Synthesis of Phospholipids Containing Polyunsaturated Fatty Acids by Phospholipase A₂-mediated Esterification with Food-compatible Reagents

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Abstract: Enzymatic synthesis of phospholipids (PLs) containing polyunsaturated fatty acids (PUFAs) was studied. The main purpose was to establish an efficient production method for PLs containing docosahexaenoic acid or eicosapentaenoic acid using only food-compatible reagents. Phospholipase A₂ (PLA₂)-mediated ester synthesis was employed to introduce the PUFAs into the *sn*-2 position of lysophospholipid (LPL) to yield PUFA-containing PLs. When LPL and the fatty acids were reacted in glycerol in the presence of porcine pancreas PLA₂, the reaction was not very effective. However, it was found that addition of certain kinds of amino acids such as glycine or L-alanine in the reaction mixture improved the reaction. After the reaction, the synthesized PLs were extracted selectively with ethanol and *n*-hexane, leaving the unreacted LPL, amino acids and the enzyme remained in the glycerol layer. It was confirmed that the enzyme remained in the glycerol layer could be reused by adding fresh substrates for the subsequent reactions.

Key words: phospholipid, polyunsaturated fatty acid, phospholipase A₂ ester synthesis

1 INTRODUCTION

Phospholipids (PLs) containing polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are attracting people's attention as these lipids show various bioactivities¹⁻⁸⁾. It is reported that DHA in the form of PL is more stable against oxidation than the ones in the forms of free fatty acid, ethyl ester, and triacylglycerol^{9, 10)}. Therefore, PUFA-containing PLs (PUFA-PLs) are of value for industrial food use. These PUFA-PLs can be obtained mainly from marine fish roe or squid skin meal. However, their industrial supply is unstable and usually very expensive.

Alternatives to produce PUFA-PLs are enzymatic syntheses. Two strategies may be possible for the enzymatic synthesis of PUFA-PLs. One is lipase (EC 3.1.1.3)-catalyzed transesterification or esterification between PLs and PUFAs (or its derivatives). Generally, lipases known so far are *sn*-1,3-position-specific, non-specific or in between. Therefore, the lipase-mediated reaction introduces the intended fatty acids into the *sn*-1-position or both the *sn*-1 and the *sn*-2-positions of the glycerol backbone of PLs, depending

on the positional specificity of the catalyst. In other words, *sn*-2-position-specific reaction is difficult by lipase-mediated reaction.

The second strategy is esterification of lysophospholipids (LPL, 1-acyl-2-lyso form) and PUFAs with the help of phospholipase A₂ (PLA₂, EC 3.1.1.4). PLA₂ is an enzyme that hydrolyzes the ester linkage of PL specifically at *sn*-2 position to generate fatty acid and LPL. PLA₂ also catalyzes the ester synthesis, the reverse reaction of the hydrolysis, between LPL and fatty acid under certain conditions. Since, generally, PUFA-PLs from natural sources contain the PUFA residues at the *sn*-2 position, esterification of LPL with PUFA specifically at the *sn*-2 position by the PLA₂-mediated reaction seems to be more promising than the lipase-catalyzed reaction, for practical purposes.

The ester synthesis by PLA₂ was first reported by Pernas *et al.* with a relatively low yield¹¹⁾. Since then, several efforts have been made to improve the reaction efficiency, and to apply the reaction for the production of PUFA-PLs¹²⁻¹⁶⁾. An interesting approach was reported by Hosokawa and co-workers¹⁴⁻¹⁶⁾, who performed the enzymatic re-

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action in glycerol in the presence of formamide as a water mimic to afford PUFA-PLs with high efficiency. However, formamide was a toxic reagent, and therefore the process was not applicable for food production.

In this study, to establish a process for production of PUFA-PL for food use, we first searched for various compounds as food-compatible additives that can activate the enzymatic reaction, and found that certain kinds of amino acids fulfilled the requirements for effective synthesis. Then, we have established a efficient method for extraction of the synthesized PLs from the reaction mixture. In addition, repeated use of the enzyme for subsequent reactions has been demonstrated.

