Increase of Oleic Acid Content in Phosphatidylcholine through Lipase-catalyzed Interesterification: Optimization by Response Surface Methodology

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Abstract: In order to obtain phosphatidylcholine (PC) with higher amount of oleic acid, the interesterification between soybean PC and Camellia oleifera oil (COO) rich in oleic acid catalyzed by lipase was studied in hexane. For this aim three commercially available immobilized lipases (Novozym 435, Lipozyme TLIM and Lipozyme RMIM) were assayed and Novozym 435 was finally selected for further optimization. The effects of the factors, such as PC concentration, substrate ratio, water amount, lipase dosage and temperature, on the oleic acid content in PC and PC recovery during the interesterification were investigated. The conditions of the interesterification were optimized using response surface methodology. The optimum conditions were as follows: lipase dosage 13 % (based on the mass of PC and COO), reaction temperature 55°C, water amount 5% (based on the mass of PC), reaction time 8 h, PC concentration 0.3g/mL (PC/hexane), PC-to-COO ratio 1:3 (acyl groups in PC/acyl groups in COO, mol/mol). Under these conditions, oleic acid content and PC recovery were 40.8 ± 0.5% and 69.0 ± 2.8%, respectively. Analysis of variance (ANOVA) showed that the regression models were adequate for predicting the interesterification. The orders of reaction variables affecting on oleic acid content and PC recovery were water amount > reaction time > lipase dosage > reaction temperature, and water amount > reaction temperature > lipase dosage > reaction time, respectively.

Key words: phosphatidylcholine, Camellia oleifera oil, lipase-catalyzed interesterification, oleic acid

1 Introduction

Phospholipids (PLs) are major constituents of cell membranes and play important roles in the biochemistry and physiology of cells<sup>1</sup>, they are widely used in the food, pharmaceutical, and cosmetic products playing the role of emulsifiers, stabilizers, and antioxidants<sup>2–4</sup>. Some oilseeds and egg yolks are particularly rich in PLs, usually fatty acid composition of plant PLs was more dependent on their source<sup>5</sup>, so there were some limitations in their properties and applications. Therefore, interest in changing the fatty acid composition of the phospholipids has significantly increased in recent years.

At present, many studies reported the incorporation of polyunsaturated fatty acids, such as linoleic acid, linolenic acid, EPA, DHA, into PLs<sup>6–9</sup>. The synthesis of PLs containing saturated fatty acids had also been reported<sup>10–11</sup>. However, there were few reports on the incorporation of monounsaturated fatty acids into phospholipids. Mustranta et al.<sup>12</sup> synthesized structured PC through lipase catalyzed interesterification between dimyristoyl PC and oleic acid. Oleic acid is the most representative monounsaturated fatty acids, some reports pointed out that monounsaturated fatty acids (mainly oleic acid) had the function of lowering serum lipids, plasma cholesterol, protecting the heart, lowering blood sugar, regulating blood fat, lowering cholesterol and preventing cardiovascular disease<sup>13,14</sup>. Incorporation of oleic acid into phospholipid may enhance the function of phospholipids. Furthermore, fatty acids could be more easily absorbed in the body as PLs than as their corresponding triglycerides or ethyl esters<sup>15</sup>. In the previous reports, the structured PLs were prepared by transesterification of PLs and free fatty acids, the free fatty acids were derived from oils by saponification and acidification<sup>6–12</sup>. If the structured PLs were produced by interesterification of PLs and oils, the saponification and acidification could be omitted, so the preparation process of the structured PLs...
was simplified.

Camellia oleifera oil (COO) is a rich source of oleic acid in the form of triacylglycerols (TAGs). In our study, we prepare PC containing higher level of oleic acid by lipase-catalyzed interesterification of soybean PC and COO. The purpose is to prepare PC as enriched in oleic acid as possible and, simultaneously, to maintain the highest PC recovery. Three commercially available immobilized lipases (Novozym 435, Lipozyme TLIM and Lipozyme RMIM) are assayed and Novozym 435 were used to catalyze the interesterification between PC and COO. The effects of the various parameters such as PC concentration, substrate ratio, water amount, lipase dosage and reaction temperature on the interesterification were investigated. The reaction system was optimized in a solvent system. Response surface methodology (RSM) was used to evaluate the effect of several variables on the oleic acid content in modified PC and PC recovery.

