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Immobilized phospholipase A1-catalyzed modification of phosphatidylcholine with *n*−3 polyunsaturated fatty acid

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ABSTRACT

n−3 Polyunsaturated fatty acids (*n*−3 PUFA)-enriched phosphatidylcholine (PC) was successfully produced with fatty acid from fish oil and PC from soybean by immobilized phospholipase A1-catalyzed acidolysis. Detailed studies of immobilization were carried out, and Lewatit VP OC 1600 was selected as a carrier for preparation of immobilized phospholipase A1, which was used for modification of PC by acidolysis. For acidolysis of PC with *n*−3 PUFA, the effects of several parameters, namely, water content, temperature, and enzyme loading on the reaction time course were investigated to determine optimum conditions. The optimum water content, temperature, and enzyme loading were 1.0%, 55 °C, and 20%, respectively. The highest incorporation (57.4 mol%) of *n*−3 PUFA into PC was obtained at 24 h and the yield of PC was 16.7 mol%. The yield of PC increased significantly by application of vacuum, even though a slight decrease of *n*−3 PUFA incorporation was observed.

1. Introduction

Phospholipids (PLs) are major constituents of cell membranes and play crucial roles in the biochemistry and physiology of the cell (Kidd & Head, 2005). PLs have been widely used in food, pharmaceutical, and cosmetic products as highly efficient emulsifiers.

In recent decades, *n*−3 polyunsaturated fatty acid (*n*−3 PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have received significant scientific attention because of their health benefits, which include improvement of immune function and prevention of heart disease and cancer (Khandelwal et al., 2013; Komprda, 2012; Kromhout, Bosschieter, & de Lezenne Coulander, 1985; Larsson, Kumlin, Ingelman-Sundberg, & Wolk, 2004). Consumption of *n*−3 PUFA has also been reported to provide important benefits with respect to functioning of the brain and retina, as well as accelerating the growth of preterm baby (Carlson, Werkman, Peeples, Cooke, & Tolley, 1993; Lanting, Fidler, Huisman, Touwen, & Boersma, 1994; Neuringer, Connor, Van Petten, & Barstad, 1984). Long-chain *n*−3 PUFAs are characteristic of marine oils and occur pervasively in the PLs of fish and marine species, with EPA and DHA commonly accounting for up to 50% of their fatty acid constituents (Haraldsson & Thorarensen, 1999). The presence of long-chain and low-melting PUFA is believed to add fluidity and mobility to cell membranes and thus, they adjust membrane integrity and function properly in lower ambient temperatures (Haraldsson & Thorarensen, 1999). In addition, fatty acids are more easily absorbed in the body as PL than as the corresponding triacylglycerols (TAGs) or ethyl esters (Galli et al., 1992). Because of the positive influence of *n*−3 PUFAs on human health, there is a growing demand for them in the pharmaceutical industry in a PL form as well as their natural TAG form (Carlson, 1991). Lemaitre-Delaunay et al. (1999) have provided evidence for higher bioavailability of DHA for incorporation into erythrocytes in human adults when DHA is provided in PL rather than in TAG. Wijendran et al. (2002) also reported that PL were about 2.1-fold more effective than TAG as substrates for accretion of brain arachidonic acid in the development of neonatal primate brain.

Although PLs containing *n*−3 PUFA is available in fish and marine products, refinement procedures, including laborious extraction and separation, are required for it to be used industrially. Thus, enzymatic modification using inexpensive plant lecithin

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(PC, phosphatidylcholine) and a rich $n-3$ PUFA source might be an effective method for obtaining $n-3$ PUFA-enriched PLs. Replacement of fatty acid residues present in a native PL by fatty acids with beneficial physiological effects can lead to tailored PLs that offer intriguing marketing opportunities for manufacturers of nutraceuticals.

Interest in the production of structured PLs containing specific fatty acid residues has also increased significantly in recent years. Vikbjerg, Mu, and Xu (2007) have reported that phospholipase A2-catalyzed synthesis of PL with caprylic acid by acyl modification of the $sn-2$ position in PLs. Hossen and Hernandez (2005) have reported that Lypozyme RM IM (from *Rhizomucor miehei*) and Lipozyme TL IM (from *Thermomyces lanuginosus*) are effective in the incorporation of conjugated linoleic acid into soybean PL. Previous research concerning the incorporation of $n-3$ PUFA into PC by phospholipase A1 (PLA1) immobilized on Duolite A568 as a carrier has been reported (Kim, Garcia, & Hill, 2010). However, although a modified PC containing $n-3$ PUFA can be produced successfully, there is a concomitant significant decrease in PC yield as a consequence of hydrolysis. Therefore, it is important to find the means to enhance the desired yield of modified PC.

In this study, detailed experiments were first conducted regarding immobilization of PLA1, and then the immobilized enzyme was employed for modification of PC. PC from soybean and highly enriched $n-3$ PUFA from fish oil were used as substrates for synthesis of structured PC by enzyme-catalyzed acidolysis. Although a response surface methodology for optimizing reaction conditions is preferred as it take into account interaction terms, to be consistent with our previous study about the modification of phospholipids, a one-factor-at-a-time approach was followed in this study. Thus, the effects of water content, temperature, and enzyme loading on incorporation of $n-3$ PUFA into PC were ascertained by monitoring reaction time course. In addition, the effect of vacuum was examined with the goal of enhancing the yield of structured PC.