2 EXPERIMENTAL

2.1 Materials

LPL (SLP-LPC70) and hydrogenated LPL (hLPL, SLP-LPC70H) from soy lecithin were provided by Tuji Oil Mill (Mie, Japan). These LPLs contains mainly lysophosphatidylcholine (ca. 70%), and the rest may include lysophosphatidylethanolamine, lysophosphatidylinositol, and lysophosphatidic acid. Diacyl-PLs were almost negligible. Fatty acid composition of the LPL was 24.8% C16:0, 7.2% C18:0, 12.0% C18:1 (oleic acid, OA), 50.1% C18:2 (linoleic acid, LA) and 5.9% C18:3, whereas that of the hLPL was 24.2% C16:0 and 75.8% C18:0. Triacylglycerol containing DHA (DHA-50G) or EPA (EPA-45G) were provided by Nippon Chemical and Feed (Hokkaido, Japan). The fatty acid composition of DHA-50G was 1.4% C14:0, 8.6 % C16:0, 2.7% C18:0, 7.2% C18:1 (OA), 2.9% C20:4, 5.8% C20:5 (EPA), and 54.9% C22:6 (DHA), whereas that of EPA-45G was 3.0% C14:0, 4.2 % C16:0, 0.5% C18:0, 5.6% C18:1 (OA), 4.1% C20:4, 48.4% C20:5 (EPA), and 14.9% C22:6 (DHA). Free DHA and EPA were prepared from DHA-50G and EPA-45G, respectively, by conventional alkaline hydrolysis. Pure EPA (purity >99%) and pure OA (purity >99%) were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Porcine pancreatic PLA₂ powders used were Lysonase (Sanyo Fine, Osaka, Japan) and Lecitase (Novozymes, Chiba, Japan). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2 Esterification reaction

Typical reaction mixture consisted of 1 g glycerol, 35 mg LPL or hLPL, 40 mg of fatty acid, 30 mg of amino acids (specified later), aqueous CaCl₂ solution (concentration and amount specified later) and 10 or 20 mg of PLA₂ powder (Lecitase or Lysonase). The mixture was first vacuumed at 80 Pa (0.6 mmHg) for 10 min at room temperature to reduce the water content. Then, the mixture was incubated at 50 or 60°C with mixing using a magnetic stirrer for 24 h

under slightly reduced pressure (7000 Pa, 52 mmHg). The details for the conditions are described in the text or in the table foot notes.

2.3 Analysis

A portion (0.05 mL) of the reaction mixture was withdrawn and mixed with 0.2 mL chloroform/methanol (2/1), and 0.5 mL saturated sodium chloride solution was added. After centrifugation, the chloroform layer was recovered and glycerol layer was extracted again with 0.2 mL chloroform/methanol. The resultant lipid solution was spotted on a TLC plate (Silica gel 60, Merck, Darmstadt, Germany), and the plate was developed with chloroform/methanol/water (65/25/4). The lipids were visualized under UV light at 365 nm after spraying 2', 7'-dichlorofluorescein solution (0.5% in ethanol). The spots of PLs together with those of LPLs were scraped off and the lipids were extracted with chloroform/methanol (2/1). The extracted lipids were converted into fatty acid methyl esters with sodium methoxide, and subjected to fatty acid composition analysis by gas chromatography.

The gas chromatograph used was Shimadzu GC-14B equipped with a flame ionization detector. A fused silica capillary column, TC-FFAP [30 m (length) × 0.53 mm (inner diameter), 1.0 μm (film thickness) (GL Science, Tokyo, Japan)]. The injector and the detector were set at 250°C. The column temperature was 180°C at 0 to 3 min, then raised to 230°C at 10°C/min, and kept at that temperature for 15 min.

The molar content of the target PL molecules were calculated from the fatty acid composition of "PL plus (h) LPL" fractions as follows, assuming that the PLA₂ esterified specifically at the *sn*-2-position of LPL and that no spontaneous acyl migration between the *sn*-1 and the *sn*-2 positions took place. In the cases of the esterification between single (pure) fatty acids and LPL or hLPL (i.e., the reaction between OA and hLPL and between pure EPA and LPL), the content of the intended fatty acid (OA or EPA) in the "PL plus (h) LPL" fraction was directly regarded as the molar content of the target molecules (i.e., OA- or EPA-containing PL molecules). This calculation was possible because the starting substrates, hLPL and LPL, did not contain OA and EPA, respectively. In these cases, the molar content of the target molecule can also measure the degree of esterification which indicates how much of the starting substrate (hLPL or LPL) was converted into PL.

