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# Cytosolic lysophosphatidylcholine/transacylase in the production of dipolyunsaturated phosphatidylcholine in bonito muscle

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**Abstract Phosphatidylcholine with docosahexaenoic acid at both *sn*-1 and *sn*-2 positions occurs in relatively high abundance in bonito muscle. To explore a possible route for the dipolyunsaturated molecular species, phosphatidylcholine formation from 2-[1-$^{14}$C]linoleoyl lysophosphatidylcholine was examined using a cytosolic fraction from bonito muscle. The formation of radiolabeled phosphatidylcholine was greatest at 15°C and did not require the presence of cofactors such as CoA and calcium. By DEAE-cellulofine column chromatography, the activity to form phosphatidylcholine was separated from that of phospholipase $A_1$, and the specific activity increased by about 100-fold. The possible involvement of cytosolic lysophosphatidylcholine/transacylase in synthesis of dipolyunsaturated phosphatidylcholine is discussed.**

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*Key words:* Dipolyunsaturated molecular species; 2-Acyl lysophosphatidylcholine; Phospholipase $A_1$; Lysophosphatidylcholine/transacylase

## 1. Introduction

The fatty acyl residues of individual phospholipids appear to be under strict metabolic regulation [1–3]. Generally, saturated fatty acids, such as palmitic and stearic acids, are esterified at the *sn*-1 position, while polyunsaturated fatty acids are commonly found at the *sn*-2 position. However, there are two exceptional phospholipid molecular species, that is, dipalmitoyl phosphatidylcholine (PC) in lung surfactant [4,5] and the didocosahexaenoate-containing molecular species of PC, phosphatidylethanolamine (PE) and phosphatidylserine in retina [6,7]. Cytosolic lysophospholipase/transacylase from lung is known as an enzyme, at least in part, responsible for the synthesis of dipalmitoyl PC [8–10]. However, to our knowledge, the synthesis process of dipolyunsaturated molecular species of phospholipids is still a matter of speculation.

Previously, we found a substantial amount of lysophosphatidylcholine (LPC), which is composed mainly of 2-acyl LPC, in bonito muscle [11], and in this connection, we found phospholipase $A_1$ ($PLA_1$) activity, which also had a transacylase function [12]. During the course of studies on the physiological meaning of $PLA_1$/transacylase, dipolyunsaturated molecular species, especially the didocosahexaenoyl (22:6/22:6) species that comprises about 10% of choline phosphoglyceride, were detected in bonito muscle. There are three routes for a remodeling of phospholipid molecular species: phospholipase coupled with acyltransferase, CoA-dependent or -independent phospholipid/transacylation, and lysophospholipase coupled with transacylase systems [3].

In the present study, we found that a novel transacylation system in a cytosolic fraction of bonito muscle produces PC using two molecules of 2-acyl LPC. This system coupled with $PLA_1$ activity might be responsible for the occurrence of dipolyunsaturated molecular species of PC in bonito muscle.

## 2. Materials and methods

### 2.1. Materials

1,2-Dipalmitoyl, 1,2-dioleoyl and 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Docosahexaenoic acid (DHA) was from Serdary Research Laboratories (London, Ont., Canada). Heat-treated venom from *Trimeresurus flavoviridis* was used as phospholipase $A_2$ ($PLA_2$). Phospholipase C (PLC) from *Bacillus cereus* and coenzyme A (CoA) were from Sigma Chemicals (St. Louis, MO, USA). All radiolabeled phospholipids were the products of Amersham International (Amersham, UK). 1,2-Di[1-$^{14}$C]palmitoyl and 1,2-di[1-$^{14}$C]oleoyl-*sn*-glycero-3-phosphocholines were diluted with the respective PCs, and their specific activities were 400 cpm/nmol. 1-Palmitoyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholines was diluted with egg yolk PC, and its specific activity was 200 cpm/nmol. The pre-coated thin-layer plates (Silica gel 60) were the product of Merck (Darmstadt, Germany). DEAE-cellulofine was from Seikagaku Kogyo (Tokyo, Japan). 1-[1-$^{14}$C]Acyl LPCs were prepared by the hydrolysis of 1,2-di[1-$^{14}$C]acyl PCs with $PLA_2$ [11], and 2-[1-$^{14}$C]acyl LPCs were prepared by the hydrolysis of PC with $PLA_1$ from bonito muscle cytosol. 2-DHA LPC was chemically prepared from beef heart choline phosphoglyceride as described previously [12].

