Conversion to Docosahexaenoic Acid-Containing Phosphatidylserine from Squid Skin Lecithin by Phospholipase D-Mediated Transphosphatidylation

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Phospholipase D (PLD)-mediated transphosphatidylation of squid skin lecithin with L-serine was examined to prepare docosahexaenoic acid-containing phosphatidylserine (DHA-PS). When a biphasic system with organic solvent and 0.2 M acetate buffer (pH 5.5) was used, PS synthesis was significantly affected by the amount of 3.4 M L-serine-containing acetate buffer. L-Serine concentration in the acetate buffer and choice of organic solvent were also crucial. In a typical reaction with 0.8 unit of PLD (*Streptomyces* sp.), 2.5 mL of ethyl acetate substrate solution containing 30 mg of squid skin lecithin in combination with 3 mL of 3.4 M L-serine-containing 0.2 M acetate buffer (pH 5.5), PS content in the recovered phospholipid fraction increased to 43.1% after 24 h. DHA composed 37.6% of fatty acids in the converted PS. This was the same DHA level as in the substrate. Phosphatidylcholine (squid skin PC, DHA 44.2%) in the squid skin lecithin was more effectively converted to PS than phosphatidylethanolamine.

Keywords: *Phosphatidylserine; docosahexaenoic acid; transphosphatidylation; phospholipase D; squid skin lecithin*

INTRODUCTION

Phosphatidylserine (PS) is one of the major phospholipids in mammalian cell membranes and plays important roles in dynamic membrane functions. It is well-known that PS is an activator of protein kinase C (Nishizuka, 1992) and that it regulates the activities of various enzymes such as Na^+/K^+-ATPase (Stekhoven et al., 1994). In clinical trials conducted in the United States and Europe, it was also indicated that PS supplemented in the diet plays important roles in the support of mental functions in the aging brain (Crook et al., 1991, 1992; Cenacchi et al., 1993). Since then, the benefits of PS in brain functions have received a great deal of attention. According to Tanaka et al. (1992), PS with docosahexaenoic acid (DHA) on the *sn*-2 position has a higher acetylcholine-releasing effect for mouse brain synaptosomes than other PS molecular species. Therefore, the beneficial effect of PS in brain functions may differ among PS molecular species. Position *sn*-2 might be more crucial than position *sn*-1 when the function of PS with the same acyl moiety is compared.

Phospholipase D (PLD) catalyzes transphosphatidylation to transfer phosphatidyl residue from phospholipids to alcohol (Kovatchev and Eibl, 1978). PS is formed by the transphosphatidylation of phosphatidylcholine (PC) with L-serine, as shown in Figure 1 (Juneja et al., 1989a; Comfurius et al., 1990; Kamata et al., 1998). Because PLD-mediated transphosphatidylation proceeds by a one-step reaction, this reaction may be very useful in obtaining DHA-bound PS using squid skin lecithin as a substrate without impairing the DHA level in fatty acid composition. However, the activity of PLD on transphosphatidylation is remarkably affected by reaction conditions employed (Juneja et al., 1987, 1989b). Taking advantage of the abundance of DHA in squid skin lecithin, we examined the optimum reaction conditions to prepare DHA-containing PS.

MATERIALS AND METHODS

Materials. PLD (EC 3.1.4.4), from *Streptomyces* sp., was a generous gift from Asahi Chemical Industry Co. (Fuji, Japan). A lecithin extracted from squid skin (squid skin lecithin) was obtained from Bizen Chemical Co. Ltd. (Okayama, Japan). PC (isolated squid skin PC) and phosphatidylethanolamine (PE) (isolated squid skin PE) were separated from squid skin lecithin by using preparative silica gel thin layer chromatography (TLC) with chloroform/methanol/water (65:25:4, v/v/v) as a developing solvent. L-Serine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents used were of analytical grade.

Transphosphatidylation. Transphosphatidylation to PS from squid skin lecithin with L-serine by PLD was carried out in a biphasic system because water miscible L-serine and immiscible squid skin lecithin were used as substrates. In this study, five solvents, ethyl acetate, diethyl ether, benzene, toluene, and *n*-hexane, were used to determine the influence of the organic solvent. Squid skin lecithin was dissolved in 2.5 mL of organic solvent and then added into various volumes (0.5−5.0 mL) of 0.2 M acetate buffer (pH5.5) containing various amounts of PLD and L-serine. The reaction was carried out at 30 °C, at 500 rpm, with a magnetic stir bar. Transphosphatidylations to PS from isolated squid skin PC or isolated squid skin PE were carried out under the biphasic system of ethyl acetate and acetate buffer.

