



## Synthesis of structured phospholipids by immobilized phospholipase A2 catalyzed acidolysis

Vikbjerg, Anders Falk; Vikbjerg, Anders Falk; Xu, Xuebing

*Published in:*  
Journal of Biotechnology

*Link to article, DOI:*  
[10.1016/j.jbiotec.2006.11.006](https://doi.org/10.1016/j.jbiotec.2006.11.006)

*Publication date:*  
2007

[Link back to DTU Orbit](#)

*Citation (APA):*  
Vikbjerg, A. F., Vikbjerg, A. F., & Xu, X. (2007). Synthesis of structured phospholipids by immobilized phospholipase A2 catalyzed acidolysis. *Journal of Biotechnology*, 128(3), 545-554.  
<https://doi.org/10.1016/j.jbiotec.2006.11.006>

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1        Synthesis of structured phospholipids by immobilized  
2                    phospholipase A<sub>2</sub> catalyzed acidolysis

3  
4                    **Anders Falk Vikbjerg\*, Huiling Mu, and Xuebing Xu**

5  
6        <sup>a</sup>*BioCentrum-DTU, Technical University of Denmark, DK 2800 Kgs. Lyngby, Denmark*

7  
8                    Running title: PLA<sub>2</sub> catalyzed synthesis of structured phospholipids

9  
10        \*Correspondence:

11        A.F. Vikbjerg, BioCentrum-DTU, Technical University of Denmark, Building 221, DK-  
12        2800 Kgs. Lyngby, Denmark

13        Tel. +45 4525 2614

14        Fax. +45 4588 4922

15        E-mail address: afv@biocentrum.dtu.dk (Anders Falk Vikbjerg)

16        Reprint request to Xuebing Xu, xx@biocentrum.dtu.dk

1 **Abstract**

2 Acyl modification of the *sn*-2 position in phospholipids (PLs) was conducted by  
3 acidolysis reaction using immobilized phospholipase A<sub>2</sub> (PLA<sub>2</sub>) as the catalyst. In the  
4 first stage we screened different carriers for their ability to immobilize PLA<sub>2</sub>. Several  
5 carriers were able to fix the enzyme and maintain catalytic activity; however the final  
6 choice of carrier for the continued work was a non-ionic weakly polar macroreticular  
7 resin. Response surface methodology was applied to evaluate the influence of substrate  
8 ratio, reaction temperature and water addition during acidolysis reaction between  
9 caprylic acid and soybean phosphatidylcholine (PC). Reaction temperature and water  
10 addition had significant effect on acidolysis reaction, however no effect was observed  
11 for substrate ratio (mol caprylic acid/mol PC) in range tested. In general an inverse  
12 relationship between incorporation of caprylic acid and PC recovery was observed.  
13 Highest incorporation obtained during acidolysis reactions was 36%. Such  
14 incorporation could be obtained under reaction temperature, 45°C; substrate ratio, 9  
15 mol/mol caprylic acid/PC; and water addition of 2%; 30 wt % immobilized enzyme; and  
16 reaction time, 48h. The yield under these conditions was however only 29%.  
17 Lysophosphatidylcholine (LPC) was the major by-product formed during the reaction.  
18 Incorporation of acyl donor into LPC was very low (<4%), which indicates that acyl  
19 migration is only a minor problem for PLA<sub>2</sub> catalyzed synthesis reaction. Conjugated  
20 linoleic acid and docosahexaenoic acid were also tested as acyl donors, and were able to  
21 be incorporated into PC with 30 and 20%, respectively.

22 *Keywords:* Immobilization; PLA<sub>2</sub> catalyzed synthesis; response surface methodology;  
23 solvent-free system; structured phospholipids.

24

## 1 **1. Introduction**

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

15 Compared to enzymatic acyl exchange at the *sn*-1 position of PLs, the enzymatic  
16 acyl exchange in the *sn*-2 position has received less attention. Porcine pancreatic  
17 phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which is the most commonly used enzyme for modification  
18 of PLs at the *sn*-2 position, is considerably more difficult for synthesis in comparison  
19 with lipases from microbial sources commonly used for modification of the *sn*-1  
20 position of PLs. Pancreatic PLA<sub>2</sub> has requirement of calcium ions and a water activity  
21 above 0.2 to be catalytically active, which means that low yields can be expected  
22 compared to lipase-catalyzed reactions that can function in nearly anhydrous reaction  
23 systems without the presence of calcium ions (Pernas et al., 1990, Adlercreutz et al,  
24 2003).

1           Despite these problems there remains a great interest in using PLA<sub>2</sub> for PL  
2 synthesis as fatty acids resided in the secondary position of PLs may have particular  
3 important influence on nutritional and medical functions (Takahashi and Hosokawa,  
4 2001).

