Phospholipase A\(_2\)-mediated synthesis of phosphatidylethanolamine containing highly unsaturated fatty acids

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Summary
Phosphatidylethanolamine (PE), which contains highly unsaturated fatty acid (HUFA) specifically in sn-2 position, was synthesized by porcine pancreatic phospholipase A\(_2\)-catalysed esterification of lysophosphatidylethanolamine with HUFA. The use of glycerol containing only a little water as solvent was the important key to this reaction. A 27% yield of up to 94.5% sn-2 eicosapentaenoic acid (EPA)-containing PE was obtained using >99% EPA as a substrate HUFA.

Keywords
Docosahexaenoic acid, eicosapentaenoic acid, ester synthesis, glycerol.

Introduction
It is well known that intake of highly unsaturated fatty acids (HUFA) can have many beneficial biological effects, such as reducing plasma lipids (Harris, 1989) and preventing thromboses (Kito et al., 1985). Recently, Yazawa et al. (1992) reported that phospholipids (PL) containing HUFA (HUFA-PL) exert a specific effect on decreasing the weight of the adipose tissue. More recently, Mori (1994) demonstrated that HUFA-PL prevents hypertension. It is therefore expected to have a use as a functional health food. Lipase and phospholipase-mediated reactions, which are characterized by high selectivity and mild reaction conditions, appear to have great potential for the synthesis of HUFA-PL. Attempts to modify PL have been made using lipase (Yagi et al., 1990) and phospholipase (Pernas et al., 1990; Svensson et al., 1992). Preparation of HUFA-containing phosphatidylcholine was first achieved by Yoshimoto et al. (1986) and more recently by Na et al. (1990), Totani & Hara (1991), Hosokawa et al. (1991, 1993), and Mutua & Akoh (1993), but there have been no other reports of preparation of HUFA containing phosphatidylethanolamine (PE) (HUFA-PE) by direct esterification of lysophosphatidylethanolamine (LPE).

The focus of the present work was to prepare HUFA containing PE at the sn-2 position by positional selectivity of porcine pancreatic phospholipase A\(_2\) (PLA\(_2\)) with food industry-acceptable glycerol as a dispersible solvent.

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Materials and methods

Materials used were soybean phosphatide extract (phosphatidylcholine >95%; Avanti Polar Lipids Inc., Alabaster, AL, USA); purified eicosapentaenoic acid (EPA; >99%) and docosahexaenoic acid (DHA, >99%; Idemitsu, Tokyo, Japan); fatty acid mixture from sardine oil (Nippon Chemical Feed Ltd, Hakodate, Japan) prepared by saponification (Osada et al., 1991); ethanolamine hydrochloride (Aldrich, Milwaukee, MI, USA); porcine pancreatic PLA2 (Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan); reagent grade (600 U/mg-1 protein, used in fatty acid binding position analysis of HUFA-PE, Sigma, St Louis, MO, USA) and phospholipase D from Streptomyces sp. (a generous gift from Toyo Jozo Co. Ltd, Tokyo, Japan).

LPE was prepared from PE obtained from soybean phosphatide extract and ethanolamine hydrochloride with phospholipase D (Juneja et al., 1988) and then hydrolysed to LPE and free fatty acid (FFA) with PLA2 (Wells & Hanahan, 1969). LPE was isolated by silica gel column chromatography with chloroform:methanol (2:3 v/v) after removal of FFA with chloroform:methanol (9:1 v/v). In order to check the purity of LPE obtained, it was applied to chromarod S III (Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform:methanol:water (10:5:3 v/v/v). The purity exceeded 95% by thin-layer chromatography [(TLC-FID; Iatron Laboratories Inc); identification was confirmed by two-way TLC, first chloroform:methanol:water (65:25:4 v/v/v), second chloroform:butanol:water (60:20:20 v/v/v)].