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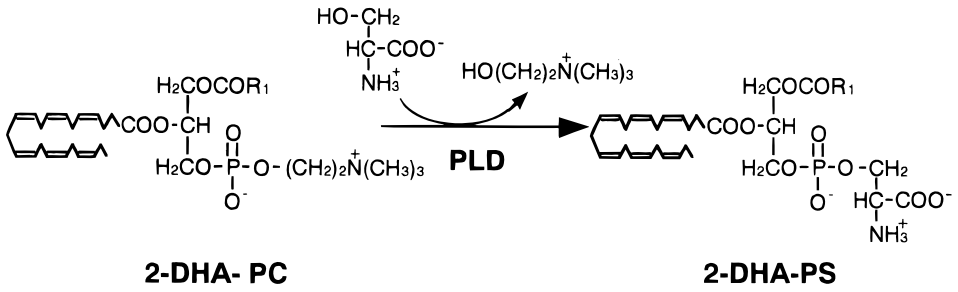

Figure 1. Reaction scheme of transphosphatidylation to DHA-PS from DHA-PC.

Hydrolysis. Hydrolytic reaction was done in the same manner with transphosphatidylation but with L-serine being omitted. Fifteen milligrams of the isolated squid skin PC or squid skin PE was dissolved in 1.25 mL of ethyl acetate and then added into 1.5 mL of 0.2 M acetate buffer (pH 5.5) containing 0.4 unit of PLD. The reaction was carried out at 30 °C and stirred with a Teflon-coated stir bar (500 rpm).

Reaction Mixture Monitoring. An aliquot sample (0.1 mL) from the reaction mixture was withdrawn and put into 5 mL of chloroform/methanol (2:1, v/v) and 0.9 mL of water. The solution was mixed well and centrifuged for 2 min at 2500 rpm, and then the lower layer was collected. Samples were dried and resolved with 0.1 mL of chloroform, and then 10 μL chloroform solutions were spotted onto a TLC plate (E. Merck Co., Darmstadt, Germany). The TLC plate was developed with chloroform/methanol/25% ammonium solution (65:25:4, v/v/v) for reaction mixtures from the squid skin lecithin or with chloroform/methanol/water (65:25:4, v/v/v) for reaction mixtures from the isolated squid skin PC and squid skin PE, and then charred at 160 °C for 20 min after being sprayed with 8% phosphoric acid containing 3% copper acetate (Fewster et al., 1969). Afterward, the plate was subjected to densitometry (model F-808, Cosmo Co. Ltd., Tokyo, Japan), and each phospholipid content was calculated from peak area. Converted PS was identified with both R_f value of the corresponding PS standard and coloration by ninhydrin reagent.

Fatty Acid Composition. The lipid fraction was separated from the 24 h reaction mixture with chloroform/methanol/water (10:5:3, v/v/v). Then an aliquot of this lipid fraction (chloroform layer) was spotted onto a preparative silica gel TLC plate (E. Merck Co.) and developed with chloroform/methanol/ water (65:25:4, v/v/v). The band corresponding to PS was scrapped off, and PS was eluted with methanol. PS thus obtained was then methylated according to the method of Christopher and Glass as described by Prevot and Mordret (1976) (Maeda et al., 1987) to analyze the fatty acid composition. An aliquot amount of PS was dissolved in 1.0 mL of *n*-hexane, and 0.2 mL of methanolic 2 N NaOH solution was added. After this mixture was shaken, it was allowed to stand for 30 s at 50 °C, and then 0.2 mL of methanolic HCl solution was added. The *n*-hexane layer was collected and concentrated. To analyze the fatty acid composition of phospholipids contained in the substrate, squid skin lecithin was first applied onto a silica Sep-Pak cartridge (Waters Associates Co. Ltd., Milford, MA) to remove nonpolar lipids. They were removed with chloroform. The remaining phospholipids in the silica Sep-Pak cartridge were then eluted with methanol. Methylation was also carried out as described above. Fatty acid composition was analyzed with a Hitachi 163 gas chromatograph equipped with a flame ionization detector (Hitachi Co. Ltd., Tokyo, Japan), G-300 column (1.2 mm × 40 m, Chemicals Inspection and Testing Institute, Tokyo, Japan), and D-2500 integrator. Helium was used as the carrier gas at a flow rate of 10 mL/min.