In the cases of the esterification between fatty acid mixture and LPL (i.e., the reaction between fatty acids from DHA-50G and LPL, and between fatty acids from EPA-45G and LPL), the molar content of the targets (DHA-PL or EPA-PL) were calculated from the fatty acid content of LA and DHA, or LA and EPA in the "PL plus LPL" fraction. The LA content in the starting substrate, LPL, was 50.1 mol%, whereas those in DHA-50G and EPA-45G were neg-

ligible (less than 1%). Therefore, the decrease in the LA content and increase in the DHA or the EPA content in the "PL plus LPL" fraction after the reaction can be a measure to estimate the molar content of the target molecule (i.e., DHA-PL or EPA-PL). The molar content of the target molecule can be calculated as follows;

$$\text{DHA-PL (mol\%)} = D \times L_1/L_2, \text{ and}$$

$$\text{EPA-PL (mol\%)} = E \times L_1/L_2$$

where, D and E are the fatty acid contents of DHA and EPA, respectively, in the "PL plus LPL" fraction after the reaction. L₁ is the LA content in the starting LPL (= 50.1%), and L₂ is the LA content in the "PL plus LPL" fraction after the reaction. It should be noted that, in these cases, the molar content of the target molecule does not measure the degree of esterification, because fatty acids other than DHA or EPA are also incorporated into LPL.

2.4 Recovery of the synthesized PLs and reuse of the enzyme

After the enzymatic reaction, 0.5 mL of ethanol and 0.5 mL of *n*-hexane were added to the reaction mixture. The mixture was separated into two layers by centrifugation. The upper (*n*-hexane) layer containing PLs was recovered.

PLs were extracted again from the lower (glycerol) layer with *n*-hexane.

To the glycerol layer, (which may contain the enzyme, the amino acids, CaCl₂ and a part of unreacted LPL), 24 mg of LPL, 40 mg fatty acids and 1.5 or 1.8 μL of 1M CaCl₂ solution were added. The mixture was vacuumed at 80 Pa for 10 min to remove water and the remaining solvents. The enzymatic reaction was restarted at 7000 Pa, for 24 h.

3 RESULTS AND DISCUSSIONS

3.1 Esterification of hLPL with OA

The purpose of the current study is to establish a method for the PLA₂-mediated incorporation of DHA or EPA into LPL without using toxic organic solvent and chemicals, because the synthesized PLs were intended for food use. Hosokawa *et al.* performed the reaction in glycerol that was a food-compatible solvent, but a toxic chemical, formamide, was required as an additive to activate the enzyme¹⁴.

Thus, we searched for food-compatible additives that could activate the reaction. The reaction was performed in

Table 1 Effects of Amino Acids on the Esterification of hLPL and OA^{a)}.

amino acid		CaCl ₂ (μmol) ^{b)}	Product yield (%) ^{c)}
type	amount(mg)		
none	—	0.6	17
L-glutamic acid · Na · H ₂ O	100	0.6	0
L-arginine	100	0.6	0
L-cysteine · HCl · H ₂ O	100	0.6	9
L-valine	100	0.6	23
L-methionine	100	0.6	24
L-phenylalanine	100	0.6	24
L-histidine	100	0.6	26
L-asparagine · H ₂ O	100	0.6	27
L-serine	100	0.6	33
L-alanine	100	0.6	35
glycine	25	0.6	34
	50	0.3	35
	50	0.6	44
	50	1.2	57
	50	2.4	63
	50	3.6	51
	50	4.8	51
	75	0.6	39
	100	0.6	33

^{a)} Reaction mixture consisted of 35 mg hLPL, 97 mg OA, 10 mg Lecitase, 1 g glycerol, amino acid (as indicated) and CaCl₂ (as indicated). Reaction was carried out at 60°C for 24 h.

^{b)} 2.5 μL of aqueous CaCl₂ solution was added to give the indicated amount.

^{c)} Molar content of OA-PL in "PL plus hLPL" fraction.

glycerol containing the enzyme, hLPL, OA and CaCl_2 . As shown in **Table 1**, we found that the yield of target PL molecule (OA-containing PL, OA-PL) was greatly improved by the addition of certain kinds of amino acids. Especially, glycine, L-alanine and L-serine strongly increased the yield. The effects of L-asparagine, L-histidine, L-phenylalanine, L-methionine and L-valine were moderate, whereas L-glutamic acid, L-arginine and L-cysteine inhibited the reaction. Since glycine is the cheapest amino acid among the ones tested, the effects of glycine was investigated in more detail. After optimizing the conditions (50 mg glycine, 2.4 μmol CaCl_2 , 60°C) for 24 h reaction, the product yield of 63.3 % was achieved.

3.2 Synthesis of PUFA-PL

Table 2 shows the effects of amino acids on the synthesis of DHA-PL and EPA-PL. In this case, the reactions were carried out at 50°C using LPL and Lysonase as the substrate and the catalyst, respectively. The reaction efficiency at 50°C was slightly lower than that at 60°C (data not shown). But we chose the reaction temperature of 50°C for the purpose of minimizing heat-inactivation of the enzyme, which would occur especially during repeated use of the catalyst.