5           Commercial product of PLA<sub>2</sub> has so far only been provided in the free form  
6 (liquid solution), but some attempts have previously been made to immobilize the  
7 enzyme (Aura et al., 1995; Doig and Diks, 2003; Härrod and Elfman, 1995; Hossen et al.  
8 2005; Lyberg et al. 2005). Main reason to use immobilized enzymes is the ability to  
9 isolate the biocatalyst from reaction mixture as well as to improve the stability. Some of  
10 the carriers selected in these previous studies would however not be suitable if having  
11 larger-scale production in mind. Enzymes immobilized on celite and certain other  
12 porous or powder inert materials have good initial activity, but are often difficult to  
13 handle or have insufficient enzymatic and physical stability in industrial processes  
14 (Eigtved, 1992). Dust formation, displacement of the enzyme from the carrier, and high  
15 pressure drops in packed bed columns are some of the problems that can occur using  
16 these types of carriers. Polymer or resin based carriers have been described, which  
17 offers strong adsorption, high activity, and stability of enzymes, which would  
18 accommodate enzymes and transport lipid substrate without major diffusion problems  
19 (Eigtved, 1992).

20           Most work described for the PLA<sub>2</sub> catalyzed synthesis of structured PLs are  
21 based on esterification of lyso-PLs in organic solvent (Adlercreutz et al., 2003; Guo et  
22 al., 2005). In order to obtain lyso-PLs for this type of reaction it would require a  
23 hydrolysis step of the PL and subsequent purification step to remove free fatty acids.  
24 Direct transesterification (acidolysis) of PL with acyl donor would avoid these

1 additional steps as reaction can be performed in a single step. Some attempts have  
2 previously been made for transesterification; however in general the incorporation of  
3 fatty acids into the *sn*-2 position is rather low (<15%) (Aura et al., 1995; Hossen et al.,  
4 2005; Park et al., 2001).

5 In this study we screened different carriers for immobilization of PLA<sub>2</sub>. A  
6 promising carrier was selected and further experiments were performed to maximize  
7 catalytic activity of the immobilized enzyme. The immobilized PLA<sub>2</sub> was subsequently  
8 used for synthesis of structured PLs under solvent-free conditions. [The reaction scheme](#)  
9 [for PLA<sub>2</sub>-catalyzed acidolysis is depicted in Fig. 1.](#) Different parameters were examined  
10 for their influence on incorporation and PL distribution during PLA<sub>2</sub> catalyzed synthesis  
11 of structured PLs. Response surface methodology was used to assist the evaluation.

12

## 13 **2. Materials and Methods**

14

### 15 *2.1. Materials*

16

17 Epikuron 200 (PC, 93%) was purchased from Degussa Texturant Systems  
18 Deutchland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition  
19 (mol%) of PC can be seen in Table 1. Caprylic acid (C8:0, purity 97%) was purchased  
20 from Riedel-de-Haen (Seelze, Germany). Conjugated linoleic acid (CLA, purity 80%)  
21 consisting of 38.8% 9c,11t isomer and 38.8% 10t,12c isomer was provided by Natural  
22 ASA (Hovdebygda, Norway). 4,7,10,13,16,19 all cis-Docosahexaenoic acid (DHA,  
23 purity 99+ %) was purchased from Loradan Fine Chemicals (Malmö, Sweden). Porcine  
24 pancreatic PLA<sub>2</sub> (Lecitase 10L, 10.000 U/ml) was supplied by Novozymes A/S

1 (Bagsvaerd, Denmark). Carrier materials and their suppliers are listed in table 2. All  
2 solvent and chemicals were of analytical grade.

3

#### 4 *2.2. Immobilization of PLA<sub>2</sub>*

5

6 Varying amounts of PLA<sub>2</sub> solution was added to 5 ml buffer (10 mM Tris-HCl,  
7 10 mM CaCl<sub>2</sub>, pH 8) followed by the addition of 250 mg carrier. The enzyme solutions  
8 containing the carrier were incubated overnight by end-over-end mixing at room  
9 temperature followed by centrifugation at 4000 rpm for 5 minutes. The fixation level  
10 was estimated subtracting the protein remaining in the supernatant after binding  
11 compared to the initial protein concentration. Protein was determined according to the  
12 method of Lowry et al. (1951) using Bovine Serum albumin (BSA) as the standard.  
13 Enzyme preparation was removed by filtration and subsequently dried overnight in  
14 fume hood. Immobilized PLA<sub>2</sub> was stored at 5°C prior to use.

15

#### 16 *2.3. Hydrolytic activity of PLA<sub>2</sub>.*

17

18 Evaluation of the catalytic activity was determined by hydrolysis of PC as  
19 described by Kim et al. (2001). Reactions were carried out in an ethanol-buffer (10 mM  
20 Tris-HCl, 10 mM CaCl<sub>2</sub>, pH 8.0) (ratio, 70:30) with 0.4 g PC/ ml. Capped flasks  
21 containing the PC solution were incubated in water bath with magnetic stirring (300  
22 rpm) at 40°C. Hydrolysis reactions were initiated by the addition of PLA<sub>2</sub>. Samples  
23 were withdrawn during progress in reaction, and analyzed by TLC-FID. The activity

1 was defined as the amount of LPC produced per min, and specific activity was defined  
2 as the amount of LPC produced per min and mg protein.

