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Enzymatic synthesis of lysophosphatidylcholine with *n*−3 polyunsaturated fatty acid from sn-glycero-3-phosphatidylcholine in a solvent-free system

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ABSTRACT

The *n*−3 polyunsaturated fatty acids (PUFA)-rich lysophosphatidylcholine (LPC) was successfully synthesized by *Thermomyces lanuginosus* lipase (TL IM)-catalyzed esterification of glycerylphosphorylcholine (GPC) and *n*−3 PUFA-rich fatty acids in a solvent-free system. Effects of reaction temperature, enzyme loading and substrate mole ratio on the yield of LPC and incorporation of *n*−3 PUFA were evaluated. The acyl-specificities of five enzymes were tested for direct esterification of *n*−3 PUFA, and Lipozyme TL IM was found to be more effective than others for production of LPC with *n*−3 PUFA. Substrate mole ratio and reaction temperature, however, had no significant effect on the incorporation. The maximal yield of LPC was obtained under the following conditions: temperature 45 °C, enzyme loading 15% by weight and substrate mole ratio (GPC/*n*−3 PUFA) 1:20. Furthermore, the composition of products were further investigated in the study. The 1-acyl-sn-glycero-3-lysophosphatidylcholine (2-LPC) was predominant in the mixtures at early stages of reaction, whereas less increment of 2-acyl-sn-glycero-3-lysopho sphatidylcholine (1-LPC) and PC was observed at later stages.

1. Introduction

Lysophospholipids (LPLs) are bioactive lipid derivatives generated via the actions of phospholipase on major membrane phospholipids (PLs) (Li, Li, Samuel, & Wang, 2015). They are all characterized by having a single carbon-chain tail (a fatty acid residue) and a polar head group (D'Arrigo and Servi, 2010). The hydrophobic tail and the hydrophilic head group determine the specific chemical structure of the LPL, and thus its unique biological functions (Richmond & Smith, 2011). Studies have demonstrated that LPLs exhibit wide-ranging biological properties, and play important roles in a variety of processes, including smooth muscle contraction, cellular proliferation, pain, inflammation, myocardial injury, and cancer cell migration and invasion (Braeuer, Zigler, Kamiya, & Bar-Eli, 2012; Camprubí-Robles, Mair, & Rukwied, 2013; Chen, Hashizume, Xiao, & Abiko, 1997; Hung, Sok, & Kim, 2012; Liao, Huang, & Lee, 2005; Monet, Gkika, Lehen'Kyi, & Bidaux, 2009; Watterson, Ratz, & Spiegel, 2005). LPLs can act as signaling mediators by binding to G protein-coupled receptors (GPCRs), and activating or inhibiting downstream secondary messengers (Geary, 2010). Lysophosphatidylcholine (LPC) is a highly abundant, bioactive, lysoglycerophospholipid that has been shown in some studies to have anti-atherogenic properties (Sevastou, Kaffe, Mouratis, & Aidinis, 2012). Contradictory findings surrounding the pro- or anti-atherogenic properties of LPC appear to be due to differences in its chain length and degree of saturation of the fatty acyl moiety (Akerele & Cheema, 2015).

Studies have suggested that the presence of *n*−3 polyunsaturated fatty acids (PUFA) at the sn-1 position of LPC modulates the inflammatory response thereby making LPC anti-atherogenic (Akerele & Cheema, 2015). Intake of *n*−3 PUFA could contribute towards the prevention of chronic inflammation and oxidative stress processes that are associated with metabolic syndrome, obesity, diabetes, and even cancer (Dasilva, Pazos, García-Egido, Gallardo, & Cela, 2015). Moreover, it was proposed by Akerele and Cheema (2015) that *n*−3 PUFA-enriched LPC has direct cardiovascular health benefits, specifically by promoting cholesterol efflux and inhibiting inflammation, thereby preventing vascular dysfunction and monocyte adhesion. We therefore synthesized *n*−3 PUFA-enriched LPC via the esterification of sn-glycero-3-phosphatidylcholine (GPC) and *n*−3 PUFA-rich fatty acids.

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LPLs can be produced through the enzyme-catalyzed hydrolysis of phosphatidylcholine (PC) at the sn-2 and sn-1 positions, using either phospholipases A1 (PLA1) or A2 (PLA2), or 1, 3-specific lipases (Baeza-Jiménez, Otero, Kim, & García, 2013; D'Arrigo and Servi, 2010; Hosokawa, Takahashi, Hatano, & Egi, 1994). The most widely used reaction is the PLA1-catalyzed hydrolysis of PC to produce LPC. A DHA/EPA-rich LPC mixture (26.3% 1-LPC, 31.4% 2-LPC, 16.5% PC) have been obtained by the transesterification of DHA/EPA-rich ethyl esters with PC using immobilized phospholipase A1. Obviously, the composition of product was quite complex. In addition, LPC has been synthesized via the Novozym 435-catalyzed esterification of GPC, using either conjugated linoleic acid (CLA) or the acid form of vinyl laureate (Hong, Kim, Kim, & Kim, 2011; Virto & Adlercreutz, 2000). Thus, the synthesis of *n*−3 PUFA-enriched LPC by esterification of GPC were proposed. The *n*−3 PUFA from fish oil were used as an acyl donor to produce LPC. In the present study, the effect of temperature, mole ratio, enzyme activity, and enzyme loading on the yield of LPC containing *n*−3 PUFA was investigated. Our anticipated long-term objective is to establish enzyme-catalyzed esterification as a method for the efficient production of *n*−3 PUFA-enriched LPC.