2 Materials and methods

2.1 Materials

Soy phosphatidylcholine (PC), Shenyang Tianfeng Biological Engineering Technology Co., Ltd; Camellia oleifera oil (COO), Yunnan Guangnan tianyun vegetable Co., Ltd, and the fatty acid composition was shown in table.1. Lipoyzoyme 435 (from Candida antarctica), Lipozyme RMIM (from Rhizomucor miehei), and Lipozyme TLIM (from Thermomycyes lanuginosus) were provided by Novozymes China (Beijing, China). Solvents (chloroform, methanol, boron trifluoride-diethyl ether, hexane and acetic acid) and other chemicals used were purchased from local factory.

2.2 Interesterification of PC and COO

In a typical procedure, 1.5 g PC, COO (PC-to-COO ratio 1:1-1:4, acyl groups in PC/acyl groups in COO, mol/mol), hexane (0.05-0.3 g/mL, PC/hexane) and water (2-6%, relative to the weight of PC) were placed into 50 mL round-bottomed flasks. The reactions were catalyzed by different enzymes (Novozym 435, Lipozyme TLIM, Lipozyme RMIM) (5-20%, relative to the weight of the PC and COO). The mixture was agitated on a magnetic-stirrer plate at 55–65 °C. The reactions were stopped at the selected time. Once the reaction was stopped, the product mixture was filtered through a membrane microfilter to remove lipase. All trials were carried out in duplicate.

2.3 Fatty Acid composition Analysis of PC

Samples (50 μL) from the product mixture were diluted with chloroform, and oleic PCs in the diluted samples were separated from the reaction resultant by thin layer chromatography (TLC) using the mixture of chloroform/methanol/water (65:35:5, v/v/v). The TLC plate was visualized under UV light after spraying with a 0.2% ethanol solution of 2,7-dichlorofluoresein sodium salt. Then the PC band was scraped off and stored in 50 mL round-bottomed flask. 6.0 mL of 0.5 M sodium hydroxide/methanol was added into the flask, and refluxed at 80°C for 7 min, and then 7.0 mL boron trifluoride/methanol was added to the flask to incubation for 1 min. Then 3 mL hexane was added. The flask was removed and an amount of saturated sodium chloride aqueous solution was added to the bottle, upside down several times and standing layer, the upper layer dried by anhydrous Na2SO4 was taken for GC analysis.

Fatty acid methyl esters were analyzed on a Agilent 6890 N gas chromatograph with a flame ionization detector and capillary column (BPX-70, 30.0 m*25 μm*0.25 μm, SGE, Australia). The column temperature was 190 °C, and the flow rate of carrier gas (N2) was 1.2 mL/min. The injector and detectors were maintained at 210 and 300 °C, respectively.

2.4 Phospholipid Profile Analysis

The phospholipid profile of the product mixtures were analyzed according to the method of Vikbjerg et al. with slight modification. The samples were diluted with chloroform, and the diluted samples (1 μL) were spotted to Chromarods SIII (Iatron Laboratories Inc., Tokyo, Japan) and developed by a mixture of chloroform/methanol/water (42:22:2.5, v:v:v). After the development, Chromarods were dried at 120°C for 5 min, and the composition of the product mixture was analyzed by TLC-FID (Iatroscan MK6s, Iatron Laboratories). Flow rates of air and hydrogen were 2 L/min and 160 mL/min, respectively. Peaks were identified by external standards.

PC recovery (%) = 100 × weight of PC in the product/original weight of PC

2.5 Experimental design

A 3-level-4-factor Box-Behnken experimental design was employed to investigate the effect of reaction variables on the interesterification. The variables and levels selected for interesterification were reaction time (2, 6, 10 h), reaction temperature (45, 55, 65°C), lipase dosage (10, 15%, 20%), and water amount (2, 4, 6%) (Table 1).

2.6 Statistical analysis

The data were analyzed by response surface methodology, the fit of the model was evaluated by coefficients of determination (R2) and the analysis of variance (ANOVA). The mathematical relationship between the variables and the responses can be calculated by the quadratic polynomial equation:

\[ y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_1x_2 + \beta_5x_1x_3 + \beta_6x_2x_3 + \beta_7x_1^2 + \beta_8x_2^2 + \beta_9x_3^2 + \epsilon \]
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Y = β₀ + ∑ᵢ βᵢXᵢ + ∑ᵢᵢ βᵢᵢXᵢ² + ∑ᵢⱼ βᵢⱼXᵢXⱼ

where Y is response (incorporation or PC recovery); Xᵢ and Xⱼ represent the independent variables; β₀ is the intercept, βᵢ are the linear coefficients, βᵢᵢ are the quadratic coefficients for the variable i, and βᵢⱼ represent the linear coefficients for interaction between variable i and j.

3 Results and Discussion

3.1 Lipase screening

Three commercial lipases were evaluated with a dosage of 20% for high content of oleic acid in to PC (Fig. 1). The lipases were screened to allow for direct large-scale production and scale-up. Novozym 435 from Candida Antarctica, which is immobilized on on macroporous acrylic resin, showed the highest activity for the interesterification. The oleic acid content of modified PC was about 38% at 8 h, increasing of oleic acid was more than 25%. Whereas Lipozyme TLIM and Lipozyme RMIM exhibited poor activity for this reaction, The oleic acid contents of PCs modified Lipozyme TLIM and Lipozyme RMIM were 17.8% and 19.6% at 8 h, respectively; and increases of oleic acid were 6.6% and 8.4%, respectively. Therefore, we selected Novozym 435 as the biocatalyst for the preparation of oleic rich PC. However, Peng et al. reported Lipozyme TLIM exhibited the highest activity for acidolysis of phospholipids and caprylyl acid comparison to Lipozyme RMIM and Novozym 435.

3.2 Effect of independent variables

With the increase of lipase dosage from 5% to 15%, oleic acid content in modified PC increased (Fig. 2a). With the increase of water addition from 2% to 6%, oleic acid content in modified PC increased (Fig. 2b). PC concentration had slight effect on the oleic acid content of the modified PC during the interesterification (Fig. 2c). With the increase of PC-to-COO ratio from 1:1 to 1:3, oleic acid content in modified PC increased (Fig. 2d), and 1:3 PC-to-COO ratio was chosen for further study. Oleic acid content in modified PC rapidly increased with reaction temperature increasing from 50°C to 55°C, however with the increase of temperature from 55°C to 65°C, oleic acid content efficiently decreased (Fig. 2e).

3.3 Model fitting

The objective was to obtain as high content of oleic acid in modified PLs as possible and, simultaneously, to maintain the highest PC recovery. RSM was an empirical method used to assay the relation a set of controllable experimental factors and the observed values. Models of the factors and the responses were performed by RSM to predict the highest possible oleic acid content in modified PC and PC recovery. The results obtained for the models were listed in Table 2. The data were analyzed by employ-

Table 1  Major fatty acids profile of PC, Camellia oleifera oil, and modified PC.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>PC</th>
<th>Camellia oleifera oil</th>
<th>Modified PCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid (16:0)</td>
<td>13.26 ± 0.13</td>
<td>7.83 ± 0.18</td>
<td>5.57 ± 0.45</td>
</tr>
<tr>
<td>Palmitooleic acid (C16:1)</td>
<td>0.07 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>4.06 ± 0.07</td>
<td>2.77 ± 0.02</td>
<td>1.74 ± 0.17</td>
</tr>
<tr>
<td>Oleic acid (C18:1)</td>
<td>11.21 ± 0.16</td>
<td>81.62 ± 0.37</td>
<td>42.32 ± 0.79</td>
</tr>
<tr>
<td>Linoleic acid (C18:2)</td>
<td>65.00 ± 0.33</td>
<td>7.38 ± 0.07</td>
<td>46.30 ± 0.69</td>
</tr>
<tr>
<td>Linolenic acid (C18:3)</td>
<td>6.39 ± 0.05</td>
<td>0.26 ± 0.01</td>
<td>3.90 ± 0.06</td>
</tr>
</tbody>
</table>

*a purified through column chromatography (silica G) eluted by chloroform-methanol-water (65:35:5, v/v/v).