#### 3 4 *2.4. Acidolysis reaction*

5  
6 Reactions between fatty acid and PC were carried out using a 1 g reaction  
7 mixture in 5 ml glass vials. Vials were incubated in a water bath with magnetic stirring  
8 (300 rpm) and reactions were initiated by the addition of 300 mg immobilized PLA<sub>2</sub>  
9 (carrier: Amberlite XAD7; 72 mg PLA<sub>2</sub>/g carrier). After reactions, samples were  
10 withdrawn from the reaction mixture for analysis. A three-level three-factor fractional  
11 experiment with 2 star points (17 experiments) was carried out. The three factors chosen  
12 were: reaction temperature (°C), water addition (wt% based on total substrate), and  
13 substrate ratio (mol/mol caprylic acid/PC). The incorporation of caprylic acid into PC,  
14 and the PL distribution (PC, LPC and glycerophosphorylcholine (GPC)) were used as  
15 responses. In table 1 are listed the factors used, the parameter ranges applied, and the  
16 responses.

#### 17 18 *2.5. Analysis methods*

19  
20 Analytical separations PL species and fatty acids were performed on Silica Gel 60  
21 thin-layer plates (20cm x 20cm, Merck, Darmstadt, Germany). After development in  
22 chloroform-methanol-water (65:35:5, v/v), the plate was sprayed with 0.2% of 2,7-  
23 dichlorofluorescein in ethanol (96%), making the lipid bands visible under UV-light.  
24 Bands representing PC and LPC were scraped off and methylated by BF<sub>3</sub> for analysis



1 on a HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany)  
2 equipped with a flame-ionization detector (FID) (Vikbjerg et al., 2005).

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

## 14 2.6. Statistical analysis

15  
16 Significance of the results was established at  $P \leq 0.05$ . Differences in the responses were  
17 determined by one-way analysis of variance, where 95% confidence intervals were  
18 calculated from pooled standard deviations (SD) using software Microsoft Office Excel  
19 2003 (Microsoft Corporation, Redmond, WA). The computer program Modde 6.0  
20 (Umetri AB, Umeå, Sweden) was used to aid the statistical design of the factorial  
21 experiments and to fit and analyze the data by multiple regressions. The fit of the  
22 models were evaluated by the coefficient of determination ( $R^2$ ) and analysis of variance  
23 (ANOVA).

### 1 **3. Results and discussion**

#### 3 *3.1. Screening for carrier materials*

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

22 Seven different carriers were examined for their ability to immobilize PLA<sub>2</sub>.  
23 Characteristics of enzyme carriers screened are presented in table 2. In all cases, the  
24 immobilization procedure was the same. Table 3 shows the protein absorption to

1 different carriers. High fixation of PLA<sub>2</sub> to the carriers was observed except for Accural  
2 EP100 and Lewatit VP1600. These two carriers were also very hydrophobic, and did  
3 not suspend in the enzyme solution as the other carriers, but floated to the top. By pre-  
4 wetting these carriers with ethanol prior to immobilization it was possible to suspend  
5 these carriers in the enzyme solution, which also resulted in an increase of the fixation  
6 level of PLA<sub>2</sub> (table 3). The **three carriers immobilized with PLA<sub>2</sub>** having the highest  
7 protein fixation (Amberlite XAD7, Duolite A568, and Superlite DAX8) were tested for  
8 their hydrolytic activity (table 3). As there was seen some differences in the enzyme  
9 fixation, the immobilized enzymes were added to the reaction mixture with similar  
10 protein loading. One-way analysis of variance showed that there was significant  
11 difference in catalytic activity of PLA<sub>2</sub> when immobilized on these different carriers  
12 ( $p < 0.01$ ). Having Amberlite XAD7 and Superlite DAX8 as carriers resulted in  
13 significant higher specific activity as compared to having Duolite A568 as the carrier;  
14 however there was no significant difference in the specific activity between Amberlite  
15 XAD7 and Superlite DAX8. Amberlite XAD7 had the highest protein fixation though,  
16 which means that lower dosage requirements were needed to obtain the same  
17 conversion degree. From considerations above Amberlite XAD7 was found to be a  
18 suitable carrier and was selected for the further study.

19

### 20 *3.2. Conversion efficiency of the immobilized enzyme*

21

22 Binding of enzyme to the carriers and the total amount bound will depend on  
23 the initial concentrations of the catalyst and the carrier, and ratio of the two components.

24 In Fig.2 the influence of initial enzyme /carrier ratio on fixation level to Amberlite

1 XAD7 is depicted. Protein binding to the carrier increased with increased ratio between  
2 enzyme and carrier. However activity only increased with increasing fixation level until  
3 a certain protein loading was reached; and the specific activity decreased with increase  
4 in fixation level of PLA<sub>2</sub> (Fig. 3A). Highest specific activity was observed at low  
5 fixation level of PLA<sub>2</sub>. At high enzyme load only a fraction of the enzyme seems to be  
6 involved in the catalytic reaction. Higher enzyme load would contribute to increased  
7 limitation of substrate diffusion and therefore decreasing efficiency. From Fig. 3A it  
8 seems that an initial enzyme/carrier ratio of approximately 100 mg/g would give the  
9 optimal fixation of PLA<sub>2</sub> in terms of activity. Influence of enzyme loading on activity  
10 and specific activity with this fixation level was examined (Fig. 3B). This was mainly to  
11 confirm that the results obtained above were valid, and that the decline in activity was  
12 not related to for example substrate limitations. As expected the activity increased with  
13 increased enzyme dosage, and the specific activity was constant. For the subsequent  
14 acidolysis reactions PLA<sub>2</sub> was immobilized to Amberlite XAD7 with an initial  
15 enzyme/carrier ratio 100mg/g (72 mg/g enzyme fixed/carrier).