### 2.2. Preparation of cytosolic fraction

Fresh bonitos were obtained from a local market in November and December, 1997. The following procedures were carried out at 4°C and centrifugation was performed at $10\,000 \times g$ for 30 min in a refrigerated centrifuge. To 100 g of the muscle, 200 ml of 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA and 0.25 M sucrose (buffer A) was added. The muscle was minced with scissors and then homogenized. After filtration through gauze, the filtrate was centrifuged twice at $10\,000 \times g$ for 15 min. The supernatant was then centrifuged at $100\,000 \times g$ for 2 h, and the resulting precipitate and supernatant were used as microsomal and cytosolic fractions. To the cytosolic fraction, solid ammonium sulfate was added to produce 60% saturation. The precipitate was collected by centrifugation and dissolved in a small volume of buffer A or 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 10% glycerol and 1 mM *N*-acetyl-L-cysteine (buffer B) for column chromatography.

### 2.3. Enzyme assay

The $PLA_1$ assay followed the method described [12]. LPC/transacylase was evaluated using the following assay conditions. The cytosolic fraction from bonito muscle was incubated with 300 nmol of 2-[1-$^{14}$C]linoleoyl LPC in the presence of 25 μmol of Tris-HCl (pH 6.5) in a total volume of 1 ml. The mixture was vigorously stirred with a Taitec TC-8 concentrator at maximum speed at 15°C for 30 min, and lipids were extracted by the method of Bligh and Dyer [13]. The extract was applied to a thin-layer plate and developed as described [12]. The regions corresponding to PC and free fatty acid (FFA) were detected, scraped off, and counted. The specific activity of $PLA_1$ is

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expressed as nmol of 2-[1-$^{14}$C]linoleoyl LPC formed per mg protein per h, and that of transacylase as nmol of PC formed per mg protein per 30 min, respectively. Data from each figure were obtained from a given cytosol preparation. However, experiments were repeated and the same trends were always seen, although different specific activities were obtained because of an instability in freezing and thawing and use of another cytosol preparation.

*2.4. Reversed-phase high-performance liquid chromatography (HPLC)*
Phospholipids were hydrolyzed with PLC and the resulting diglycerides were derivatized to dinitrobenzoyl derivative. They were separated by reversed-phase HPLC with a ODS 120-T column using a mixture of acetonitrile:2-propanol (90:10, v/v) as the eluting solution [14].

*2.5. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry*
Spectra were obtained in the positive ion mode on a PerSeptive Voyager Elite TOF mass spectrometer using a nitrogen laser. Phospholipid dissolved with acetonitrile was analyzed for molecular weight using 2,5-dihydrobenzoic acid (Aldrich, Milwaukee, WI, USA) as matrix.
The protein concentration was determined by the method of Lowry et al. [15], and the phosphorus concentration was determined according to the procedure of Bartlett [16].

## 3. Results

Phospholipid is the most abundant lipid class, being 7.7 μmol/g fresh bonito muscle. In the phospholipid class, PC is the highest proportion, being two thirds of total phospholipids, and 93.9% of PC was a diacyl subclass. The major fatty acids in PC were DHA (33.7%) and palmitic acid (24.2%). The intramolecular distribution of PC fatty acid showed that one third of DHA occupied the *sn*-1 position; however, it was only 6.0% of PC from liver, although total DHA contents between muscle (33.7%) and liver (32.2%) were similar. Fig. 1 shows representative elution profiles of molecular species of DNB derivatives of PC from bonito muscle and liver. The peak corresponds to 22:6/22:6 species, which comprised 9.6% in muscle. This was scarcely observed in liver. Interestingly, only one third of this amount of 22:6/22:6 species was detected in muscle PE (data not shown).

