ethanol (96%), making the lipid bands visible under UV-light. Bands representing PC and LPC were scraped off and methylated by BF3 for analysis.
on a HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame-ionization detector (FID) (Vikbjerg at al., 2005).

Phospholipid profile analysis was performed on product mixtures from acidolysis reactions using thin layer chromatography coupled with flame ionization detection (TLC-FID). Samples were spotted onto silica gel chromarods (Chromarod SIII, Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (42:22:3, v/v/v). After developing, chromarods were dried at 120°C for 5 min. Chromarods were then placed into the TLC-FID analyzer (Iatroscan MK6s, Iatron Laboratories Inc., Tokyo, Japan) and scanned at a rate of 30s/rod. Flow rates of 160 ml/min for hydrogen and 2 l/min for air were used during analysis. Peaks were identified by external standards.

2.6. Statistical analysis

Significance of the results was established at $P \leq 0.05$. Differences in the responses were determined by one-way analysis of variance, where 95% confidence intervals were calculated from pooled standard deviations (SD) using software Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA). The computer program Modde 6.0 (Umetri AB, Umeå, Sweden) was used to aid the statistical design of the factorial experiments and to fit and analyze the data by multiple regressions. The fit of the models were evaluated by the coefficient of determination ($R^2$) and analysis of variance (ANOVA).
3. Results and discussion

3.1. Screening for carrier materials

In order to have a practical approach for PLA2 catalyzed production of structured PLs the enzyme is preferred in the immobilized form. This would make it possible in sight to develop a continuous process as the enzyme can easily be recovered and reused, and would make the process more economically feasible. Of the various methods for immobilization physical absorption of the enzyme onto solid support remains the simplest, least expensive, and least labour-intensive procedure. Secreted PLA2 requires Ca$^{2+}$ as co-factor; however the concentration of Ca$^{2+}$ strongly influences the synthetic activity of these enzymes (Pernas et al., 1990). High concentrations of Ca$^{2+}$ give rise to sever inhibition of synthesis reactions. In some cases the dependence of Ca$^{2+}$ is simply overcome by doing the immobilization in buffer containing CaCl$_2$ (Egger et al., 1997; Aura et al., 1995; Lyberg et al. 2005). Pernas et al. (1990) reported that initial rate of PL synthesis conducted in organic solvent was dependent on the pH of the last aqueous solution in which the enzymes were exposed; however the maximum conversion was not dependent on the pH in the range 4-11. In most cases buffer has been adjusted to pH 8, when porcine pancreatic PLA2 have been used as catalyst. Conditions for the buffer used in the current study were selected based on recommendations from the previous studies mentioned above.

Seven different carriers were examined for their ability to immobilize PLA2. Characteristics of enzyme carriers screened are presented in table 2. In all cases, the immobilization procedure was the same. Table 3 shows the protein absorption to
different carriers. High fixation of PLA$_2$ to the carriers was observed except for Accural EP100 and Lewatit VP1600. These two carriers were also very hydrophobic, and did not suspend in the enzyme solution as the other carriers, but floated to the top. By pre-wetting these carriers with ethanol prior to immobilization it was possible to suspend these carriers in the enzyme solution, which also resulted in an increase of the fixation level of PLA$_2$ (table 3). The **three carriers immobilized with PLA$_2$** having the highest protein fixation (Amberlite XAD7, Duolite A568, and Superlite DAX8) were tested for their hydrolytic activity (table 3). As there was seen some differences in the enzyme fixation, the immobilized enzymes were added to the reaction mixture with similar protein loading. One-way analysis of variance showed that there was significant difference in catalytic activity of PLA$_2$ when immobilized on these different carriers (p<0.01). Having Amberlite XAD7 and Superlite DAX8 as carriers resulted in significant higher specific activity as compared to having Duolite A568 as the carrier; however there was no significant difference in the specific activity between Amberlite XAD7 and Superlite DAX8. Amberlite XAD7 had the highest protein fixation though, which means that lower dosage requirements were needed to obtain the same conversion degree. From considerations above Amberlite XAD7 was found to be a suitable carrier and was selected for the further study.