16

### 17 *3.3. PLA<sub>2</sub> catalyzed acidolysis reaction*

18

19 Reactions were performed in a single step, having both hydrolysis and  
20 esterification reactions that occur simultaneously. The fatty acids resided in the *sn*-2  
21 position of PLs will therefore be a mixture of original fatty acids and the ones to be  
22 incorporated. Theoretically the presence of original fatty acids can be minimized by  
23 having high substrate ratio (mol acyl donor/mol PL). A preliminary study was  
24 conducted to evaluate incorporation and PL distribution during the time course of

1 acidolysis reaction between PC and caprylic acid. Reaction conditions selected were a  
2 substrate ratio of 6 mol/mol caprylic acid/PC, together with 30% enzyme dosage at  
3 40°C. Some water was added to the reaction mixture (0.75%), as this enzyme requires  
4 some water to main activity (Adlercreutz et al., 2003). The results showed that, after  
5 72h, it was possible to have 15% incorporation of caprylic acid into PC (Fig. 4A).  
6 However with increasing incorporation, the recovery of PC decreased. Complexity of  
7 the acidolysis reaction makes it difficult to predict the influence of different parameters  
8 on incorporation and PL distribution. A statistical experimental design was therefore set  
9 up with the assistance of response surface methodology (RSM) to evaluate the influence  
10 of individual parameters, as well as their interactions, on incorporation and PL  
11 distribution. Reaction temperature, substrate ratio and water addition were selected as  
12 variables, whereas enzyme dosage and reaction time were held constant in the current  
13 study. From Fig. 4B it can be observed that with a reaction more than 48h there was  
14 only seen a small progress in the reaction. From a process point of view it would be  
15 desirable to have as low a reaction time as possible. Responses and variable settings in  
16 Table 4 were fitted to each other with multiple regressions. The best-fitting models were  
17 determined by multiple regression and backward elimination, whereby insignificant  
18 factors and interactions were removed from the models. The statistics for the model  
19 coefficients and probability values for response variables are presented in table 5. The  
20 coefficient of determination ( $R^2$ ) of the models were 0.95, 0.99, 0.98, 0.67 for the four  
21 responses, i.e. incorporation into PC, PC content, LPC content and GPC content,  
22 respectively. Models with acceptable qualities should have  $R_2 > 0.8$ . Most of models  
23 therefore represent real relationship between responses and the reaction parameters.  
24 According to the analysis of variance there was no lack of fit for the generated models.

1 Observed and predicted values were sufficiently correlated except for experiment no.1,  
2 which was treated as an outlier.

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

18 Reaction temperature also had significant effect on the acidolysis reaction.  
19 Maximum incorporation was observed at 45°C (Fig.6A). At higher and lower  
20 temperatures there was a decrease in the incorporation of caprylic acid into PC. The  
21 lowest yield was obtained at 45°C (Fig.6B). At higher and lower temperatures PC  
22 content increased. In general an increase in temperature increases the rate of all  
23 chemical reactions, including those catalyzed by enzymes, but at the same time it  
24 increases the rate of denaturation of enzyme protein. These processes probably explain

1 the characteristic temperature profile of PLA<sub>2</sub> and high value for the second order value  
2 in the models. Park et al. (2000) examined the effect of reaction temperature on  
3 transesterification of PC and ethyl esters of EPA in toluene, and found that maximum  
4 reaction rate and yield were at 50°C. Enzyme activity was observed to drop sharply  
5 above 50°C. Egger et al. (1997) reported that during synthesis of PC from LPC highest  
6 reaction rate was observed at 40°C. At this temperature there was however observed a  
7 decrease in the amount of PC and LPC during the enzymatic reaction. This decrease  
8 was found to be due to formation of GPC. It was claimed that at this high temperature  
9 GPC formation occurred due to acyl migration. In this study the temperature had an  
10 effect on formation of GPC. Highest content of GPC was at 45°C. With higher LPC  
11 content in reaction system formation of GPC seems to increase especially at elevated  
12 temperatures.

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

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

23

24

### 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. PLA<sub>2</sub> 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 PLA<sub>2</sub> catalyzed acidolysis. Water addition and reaction temperature were shown to have



1 significant effect on both incorporation and yield. Both reaction temperature and water  
2 addition had an inverse relationship between incorporation and recovery of PC.  
3 Substrate ratio showed no effect on the PL distribution. Incorporation of caprylic acid  
4 into PC could reach 36% accounting for 29% of the PL fraction. Incorporation of new  
5 fatty acids was shown to depend on acyl donor. Polyunsaturated fatty acids DHA and  
6 CLA were incorporated into PC with 30 and 20%, respectively.

7

### 8 **Acknowledgements**

9

10 This project was financially supported by the Danish Technical Research Council  
11 (STVF) and the Center for Advanced Food Studies (LMC).