HUFA-PE synthesis was carried out in 50-ml glass-stoppered flasks controlled at 25°C and stirred (teflon-coated stir bar, 600–800 r.p.m.) under argon for 48 h. Reactions were initiated by adding dialysed freeze-dried PLA2 [25 mg (4785 U)] in 0.3–1.0 ml of 0.2 M Tris–HCl buffer (pH 8.0) containing 3 μmole CaCl2 to the LPE, FFA and glycerol. Reaction was terminated by addition of chloroform:methanol:water (10:5:3, v/v/v) and products recovered from the chloroform layer were separated on silica (Sep-Pak; Waters, Millipore, Milford MA) columns. FFA were eluted by chloroform:methanol (10:1 v/v) and residual LPE and HUFA-PE were eluted with methanol and measured gravimetrically. Yields of HUFA-PE were obtained by separation on chromarod S III developed with chloroform:
Synthesis of phospholipid by phospholipase A$_2$

Figure 2. Effect of volume of buffer on HUFA-containing PE synthesis from LPE (individual samples).

*Yield(%) = HUFA-PE/substrate LPE $\times$ 100 (w/w)

methanol : water (65:25:4 v/v/v) and analysis by TLC-FID as above using standard curves constructed from known quantities of LPE and HUFA-PE; the yield was calculated from the PL weight and the peak areas on the TLC-FID chromatograms.

The fatty acid composition of HUFA-PE was measured by gas chromatography (GC) on methyl esters on methanol-eluted scraped-off bands from silica gel TLC developed with chloroform : methanol : water (65:25:4 v/v/v). The analytical conditions were Unisole 3000 3 m x 3 m glass column at 220°C using N$_2$ 30 ml min$^{-1}$ as carrier gas.

For fatty acid positional analysis of HUFA-PE, a sample (8 mg) was dissolved in 2.6 ml of 0.2 M Tris–HCl buffer (pH 8.0) containing 6 mM CaCl$_2$ and 27.5 mM sodium deoxycholate, reagent grade PLA$_2$ (25 U) was added and the mixture incubated at 40°C for 45 min. After termination with chloroform : methanol : water (10:5:3 v/v/v), LPE and FFA were recovered from the chloroform layer and analysed by TLC and GC, respectively, as above.

Results and discussion

As it is known that the water content in a reaction system is critical to the PLA$_2$-mediated esterification process (Na et al., 1990), we investigated it by varying the volume of added buffer. In a typical synthesis, 110 mg of LPE and 180 mg of sardine oil FFA were combined with 5500 mg of glycerol containing 0.3–1.0 ml of buffer. Results of single syntheses (Fig. 2) demonstrated that the optimum content of added buffer was 0.4–0.5 ml; with 1.0 ml the yield was very low, presumably due to increased hydrolysis.

The influence of varying the FFA content on HUFA-PE synthesis (Fig. 3) under identical reaction conditions at a buffer content of 0.5 ml demonstrated increased yield with an increase of FFA content up to 180 mg, and then no further increase. This composition was used for further HUFA-PE syntheses using purified EPA (>99%) and DHA (>99%) as substrates, to establish total and positional fatty acid composition of the HUFA-PE product. The yields of EPA- and DHA-containing PE were 27.2% and 29.7%, respectively, with incorporation rates of 46.5% (almost the maximum of the theoretical incorporation rate; PLA$_2$ esterifies FFA exclusively at the sn-2 position of LPE) and 46.6%, respectively. Na et al. (1990) reported the EPA incorporation of 36.6% in the microemulsion system (theoretical maximum: 45%).
We have previously demonstrated that HUFA resides exclusively at the sn-2 position of phosphatidylethanolamine after PLA₂-mediated esterification of LPC (Hosokawa et al., 1991). In the present syntheses, substrate EPA was also incorporated almost exclusively in the sn-2 position of PE (Table 1), and confirms the selectivity of PLA₂ in esterifying lysophospholipid with HUFA. Specific incorporation of HUFA to the sn-2 position of phospholipid can be exploited to produce various added levels without some of the deleterious effects compared to chemical synthesis.

Acknowledgment
This work was partly supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture of Japan.

Table 1. Total fatty acid composition of the lysophosphatidylethanolamine substrate and the highly unsaturated fatty acid-containing phosphatidylethanolamine, and positional analysis of eicosapentaenoic acid-containing phosphatidylethanolamine

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<th>Fatty acid (%)</th>
<th>Yield (%)*</th>
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<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
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<td>12.2</td>
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<td>27.3</td>
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* Yield (%) = HUFA-PE/substrate LPE × 100 (w/w).

¹ Duplicate samples.
References


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