The addition of glycine or L-alanine was effective also for the PUFA-PL synthesis. L-Glutamate and L-lysine rather inhibited the reaction. Addition of both glycine and L-alanine gave somewhat better results than added separately, giving DHA-PL content of 19 mol%. The synthesis of EPA-PL was also successful. When the EPA of 48% purity (derived from EPA-48G) was used in the presence of glycine and L-alanine, the EPA-PL content reached to 26%. When

the pure EPA (>99%) was used, the EPA-PL amounted to 49 mol%.

Oxidation of the PUFA residues during the enzymatic reaction will become a serious problem, which should be minimized. Although we did not check the oxidation of the synthesized PLs in this study, it was expected that performing the reaction under reduced pressure will help preventing the oxidation of the product to some extent. Of course, it will be an effective option to perform the reaction in the presence of an appropriate antioxidant such as tocopherols.

3.3 Extraction of the synthesized DHA-PL and reuse of the enzyme

The synthesized DHA-PL was extracted with ethanol and *n*-hexane. **Figure 1** shows the lipid profile of the recovered *n*-hexane layer and the glycerol layer. For this analysis, the lipid remained in the glycerol layer was extracted with chloroform and methanol. It was revealed that the *n*-hexane layer was rich in the synthesized PL (diacyl form), while the glycerol layer was rich in the unreacted LPL. The fatty acid composition analysis also supported the above results; the molar content of DHA-PL in the whole reaction mixture (extracted directly with chloroform and methanol) was 19%, whereas those in the *n*-hexane and the glycerol layers were 27% and 3%, respectively. These results are of advantage, as the target DHA-PL could be selectively recovered from the reaction mixture, leaving a part of the unreacted LPL remained in the glycerol layer. The *n*-hexane layer also contained the unreacted fatty acids, which will be separated from the PLs by washing with acetone.

Table 2 Synthesis of PUFA-PL from LPL and PUFA

Fatty acid	Amino acids	PUFA-PL (mol%)
DHA (54.9%)	None	10
	L-lysine · HCl	0
	L-glutamic acid · Na · H ₂ O	6
	L-glutamine	11
	L-phenylalanine	12
	L-methionine	13
	glycine	17
	L-alanine	17
	glycine + L-alanine	19
EPA (48.4%)	None	7
	glycine + L-alanine	26
pure EPA (99%)	glycine + L-alanine	49

Reaction mixture; 35 mg LPL, 40 mg fatty acids, 30 mg each amino acid, 20 mg Lysonase, 1 g glycerol, 3 μmol calcium chloride (3 μL of 1 M solution). Reaction was carried out at 50°C for 24 h.

Fig. 1 TLC Pattern of the Lipids Extracted from the Reaction Mixture of DHA-PL Synthesis. Lane 1, *n*-hexane layer; lane 2, glycerol layer. “LPL” indicates unreacted LPL which is mainly LPC. “PL(PC)” stands for the generated phosphatidylcholine, while “PL (others)” for other (diacyl)-PLs, which may include phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid (not identified). “FA” indicates the unreacted fatty acid, which is dimly visible as a white spot over the gray background. “O” and “F” denote the origin and the solvent front of the development, respectively. The lipids were visualized with Dittmer-Lester’s reagent.

Hence, we wondered that if the enzyme was also remained in the glycerol layer and was still active, the reaction could be repeated for several times without adding fresh enzyme, and thereby reducing the production cost. To check the reusability of the enzyme, the DHA-PL synthesis was performed in three batches. After 24 h reaction (first reaction), one of the three batches was extracted with chloroform and methanol and analyzed. The other two batches were extracted with ethanol and *n*-hexane. The glycerol layer was mixed with fresh LPL, fatty acids and CaCl₂ solution, vacuum dried, and then the reaction was allowed to restart in two batches (second reaction). After 24 h reaction (second reaction), one of the two batches was used for analysis, while the other batch was extracted again with ethanol and *n*-hexane, and used similarly for the third reaction.

The DHA-PL contents after the first, the second and the third reactions were, 19, 18 and 15%, respectively, confirming the reusability of the enzyme. We found that addition of CaCl₂ after each reaction was indispensable (1.8 μmol for the second reaction, 1.5 μmol for the third reaction). This may be because calcium ion, which is necessary for PLA₂ activity, was removed into the *n*-hexane layer as a complex with the PLs.

4 CONCLUSION

We have established a production scheme for PUFA-PL, including enzymatic synthesis, product recovery and catalyst recycling. Since all the reagents used are food-compatible, the process is promising for industrial production of food grade PUFA-PLs. However, there is still much room for improving the process with respect to yield and cost. Attempts for the process improvement and the scale-up are now in progress.

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