Fig. 1  Oleic acid content in modified PC through the interesterifications catalyzed by various lipases. Temperature 55°C, 50°C, 50°C for Novozym 435, Lipozyme TLIM and Lipozyme RMIM respectively, lipase dosage 20% (based on the mass of PC and COO), ratio of PC to COO 1:2 (mol/mol), PC concentration 0.2 (g/mL), water amount 4% (based on the mass of PC).
Fig. 2 Effects of the lipase dosage, water amount, PC concentration, PC to COO ratio, and temperature on oleic acid content in modified PC through Novozym 435 catalyzed interesterification. a reaction temperature 55°C, water amount 4% (based on the mass of PC), PC concentration 0.2 g/mL (PC/hexane), PC-to-COO ratio 1:2 (acyl groups in PC/acyl groups in COO, mol/mol). b lipase dosage 15% (based on the mass of PC and COO), reaction temperature 55°C, PC concentration 0.2 g/mL (PC/hexane), PC-to-COO ratio 1:2 (acyl groups in PC/acyl groups in COO, mol/mol). c lipase dosage 15% (based on the mass of PC and COO), reaction temperature 55°C, water amount 4% (based on the mass of PC), PC-to-COO ratio 1:2 (acyl groups in PC/acyl groups in COO, mol/mol). d lipase dosage 15% (based on the mass of PC and COO), reaction temperature 55°C, water amount 4% (based on the mass of PC), PC concentration 0.3 g/mL (PC/hexane), PC-to-COO ratio 1:3 (acyl groups in PC/acyl groups in COO, mol/mol).
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ing a multiple regression technique to evaluate the true relationship between the variables and the responses. Two second-order models for Ia of oleic acid and PC recovery were satisfactorily established. Y₁ and Y₂ were the predicted values for the oleic acid content (%) and PC recovery (%), respectively, and X₁, X₂, X₃, and X₄ were the coded variables as described in Table 2.

\[
Y₁ = 37.71 + 4.37X₁ + 1.87X₂ + 8.61X₃ + 5.32X₄ - 1.66X₁X₂ - 0.76X₁X₃ - 0.37X₁X₄ - 2.93X₂X₃ - 1.01X₂X₄ - 2.06X₃X₄ - 3.49X₁² - 3.08X₂² - 4.80X₃² - 3.99X₄² \quad (1)
\]

\[
Y₂ = 78.59 - 1.08X₁ - 3.31X₂ - 15.40X₃ + 0.096X₄ + 4.51X₁X₂ - 5.08X₁X₃ + 0.77X₁X₄ + 0.45X₂X₃ + 0.95X₂X₄ - 2.09X₃X₄ - 5.50X₁² - 3.62X₂² + 1.54X₃² - 4.82X₄² \quad (2)
\]

The statistical significance of regression equation was checked by F-test, and ANOVA for response surface quadratic polynomial models were summarized in Table 3 and Table 4. The high F-values (27.22 and 6.18 for oleic acid content and PC recovery, respectively) and low p-values (p < 0.0001 for oleic acid content and 0.0008 for PC recov-
Table 3 Analysis of variance (ANOVA) for response surface quadratic model pertaining to the predicted oleic acid content.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degree of freedom</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>1825.68</td>
<td>14</td>
<td>130.41</td>
<td>27.22</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>Lipase dosage (X₁)</td>
<td>228.9</td>
<td>1</td>
<td>228.9</td>
<td>47.78</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>reaction temperature (X₂)</td>
<td>41.78</td>
<td>1</td>
<td>41.78</td>
<td>8.72</td>
<td>0.0105</td>
<td>*</td>
</tr>
<tr>
<td>Water amount (X₃)</td>
<td>889.41</td>
<td>1</td>
<td>889.41</td>
<td>185.64</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>reaction time (X₄)</td>
<td>339.31</td>
<td>1</td>
<td>339.31</td>
<td>70.82</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>X₁ X₂</td>
<td>11.09</td>
<td>1</td>
<td>11.09</td>
<td>2.31</td>
<td>0.1504</td>
<td></td>
</tr>
<tr>
<td>X₁ X₃</td>
<td>2.33</td>
<td>1</td>
<td>2.33</td>
<td>0.49</td>
<td>0.4974</td>
<td></td>
</tr>
<tr>
<td>X₁ X₄</td>
<td>0.53</td>
<td>1</td>
<td>0.53</td>
<td>0.11</td>
<td>0.7437</td>
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</tr>
<tr>
<td>X₂ X₃</td>
<td>34.46</td>
<td>1</td>
<td>34.46</td>
<td>7.19</td>
<td>0.0179</td>
<td>*</td>
</tr>
<tr>
<td>X₂ X₄</td>
<td>4.1</td>
<td>1</td>
<td>4.1</td>
<td>0.86</td>
<td>0.3706</td>
<td></td>
</tr>
<tr>
<td>X₃ X₄</td>
<td>16.97</td>
<td>1</td>
<td>16.97</td>
<td>3.54</td>
<td>0.0808</td>
<td></td>
</tr>
<tr>
<td>X₁²</td>
<td>78.8</td>
<td>1</td>
<td>78.8</td>
<td>16.45</td>
<td>0.0012</td>
<td>**</td>
</tr>
<tr>
<td>X₂²</td>
<td>61.45</td>
<td>1</td>
<td>61.45</td>
<td>12.83</td>
<td>0.003</td>
<td>**</td>
</tr>
<tr>
<td>X₃²</td>
<td>149.32</td>
<td>1</td>
<td>149.32</td>
<td>31.17</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>X₄²</td>
<td>103.03</td>
<td>1</td>
<td>103.03</td>
<td>21.5</td>
<td>0.0004</td>
<td>**</td>
</tr>
<tr>
<td>Residual</td>
<td>67.07</td>
<td>14</td>
<td>4.79</td>
<td></td>
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</tr>
<tr>
<td>Lack of Fit</td>
<td>62.14</td>
<td>10</td>
<td>6.21</td>
<td>5.03</td>
<td>0.0667</td>
<td></td>
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<tr>
<td>Pure Error</td>
<td>4.94</td>
<td>4</td>
<td>1.23</td>
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<tr>
<td>Cor Total</td>
<td>1892.76</td>
<td>28</td>
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</tr>
</tbody>
</table>

