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STUDIES ON THE LIPIDS OF ABALONE(Ⅱ)

The Aldehyde Composition of Plasmalogen From Abalone and Some Marine Molluscs

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전복의 脂質에 關한 硏究(Ⅱ)

전복 및 數種의 貝類의 프라스마로겐의 알데하이드의 組成

趙 鏞 桂* · 秦 滿 夫**

1. 전복, 소라, 북방조개, 가리비의 重要한 燐脂質은 choline phosphatides, ethanolamine phlosphatides, serine phosphatides, cardiolipin, sphingomyelin 및 lyso choline phosphatides 였다.
2. 이들 plasmalogen은 거의 대부분이 ethanolamine phosphatides에 存在하고, choline phosphatides에는 少量存在하였으며, serine phosphatides에는 全然發見되지 않았다.
3. 전복의 ethanolamine plasmalogen 含量은 1월에 採取한 것에는 3.5%, 7월에 採取한 것에는 17.2%였다. 또, 소라, 북방조개에는 각각 그 含量이 20.7%, 15.1%였다. 가리비에는 ethanolamine phosphatides에 plasmalogen이 18.8% 존재하였다.
4. 전복 및 소라의 重要한 plasmalogen의 aldehyde 組成은 stearyl aldehyde ($C_{18:0}$), palmityl aldehyde ($C_{16:0}$), Palmitoleyl aldehyde ($C_{16:1}$) 및 oleyl aldehyde ($C_{18:1}$) 인데 대하여, 二枚貝인 북방조개, 가리비에는 eicosamonoenal ($C_{20:1}$)이 14—22% 程度含有 되어 있어 巻貝와 對照的이 있다.

INTRODUCTION

There are several papers reported on the subject of phospholipids in marine organisms by Yasuda[1], Katada[2),3)], Zama[4], Hatanos[5] and Joh[6]. According to their results, choline, ethanolamine and serine phospholipids were contained mainly in the phospholipids from all species of bivalves and snails, while inositol phospholipid, sphingomyelin and cardiolipin were found as minor components.

Plasmalogen is generally considered to be the material in which the fatty acid in the 1-position of the diacyl glycerophosphatide is replaced by a fatty aldehyde in an unsaturated ether linkage[7] (or vinylic ether bond).

Plasmalogen is distributed[8] in the central and peripheral nervous system, kidney, heart and other various tissues of animals. Is is resistant against radiation[8] and regulates[9] the body temperature of poikilothermic animals in response to enviromental temperature.

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Recently Gambal and Monty[10] reported the incorporation of palmitic acid-1-C^{14} into the aldehydogenic moiety of plasmalogens. Bell and white[20] presented evidence that long-chain aldehydes are direct precursors in the formation of the vinyl ether linkage and oxidation of the aldehyde to the acid is not a prerequisite to incorporation into the phospholipid molecule in the developing rat brain.

Rapport[12] indicated that the concentration of plasmalogen in the lipids of many marine invertebrates was as high as that in the lipids of mammalian brain(greater than 10% of the total lipid), and amount of choline plasmalogen in the phospholipids of 11 animals examined was considerably lower than that of phosphatidal ethanolamine(plus phosphatidal serine. De Koning[11] indicated that abalone, *Haliotis midae*, contained plasmalogen in the phospholipid fractions to a greater extent. Plasmalogen was by far the most predominant component among phospholipid fractions in the abalone.

Lee[14]showed that alcohols of wax esters from zooplankton incorporated into aldehyde moieties of plasmalogen in salmon.

Reviewing the biological role and metabolism of plasmalogen in the phospholipids, the composition of aldehydes attached to plasmalogen of marine invertebrates seemed to vary with differences of their feed.

This study was aimed to investigate the differences in the aldehyde components of plasmalogens between the snails, sea-weed feeder, and the bivalves, plankton feeder. The snails used in this work, were abalone, *Haliotis discus hannai* Ino, and *Turbo cornutus*, while the bivalves were *Spisula sachalinensis* and *Placopecten magellanicus*.

MATERIALS AND METHODS

Abalone, *Haliotis discus hannai* Ino, was purchased on January 31 and also July 12, in 1974 on Onagawa and Kitasendai Fish Market, Miyagi Prefecture, Japan, while *Spisula sachalinensis*, *Turbo cornutus*, *Placopecten magellanicus* were collected on July 6 in 1974 on Kitasendai Fish Market.

Total lipids were extracted according to the method of Bligh & Dyer[16]. The chloroform layer was evaporated on a rotary evaporator under vacuum. All the solvents were distilled and the lipid was manipulated under an atmosphere of nitrogen.

The phospholipids were separated from the non-polar lipid fraction by column chromatography on silicic acid(Mallinckrodt, 60-100 mesh) according to the procedure of Lovern[21]. Polar lipids eluted from column chromatography were applied to thin layer chromatography(TLC). The TLC plate was impregnated with Kiesel gel H, activated at 120°C for 5 hours prior to use. Small amounts of phospholipids were applied on the spot, 3 *cm* above the bottom edge of the plate, which were then developed in the first direction with chloroform-methanol-water(65:25:4, V/V)[22] and were redeveloped in the same direction to avoid contaminating with the non-polar lipids with chloroform-ethylacetate(6:3, V/V)[23]. After being air-dried at room temperature for 20 minutes, the plates were run in the second direction with chloroform-methanol-7N ammonia water(230:90:15, V/V/V), and the identification methods were employed to confirm the identity of theespots by spraying Dittmer reagent[24], 0.1% ninhydrinacetone solution, and Dragendorff reagent[24] and by comparing the Rf values of the spots with those of the standard materials.

Plasmalogen detection

As shown in Fig. 1 small amounts of phospholipids applied to the plates prepared as described previously had run in the solvent system as chloroform-methanol-water(65:25:4, V/V/V) until the solvent front reached 3 *cm* below the upper part of the plate. The plate was redeveloped in the same direction with chloroform-ethylacetate(6:3, V/V) to separate the non-polar

lipids from the polar lipids.

After spraying 3% 2, 4-DNP hydrazine-HCl on the sample run lane of the plate (the rest parts of plate were covered with sheets of paper to prevent getting wet by spraying reagent)[19], and was allowed to evaporate most of solvent of the plate for 30 minutes. The plate then was run in the other direction with petroleum ether-diethylether-acetic acid mixture (80:20:1, V/V/V).

Separation and quantification of plasmalogen by TLC

As shown Fig. 2-a, b small quantities of phospholipids were applied to TLC. Two blots were spotted on 1 *cm* band; one from the 3 *cm* above the bottom edge at the left side, and other also 3 *cm* above the bottom edge at the right side. The plates then were developed in the first direction in the manner used in the detection of plasmalogens.

Before the second running, the one developed on the lane of the plate was sprayed with 5mM $HgCl_2$ [15] (the rest parts of the plate were covered with sheets of paper to prevent getting wet by spraying reagent), and allowed for approximately 20 minutes for the complete reaction with plasmalogen, and afterwards desiccated thoroughly in a vacuum desiccator. The plate was then developed in the second direction with chloroform-methanol-7N ammonia water(230:90:15, V/V/V). The spots were detected with iodine vapor and encircled with a fine needle. The rest of the iodine to disappear before the removal of the spots. A drop of water [25] was gently added to the silica gel area in order to be removed for analysis and silica gel was transferred to a 50 *ml* centrifuge tube by a thin knife spatula. The whole spot could be transferred quantitatively because water cohered the silica gel. The areas in which there was no lipid were taken in such a size as lipid contained spots as references. The silica gel of each spot collected from 5 TLC plates was suspended with 20*ml* of chloroform-methanol (4:1, V/V) and centrifuged at 5×10^3 rpm for 30 minutes. After centrifuging the solvent was removed with a capillary pipette. The second, third and fourth elution were made with a series of chloroform-methanol mixtures, 3:2, 2:3 and 1:5 by volume, respectively. All the sample elutes were evaporated under nitrogen approx. 1 *ml*, and transferred to 30 *ml* test tubes.

Phosphorus determination

Phosphorus was determined according to the methods of Bartlett[13]; the test tube added with 0.5*ml* of 10N H_2SO_4 was subjected to be digested in a dry oven adjusted to 150°C-160°C for three hours. After cooling it at room temperature for a few seconds, 2-3 drops of 3% hydrogen peroxide were added to the test tube prior to heating in the same drying oven.

When all the peroxides in the test tube were decomposed, 4.6*ml* of 0.22% ammonium-molybdate and 0.2*ml* of Fiske-Subbarow reagent[13] were added to one after the other. The solution was allowed to be coloured in a boiling water bath for 7 minutes and the color was read at 830*mμ*.

Dimethylacetals(DMAs) preparation

Ethanolamine phosphatides and choline phosphatides fractions were methylated with 50-80 volume of 3% HCl in anhydrous methanol in sealed ampoules at 80°C for 5-8 hours. Prior to sealing the ampoules were flushed with nitrogen and cooled with dry ice and acetone.

The DMAs and methylesters of fatty acids were recovered by adding 5 volumes of n-hexane. The n-hexane extracts were washed repeatedly with 5 volumes of ethanol-water(1:3, V/V). The DMAs and methylesters were then separated according to the method of O'brien [26]. The methylesters were converted to their sodium salts by saponifying with 3% KOH-ethanol at 80°C under reflux for 2 hours. The saponification mixture was extracted twice with

equal volumes of n-hexane, and the unsaponifiable DMAs were recovered in the n-hexane layer with the soaps in the aqueous phase.

Purification [26]

During the process of preparing DMAs, it could not be excluded the possibility that hemiacetals and alcohols as well as methylesters were contaminated to seme degree as impurities. The prepared DMAs were purified on TLC (Wakogel B-5) with xylene. The DMAs so obtained then were tested for confirmation by IR spectra.

Preparation of DMA references[27,34]

Dimethylacetals like 1,1-dimethoxytetradecane, and 1, 1-dimethoxyhexadecane and 1, 1-dimethoxyoctadecane were used as references. The 1,1-dimethoxyhexadecane and the 1,1-dimethoxyoctadecane were prepared from n-hexadecanal along with n-octadecanal reduced from cetyl alcohol and stearyl alcohol respectively. The condition of standard DMAs preparation was the same as that of DMAs preparation from ethanolamine phosphatides and choline phosphatides.

GLC of DMAs[26]

GLC analysis was made on a Hitachi Model GC Chromatograpa fitted with a flame ionization detector. The samples were run on the same column (3*m* x 3*mm*) packed with 10% DEGS on Chromosorb W(80-100 mesh). The column temperature was 180°C.

RESULTS AND DISCUSSION

The shell length and shell content are shown in Table 1. The phospholipids of the abalone are composed of cardiolipin, ethanolamine phosphatides, serine phosphatides, choline phosphatides, sphingomyelin and lyso-choline phosphatides. This pattern is common to the other molluscs examined in this study.

Table 1. Samples used in the study

Sample	Place caught	Datum caught	Number	Shell length (*cm*)	Contents (*g*)
Haliotis discus hannai, A	Onagawa, Miyagi Pref., Japan	Jan. 31, 1974	4	9.4	348
Haliotis discus hannai, B	Kitasendai Fish Market, Miyagi Pref., Japan	July 12, 1974	3	8.2	330
Turbo cornutus	〃	July 6, 1974	9	12.5	673
Spisula sachalinensis	〃	July 6, 1974	5	7.9	174
Placopecten magellanicus	〃	July 6, 1974	7	10.1	566

Table 2. The plasmalogen content of the phospholipids isolated from ***H. discus hannai*** (INO), ***T. cornutus***, ***Sachalinensis*** and ***M. sulcataria*** (% as phosphorus content to that of total phospholipids)

Sample		a	a′	b	b′
Haliotis discus hannai	A	3.5	16.3		52.3
	B	17.2	15.1	0.3	60.9
Turbo cornutus		20.6	13.4	0.1	49.3
Spisula sachalinensis		14.9	15.4	0.2	45.4
Mactra sulcataria		18.8			

a: phosphatidal ethanolamine(ethanolamine plasmalogen) a′: phosphatidyl ethanolamine
b: phosphatidal choline(choline plasmalogen) b′: phosphatidyl choline

De koning [11] reported the presence of inositol phosphatides (5%) of total phospholipids and 2-amino phosphatides in the phospholipids from the abalone, *Haliotis midae*. But neither of the two phospholipids are detected in all the samples investigated in this study(Table 2). According to him, almost all of the plasmalogens of the abalone are present in the ethanolamine phosphatides, and their content amounted up to 47% of the total phospholipids is much higher than that from the abalone used in this study (17.5%).

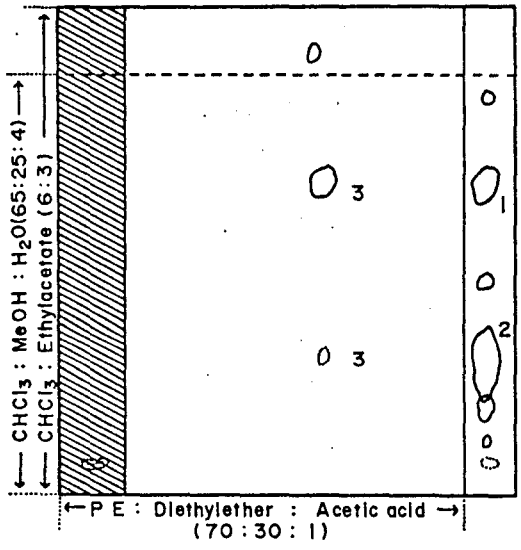

Fig. 1. Detection of plasmalogen in the phospholipids from abalone with spraying of 2,4-DNP hydrazine-HCl solution. Shaded area sprayed with 3% 2,4-DNP hydrazine-HCl
1: ethanolamine phosphatides
2: choline phosphatides
3: 2,4-DNP hydrazone

As shown in Fig. 1 on the TLC plates sprayed with 2, 4-DNP hydrazine-HCl solution for the aldehyde moieties of plasmalogen, the ethanolamine and choline glycerophosphatides bands from the abalone showed a positive reaction signifying that plasmalogen are distributed in the two fractions. The reaction given by the ethanolamine phosphatide band is very strong and it was given by the choline phosphatide band.

On the TLC chromatogram of the phospholipids from the abalone, which was sprayed with 5mM $HgCl_2$ solution, the spots 3b and 5b corrseponed with lyso phosphatidal ethanolamine and lysophosphatidal choline, which are dealkenylated at 1-position of phosphatidal ethanolamine (ethanolamine plasmalogen) (Fig. 2).

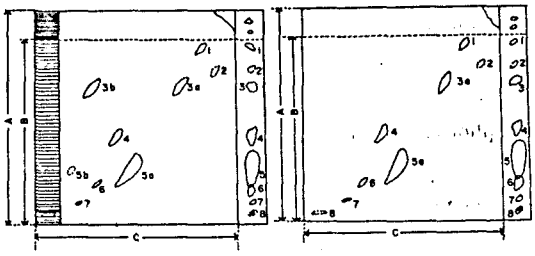

Fig. 2. Fractionation of the phospholipids and the plasmalogen from them of abalone.
2-a(left figure): TLC plate not sprayed with 5mM $HgCl_2$
2-b(right figure): TLC plate sprayed with mM $HgCl_2$
1, Cardiolipin
2. Unidentified
3. Ethanolamine phosphatides
3a. Phosphatidyl ethanolamine
3b. 1-deak-1-enylated ethanolamine lysophosphatide
4. Serine phosphatide
5. Choline phosphatides
5a. Phosphatidyl choline
5b. 1-deak-1-enylated choline lysophosphatide
6. Sphingomyelin
7. Choline lysophosphatide
8. Origin
Developing solvents
A. Chloroform: Ethylacetate(6:3 V/V)
B. Chloroform: Methanol: Water(65:25:4 V/V)
C. Chloroform: Methanol: Ammonia(230: 90:15 V/V)
Shaded area sprayed with $HgCl_2$

The contents of phosphatidal ethanolamine was very much higher than those of phosphatidal choline in all the molluscs investigated in this study. The marine invertebrates[12] studied

so far were found to be especially rich in plasmalogen, most of which exist in phosphatidal ethanolamine. This situation is also analogous to that observed in mammalian brain[29] and[30] nervous tissues, and in goldfish nervous tissues[9], though Dumont[12] indicated that the posterior gills of *Eriocheir sinensis* contained only choline plasmalogen, and Bergmann & Landowne[12] also insisted only choline plasmalogen was present in *Anthopleura elegantissima.*

Thiele [32] has found that in *Helix pomatia* the plasmalogen content varies from 12.8% of lipid phosphorus in March down to 4.4% of lipid phosphorus in June (breeding time). According to Roots & Jonston[9], environmental temperature has a striking effect on levels of alk-1-enyl glycerolipids in goldfish brains, and plasmalogen values(moles aldehyde/ moles phosphorus x 100) for fish acclimated at 5°C were 35.9±1.37, but rose to 45.73±3.51 at 30°C. But Thompson & Hanahan[32] showed plasmalogen level remained constant at 13% of lipid phosphorus in both adult and immature body of a slug, *Arion ater.*

In this experiment the plasmalogen content of abalone varied considerably with change of season; 3.5% to total phospholipids as phosphorus in January, 17 and 2% in June.

Considerable amounts of serine plasmalogen

Table 3. The aldehyde composition of the plasmalogens isolated from *H. discus hannai* (INO), *T. cornutus*, *S. sachalinensis* and *M. sulcataria*

C	Rrt**	A*		B		C		D	
		I	II	I	I	II	II	I	II
14:0	0.52	2.4	2.2	0.5	1.1	2.5	1.2	0.6	1.1
1	0.63	2.0	1.2	0.6	0.7	1.6	1.2	0.9	0.6
15:0	0.70	2.5	2.2	3.0	1.9	0.9	1.7	0.7	1.0
1	0.74	1.4	1.6	2.7	1.6	2.0	1.9	0.4	1.6
16b?	0.83							2.2	
0	1.00	22.6	26.0	21.4	21.4	12.3	18.4	11.0	10.2
1	1.16	14.9	9.8	16.3	11.9	8.7	6.7	11.6	8.9
?	1.30	8.4	5.9	11.7	14.1	2.2	2.5	3.3	3.9
?	1.42	0.3	6.0	4.2	3.4	0.3	0.4	1.1	0.5
18b?	1.50	1.0				7.7	5.6	5.0	6.1
0	1.67	28.1	30.9	35.7	39.9	19.3	22.7	24.5	25.1
1	1.79	7.1	3.6	2.4	2.6	8.4	8.1	10.7	9.8
2	1.86					1.1	1.2	2.5	2.4
19b?	1.92		3.0		trace		1.4	1.0	1.0
0	2.03	3.4	1.2	0.7	0.2	9.4	7.0	8.8	7.6
1	2.25	0.5	0.3	0.2	0.8	0.4	0.6	0.5	1.0
20b?	2.45							0.3	
0	2.59	trace	0.2	0.2	0.3	0.6	0.5	0.3	0.7
1	2.86	5.4	5.8	0.4	trace	21.8	16.7	13.6	18.5
?	3.02					0.7	2.2	trace	trace
?	3.29							0.7	
?	3.93							0.3	

A; *Haliotis discus hannai*(INO) B; *Turbo cornutus* C; *Spisula sachalinensis* D; *Mactra sulcataria*
I; Phosphatidal ethanolamine(ethanolamine plasmalogen)
II; Phosphatidal choline (choline plasmalogen)

occurred in goldfish[9], a strictly anaerobic bacterium, *Megasphaera elsdenii*[31], and human brain myelin[20]. But no traces of serine plasmalogen in the phospholipids of all the molluscs used in this study were detected on TLC.

IR spectrum of DMAs purified on TLC showed no absorption peaks at near 3200--3500cm^{-1}, and at near 1700 cm which are characteristic of -OH group and-CO group. This fact in dicates that DMAs for GLC are not contaminated with impurities such as hemiacetals, alcohols, esters and carbonyl & carboxyl compounds. On this spectrum, several peaks at near 1,000-1,200 cm^{-1} [28] are specific to DMAs (Fig. 3).

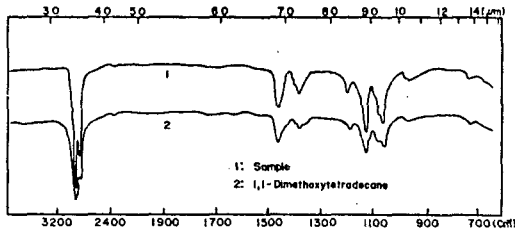

Fig. 3 IR spectrum of DMAs from the ethanolamine plasmalogen of abalone.

Gas chromatographic analysis of the DMAs derived from the plasmalogen of the snails, seaweed feeder, revealed that the major fatty aldehydes are octadecanal($C_{18:0}$), hexadecanal ($C_{16:0}$) and hexadecenal($C_{16:1}$) (Table 3).

In contrast to those of the snails, the major aldehydes of the bivalves, plankton feeder were octadecanal ($C_{18:0}$), eicosamonoenal ($C_{20:1}$), hexadecanal ($C_{16:0}$). As minor components, $C_{14:0}$, $C_{14:1}$, $C_{15:1}$ and tentatively identified $C_{16:Br}$, $C_{18:Br}$, $C_{20:Br}$ occurred in all the molluscs.

It is noteworthy that the plasmalogen in the bivalves were much richer in eicosamonoenal ($C_{20:1}$) than those in the snails, and also contained 1.1-2.5% octadecadienal ($C_{18:2}$) which was not detected in the snails.

Roots and Johnston[9] insisted that the major fatty aldehydes of ethanolamine plasmalogen from goldfish were found to be stearyl aldehyde ($C_{18:0}$), oleylaldehyde($C_{18:1}$) and palmitylaldehyde($C_{16:0}$), but the major fatty aldehyde of choline plasmalogen was found to be palmityl ($C_{16:0}$). But no significant difference of aldehyde composition between ethanolamine plasmalogen and choline plasmalogen was made in the four molluscs. It is also of great interest that the plasmalogens of the bivalves contained a larger amount of $C_{19:0}$ fatty aldehyde.

Shimma & Taguchi[33] suggested that the difference of fatty acid composition between gastropoda and pelecypoda made be due to their feed. Brockerhoff and co-workers[8] postulated that marine fish may synthesize triacylglycerols from a 2-acylglycerols structure containing high proportions of C_{20} and C_{22} polyenoic acids that is carried throughthe food chain essentially unchanged. This structure may have its origin in the phytoplankton, where synthesis of the polyenoic chains takes place.

Precursor roles for fatty aldehydes and alkylglycerols have been considered in the biosynthesis of alk-1-enyl glycerolipids. The fatty aldehydes have not been extensively studied in the biosynthesis of alk-1-enyl glycerolipids of marine origin, but Ellingboe and Karnovsky[20] have shown that aldehyde was a better precursor than fatty alcohol and fatty acid in the formation of alk-1-enyl glycerolipids of the starfish, *Asterias forbesi*. