Optimization of High EPA Structured Phospholipids Synthesis from ω-3 Fatty Acid Enriched Oil and Soy Lecithin

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Abstract: The molecular structure of phospholipids can be changed enzymatically to obtain different tailor-made phospholipids. Incorporation of ω-3 fatty acids into phospholipids structure increased their oxidative stability, suggesting more health beneficial phospholipids. This study aimed to optimize eicosapentaenoic acid (EPA) incorporation into phospholipids structure by acidolysis reaction using free lipase (EC 3.1.1.3) from Rhizomucor miehei. Deoiled soy lecithin from anjasmoro variety was used as phospholipids source, while ω-3 fatty acid enriched oil was used as acyl source. Oil enriched with ω-3 fatty acids was obtained from low temperature solvent crystallization of lemuru (Sardinella longiceps) by-product. Response surface methodology (RSM) was used in this study to determine the relationship between the three factors (enzyme concentration, reaction time and substrate ratio) and their effects on EPA incorporation into soy lecithin structure. The results showed that the relation between EPA content with three factors (reaction time, enzyme concentration and substrate ratio) was quadratic. The significant factors were substrate ratio and reaction time. Optimum conditions at a ratio of 3.77:1 between ω-3 fatty acids enriched oil and soy lecithin, 30% lipase concentration, and 24.08 h reaction time, gave 22.81% of EPA content of structured phospholipids.

Key words: Structured phospholipids, enzymatic acidolysis, EPA, lipase, deoiled soy lecithin, ω-3 fatty acids enriched oil, lemuru.

1. Introduction

Soy lecithin or phospholipids (PL) is widely used as an emulsifier in food industries. Soy lecithin comprises of 18% phosphatidylcholine (PC), 14% phosphatidylethanolamine (PE), 9% phosphatidylinositol (PI), 2% phosphatidic acid (PA), other minor phospholipids, 11% glycolipid, and 27% neutral lipid [1]. Some studies reported that the major fatty acid in soy lecithin was linoleic acid [2, 3]. Modification is usually performed to obtain desired functional properties of lecithin. The molecular structure of phospholipids can be changed enzymatically or chemically to obtain different tailor-made technological and/or physiological properties compared to those of natural substrate. Enzymatic modification being of interest as enzymes can be used to modify phospholipids in different ways [4].

Structured phospholipids with certain fatty acid profile can be obtained by enzymatic synthesis [5]. This synthesis process gives benefits as the reaction conditions are not extreme and the enzyme is highly specific [6]. Incorporation of ω-3 fatty acids into phospholipids structure increases their oxidative stability [7], suggesting more health beneficial
phospholipids. The health beneficial effects of ω-3 PUFA are currently well known and have been almost exclusively attributed to cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) [8, 9]. The beneficial effects of EPA include reductions in heart diseases severity and various inflammatory disorders [10, 11]. EPA has been used in the prevention and treatment of atherosclerosis, arthritis, thrombosis, inflammation, diabetes, and cancer [12]. EPA also exhibits various physiological functions on being incorporated into membrane phospholipids [13, 14].

Some studies showed that enzymatic incorporation of ω-3 fatty acids into phospholipids structure used lipase or phospholipase. A mobile lipase from Rhizomucor meihei (Lipozyme TM) with 1,3 regiospecific had been used by Haraldsson and Thorarensen [15] in PC containing EPA and DHA synthesis. Recent research suggested egg yolk was used as a source of PC and acyl source was ω-3 fatty acids concentrates. However, information on structured phospholipids synthesis using specific EPA incorporation is still lacking.

Almost all of the acidolysis studies in structured phospholipids synthesis used certain type of phospholipids, such as PC as a substrate [6, 15-20]. However, studies on structured phospholipids synthesis using a mixture of phospholipids such as soy lecithin are still limited. Therefore, there is a call for research in different properties of phospholipid forms in soy lecithin.

In general, enzyme used in structured phospholipids synthesis is specific 1,3-lipase and PLA2, which can modify fatty acid in sn-1 and sn-2 positions. Incorporation of such fatty acid into phospholipids structure can be controlled during reaction [21]. Some factors that may affect acidolysis reaction in ω-3 fatty acids incorporation into phospholipids structure are enzyme concentration, reaction time, and ratio of acyl to phospholipids [15]. While enzymes used for structured phospholipids synthesis are lipase that may derive from Rhizomucor meihei [15, 17] or Thermomyces lanuginose [5, 21], and phospholipase A1 [12].

This study aimed to optimize EPA incorporation into soy lecithin from anjasmoro soybean variety. Anjasmoro is one of improved varieties cultivated in Indonesia that has high productivity (2.3 t/ha) and tolerant to puddle. As a acyl source, we used ω-3 fatty acid enriched oil that was prepared from a by-product of Lemuru (Sardinella longiceps) canning processing using low temperature solvent crystallization. This study focused on the generation of soy lecithin that is highly enriched with EPA from inexpensive EPA source. Response surface methodology (RSM) was used in this study to determine the relationship between the three factors (enzyme concentration, reaction time and substrate ratio) and their effect on EPA incorporation into soy lecithin structure during lipase-catalyzed acidolysis.

2. Materials and Methods

2.1 Materials

Crude soy lecithins were obtained from water degumming of soybean oil extracted from anjasmoro variety. Lemuru (Sardinella longiceps) oil as a by-product of fish meal processing was purchased from a local fish meal plant. ω-3 fatty acids enriched oil was obtained using low solvent crystallization of hydrolyzed lemuru oil. Other materials were lipase derived from Rhizomucor meihei, fatty acid methyl ester mixture, phospholipids standard, and BF3-methanol 14% (Sigma Co.), other chemical reagents, and TLC plate (silica gel G60 F254) (Merck).

2.2 Soy Lecithin Preparation from Anjasmoro Soybean Variety

Lecithin extraction was performed according to Eshratabadi [22] with a minor modification. Lecithin was extracted from soybean oil using water degumming. About 20 g ± 0.1 mg of soybean oil was placed in a beaker glass and 10% (w/v reaction mixture) distilled water and 3 mL of hexane was
added. The mixture was stirred and heated at 60 °C for 30 min. Oil and water phase were separated by centrifugation at 300 rpm for 30 min. Gum or crude lecithin was formed in the bottom layer (subnatant) and then dried in a vacuum oven at 25 °C and 1 atm for 1 h.

This dried crude lecithin was then purified using the method employed by Nasir et al. [23] to remove neutral oil. Acetone was added into the crude lecithin with the ratio of 6:1 (v/w). The mixture was stirred for 1 h. The solvent was decanted to separate the lecithin. This treatment was repeated until the solvent was colorless. Lecithin was flushed with nitrogen to discard residual solvent.

2.3 Acidolysis Reaction of Oil Enriched with ω-3 Fatty Acids and Deoiled Soy Lecithin

This activity was referred to Haraldsson and Thorarensen [15] with a slight modification. Oil enriched with ω-3 fatty acids and soy lecithin (phospholipids) were put into a number of reaction tubes at different ratios as shown in Table 1. Free enzyme of Lipzyme (*Rhizomucor miehei*) at different concentrations (Table 1) were subsequently added. The mixture was shook slowly at 300 rpm in a shaking water bath with temperature of 40 °C for different reaction times (Table 1). Afterwards, the mixture was centrifuged at 1,500 rpm for 15 min to precipitate structured phospholipids. Then the precipitate was washed with 1 mL hexane to discard residual free fatty acid. The optimized response in this study was EPA content in structured phospholipids. Data were analyzed using Design Expert 7.0 Trial Version. Structured phospholipids from optimum conditions were analyzed for Hydrophilic Lipophilic Balance (HLB) (saponification number and acid value) according to the method of AOCS [24], phospholipids profile, and fatty acid profile.

2.4 Analysis of Phospholipids

Phospholipids analysis was performed using TLC according to Nzai and Proctor’s method [25] with

<table>
<thead>
<tr>
<th>No.</th>
<th>Actual variable</th>
<th>Coded variable</th>
<th>Response of EPA content (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Substrate ratio*</td>
<td>Enzyme concentration**</td>
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<tr>
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</tr>
<tr>
<td>20</td>
<td>3.5:1</td>
<td>20</td>
<td>13.75</td>
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</table>

*oil enriched with ω-3 fatty acids to deoiled soy lecithin, ***% w/w substrate.
slight modification. About 1 mg of sample or standard was dissolved in chloroform:methanol (95:5 v/v) and 10 µL of sample or standard solution was spotted into TLC plate. The TLC plate was activated by heating for 10 min at 100 °C prior to analysis. The plates were developed for 40 min in a mobile phase solution containing chloroform, methanol, and distilled water at a ratio of 75:25:3 (v/v/v). Visualization was performed by charring using H$_2$SO$_4$:distilled water (1:1), and quantification by TLC scanner at 500 nm wavelength (Dual Wave Length Chromato Scanner CS-930 and Data Recorder DR-2 Shimadzu).

2.5 Fatty Acid Analysis

Fatty acid profile of ω-3 fatty acids enriched oil, deoiled soy lecithin, and structured phospholipids from optimum conditions of acidolysis reaction were analyzed using gas chromatography (Shimadzu GC 8A). The column used for separation was capillary CBP20 0.25 µm bonded silica column with dimension of 50 mm in length, i.d. 0.22 mm and o.d. 0.33 mm. Nitrogen was used as a gas carrier with a pressure of 200 kg/m$^2$, while for supporting and burning gas, air and hydrogen were used with a pressure of 0.15 kg/cm$^2$ and 0.6 kg/cm$^2$, respectively. Injector, column, and detector temperature was 230 °C, 250 °C and 230 °C, respectively. Samples and standard were injected at the volume of 2 µL. Methylation of phospholipids was performed according to Park and Goin’s method [26]. Analysis of fatty acid composition of phospholipids was conducted after phospholipids separation using TLC. Each spot was scrapped off and extracted according to Christie [27]. Derivatization prior to GC analysis was performed using Park and Goin’s method [26].

3. Results and Discussions

The predominant fatty acids in ω-3 fatty acids enriched oil were EPA and DHA (Table 2). In original lemuru oil, the EPA and DHA content were 15.87% and 13.58%, respectively. Meanwhile, EPA and DHA content in enriched oil were 1.79 and 1.72 times higher than the original oil. Lemuru oil is a good source of EPA rather than DHA. According to Howe et al. [28], fish oil generally contains higher EPA than DHA, except for tuna oil. Hence, in this study, we used lemuru oil as the source of EPA in the preparation of high EPA structured phospholipids from soy lecithin.

Linoleic acid was a predominant fatty acid obtained in soybean lecithin derived from Anjasmoro variety (Table 2). Normally, soybean oil has high linoleic acid

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Fatty acids profile of ω-3 fatty acids enriched oil, deoiled soy lecithin, and high EPA structured phospholipids.</th>
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</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>De-oiled soy lecithin</td>
</tr>
<tr>
<td>Lauric acid (C12:0)</td>
<td>0.60</td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>1.29</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
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<td>Palmitoleic acid (C16:1ω-7)</td>
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</tr>
<tr>
<td>Stearic acid (C18:0)</td>
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</tr>
<tr>
<td>Oleic acid (C18:1ω-9)</td>
<td>11.86</td>
</tr>
<tr>
<td>Linoleic acid (C18:2ω-6)</td>
<td>48.64</td>
</tr>
<tr>
<td>Linolenic acid (C18:3ω-3)</td>
<td>5.26</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4ω-3)</td>
<td>1.37</td>
</tr>
<tr>
<td>Docosanoic acid (C22:0)</td>
<td>3.59</td>
</tr>
<tr>
<td>Docosaenoic acid (C22:1)</td>
<td>1.40</td>
</tr>
<tr>
<td>Eicosapentaenoic acid/EPA (C20:5ω-3)</td>
<td>nd</td>
</tr>
<tr>
<td>Docosahexaenoic acid/DHA (C22:6ω-3)</td>
<td>nd</td>
</tr>
<tr>
<td>EPA + DHA</td>
<td>nd</td>
</tr>
</tbody>
</table>
| nd = not detected.
content that may comprise 55.83% of the total fatty acids [29]. Soybean lecithin consisted of 32.63% PI, 29.18% PC, 27.15% PE and 11.01% PA with the order PI > PC > PE > PA (Table 3). Wu and Wang [1] reported that soy lecithin had 18% PC, 14% PE, 9% PI, 2% PA, 2% minor PL, 11% glycolipid, 5% sugar complex and 37% neutral lipid. Commercial lecithin has different phospholipids composition with a predominant phospholipids is PC or PE [30]. Higher PC or PE concentration in commercial lecithin is due to fractionation treatment. Wang [3] reported that predominant fatty acid in PE or PC was linoleic acid, followed by palmitic acid.