3.2. Conversion efficiency of the immobilized enzyme

Binding of enzyme to the carriers and the total amount bound will depend on the initial concentrations of the catalyst and the carrier, and ratio of the two components. In Fig.2 the influence of initial enzyme /carrier ratio on fixation level to Amberlite
XAD7 is depicted. Protein binding to the carrier increased with increased ratio between enzyme and carrier. However activity only increased with increasing fixation level until a certain protein loading was reached; and the specific activity decreased with increase in fixation level of PLA$_2$ (Fig. 3A). Highest specific activity was observed at low fixation level of PLA$_2$. At high enzyme load only a fraction of the enzyme seems to be involved in the catalytic reaction. Higher enzyme load would contribute to increased limitation of substrate diffusion and therefore decreasing efficiency. From Fig. 3A it seems that an initial enzyme/carrier ratio of approximately 100 mg/g would give the optimal fixation of PLA$_2$ in terms of activity. Influence of enzyme loading on activity and specific activity with this fixation level was examined (Fig. 3B). This was mainly to confirm that the results obtained above were valid, and that the decline in activity was not related to for example substrate limitations. As expected the activity increased with increased enzyme dosage, and the specific activity was constant. For the subsequent acidolysis reactions PLA$_2$ was immobilized to Amberlite XAD7 with an initial enzyme/carrier ratio 100mg/g (72 mg/g enzyme fixed/carrier).

3.3. PLA$_2$ catalyzed acidolysis reaction

Reactions were performed in a single step, having both hydrolysis and esterification reactions that occur simultaneously. The fatty acids resided in the $sn$-2 position of PLs will therefore be a mixture of original fatty acids and the ones to be incorporated. Theoretically the presence of original fatty acids can be minimized by having high substrate ratio (mol acyl donor/mol PL). A preliminary study was conducted to evaluate incorporation and PL distribution during the time course of
acidolysis reaction between PC and caprylic acid. Reaction conditions selected were a 
substrate ratio of 6 mol/mol caprylic acid/PC, together with 30% enzyme dosage at 
40ºC. Some water was added to the reaction mixture (0.75%), as this enzyme requires 
some water to main activity (Adlercreutz et al., 2003). The results showed that, after 
72h, it was possible to have 15% incorporation of caprylic acid into PC (Fig. 4A). 
However with increasing incorporation, the recovery of PC decreased. Complexity of 
the acidolysis reaction makes it difficult to predict the influence of different parameters 
on incorporation and PL distribution. A statistical experimental design was therefore set 
up with the assistance of response surface methodology (RSM) to evaluate the influence 
of individual parameters, as well as their interactions, on incorporation and PL 
distribution. Reaction temperature, substrate ratio and water addition were selected as 
variables, whereas enzyme dosage and reaction time were held constant in the current 
study. From Fig. 4B it can be observed that with a reaction more than 48h there was 
only seen a small progress in the reaction. From a process point of view it would be 
desirable to have as low a reaction time as possible. Responses and variable settings in 
Table 4 were fitted to each other with multiple regressions. The best-fitting models were 
determined by multiple regression and backward elimination, whereby insignificant 
factors and interactions were removed from the models. The statistics for the model 
coefficients and probability values for response variables are presented in table 5. The 
coefficient of determination ($R^2$) of the models were 0.95, 0.99, 0.98, 0.67 for the four 
responses, i.e. incorporation into PC, PC content, LPC content and GPC content, 
respectively. Models with acceptable qualities should have $R^2 > 0.8$. Most of models 
therefore represent real relationship between responses and the reaction parameters. 
According to the analysis of variance there was no lack of fit for the generated models.
Observed and predicted values were sufficiently correlated except for experiment no.1, which was treated as an outlier.