2. Materials and methods

2.1. Materials

PC (purity 95%, from soybean) was obtained from Avanti Polar-Lipids, Inc. (Alabaster, Al, USA). GPC, LPC and PC standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Highly enriched *n*−3 PUFA from fish oil and soy-PC was obtained from Xiamen Huisheng Biological Ltd. Immobilized lipozyme RM IM, immobilized TL IM and immobilized 435 were donated by Novozymes (TianJin, China). All solvents and chemicals were analytical grade. Lipozyme TL IM (the immobilized lipase from *Thermomyces lanuginosus*), Lipozyme RM IM (the immobilized lipase from *Rhizomucor miehei*), Novozym 435 (the immobilized lipase from *Candida antarctica*), Phospholipase A1 (Lecitase® Ultra) from *Thermomyces lanuginosus*/*Fusarium oxysporum*, and phospholipase A2 (Lecitase® 10L) from porcine pancreas, were purchased from Novo Nordisk Bioindustry Ltd. (Seoul, Korea). For the present purposes, we defined *n*−3 PUFA as the sum of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). The fatty acid composition in fish oil was listed in Table 1. Eicosapentaenoic acid, docosahexaenoic acid, linoleic acid (LA), oleic acid (OA), stearic acid (SA), α-linolenic acid (ALA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Enzymatic synthesis of LPC

In this work, LPC were synthesized by direct esterification of GPC with *n*−3 PUFA. These esterification reactions catalyzed by lipases were carried out to examine the impact of enzyme, temperature, substrate ratio and vacuum on the yield of LPC. GPC was mixed with different amount of *n*−3 PUFA (1:2, 1:4, 1:8, 1:10, 1:20 and 1:40, substrate mole ratio of DHA to GPC). Enzyme (5, 10, 15, 20, 25% by weight based on total substrates) were subsequently added to a mixture of GPC and *n*−3 PUFA. The mixture was incubated at various temperatures (20, 30, 40, 45, 50, 60, 70 °C) in a shaking air bath at 200 r/min under a defined vacuum.

2.3. Analysis of fatty acid composition

Once the reaction was complete, the mixture were applied to thin layer chromatography (TLC) plates (10 × 20 cm), and was developed in a TLC tank. The developing solvent was chloroform/methanol/acetic acid/water (75:40:8:3, v/v/v). The bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under ultraviolet (UV) light. The PC and LPC band was scraped off and extracted by chloroform/methanol (1:1, v/v). The extract was separated and dried after centrifugation (1914*g*) for 20 min. Samples such as PC and LPC band were methylated to FA methyl esters and then were analyzed by using a 6890 N gas chromatograph (Agilent, American) equipped with a flame-ionization detector and HP-INNOWax column. Peaks were identified by comparison of retention times with known fatty acid methyl ester standards and quantified using an internal standard (heptadecanoic acid). In this study, the incorporation of *n*−3 PUFA (%) was calculated as follows:

$$\text{Incorporation of n-3 PUFA} = \frac{\text{the mole of (ALA + EPA + DPA + DHA) in LPC}}{\text{the mole of total FA content in LPC}} \times 100$$

2.4. Analysis of PC, GPC, 1-LPC and 2-LPC yield in reaction products by ^{31}P NMR

Once the reaction was complete, products were dissolved in a mixture of chloroform and methanol (5:1, v/v) which adjust the pH to 4.0 using a boric acid solution, and then its composition was analyzed by ^{31}P NMR as previously described by Li et al. (2014). All NMR experiments were conducted on a Bruker Avance Spectrometer 500 (Bruker, Germany) operating at 243 MHz, at 25 °C using temperature stabilization. An amount of 5 mg of the extracted phospholipids precipitation obtained from reaction mixtures was dissolved in 0.6 ml of $CDCl_3$/MeOH (2:1, v/v). The mixture was added directly into the 5 mm NMR tube. Parameters were as follows: pulse width, 12 μs; acquisition time, 2.3 s; repetition time (relaxation delay + acquisition time), 7.3 s; number of scans, 96. The relative integrated intensity (I) of each peak was used to calculate the composition of the product mixture. The large sweep width was dictated by the ^{31}P chemical shift (δ:−17.1) of the internal standard triphenylphosphate (TPP). Typical chemical shift values obtained (relative to TPP as an internal standard) were as follows: δ −17.1 (TPP), −0.56 (PC), −0.14 (1-LPC), 0.21 (2-LPC), and 0.40 ppm (GPC). The yield of PC, GPC, 1-LPC and 2-LPC was calculated as the ratio of PC (1-LPC, 2-LPC, or GPC)/initial PC (mol/mol).

2.5. Statistical analysis

All experiments were performed in triplicate and results were given as mean ± SD. The significance of difference at *P*-value of 0.05 was determined by using an ANOVA procedure.

Table 1
Yield of LPC and PC in LPC containing *n*−3 PUFA produced by different enzymes.*

Yield (mol%)	TL IM	Nov 435	RM IM	PLA1	PLA2
PC	1.34 ± 0.14	1.21 ± 0.12	8.23 ± 0.41	ND	ND
LPC	67.31 ± 2.45	35.65 ± 0.921	27.41 ± 1.21	9.41 ± 0.32	6.12 ± 0.24

* Reaction performed at a mole ratio of 1:10 (PC:*n*−3 PUFA), 40 °C and enzyme loading at 15% (w/w, based on total substrate weight); and bar, mean ± standard deviations (n = 3).