The results of optimization revealed that the suitable model for acidolysis reaction with three factors (enzyme concentration, reaction time, ratio of oil enriched with ω-3 fatty acids to soy lecithin) was quadratic. However, the model was not significant. Factors that affected the response in quadratic manner were the ratio of ω-3 fatty acids enriched oil to soy lecithin and reaction time, while the effect of enzyme concentration was not significant.

The effect of substrate ratio and enzyme concentration was quadratic (Fig. 1). At high enzyme concentration, enzyme was sufficient for hydrolysis of fatty acid from original phospholipids, and subsequently esterification of ω-3 fatty acid enriched oil into phospholipids structure. At low enzyme concentration, it was supposed that enzyme would hydrolyze fatty acid from phospholipids structure, however, the synthesis was limited. This led to low EPA content of structured phospholipids. Similarly, increasing substrate ratio also enhanced EPA content, but then it decreased. According to Vikbjerg et al. [5], fatty acid composition of structured phospholipids depended on the substrate ratio (mole of acyl donor/mole of phospholipids) and incorporation of acyl increased a long with the availability of acyl or mole substrate ratio. This study showed that increasing acyl donor decreased incorporation of EPA into phospholipids structure. Previous study reported that

| Table 3 | Phospholipids profile of deoiled soy lecithin and structured phospholipids. |
|---------|-------------------------------|-----------------|
| Phospholipids | Deoiled soy lecithin | High EPA structured phospholipids |
| Phosphatidylinositol | 32.63 | 22.96 |
| Phosphatidylcholine | 29.18 | 25.85 |
| Phosphatidylethanolamine | 27.15 | 22.32 |
| Phosphatidic acid | 11.01 | 28.87 |

through different ratios of PC to palmitic acid as substrate (1:2 to 1:10) and 20% enzyme used, the maximum incorporation obtained at the ratio of 1:5. Increasing substrate ratio from 1:5 to 1:10, however, did not increase the incorporation level [6].

This study showed that high amounts of acyl donor did not always increase the level of incorporation. This was due to the preference of enzyme to hydrolyze and esterify acyl from and into phospholipids structure. At high acyl concentration, reaction tended to synthesize structured phospholipids. However, an increase of acyl availability could limit the hydrolysis due to inhibition effect of acyl [31].

The effect of substrate ratio and reaction time on the EPA incorporation into structured phospholipids was quadratic (Fig. 2). Maximum incorporation obtained at certain reaction time. Longer reaction time did not increase EPA incorporation into phospholipids structure. Reddy et al. [6] observed that incorporation of palmitic acid and stearic acid into PC was maximum at reaction time of 24 h. Extended reaction time did not increase the level of incorporation. That finding was similar to those results obtained in this study. After maximum reaction time, longer reaction time was supposed to lead the hydrolysis, including hydrolysis of incorporated EPA from structured phospholipids. Enzymatic reaction was hydrodynamic process and lipase was able to hydrolyze and synthesize depending on particular factors. One of them was water availability that stimulated reaction into hydrolysis. The availability of water in this study was limited to 10% (w/w substrate) that aimed to promote esterification reaction.
Optimization of High EPA Structured Phospholipids Synthesis from ω-3 Fatty Acid Enriched Oil and Soy Lecithin

Fig. 1  Response surface (a) and contour (b) of enzyme concentration and substrate ratio in acidolysis reaction of high EPA structured phospholipids synthesis.

