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Synthesis of Novel Phosphatidyldihydroxyacetone *via* Transphosphatidylation Reaction by Phospholipase D

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Phospholipase D (PLD) from *Streptomyces* sp. catalyzed the transfer reaction of the dipalmitoylphosphatidyl residue of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) to dihydroxyacetone (DHA) in a biphasic system, to afford 1,2-dipalmitoyl-3-*sn*-phosphatidyldihydroxyacetone (DPP-DHA). The structure of DPP-DHA was identified by NMR, FAB-mass, IR, and UV spectra. PLD also catalyzed the transphosphatidylation reaction of DPP-DHA to choline, and DPPC was reproduced. In the presence of phospholipase C (PLC) from *Bacillus cereus*, DPP-DHA was hydrolyzed to 1,2-dipalmitoylglycerol (DPG). DPP-DHA might be another source of DHA phosphate.

Dihydroxyacetone (DHA) was first used as a constituent of an after-shave lotion, but was claimed to produce tanning of the skin. Thereafter, DHA was recommended as a tanning agent in cosmetic preparations including lotions and creams.[1-3] DHA among other α-hydroxymethylketones was found to react with free amino groups in proteins, such as keratin, to form Schiff base analogues that condense and polymerize to colored melanidins.[4] Furthermore it has been reported that DHA has properties as an antioxidant and free radical scavenger.[1,5] In spite of these properties, aqueous solution of DHA do not spread well, produce a weak tan, and have a further disadvantage of producing irregular effects. It is desirable to improve the physical properties of DHA and modify its biological activities.

For this purpose, phosphatidylation may be a suitable method, because the phosphatidyl residue is well characterized as a non-toxic carrier moiety that has a high affinity for cell membranes and gives stability to hydrophilic compounds in water systems by forming lipid-microsphere-like bodies.

On the other hand, in metabolites of human body DHA phosphate is a key intermediate in carbohydrate synthesis. It has been seen that aldolases catalyze the synthesis of ketose-1-phosphate from DHA phosphate and a variety of aldehydes.[6] Though DHA phosphate has been prepared by chemical methods[7,8] and enzymatic phosphorylation of DHA with glycerol kinase and ATP,[9] the availability of DHA phosphate has not been improved and the practical use has been restricted. If phosphatidyl DHA is prepared by a convenient method, it may possibly be another source for DHA phosphate, because phosphatidyl DHA can be hydrolyzed to DHA phosphate and diacylglycerol by phospholipase C (PLC).

A transfer reaction of the phosphatidyl residue from phosphatidylcholine to primary lower alkanols has been seen using PLD first from cabbage leaves[10-14] and recently from microorganisms. A large variety of acceptors for transphosphatidylation have been reported.[15-20] Among them, ethanol,[11] ethanolamine,[21] serine,[22] and glycerol,[12] are excellent acceptors, and a water-soluble vitamin,[23] nucleosides,[17,18] and primary higher alkanols with more than 6-carbon chains[24] are also good acceptors. Transphosphatidylation to DHA has not yet been reported.

This paper describes the enzymatic synthesis of DPP-DHA by PLD, together with the transphosphatidylation reaction of DPP-DHA to choline, and the possibility for DPP-DHA to be a source of DHA phosphate.

Materials and Methods

Enzymes and chemical reagents. PLD (EC 3.1.4.4) from *Streptomyces* sp. was purchased from Asahi Chemical Industry Co., (Shizuoka, Japan), and PLC (EC 3.1.4.3) from *Bacillus cereus* was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). DHA dimer and choline chloride were obtained from Nacalai Tesque, Inc., (Kyoto, Japan). DPPC was purchased from Nippon Fine Chemical Co., Ltd., (Osaka, Japan). L-α-Dipalmitoylphosphatidic acid (1,2-dipalmitoyl-3-*sn*-phosphatidic acid) sodium salt (DPPA-Na) and 1,2-dipalmitoyl-*rac*-glycerol (DPG) were obtained from Sigma Chemicals Co., (St. Louis, MO, U.S.A.). Other reagents used were of analytical grade from commercial sources.

Analyses of phosphatidyl compounds and DPG with thin-layer chromatography (TLC). TLC was done by using a silica gel $60F_{254}$ plate (E. Merck Co., Darmstadt, Germany) and $CHCl_3$–MeOH–water (30 : 10 : 1, v/v) or $CHCl_3$–MeOH (20 : 1, v/v) as the solvent. Phosphatidyl compounds and DPG were detected by spraying with 5% phosphomolybdic acid in ethanol, followed by heating, and further analyzed by a Shimazdu Flying-Spot Scanner CS-9000 (absorbance at 500 nm). The contents of these compounds were calculated according to the area observed on the scanner.

Instrumental analyses. UV spectra were recorded with a Hitachi U-3210 spectrophotometer using $CHCl_3$ as solvent. NMR spectra were recorded with a JEOL GSX-270 spectrometer with tetramethylsilane (TMS) as an internal standard in $CDCl_3$–CD_3OD (2 : 1, v/v). FAB mass spectra (pos.) were measured on a JEOL JMS-HX100 mass spectrometer with a matrix of glycerol and *m*-nitrobenzyl alcohol (1 : 1, wt/wt). IR spectrum was recorded with a JEOL JIR-5500 IR spectrometer.

Results

Transphosphatidylation reaction of DPPC to DHA by PLD

DPPC (6 mg, 8 μmol) and DHA dimer (18 mg, 200 μmol) were incubated with 2.5 units of PLD in a biphasic system

Abbreviations: DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DPPA-Na, 1,2-dipalmitoyl-3-*sn*-phosphatidic acid sodium salt; DPP-DHA, 1,2-dipalmitoyl-3-*sn*-phosphatidyldihydroxyacetone; DPG, 1,2-dipalmitoylglycerol; PLD, phospholipase D; PLC, phospholipase C; DHA, dihydroxyacetone; TLC, thin-layer chromatography.

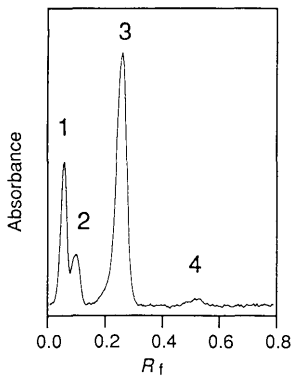

Fig. 1. TLC Analysis of Transphosphatidylation Reaction.

1, 2, 3, and 4 correspond to DPPC, DPPA-Na, DPP-DHA, and DHA dimer, respectively. These were confirmed by TLC analyses of authentic samples under the same conditions.

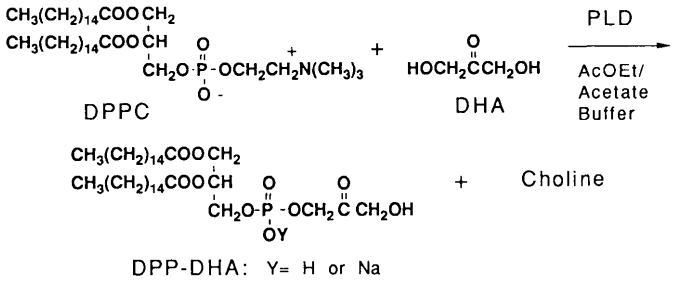

Fig. 2. Scheme of PLD-Catalyzed Transphosphatidylation Reaction of DPPC to DHA.

of 0.5 ml of ethyl acetate (AcOEt) and 0.5 ml of 0.2 M sodium acetate buffer (pH 5.6) at 20°C under stirring. After 6 h of incubation, a 50 μl sample was drawn from the well-mixed reaction mixture. To the sample, 50 μl of 1 N HCl and 100 μl of $CHCl_3$–MeOH (3 : 1, v/v) were added and well mixed. The organic layer was extracted and analyzed with TLC, using $CHCl_3$–MeOH–water (30 : 10 : 1, v/v) as the solvent. As shown in Fig. 1, a new spot (corresponding to peak 3) having a lower R_f than that of DHA dimer was observed. Because this new spot was not formed in the reaction without DPPC, without DHA dimer, or without PLD, it was suggested to be a phosphatidyl DHA. The isolation and identification of the products were attempted as follows.

Isolation and identification of 1,2-dipalmitoyl-3-sn-phosphatidyldihydroxyacetone (DPP-DHA)

DPPC (400 mg) and DHA dimer (800 mg) were added to a mixture of 50 ml of AcOEt and 50 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 30 units of PLD and incubated at 20°C for 5 h with stirring. To the reaction mixture, 50 ml of 1 N HCl were added and the mixture was extracted with 100 ml of $CHCl_3$–MeOH (3 : 1, v/v). The organic layer was washed three times with water saturated with NaCl, dried over anhydrous sodium sulfate, concentrated, and chromatographed on a silica gel (Wakogel C-200) column. From the eluate with $CHCl_3$–MeOH (3 : 1, v/v), 254 mg of DPP-DHA was obtained as a colorless waxy mass (65% yield based on DPPC). The eluate with $CHCl_3$–MeOH–water (20 : 10 : 2, v/v) gave 25 mg of 1,2-dipalmitoyl-3-*sn*-phosphatidic acid sodium salt (DPPA-Na).

The structure of DPP-DHA was identified as shown in Fig. 2 by the following spectral analyses. The FAB mass spectrum (pos.) showed *m/z* 743 (M+Na). The UV absorption spectrum showed λ_{max} at 280 nm in MeOH (DHA, λ_{max} 274 nm). The IR spectrum had carbonyl signals at 1736 cm^{-1} and 1718 cm^{-1}(s). The ^{1}H-NMR spectrum was as follows; 5.22 (1H, m, glycerol CH), 4.59 (2H, d, J=3.4 Hz, DHA CH_2), 4.41 (1H, m, glycerol CH_2), 4.38 (2H, s, DHA CH_2), 4.16 (1H, m, glycerol CH_2), 3.98 (2H, m, glycerol CH_2), 2.28 (4H, m, palmitoyl $COCH_2$), 1.60 (4H, m, palmitoyl CH_2), 1.27 (48H, m, palmitoyl CH_2), 0.88 (6H, t, palmitoyl CH_3). The ^{13}C-NMR spectra were

Table ^{13}C Chemical Shifts of DPP-DHA, DPPC, and DHA

Position		δ_C		
		DPPC[a]	DPP-DHA[b]	DHA[c]
Palmitoyl	CH_3	14.0	13.7	
	CH_2	22.5–34.0	22.4–33.9	
	C=O	173.0	173.4	
		173.4	173.4	
Glycerol	1-CH_2	62.9	62.2	
	3-CH_2	63.2	63.4	
	2-CH	70.4	70.1	
DHA	CH_2		65.6	66.6
			68.2	
	C=O		208.9	211.4

Chemical shifts are indicated in ppm. Solvents: [a] $CDCl_3$, [b] $CDCl_3$–CD_3OD (2 : 1, v/v), [c] CD_3OD.

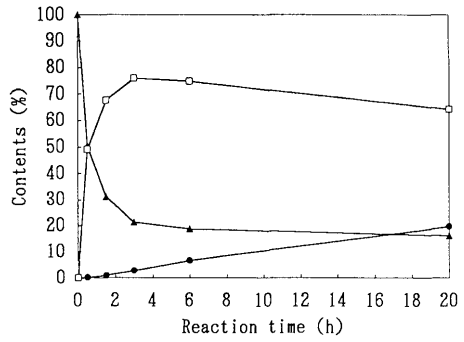

Fig. 3. Course of the Transphosphatidylation Reaction.

In the reaction described in Fig. 1, samples were drawn from the reaction mixture and analyzed by TLC. —□—, DPP-DHA; —▲—, DPPC; —●—, DPPA-Na.

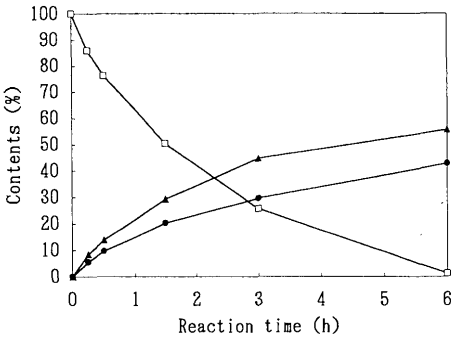

Fig. 4. Transphosphatidylation Reaction from DPP-DHA to Choline Chloride with PLD.

The samples were drawn from the reaction mixture and analyzed by TLC as described in Fig. 1. —□—, DPP-DHA; —▲—, DPPC; —●—, DPPA-Na.

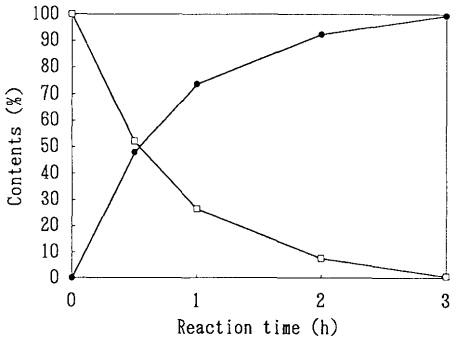

Fig. 5. PLC-Catalyzed Hydrolysis of DPP-DHA.

The samples were drawn from the reaction mixture and analyzed by TLC as described in Fig. 1. —□—, DPP-DHA; —●—, DPG.

summarized in the Table comparing with those of DPPC and DHA. In the ^{13}C-NMR spectra, the CH_2 carbon in DHA moiety of DPP-DHA was splitted to two signals, 65.6 ppm and 68.2 ppm from 66.6 ppm of that in DHA. DPPA-Na was identified by comparison with the authentic 1,2-dipalmitoyl-3-*sn*-phosphatidic acid sodium salt.

Course of transphosphatidylation reaction

The course of the reaction of DPPC and DHA dimer with PLD is shown in Fig. 3. While a rapid formation of DPP-DHA and its slight decrease after 4 h were observed, the hydrolysis to DPPA-Na gradually increased. Even on the prolonged incubation, DPPC was not consumed any more at around 20% content.

Transphosphatidylation reaction of DPP-DHA to choline with PLD

DPP-DHA (5 mg, 6.9 μmol) and choline chloride (1.67 mg, 11.9 μmol) were added to a mixture of 1 ml of AcOEt and 1 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 1 unit of PLD and incubated at 20°C. The course of the reaction was analyzed with TLC and shown in Fig. 4. As DPP-DHA was consuming, DPPC was produced and the hydrolysis to DPPA-Na proceeded. After 6 h of incubation, DPP-DHA almost completely disappeared.

Hydrolysis of DPP-DHA with PLC

According to the described method,[25] DPP-DHA (1 mg, 1.4 μmol) in a mixture of 1 ml of diethyl ether and 1 ml of 0.1 M Tris buffer (pH 7.2) containing 20 mM $CaCl_2$ was incubated with 30 units of PLC at 37°C with stirring. As shown in Fig. 5, after 3 h of incubation DPP-DHA was almost completely hydrolyzed to DPG, which was identified by comparison with the authentic 1,2-dipalmitoyl-*rac*-glycerol. DPG showed $R_f = 0.8$ on TLC using $CHCl_3$–MeOH (20 : 1, v/v) as the solvent.

Discussion

The reaction of DPPC and DHA with PLD in a biphasic system was very simple. The analysis of the reaction mixture with TLC indicated that products containing phosphatidyl residues were DPP-DHA and DPPA-Na (Fig. 1). The transphosphatidylation reaction providing DPP-DHA was accompanied with the hydrolysis to DPPA-Na. Since the course of the reaction (Fig. 3) showed that the contents of DPP-DHA had a maximal point at about 4 h followed by a gradual decrease and that DPPC did not consumed any more at around 20% content even on prolonged incubation, the increase of DPPA-Na may come from the enzymatic degradation of DPP-DHA produced at an early stage of the reaction.

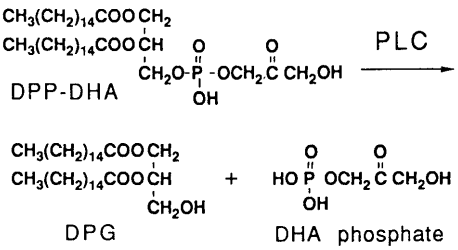

Fig. 6. Scheme of the PLC-Catalyzed Hydrolysis of DPP-DHA.

To study the secondary reaction of DPP-DHA, we reacted DPP-DHA and choline chloride with PLD. As shown in Fig. 4, the transphosphatidylation from DPP-DHA to choline took place to form DPPC, and at the same time the hydrolysis to DPPA-Na was observed. Therefore it can be concluded that DPP-DHA liberates DHA by PLD in the presence of an acceptor and/or water. It is expected that because DPP-DHA is highly hydrophobic and has an affinity for cell membranes, DPP-DHA easily transfers through the dermal layer and penetrates the dermal cell membranes, liberates DHA by the action of PLD on the membranes, and has the biological and chemical effects of DHA in the cells. Moreover, it is also expected that DPP-DHA has a good stability in water systems by forming lipid-microsphere-like bodies.

It has already been demonstrated that PLC catalyzes the hydrolysis of the linkage between diacylglycerol and phosphate residue on phosphatidylcholine[25] and phosphatidylinositol.[26] When DPP-DHA was incubated with PLC in a biphasic system of diethyl ether and Tris buffer, DPP-DHA was almost completely hydrolyzed to DPG (Fig. 5). Therefore it is suggested that DHA phosphate can be prepared from DPP-DHA by PLC. The enzymatic formation of DPP-DHA by PLD followed by the hydrolysis of DPP-DHA with PLC is another pathway for the synthesis of DHA phosphate (Fig. 6). We are now isolating DHA phosphate from the reaction mixture of DPP-DHA with PLC.

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