* Significant at the 5% level; ** Significant at the 1% level

The relationship between independent variables and response can be better understood by examining the planned series of 2D contour plots (Fig. 3 and 4) generated from the predicted model. Figure 3a and 4a show the effect of lipase dosage, reaction temperature, and their mutual interaction on oleic acid content and PC recovery, respectively. The maximum value of oleic acid content appears in the temperature range of 45-55°C and lipase dosage range of 12-25%, whereas the oleic acid content increases, and then decreases. Figure 3b and 4b show the effect of lipase dosage, reaction time, and their mutual interaction on oleic acid content and PC recovery, respectively. At the same lipase dosage, with the increase of water amount, the PC recovery decreases, whereas the oleic acid content increases, and then decreases.
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3.4 Attaining optimum condition and model verification

Reaction conditions optimized by RSM were as follows: lipase dosage 12.8%, reaction temperature 54.5°C, water amount 4.8%, reaction time 8 h. In the actual work, these variables values were corrected as follows: lipase dosage 13%, reaction temperature 55°C, water amount 5%, reaction time 8 h. Under these conditions, a high oleic acid content (40.8 ± 0.5%) and a relatively high PC recovery (69.0 ± 2.8%) were achieved, which were well accorded with the predicted results 40.6% and 71.6%, respectively. The results indicated the feasibility and accuracy of the quadratic regression model.

4 Conclusions

PC containing higher level of oleic acid was successfully prepared through interesterification of PC and COO catalyzed by Novozym 435 in hexane. The optimum conditions were as follows: lipase dosage 13% (based on the mass of PC and COO), reaction temperature 55°C, water amount 5% (based on the mass of PC), reaction time 8 h, PC content increases, then decreases.
Fig. 3 Contour plots showing the effects of variables on oleic acid content. (a) lipase dosage and reaction temperature with water amount 4% at 6 h. (b) lipase dosage and water amount with temperature 55°C at 6 h. (c) lipase dosage and reaction time with temperature 55°C and water amount 4%. (d) reaction temperature and water amount with lipase dosage 15% at 6 h. (e) reaction temperature and reaction time with lipase dosage 15% and water amount 4%. (f) water amount and reaction time with lipase dosage 15% and temperature 55°C.
Fig. 4  Contour plots showing the effects of variables on PC recovery. (a) lipase dosage and reaction temperature with water amount 4 % at 6 h. (b) lipase dosage and water amount with temperature 55°C at 6 h. (c) lipase dosage and reaction time with temperature 55°C and water amount 4 %. (d) reaction temperature and water amount with lipase dosage 15% at 6 h. (e) reaction temperature and reaction time with lipase dosage 15% and water amount 4 %. (f) water amount and reaction time with lipase dosage 15% and temperature 55°C.
centration 0.3 g/mL (PC/hexane), PC-to-COO ratio 1:3 (acyl groups in PC/acyl groups in COO, mol/mol). Under these conditions, oleic acid content and PC recovery were 40.8 ± 0.5% and 69.0 ± 2.8%, respectively. The orders of reaction variables affecting on oleic acid content and PC recovery were water amount, reaction time, lipase dosage, reaction temperature, and water amount, reaction time, respectively.

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