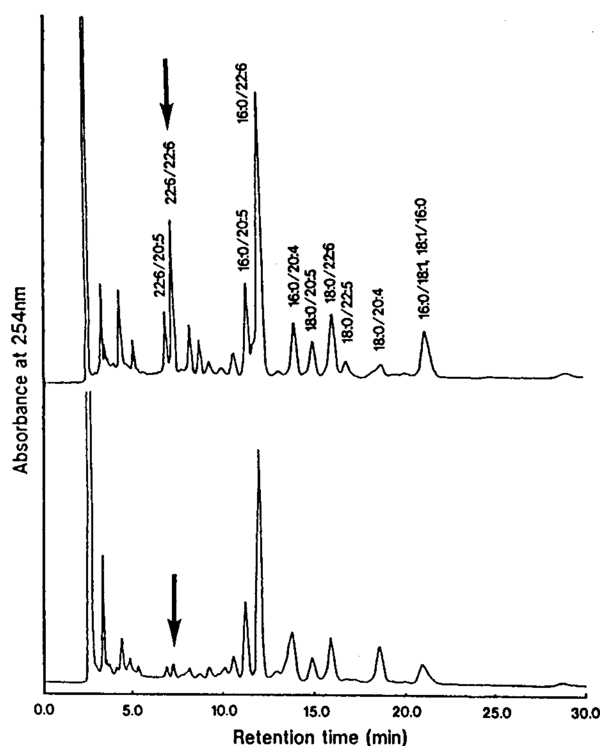


Fig. 1. HPLC chromatograms of DNB derivatives of PC from bonito muscle (upper panel) and liver (lower panel). Phospholipids were obtained by a previously described procedure [11]. 1 μmol of phospholipid was hydrolyzed with PLC and derivatized to dinitrobenzoyl derivative. Arrows indicate the position where standard 1,2-didocosahexaenoyl PC was eluted.

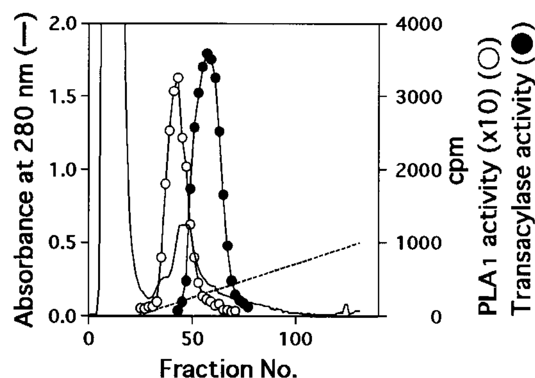


Fig. 2. Column chromatography of bonito cytosolic fraction by DEAE-cellulofine. The $PLA_1$ (○) and PC formation (●) activities were determined using 200 μl and 500 μl aliquots of each fraction, respectively. Absorbance at 280 nm (solid line); KCl concentration (dotted line).

In bonito muscle, $PLA_1$ activity was detected, and the muscle LPC was abundant in 2-acyl LPC, which was predominantly polyunsaturated fatty acid such as DHA [11]. Therefore, it was hypothesized that microsomal acyltransferase carries DHA to 2-acyl LPC, and thus dipolyunsaturated species of PC, such as 22:6/22:6 species, are formed. Incorporation of [1-$^{14}$C]DHA into LPC from bonito muscle was examined using the bonito muscle microsomal fraction. [1-$^{14}$C]DHA was incorporated into LPC time-dependently. However, radioactivity was detected exclusively in the *sn*-2 position of glycerol, indicating that the 2-acyl LPC/acyltransferase system could not be a reason for the synthesis of 22:6/22:6 species of PC (data not shown).

In lung cytosol, there is an enzyme responsible for synthesis of dipalmitoyl PC using two molecules of 1-palmitoyl LPC [9]. By analogy, we speculated that a muscle cytosol may synthesize dipolyunsaturated species using 2-acyl LPC. In a preliminary experiment, 2-[1-$^{14}$C]linoleoyl LPC was incubated at room temperature with 5.4 mg of cytosolic fraction from bonito muscle at pH 6.5 for 1 h. PC (2662 cpm) as well as FFA (1775 cpm) were formed but not without the addition of cytosol. Treatment of the formed PC with $PLA_2$ showed radioactivity was distributed equally between *sn*-1 and *sn*-2 positions. A concomitant production of glycerophosphocholine was confirmed using 2-palmitoyl-3-phospho-[*N-methyl*-$^{3}$H]choline as substrate (data not shown). Further characterization of cytosolic PC formation demonstrates that the optimum temperature was 15°C and the optimum pH was 6.5–7.0, although reaction was not done at pH values higher than 7.0, because migration of the acyl residue to the *sn*-1 position is possible and it was prevented within 5% at pH 6.5. The formation of PC increased time-dependently until 30 min, and then decreased slightly, probably due to hydrolysis of PC by $PLA_1$. Accordingly, formation of PC from 2-[1-$^{14}$C]linoleoyl

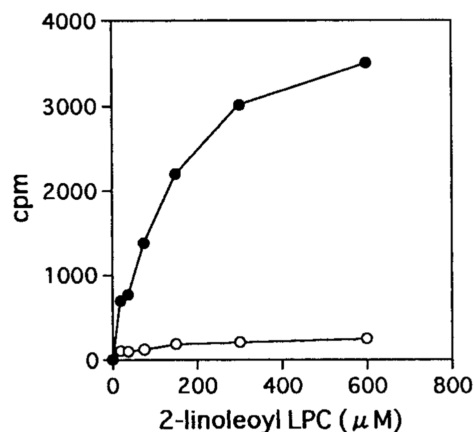


Fig. 3. Lysophospholipase/transacylase activity on increasing substrate concentration. Assay was performed as described in Section 2 using 137 μg of the DEAE fraction in Fig. 2. Radioactivities in PC (●) and FFA (○) fractions were counted.