12

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- 9

1 Table 1 Fatty acid distribution in PC and structured PCs (mol%)

| Fatty acids       | Soybean PC | Structured PC <sup>a</sup> |                 |                 |
|-------------------|------------|----------------------------|-----------------|-----------------|
|                   |            | Caprylic acid enriched PC  | CLA enriched PC | DHA enriched PC |
| 8:0               | -          | 25.3                       | -               | -               |
| 16:0              | 12.8       | 13.0                       | 13.0            | 12.2            |
| 18:0              | 3.9        | 3.2                        | 3.0             | 3.2             |
| 18:1              | 9.4        | 7.5                        | 9.1             | 8.3             |
| 18:2              | 65.5       | 45.3                       | 38.6            | 48.9            |
| 18:3              | 8.1        | 5.7                        | 6.4             | 7.3             |
| CLA (all isomers) | -          |                            | 30.0            | -               |
| 22:6              | -          |                            | -               | 20.2            |

2

3 <sup>a</sup> Reaction conditions: Reaction temperature, 45°C, Water addition, 2%; Substrate ratio, 3 mol/mol,  
 4 enzyme dosage, 30%; Reaction time, 48h.

5

1 Table 2. Carriers screened and their characteristics

| Carrier          | Supplier                                      | General description  |
|------------------|---|--|
| Amberlite XAD7   | Sigma-Aldrich Chemie GmbH, Steinheim, Germany | Nonionic weakly polar macroreticular resin (matrix: acylic ester), Particle size: 0.25-0.85 mm (wet)                               |
| Superlite DAX8   | Supelco, Bellefonte, USA                      | Resin with moderate polarity (matrix: acrylic ester), Particle size: 0.25-0.45mm   |
| Celite 545       | BHD, Poole, UK                                | Diatomaceous Earth, Particle size: 0.02-0.1 mm   |
| Dowex 50W        | Dow Chemical Company, Michigan, USA           | Strongly acidic cation exchange (maxtrix:resinstyrene-divinylbenzene; functional group: sulfonic acid), Particle size: 0.15-0.30mm |
| Lewatit VPOC1600 | Lanxess AG, Leverkusen, Germany               | Divinyl benzene crosslinked polymer (Marix: methacrylate), Particle size: 0.3-1.2 mm   |
| Duolite A568     | Rohn and Haas, Chauny, France                 | Polymerized phenol-formaldehyde anionic exchange resin, Particle size: 0.15-0.85 mm  |
| Accurel EP 100   | Akzo, Obernburg, Germany                      | Macroporous polypropylene, Particle size: 0.6-0.8 mm   |

2

3

1 Table 3. Fixation level of PLA2 on different carriers, and corresponding enzyme loading and specific  
 2 activity.

| Carrier <sup>a</sup> | Enzyme loading <sup>b</sup><br>(mg protein/g support) | Specific activity <sup>c</sup><br>( $\mu\text{mol mg}^{-1} \text{min}^{-1}$ ) |
|----------------------|---|---|
| Amberlite XAD7       | 49.0  | 0.30  |
| Superlite DAX8       | 44.4  | 0.28  |
| Duolite A568         | 43.3  | 0.24  |
| Dowex 50W            | 7.8   | -   |
| Celite 545           | 3.6   | -   |
| Accural EP 100       | 1.9   | -   |
| + Prewetting         | 42.5  | -   |
| Lewatit VPOC 1600    | 1.3   | -   |
| + Prewetting         | 40.3  | -   |

3  
 4 <sup>a</sup> Pre-wetting of Accural EP 100 and Lewatit VPOC 1600 were done by addition of 0.5 ml 96% ethanol/  
 5 g support immediately before immobilization; <sup>b</sup> Pooled SD = 1.0 mg protein/g support; <sup>c</sup> Pooled SD =  
 6 0.015  $\mu\text{mol mg}^{-1} \text{min}^{-1}$ ; - n.d., not determined.

1 Table 4 Settings of the RSM generated experimental design for the PLA<sub>2</sub> catalyzed acidolysis and  
 2 measured responses.

| Experiment no. | Factors        |                |                | Responses <sup>a</sup> |      |      |      |
|----------------|----------------|----------------|----------------|------------------------|------|------|------|
|                | T <sub>e</sub> | W <sub>a</sub> | S <sub>r</sub> | Inc.                   | PC   | LPC  | GPC  |
| 1              | 35             | 1              | 6              | 8.0                    | 70.0 | 22.1 | 7.9  |
| 2              | 55             | 1              | 6              | 5.9                    | 72.1 | 19.8 | 8.1  |
| 3              | 35             | 3              | 6              | 28.3                   | 20.6 | 62.4 | 17.0 |
| 4              | 55             | 3              | 6              | 32.9                   | 24.1 | 64.9 | 11.0 |
| 5              | 35             | 1              | 12             | 11.3                   | 62.4 | 28.1 | 9.5  |
| 6              | 55             | 1              | 12             | 7.2                    | 76.0 | 16.8 | 7.2  |
| 7              | 35             | 3              | 12             | 32.1                   | 22.4 | 63.8 | 13.8 |
| 8              | 55             | 3              | 12             | 28.6                   | 25.1 | 58.3 | 16.6 |
| 9              | 25             | 2              | 9              | 9.8                    | 54.8 | 40.2 | 5.0  |
| 10             | 65             | 2              | 9              | 3.1                    | 74.0 | 19.4 | 6.6  |
| 11             | 45             | 0              | 9              | 0.6                    | 90.0 | 3.0  | 7.0  |
| 12             | 45             | 4              | 9              | 30.5                   | 17.7 | 65.0 | 17.3 |
| 13             | 45             | 2              | 3              | 25.3                   | 29.2 | 62.3 | 8.5  |
| 14             | 45             | 2              | 15             | 35.0                   | 25.2 | 56.2 | 18.6 |
| 15             | 45             | 2              | 9              | 33.5                   | 30.7 | 56.4 | 12.9 |
| 16             | 45             | 2              | 9              | 35.9                   | 28.7 | 58.3 | 13.0 |
| 17             | 45             | 2              | 9              | 33.5                   | 30.3 | 60.4 | 9.3  |

3  
 4 Abbreviations: T<sub>e</sub>, Reaction temperature (°C); W<sub>a</sub>, water addition (wt% based on total substrate); S<sub>r</sub>,  
 5 substrate ratio (mol Caprylic acid/mol PC), Inc., Incorporation of caprylic acid (mol%), PC,  
 6 phosphatidylcholine content; LPC, lysophosphatidylcholine content; GPC, glycerophosphorylcholine  
 7 content. <sup>a</sup>Values reported for the PL distribution are based on weight percentages of PC + LPC+ GPC

8

1 Table 5 Regression coefficients and P-values describing the influence of different parameters on  
 2 incorporation of caprylic acid into PC and PL distribution<sup>a</sup>.

| Term     | Incorporation of caprylic acid<br>into PC (mol%) |                        | PL distribution (wt%) |                        |             |                        |             |                       |
|----------|--|------------------------|-----------------------|------------------------|-------------|------------------------|-------------|-----------------------|
|          | Regression                                       |                        | PC                    |                        | LPC         |                        | GPC         |                       |
|          | coefficient                                      | P-value                | coefficient           | P-value                | coefficient | P-value                | coefficient | P-value               |
| Constant | 32.38  | $9.97 \times 10^{-10}$ | 28.37                 | $1.45 \times 10^{-9}$  | 58.72       | $3.58 \times 10^{-12}$ | 12.91       | $9.68 \times 10^{-7}$ |
| Te       | -2.07  | 0.06                   | 5.22                  | $1.52 \times 10^{-4}$  | -4.81       | $6.95 \times 10^{-4}$  | -0.40       | 0.62                  |
| Wa       | 8.50   | $5.32 \times 10^{-6}$  | -19.38                | $8.81 \times 10^{-10}$ | 16.55       | $1.32 \times 10^{-8}$  | 2.83        | $5.13 \times 10^{-3}$ |
| Te x Te  | -6.51  | $7.83 \times 10^{-6}$  | 8.83                  | $2.01 \times 10^{-7}$  | -7.18       | $4.10 \times 10^{-6}$  | -1.65       | 0.03                  |
| Wa x Wa  | -4.33  | $2.38 \times 10^{-4}$  | 6.19                  | $5.34 \times 10^{-6}$  | -6.16       | $1.59 \times 10^{-5}$  | -0.03       | 0.97                  |
| Te x Wa  | 1.87   | 0.22                   | -3.72                 | 0.02                   | 3.60        | 0.03                   | 0.12        | 0.92                  |

3  
 4 <sup>a</sup>Values reported for the PL distribution are based on weight percentages of PC + LPC+ GPC. The effect  
 5 of each factor (linear and quadratic) and interaction effects are statistically significant when P-value<0.05.



1 **Figure legend:**

- 2 Figure 1: Schematic presentation of PLA<sub>2</sub>-catalyzed acidolysis of phospholipid  
3 with free fatty acid. R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> refer to fatty acids and x refers to  
4 phospholipid head group (e.g. choline).
- 5 Figure 2: Influence of initial enzyme/support ratio on fixation level to Amberlite  
6 XAD7. Varying amounts PLA<sub>2</sub> were incubated in the presence of 250  
7 mg carrier. Bars represents mean ± pooled SD.
- 8 Figure 3: Bioconversion efficiency of PLA<sub>2</sub> immobilized Amberlite XAD7. A)  
9 Influence on enzymatic loading on activity and specific activity of  
10 immobilized system with different fixation level (mg enzyme per g  
11 support). B) Influence on enzymatic loading on activity and specific  
12 activity of immobilized system with same fixation level. Enzymatic  
13 assay and PLA<sub>2</sub> activity measurement were performed according to  
14 procedure described in material and methods. Bars represent mean ±  
15 pooled SD (n=2).
- 16 Figure 4: Time course for acidolysis reaction between PC and caprylic acid in  
17 solvent free system. Reaction conditions: substrate ratio, 6 mol/mol  
18 caprylic acid/PC, water addition, 0.75%; dosage of immobilized enzyme,  
19 30 wt%; and reaction temperature, 40°C. A) Incorporation of caprylic  
20 acid into PC and B) PL distribution. Bars represent mean ± pooled SD  
21 (n=2).
- 22 Figure 5: Effect of water addition on PLA<sub>2</sub> catalyzed acidolysis reaction when  
23 varied from low to a high level with all other factors being on their

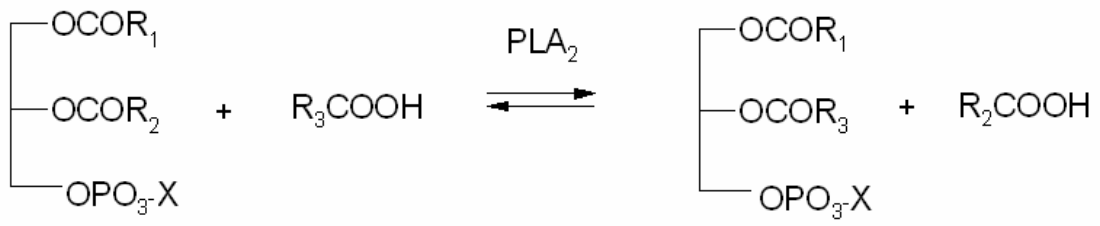
1 average. A) Incorporation of caprylic acid into PC and B) PL  
2 distribution. Error bars indicate 95% confidence interval.

3 Figure 6: Effect of reaction temperature on PLA<sub>2</sub> catalyzed acidolysis reaction  
4 when varied from low to a high level with all other factors being on their  
5 average. A) Incorporation of caprylic acid into PC and B) PL  
6 distribution. Bars indicate 95% confidence interval.

7

8

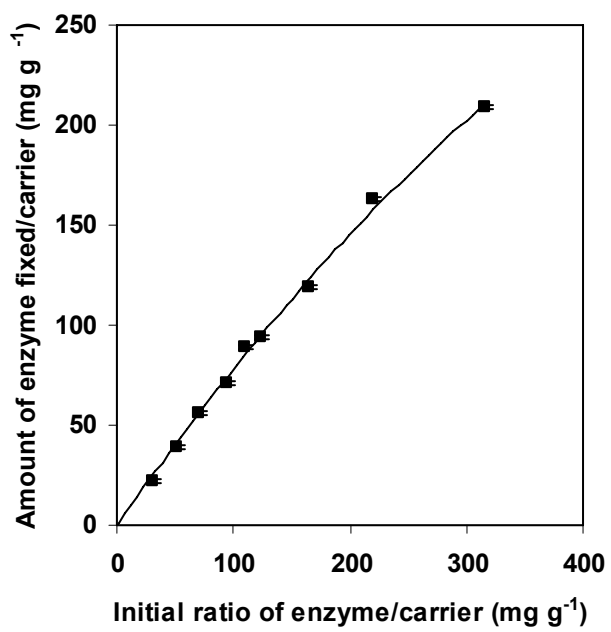
1 Figure 1



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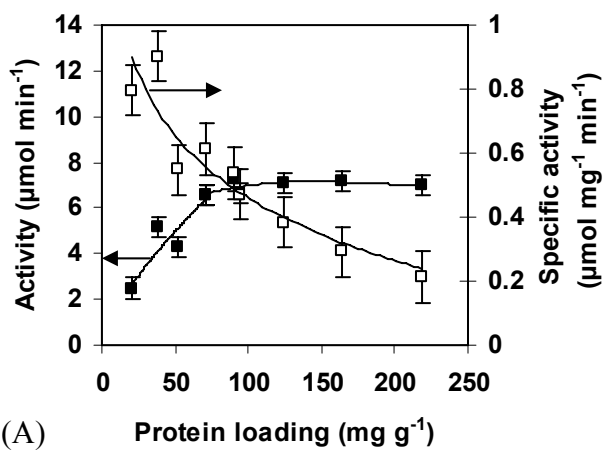
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1 Figure 2