Fig. 2  Response surface (a) and contour (b) of reaction time and substrate ratio in acidolysis reaction of high EPA structured phospholipids synthesis.

The effect of reaction time and enzyme concentration on EPA incorporation into structured phospholipids was also quadratic (Fig. 3). High incorporation of EPA tended to occur at high enzyme concentration. At low enzyme concentration, increasing reaction time enhanced EPA incorporation. However, longer reaction time decreased incorporated EPA of structured phospholipids. It was supposed that at a low enzyme concentration, hydrolysis of fatty acid from original phospholipids occurred at the beginning of reaction, followed by esterification of EPA. At long reaction time, incorporated EPA can be hydrolyzed from the structured phospholipids. Optimum incorporation of EPA was supposed to be higher than enzyme concentration of 30% and reaction time about 24 h (Fig. 3).

Among the three factors, ratio of substrate and reaction time gave significant effect on EPA content and the relation was quadratic (Fig. 2). The quadratic equation to predict the response was $Y = -36.76 - 9.059X_1 + 2.05X_2 + 3.78X_3 - 0.033X_1X_2 + 0.19X_1X_3 - 0.031X_2X_3 + 0.70X_1^2 - 0.029X_2^2 - 0.056X_3^2$ with $X_1 = \text{ratio of oil enriched with ω-3 fatty acids to soy lecithin}$, $X_2 = \text{enzyme concentration}$, $X_3 = \text{reaction time}$, and $Y = \text{EPA content (%) of structured phospholipids}$. Optimum conditions were obtained at a ratio of oil enriched with ω-3 fatty acids to soy lecithin 3.77:1, enzyme concentration 30%, and reaction time 24.08 h. At these optimum conditions, the response was predicted to be 22.81% of EPA. Verification showed that the response of EPA content of structured phospholipids was 24.23%.
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There was an interesting phenomenon in fatty acid composition of structured phospholipids (Table 2). Incorporation of EPA decreased palmitic acid content sharply, while linoleic acid increased. This reflected that palmitic acid was replaced by EPA in phospholipids structure. Acidolysis reaction using lipase was reversible [32]. At the beginning, lipase hydrolyzed fatty acids from phospholipids structure. Afterwards, EPA from ω-3 fatty acids enriched oil was esterified. Therefore, the increase of linoleic acid content was due to hydrolysis of other fatty acids rather than linoleic acid from phospholipids structure.

HLB value of structured phospholipids was 8.24, while original soy lecithin had HLB of 5.0. This suggested that high EPA structured phospholipids was suitable for oil in water emulsion. Fatty acid composition of structured phospholipids would influence the HLB value [33]. Increasing desaturation of fatty acid attached to phospholipids structure by incorporation of EPA, increased the polarity, as well as the HLB value. Additionally, fatty acid composition of structured phospholipids may affect its functional properties [34].

4. Conclusions

Factors affecting high EPA structured phospholipids synthesis were ratio of substrates and reaction time. Relation of three factors (ratio of substrates, reaction time, and enzyme concentration) was quadratic. EPA was supposed to replace palmitic acid in phospholipid structure. Optimum condition was achieved at ratio of oil enriched with ω-3 fatty acids to soy lecithin 3.77:1, enzyme concentration 30%, and reaction time 24.08 h. At these optimum conditions, the response was predicted to be 22.81% of EPA, meanwhile, verification showed 24.23%. Further work is needed to know degree of EPA incorporation into phospholipids classes as well as physiological effects of high EPA structured phospholipids ingestion.

Acknowledgments

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