LPC was examined at 15°C and pH 6.5 for 30 min. PC formation increased with increasing cytosolic protein. No change in PC formation was observed due to addition of $Ca^{2+}$ in a final concentration of 5 mM. Additions of ATP, CoA and $Mg^{2+}$ in final concentrations of 750 nM, 40 nM and 5 mM also had no effect on PC formation, indicating that the transacylation was CoA-independent.

The cytosolic fraction was applied to a DEAE-cellulofine column. After large amounts of unabsorbed materials were washed out, protein was eluted from the column with a gradient of KCl from 0 to 0.5 M. As shown in Fig. 2, $PLA_1$ activity, which produces 2-[1-$^{14}$C]linoleoyl LPC in the presence of sodium deoxycholate, and transacylase activity, which

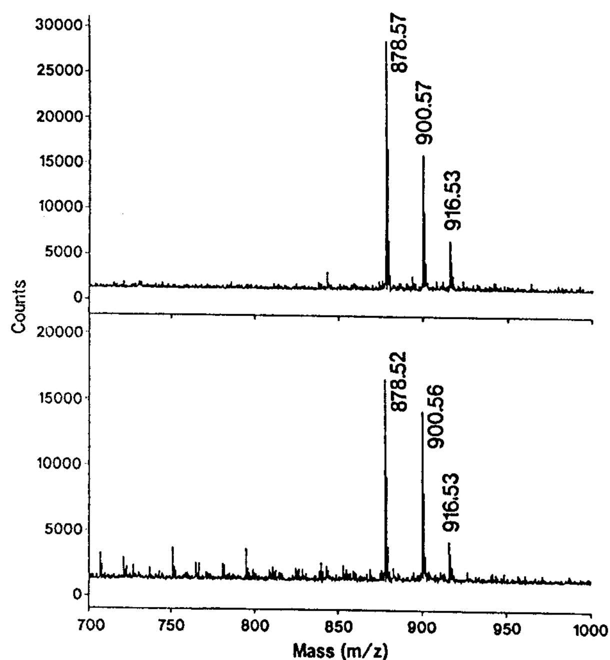


Fig. 4. Mass spectra of standard 1,2-didocosahexaenoyl PC and a product by the cytosolic transacylase. Standard (250 nmol, upper panel) and an enzymatic product using 3.5 mg of the DEAE fraction in Table 1 (lower panel) in 100 μl acetonitrile were mixed with dihydroxybenzoic acid (10 mg/ml acetonitrile) in a ratio of 1:20, and 1 μl of each was spotted on a sample plate.

Table 1
Effect of fatty acid and the position of lysophosphatidylcholine on phosphatidylcholine formation

| | PC formed (nmol/mg/30 min) |
|---|---|
| 1-Palmitoyl LPC | 2.5 |
| 2-Palmitoyl LPC | 5.6 |
| 1-Oleoyl LPC | 38.0 |
| 2-Oleoyl LPC | 42.0 |

The PC formation was performed as described in Section 2 using 137 μg of the DEAE fraction (specific activity, 97 nmol/mg/30 min).

forms radioactive PC from 2-[1-$^{14}$C]linoleoyl LPC in the absence of the detergent, appeared separately. The specific activity of each peak was 1574 nmol/mg/h for $PLA_1$ and 64 nmol/mg/30 min for PC formation. Thus, about 100-fold purification in PC formation activity was achieved from the starting cytosolic fraction.

Fractions 55–63, which were devoid of $PLA_1$ activity, were combined. Fig. 3 presents the velocities of lysophospholipase and LPC/transacylase with increasing substrate concentration. The LPC/transacylase activity tends toward a saturation pattern, although maximum velocity was not reached at a concentration of 600 μM. The conversion rate was about 20% around 20 μM of substrate, and the rate decreased with increasing concentration of the substrate. In contrast, the release of FFA was scarcely observed at any concentration of substrate. Because a higher rate of transacylation is attained at a lower concentration of 2-acyl LPC, the LPC/transacylase fraction was incubated with 2-DHA LPC at a concentration of 150 μM in a preparative scale. From 2.3 μmol of 2-DHA LPC, 150 nmol of 22:6/22:6 PC, which was determined by the HPLC analysis using 1,2-dioleoyl PC as an internal standard, was formed. In a parallel experiment, 22:6/22:6 PC formed was directly analyzed by MALDI-TOF mass spectrometry. A typical MALDI mass spectrum is shown in Fig. 4. Protonated $[MH]^+$ molecular ion at *m/z* 878 as well as its $Na^+$ and $K^+$ adducts coincide with those of standard 22:6/22:6 PC.

The effects of fatty acid and its position on LPC in PC formation are shown in Table 1. The cytosolic LPC/transacylase also utilizes palmitoyl and oleoyl LPC; however, palmitoyl LPC was not as preferred a substrate as oleoyl LPC. As far as the position of fatty acid is concerned, 1-acyl LPC was also a substrate for PC formation, but higher LPC/transacylase activity was observed in 2-acyl LPC than in its positional isomer.

## 4. Discussion

Dipolyunsaturated fatty acid molecular species of phospholipid are often observed in neural tissues, especially in retina [6,7]. Recently, Bell and Dick reported finding a substantial amount of 22:6/22:6 species of phospholipids in cod muscle in addition to retina; however, this species was not abundant in cod liver [17]. In bonito muscle PC, 22:6/22:6 species is a major molecular species, second to 16:0/22:6 species, as shown in Fig. 1. This is consistent with the cod muscle finding, but this species was not found so much in PE as in PC. In the cod muscle PE, this species was the most abundant and comprised 20%. Recently, we reported that there is $PLA_1$ activity in bonito muscle and speculated that this activity might be involved in the formation of 22:6/22:6 species [12]. As a test, we examined a deacylation/reacylation pathway

coupled with $PLA_1$ activity. However, muscle acyltransferase carries DHA exclusively to the *sn*-2 position, which is a general specificity of acyltransferase for polyunsaturated fatty acid [18]. Recently, it has been shown that polyunsaturated fatty acids are also incorporated de novo into the *sn*-1 position of phospholipids in rat hepatocytes. However, this is limited to situations where the concentration of exogenous polyunsaturated fatty acids used are high [19].

Next, we examined a LPC/transacylase system to form PC from 2-acyl LPC. The incubation of 2-[1-$^{14}$C]linoleoyl LPC with a bonito muscle cytosolic fraction resulted in PC production. At this point we theorized that the PC formation was performed by $PLA_1$ because it has been reported that the group VI $Ca^{2+}$-independent cytosolic $PLA_2$ from $P388D_1$ macrophages has lysophospholipase activity, and it also has lysophospholipid/transacylase activity [20]. However, PC formation using 2-[1-$^{14}$C]linoleoyl LPC was separated from the $PLA_1$ activity by DEAE-cellulofine column chromatography (Fig. 2). The formation of PC (LPC/transacylase activity) overwhelmingly exceeded fatty acid release (lysophospholipase activity) even at as low a concentration of substrate as around 20 μM (Fig. 3). This is in striking contrast to the lysophospholipase/transacylase from rat lung. In lung, the ratio of lysophospholipase to transacylase activity depends on the substrate concentration, that is, hydrolysis predominated at low substrate concentrations, while esterification was stimulated at a micelle-forming concentration [9]. The transacylase from rabbit myocardium acts on the submicellar concentration of 1-acyl LPC, which would protectively scavenge LPC in the membrane [21]. In this respect, the property of LPC/transacylase from bonito muscle is similar to that from rabbit myocardium. The LPC/transacylase utilizes 1-acyl LPC as well as 2-acyl LPC. Thus, it is possible to speculate that 1-acyl LPC formed by acyl migration from 2-acyl LPC during the preparation [22] and reaction might be preferentially utilized and PC is formed. However, more PC formation was observed in 2-acyl LPC, especially in palmitoyl LPC (Table 1). Though it is necessary to elucidate the further mechanism of transacylation, it is obvious at present that the LPC/transacylase is different from that from rat lung; that is, the former prefers to utilize unsaturated acyl LPC, whereas the latter prefers palmitoyl LPC [9].

$PLA_1$ may function to release saturated fatty acids, which are effectively utilized by β-oxidation for energy production, and the resulting 2-unsaturated acyl LPC, which is the preferred substrate for the present LPC/transacylase, is converted to PC with no significant FFA production. This tandem system might be a mechanism to produce dipolyunsaturated molecular species in bonito muscle.

Purification and further characterization of the transacylation system responsible for synthesis of dipolyunsaturated species are now in progress.

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