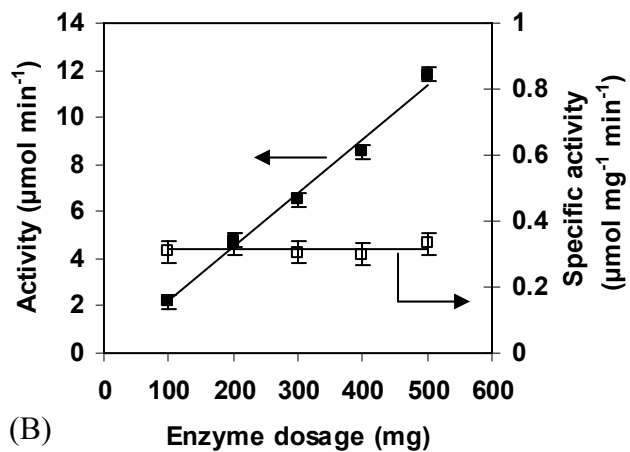


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1 Figure 3



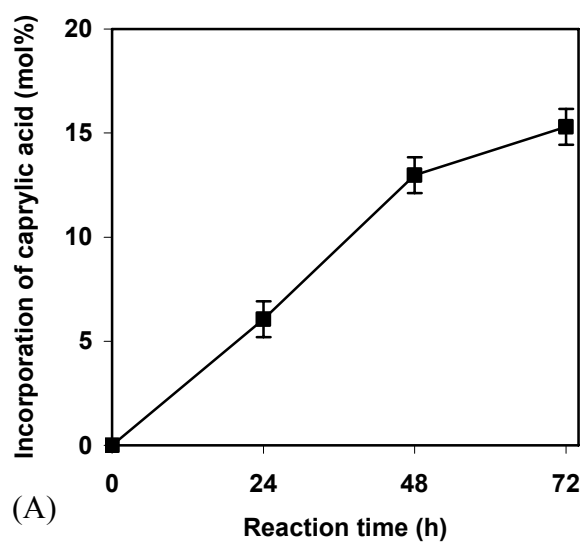
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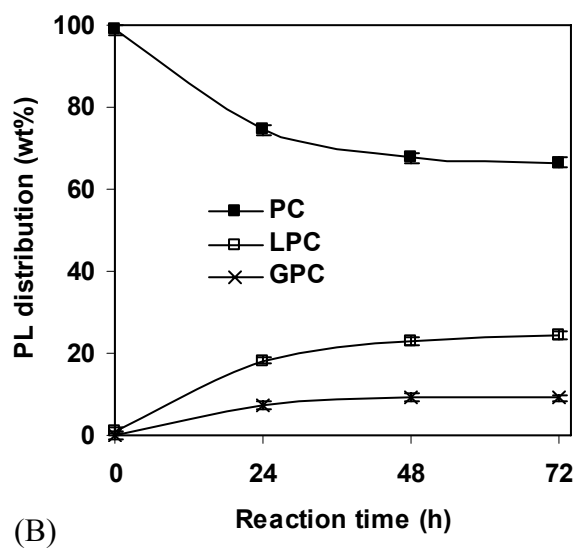
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1 Figure 4



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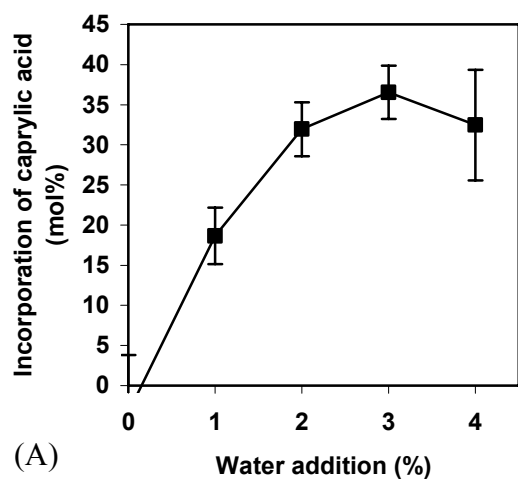
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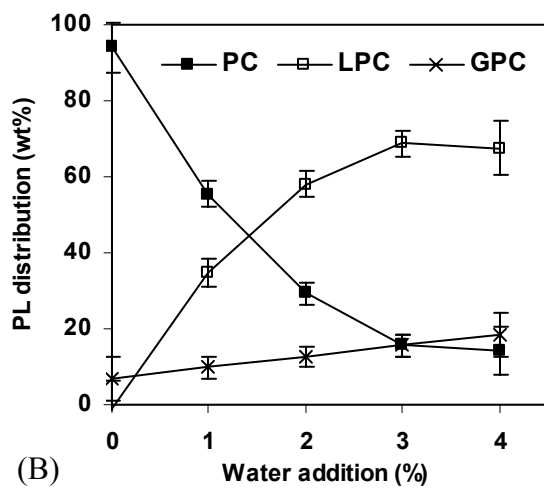
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1 Figure 5



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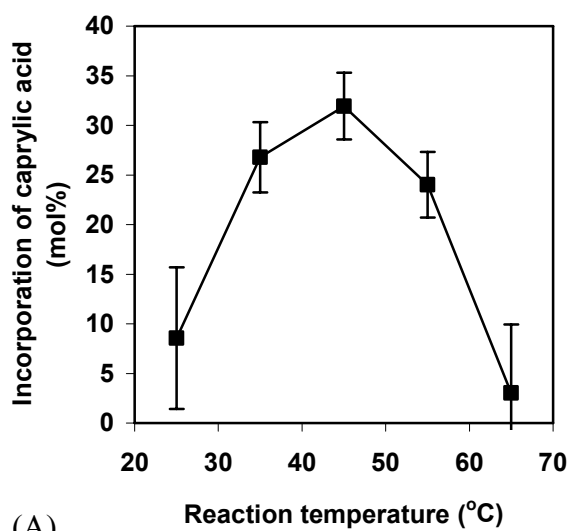
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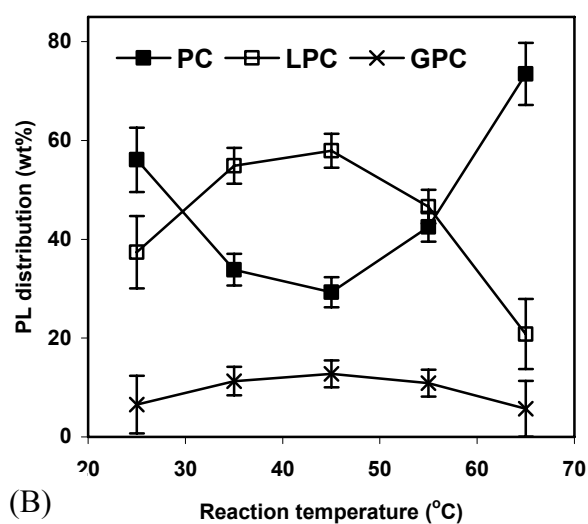
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1 Figure 6



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