Water addition was the most significant factor on the PLA₂ catalyzed acidolysis reactions in terms of incorporation and recovery (table 5). A continuous increase in the incorporation was observed until water level of 2% (Fig. 5A). Higher water addition had no significant effect on incorporation. The recovery of PC decreased with increased water addition (Fig. 5B). With increase of water in the reaction system both LPC and GPC increased. GPC forms if acyl chain of LPC molecule migrates from the \textit{sn}-1 position to the \textit{sn}-2 position, and the formed 2-acyl LPC is hydrolyzed by PLA₂. It was previously demonstrated that water content had no effect on the incorporation in solvent-free system during lipase-catalyzed acidolysis reaction (Vikbjerg et al., 2005), which is in contrast to PLA₂ catalyzed acidolysis reaction. With both types of enzyme, the recovery of PC decreases with increasing water content due to parallel hydrolysis reaction. Water seems to have a complex role in terms of compromising enzyme activity, hydrolysis side reactions, reaction rate, and extent of incorporation. As PLA₂ require a higher water activity to function as compared to lipases, the yield is expected to be lower (Adlercreutz et al., 2003).

Reaction temperature also had significant effect on the acidolysis reaction. Maximum incorporation was observed at 45°C (Fig. 6A). At higher and lower temperatures there was a decrease in the incorporation of caprylic acid into PC. The lowest yield was obtained at 45°C (Fig. 6B). At higher and lower temperatures PC content increased. In general an increase in temperature increases the rate of all chemical reactions, including those catalyzed by enzymes, but at the same time it increases the rate of denaturation of enzyme protein. These processes probably explain
the characteristic temperature profile of PLA₂ and high value for the second order value in the models. Park et al. (2000) examined the effect of reaction temperature on transesterification of PC and ethyl esters of EPA in toluene, and found that maximum reaction rate and yield were at 50°C. Enzyme activity was observed to drop sharply above 50°C. Egger et al. (1997) reported that during synthesis of PC from LPC highest reaction rate was observed at 40°C. At this temperature there was however observed a decrease in the amount of PC and LPC during the enzymatic reaction. This decrease was found to be due to formation of GPC. It was claimed that at this high temperature GPC formation occurred due to acyl migration. In this study the temperature had an effect on formation of GPC. Highest content of GPC was at 45°C. With higher LPC content in reaction system formation of GPC seems to increase especially at elevated temperatures.

Substrate ratio had no significant effect on either incorporation of caprylic acid or the PL distribution, and no interaction was seen for this factor. Even though no differences are seen in the relative PL distribution, it should be remembered that the PL concentration is higher at lower substrate ratios. In terms of production it would be recommended to have low substrate ratio.

Highest incorporation was obtained by having reaction temperature, 45°C; water addition 2%; and substrate ratio, 9 mol/mol caprylic acid/PC. Under these conditions the PC accounted for 29% of the PL fraction. The incorporation of caprylic acid into LPC was also examined, however was less than 4% for all samples (data not shown), and therefore no attempts were made to model these data.
3.4. Reactivity of different fatty acids

Different fatty acids may be applied as acyl donor for acidolysis reaction. However the fatty acids usually result in different reactivity, due to fatty acid specificity or possible inhibition effects. Under the same conditions, different fatty acids often result in different incorporation into PLs or different yields. Reaction rates have been reported to be the same for saturated fatty acids of length between 6 and 12 carbon atoms, but they were lower for myristic and palmitic acids (Egger et al. 1997). Highest reaction rate was obtained with oleic acid, but higher degree of unsaturation resulted in lower reaction rates. In this study we compared the incorporation of DHA and CLA with that of caprylic acid under similar reaction conditions (Te, 45°C; Wa, 2%; Sr, 3 mol/mol fatty acid/PL). The incorporations of the different fatty acids into PC are presented in table 1. CLA resulted in the highest degree of incorporation, followed by caprylic acid and DHA. PLA2 showed little discrimination toward the two main isomers of CLA (data not shown). With CLA as acyl donor the PL distribution after reaction was 21, 74, and 5% for PC, LPC and GPC, respectively. With DHA as acyl donor the PL distribution was 22, 77, and 1% for PC, LPC and GPC respectively. Yields were thus lower when using CLA and DHA as acyl donors, however the formation of GPC was also lower as compared to reactions performed with caprylic acid (see table 4, experiment no.13). The results indicate that caprylic acid may cause more acyl migration in the reaction system compared to DHA and CLA, however further experiments would be required to verify this observation.