RESULTS AND DISCUSSION

Lipid Composition of the Substrate Squid Skin Lecithin. The phospholipid content of squid skin lecithin was 82.4% when gravimetrically determined as shown in Table 1. The major phospholipid classes were PC (44.2%), PE (29.4%), and lysophosphatidylethanolamine (LPE; 12.2%). PS was low (3.3%), and phosphatidic acid (PA) was not detected. The DHA level in the phospholipids of squid skin lecithin was 36.8% as seen in Table 2. That of eicosapentaenoic acid (EPA) was 15.1%, and $n-3$ highly unsaturated fatty acid occupied >50% in the fatty acids of substrate phospholipids. Arachidonic acid was 3.2%. In the squid skin PC and PE isolated from the squid skin lecithin, DHA levels were 44.2 and 23.3%, respectively.

Table 1. Phospholipid Content and Phospholipid Composition of Substrate Squid Skin Lecithin

	phospholipid content[a] (%)	phospholipid composition (%)				
		PC	PE	PS	LPE	others[b]
squid skin lecithin	82.4	44.2	29.4	3.3	12.2	10.9

[a] Phospholipid weight percent to squid skin lecithin. [b] Other phospholipids.

Table 2. Fatty Acid Composition of Phospholipid in Substrate Squid Skin Lecithin and Isolated Squid Skin PC and PE

	16:0	18:0	18:1	20:4 (AA)	20:5 (EPA)	22:6 (DHA)	others
squid skin lecithin	30.0	3.8	2.9	3.2	15.1	36.8	8.2
squid skin PC	36.9	1.6	2.0	0.9	8.0	44.2	6.4
squid skin PE	9.8	6.2	3.9	9.6	35.2	23.3	12.0

Table 3. Effect of Organic Solvent on Conversion to PS from Squid Skin Lecithin by PLD[a]

	relative PS content (%) at reaction time of	
organic solvent	3 h	24 h
ethyl acetate	30.0	35.2
diethyl ether	20.0	36.2
benzene	23.7	32.7
toluene	17.0	33.4
n-hexane	3.7	9.7

[a] Reaction conditions: 0.8 unit of PLD, 30 mg of squid skin lecithin, 2.5 mL of organic solvent, 1.0 mL of 3.4 M L-serine-containing 0.2 M acetate buffer (pH 5.5), 30 °C, 500 rpm.

Effect of Organic Solvent Choice on Transphosphatidylation. It is known that the transphosphatidylation activity of PLD is affected by the choice of organic solvent (Juneja et al., 1988). We found that in a biphasic reaction system consisting of 2.5 mL of organic solvent with 30 mg of squid skin lecithin in addition to 0.8 unit of PLD dissolved in 1.0 mL of acetate buffer containing 3.4 M L-serine, ethyl acetate or diethyl ether seemed to be desirable for carrying out transphosphatidylation. The PS content increased to >35% within 24 h when ethyl acetate or diethyl ether was used as shown in Table 3. Between these two, ethyl acetate was the best solvent because the PS content increased up to 30.0% within 3 h. These results were in agreement with the

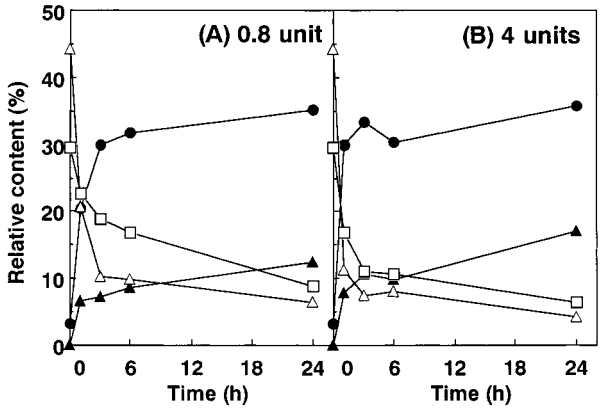

Figure 2. Time courses of conversion to PS from squid skin lecithin by PLD. Reaction conditions: (A) 0.8 unit of PLD; (B) 4 units of PLD, 1.0 mL of 0.2 M acetate buffer (pH 5.5) containing 3.4 M L-serine, 2.5 mL of ethyl acetate, 30 mg of squid skin lecithin, 30 °C, 500 rpm; (●) PS; (△) PC; (▲) PA; (□) PE.

findings of Juneja et al. (1989a), who carried out the transphosphatidylation of soy PC to PS. When benzene and toluene were used, PS contents were also comparable but slightly lower (Table 3). In the case of *n*-hexane, PS contents did not exceed 10%. Thus, we concluded that ethyl acetate is the best organic solvent for transphosphatidylation of highly unsaturated lecithin.

Effect of Squid Skin Lecithin and Phospholipase D Contents on Transphosphatidylation in a Biphasic Reaction System. The time course of transphosphatidylation by PLD in the biphasic system of 2.5 mL of ethyl acetate and 1.0 mL of acetate buffer is shown in Figure 2A. In the reaction system with 0.8 unit of PLD, the PS content increased rapidly in the first 3 h and then reached 35.2% after 24 h. Conversely, PC and PE were hydrolyzed to 6.5 and 8.8%, respectively. During this reaction, PA increased to 12.5% after 24 h. The occurrence of PA should be the result of the unavoidable hydrolytic side reaction. LPE remained almost unchanged throughout the reaction (data not shown). When PLD was 4 units, the initial rate of PS synthesis was accelerated, but the ultimate PS content did not increase as shown in Figure 2B.

When transphosphatidylation was carried out under twice the amount (60 mg) of squid skin lecithin with 0.8 unit of PLD, the PS content increased to 34.0% after 24 h (data not shown). However, when 5 times the amount (150 mg) of squid skin lecithin was used with the same number of PLD units, the level of conversion to PS did not exceed 20% (Figure 3A). When PLD was increased to 4 units, the PS content was enhanced up to ~30%, but the PS content did not go over 30% even though PLD was fortified to 20 units. Only the PA formation was increased with the excess of PLD (Figure 3B, C). Juneja et al. (1992) reported that choline released from PC inhibits the transphosphatidylation activity of PLD. In the reaction system with 150 mg of squid skin lecithin, choline released from PC may impair the synthetic reaction of PS from PA and L-serine because PA itself was proportionally increased with the amount of PLD.

Effect of the Amount of L-Serine-Containing Buffer on Transphosphatidylation. As shown in Figure 4, we found that transphosphatidylation is remarkably affected by the amount of 0.2 M acetate

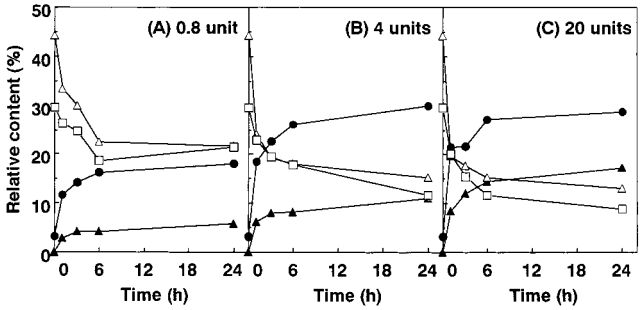

Figure 3. Effect of PLD content on conversion to PS from squid skin lecithin (150 mg). Reaction conditions: (A) 0.8 unit of PLD; (B) 4 units of PLD; (C) 20 units of PLD, 1.0 mL of 0.2 M acetate buffer (pH 5.5) containing 3.4 M L-serine, 2.5 mL of ethyl acetate, 150 mg of squid skin lecithin, 30 °C, 500 rpm; (●) PS; (△) PC; (▲) PA; (□) PE.

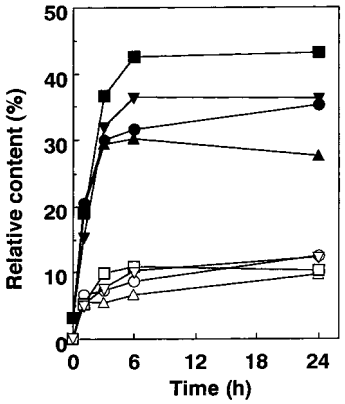

Figure 4. Effect of 3.4 M L-serine-containing acetate buffer content on conversion to PS by PLD. Reaction conditions: 0.8 unit of PLD; (▲, △) 0.5 mL; (●, ○) 1.0 mL; (■, □) 3.0 mL; (▼, ▽) L-serine-containing 0.2 M acetate buffer (pH 5.5), 2.5 mL of ethyl acetate, 30 mg of squid skin lecithin, 30 °C, 500 rpm; (solid symbols) PS; (open symbols) PA.

buffer (3.4 M L-serine, pH 5.5). The optimum acetate buffer content was 3 mL, which gave the highest PS content (43.1%) after 24 h. When 5 mL of L-serine-containing buffer was employed, the ultimate PS content after 24 h decreased compared to that of the 3 mL buffer system. Comfurius et al. (1990) reported that some detergents can enhance PS synthesis in a one-phase transphosphatidylation system. We also observed that the addition of $CaCl_2$ and bovine serum albumin can increase PS synthesis (data not shown). Albumin obviously affects the interfacial environment in a biphasic system. Thus, it is inferred that transphosphatidylation by PLD is crucially affected by the interface environment. It is presumed that the interfacial environment in our biphasic system was influenced by the amount (the ratio against organic solvent) of the L-serine-containing buffer.

On the other hand, when the volume of acetate buffer was increased by keeping the absolute amount of L-serine to a constant level (3.4 mmol), PS synthesis decreased while PA, which is a hydrolysate, increased (Figure 5). This indicates that the equilibrium of the reaction might be shifted to a hydrolytic side reaction in accordance with the decrease in L-serine concentration in the acetate buffer (from 3.4 to 0.68 M). From these results, we concluded that not only the volume of L-serine-containing buffer but also the L-serine concentration in the buffer are important to obtain high PS

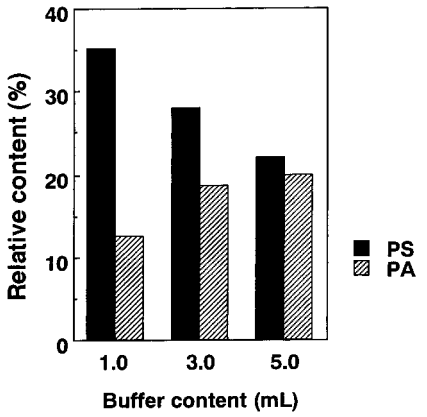

Figure 5. Effect of acetate buffer content on conversion to PS by PLD by keeping the L-serine amount constant/system. Reaction conditions: 0.8 unit of PLD, various 0.2 M acetate buffer (pH 5.5) volumes with L-serine level constant at 3.4 mmol, 2.5 mL of ethyl acetate, 30 mg of squid skin lecithin, 30 °C, 500 rpm, 24 h.

synthesis when the proposed reaction system for squid skin lecithin is carried out.

Transphosphatidylation and Hydrolysis of Isolated Squid Skin Phospholipid Classes. We compared the conversions of individual phospholipid classes to PS. As shown in Figure 2, the decrease of PC was more rapid than that of PE at the early stage of the reaction. It is known that substrate specificities of PLD vary depending on the source of PLD (Juneja et al., 1989b). To compare the susceptibility against transphosphatidylation between squid skin PC and PE, which are the main components in squid skin lecithin, we carried out the reactions with squid skin PC and PE obtained from the same source. As shown in Figure 6A, PC was converted to PS more effectively than PE. PS content reached 60% within 24 h. PC was hydrolyzed but partly remained as PA at a level of 13.6%. On the other hand, PS synthesis from squid skin PE was poor. Considerable amounts of PE remained unhydrolyzed even after 24 h of reaction. Thus, PLD from *Streptomyces* sp. used in this study exhibited a higher activity for squid skin PC transphosphatidylation than it did for PE. Squid skin PC may first be hydrolyzed more efficiently than PE by PLD, and with this sufficient level of PA, L-serine should be easily incorporated into PA to produce PS (Figure 6B). It is the hydrolytic specificity of PLD that might be crucial for transphosphatidylation of highly unsaturated phospholipids. Because squid skin PC was a more suitable substrate for transphosphatidylation than PE, in the proposed reaction system it might be desirable to increase the PC content in the squid skin lecithin beforehand to enhance PS synthesis.

Fatty Acid Composition of the Converted PS. The DHA content of PS prepared from squid skin lecithin was 37.6%, and that of the isolated PC was 43.3% (Table 4). This study confirmed that DHA, EPA, AA, and other fatty acids remain unchanged throughout the PLD-mediated transphosphatidylation and that highly unsaturated fatty acid content in the converted PS is dependent on the highly unsaturated fatty acid content in substrate phospholipid.

Conclusion. DHA-containing PS can be prepared easily from squid skin phospholipid, which contains abundant DHA. The crucial points are the ratio of L-serine-containing buffer against organic solvent, the saturation of L-serine in buffer, the choice of an organic solvent that is moderately immiscible to water, and the proper ratio of PLD against substrate. PS with $n-3$ highly unsaturated fatty acids, DHA in particular, would have high potential for nutritional and pharmaceutical uses. It is hoped that this study will stimulate further investigations on the physiological activities of DHA-containing PS.

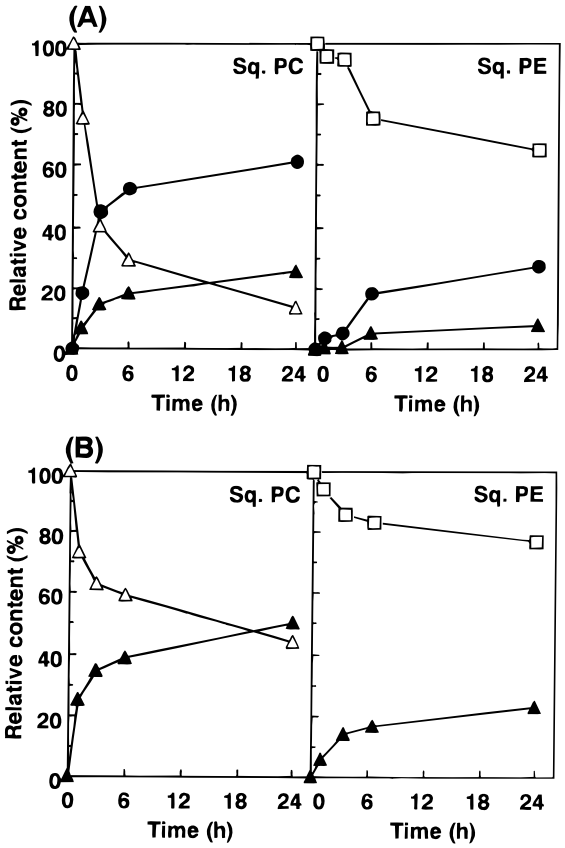

Figure 6. The transphosphatidylation (A) and the hydrolytic reaction (B) of isolated squid skin phosphatidylcholine (Sq. PC) and phosphatidylethanolamine (Sq. PE) by PLD. Reaction conditions: (A) 0.8 unit of PLD, 3.0 mL of 3.4 M L-serine-containing 0.2 M acetate buffer (pH 5.5), 2.5 mL of ethyl acetate, 30 mg of squid skin PC or PE, 30 °C, 500 rpm; (B) 0.4 unit of PLD, 1.5 mL of 0.2 M acetate buffer (pH 5.5), 1.25 mL of ethyl acetate, 15 mg of squid skin PC or PE, 30 °C, 500 rpm; (●) PS; (△) PC; (▲) PA; (□) PE.

Table 4. Fatty Acid Composition of Converted Phosphatidylserine

	16:0	18:0	18:1	20:4 (AA)	20:5 (EPA)	22:6 (DHA)
PS converted from squid skin lecithin	31.4 (30.0)[a]	4.1 (3.8)[a]	3.2 (2.9)[a]	2.6 (3.2)[a]	13.1 (15.1)[a]	37.6 (36.8)[a]
PS converted from isolated squid skin PC	38.0 (36.9)[b]	1.7 (1.6)[b]	1.9 (2.0)[b]	1.1 (0.9)[b]	8.9 (8.0)[b]	43.3 (44.2)[b]

[a] That of the phospholipid of the substrate squid skin lecithin. [b] That of the substrate isolated squid skin PC.

ABBREVIATIONS USED

PS, phosphatidylserine; DHA, docosahexaenoic acid; PLD, phospholipase D; squid skin PC, squid skin phosphatidylcholine; squid skin PE, squid skin phosphatidylethanolamine; TLC, thin layer chromatography; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; EPA, eicosapentaenoic acid.

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