2. Materials and methods

2.1. Materials

PLA1 (Lecitase® Ultra) from *T. lanuginosus*/*Fusarium oxysporum* was obtained from Novozymes (Franklinton, NC, USA) as an aqueous solution of 57.0% water (by vol.). Fatty acid standards were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Lewatit VP OC 1600, Accurel MP 1000, and Duolite A568 were purchased from Lanxess Energizing Chemistry (Leverkusen, Germany), Membrana GmbH (-Accurel System, Obernburg, Germany), and Rohm and Haas France SAS (Chauny, France), respectively. Amberlite XAD 7HP, Celite 545, Dowex 50w x8, Amberlite XAD4, and Octyl silica were purchased from Sigma–Aldrich, Inc. Granulated PC (purity 98%, from soybean) was obtained from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA). The primary fatty acid residues in the soybean PC were those of linoleic acid, 18:2$n-6$ (64.9 mol%), palmitic acid, 16:0 (14.0 mol%), oleic acid, 18:1$n-9$ (10.4 mol%), linolenic acid, 18:3$n-3$ (6.2 mol%) and stearic acid, 18:0 (3.3 mol%). Highly enriched $n-3$ PUFA from fish oil was donated by Ilshinwells Co., Ltd. (Seoul, Republic of Korea). The primary species in the highly enriched $n-3$ PUFA from fish oil were those of oleic acid (2.5 mol%), EPA, 20:5$n-3$ (14.7 mol%), docosapentaenoic acid, 22:5$n-3$ (DPA, 6.0 mol%) and DHA, 22:6$n-3$ (71.2 mol%). For the present purposes, we defined $n-3$ PUFA as the sum of EPA, DPA, and DHA. The TLC plates were coated with silica gel (layer thickness 0.25 mm, Kieselgel 60 F254, Merck KGaA, Darmstadt, Germany). All solvents and chemicals were analytical grade.

2.2. Immobilization of PLA1

For the immobilization of PLA1, a free type PLA1 was mixed with sodium phosphate buffer solution (50 mM, pH 7.5) at various ratios. The enzyme suspension was stirred at 300 rpm for 30 min and its soluble protein concentration was determined according to Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as the standard. For hydrophobic carriers, one gram of each carrier was wetted with 20 mL of ethanol for 2 h until the carrier sedimented. After decanting ethanol, the carrier was washed with 40 mL sodium phosphate buffer and subsequently placed in a flask with 10 mL of enzyme suspension. Immobilization was performed in a water bath shaker operating at 200 rpm and 30 °C. After shaking for 16 h, the suspension was filtered to recover the resulting immobilized enzyme preparation, which was then rinsed with 40 mL of sodium phosphate buffer and then dried at 45 °C in a vacuum oven overnight, and stored at 4 °C until use. The fixation level (wt%) and the amount of protein bound in immobilized enzyme (mg/g) were estimated by subtracting the protein remaining in the enzyme suspension after immobilization compared with the initial protein concentration.

Fixation level (%) and amount of protein adsorbed onto the carrier (mg/g) were calculated by following equations:

$$\text{Fixation level } (\%) = \frac{a-b}{a} \times 100$$

$$\text{Protein amount in immobilized enzyme } (\text{mg/g}) = \frac{a-b}{c+a-b} \times 100$$

a: amount of initial protein in enzyme suspension (mg).
b: amount of unbound protein in enzyme suspension after immobilization (mg).
c: amount of carrier used in immobilization (g).

2.3. Activity test for immobilized PLA1

Enzymatic modification of PC was evaluated by performing activity tests. A 1.2 g sample of highly enriched $n-3$ PUFA from fish oil (8 mol) and 2.8 g of PC (1 mol) were placed in a 50 mL water-jacketed glass vessel, mixed at 250 rpm and preheated to 55 °C. Then, 0.5 g of the enzyme (10% of total substrate weight) was added. Samples (50 mg) of the product mixture were taken at regular intervals, dissolved in chloroform, and applied to silica-coated preparative TLC plates and developed to obtain the PC fraction. After saponification and methylation, the resulting fatty acid methyl esters (FAMEs) were subjected to gas chromatographic analysis.

The apparent activity was defined as the initial reaction rate divided by the amount of immobilized enzyme. The specific activity was defined as the initial reaction rate divided by the amount of protein. The acidolysis reaction involves concurrent decomposition of PC and addition of fatty acid to the molecule. Since esterification of fatty acid should be preceded by hydrolysis of PC, hydrolytic activity of the enzyme was used to measure the initial reaction rate. The apparent activity and the specific activity were calculated as follows:

$$\text{Apparent activity } (\mu\text{mol/g particle/min}) = \frac{d}{e \times f}$$

$$\text{Specific activity } (\mu\text{mol/g protein/min}) = \frac{d}{g \times f}$$

d: hydrolyzed PC (μmol).
e: amount of immobilized enzyme used in the reaction (g).