In conclusion PC with modified fatty acid profile can be produced by PLA2 catalyzed acidolysis. Water addition and reaction temperature were shown to have
significant effect on both incorporation and yield. Both reaction temperature and water
addition had an inverse relationship between incorporation and recovery of PC.
Substrate ratio showed no effect on the PL distribution. Incorporation of caprylic acid
into PC could reach 36% accounting for 29% of the PL fraction. Incorporation of new
fatty acids was shown to depend on acyl donor. Polyunsaturated fatty acids DHA and
CLA were incorporated into PC with 30 and 20%, respectively.

Acknowledgements

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Phospholipase A2 catalyzed synthesis of phosphatidylcholine. Biochim. Biophys.
Acta 1343, 76-84.


<table>
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<th>Fatty acids</th>
<th>Soybean PC</th>
<th>Caprylic acid enriched PC</th>
<th>CLA enriched PC</th>
<th>DHA enriched PC</th>
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<tr>
<td>8:0</td>
<td>-</td>
<td>25.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16:0</td>
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<td>13.0</td>
<td>13.0</td>
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<td>3.2</td>
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<tr>
<td>22:6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20.2</td>
</tr>
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</table>

*a Reaction conditions: Reaction temperature, 45°C; Water addition, 2%; Substrate ratio, 3 mol/mol, enzyme dosage, 30%; Reaction time, 48h.*
### Table 2. Carriers screened and their characteristics

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Supplier</th>
<th>General description</th>
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<tbody>
<tr>
<td>Amberlite XAD7</td>
<td>Sigma-Aldrich Chemie GmbH, Steinheim, Germany</td>
<td>Nonionic weakly polar macroreticular resin (matrix: acylic ester), Particle size: 0.25-0.85 mm (wet)</td>
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<tr>
<td>Superlite DAX8</td>
<td>Supelco, Bellefonte, USA</td>
<td>Resin with moderate polarity (matrix: acrylic ester), Particle size: 0.25-0.45mm</td>
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<tr>
<td>Celite 545</td>
<td>BHD, Poole, UK</td>
<td>Diatomaceous Earth, Particle size: 0.02-0.1 mm</td>
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<tr>
<td>Dowex 50W</td>
<td>Dow Chemical Company, Michigan, USA</td>
<td>Strongly acidic cation exchange (matrix: resin-styrene-divinylbenzene; functional group: sulfonic acid), Particle size: 0.15-0.30mm</td>
</tr>
<tr>
<td>Lewatit VPOC1600</td>
<td>Lanxess AG, Leverkusen, Germany</td>
<td>Divinyl benzene crosslinked polymer (Matrix: methacrylate), Particle size: 0.3-1.2 mm</td>
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<tr>
<td>Duolite A568</td>
<td>Rohn and Haas, Chauny, France</td>
<td>Polymerized phenol-formaldehyde anionic exchange resin, Particle size: 0.15-0.85 mm</td>
</tr>
<tr>
<td>Accurel EP 100</td>
<td>Akzo, Obernburg, Germany</td>
<td>Macroporous polypropylene, Particle size: 0.6-0.8 mm</td>
</tr>
</tbody>
</table>
Table 3. Fixation level of PLA2 on different carriers, and corresponding enzyme loading and specific activity.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Enzyme loading $^b$ (mg protein/g support)</th>
<th>Specific activity $^c$ ($\mu$mol mg$^{-1}$ min$^{-1}$)</th>
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<tr>
<td>Amberlite XAD7</td>
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<td>Superlite DAX8</td>
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<td>Duolite A568</td>
<td>43.3</td>
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<td>Dowex 50W</td>
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<td>Celite 545</td>
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<tr>
<td>Accural EP 100</td>
<td>1.9</td>
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<tr>
<td>+ Prewetting</td>
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<tr>
<td>Lewatit VPOC 1600</td>
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<td>-</td>
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<tr>
<td>+ Prewetting</td>
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<td>-</td>
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</table>

$^a$ Pre-wetting of Accural EP 100 and Lewatit VPOC 1600 were done by addition of 0.5 ml 96% ethanol/g support immediately before immobilization; $^b$ Pooled SD = 1.0 mg protein/g support; $^c$ Pooled SD = 0.015 $\mu$mol mg$^{-1}$ min$^{-1}$; - n.d., not determined.
Table 4 Settings of the RSM generated experimental design for the PLA$_2$ catalyzed acidolysis and measured responses.

<table>
<thead>
<tr>
<th>Experiment no.</th>
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Abbreviations: $T_e$, Reaction temperature (°C); $W_a$, water addition (wt% based on total substrate); $S_r$, substrate ratio (mol Caprylic acid/mol PC), Inc., Incorporation of caprylic acid (mol%), PC, phosphatidylcholine content; LPC, lysophosphatidylcholine content; GPC, glycerophosphorylcholine content. $^a$Values reported for the PL distribution are based on weight percentages of PC + LPC + GPC
Table 5 Regression coefficients and P-values describing the influence of different parameters on incorporation of caprylic acid into PC and PL distribution.

<table>
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<th>Term</th>
<th>Incorporation of caprylic acid into PC (mol%)</th>
<th>PL distribution (wt%)</th>
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<td>Regression coefficient</td>
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<td>Te x Wa</td>
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*Values reported for the PL distribution are based on weight percentages of PC + LPC+ GPC. The effect of each factor (linear and quadratic) and interaction effects are statistically significant when P-value<0.05.
Figure legend:

Figure 1: Schematic presentation of PLA₂-catalyzed acidolysis of phospholipid with free fatty acid. \( R_1, R_2 \) and \( R_3 \) refer to fatty acids and \( x \) refers to phospholipid head group (e.g. choline).

Figure 2: Influence of initial enzyme/support ratio on fixation level to Amberlite XAD7. Varying amounts PLA₂ were incubated in the presence of 250 mg carrier. Bars represents mean ± pooled SD.

Figure 3: Bioconversion efficiency of PLA₂ immobilized Amberlite XAD7. A) Influence on enzymatic loading on activity and specific activity of immobilized system with different fixation level (mg enzyme per g support). B) Influence on enzymatic loading on activity and specific activity of immobilized system with same fixation level. Enzymatic assay and PLA₂ activity measurement were performed according to procedure described in material and methods. Bars represent mean ± pooled SD (n=2).

Figure 4: Time course for acidolysis reaction between PC and caprylic acid in solvent free system. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PC, water addition, 0.75%; dosage of immobilized enzyme, 30 wt%; and reaction temperature, 40°C. A) Incorporation of caprylic acid into PC and B) PL distribution. Bars represent mean ± pooled SD (n=2).

Figure 5: Effect of water addition on PLA₂ catalyzed acidolysis reaction when varied from low to a high level with all other factors being on their
average. A) Incorporation of caprylic acid into PC and B) PL
distribution. Error bars indicate 95% confidence interval.

Figure 6: Effect of reaction temperature on PLA$_2$ catalyzed acidolysis reaction
when varied from low to a high level with all other factors being on their
average. A) Incorporation of caprylic acid into PC and B) PL
distribution. Bars indicate 95% confidence interval.
Figure 1

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\[
\begin{align*}
& \text{OCOR}_1 \quad \text{PLA}_2 \quad \text{OCOR}_1 \\
& \text{OCOR}_2 + R_3\text{COOH} \quad \leftrightarrow \quad \text{OCOR}_3 + R_2\text{COOH} \\
& \text{OPO}_3\text{X} \quad \text{OPO}_3\text{X}
\end{align*}
\]```

Figure 2

![Graph showing the relationship between initial ratio of enzyme/carrier (mg g\(^{-1}\)) and amount of enzyme fixed/carrier (mg g\(^{-1}\)).]
Figure 3

(A) Protein loading (mg g\textsuperscript{-1})

(B) Enzyme dosage (mg)
Figure 4

(A) Incorporation of caprylic acid (mol%) vs. Reaction time (h)

(B) PL distribution (wt%) vs. Reaction time (h)

- PC
- LPC
- GPC
Figure 5

(A) Incorporation of caprylic acid (mol%) vs Water addition (%)

(B) PL distribution (wt%) vs Water addition (%)

- PC
- LPC
- GPC
Figure 6

(A) Incorporation of caprylic acid (mol%) vs. reaction temperature (°C)

(B) PL distribution (wt%) vs. reaction temperature (°C)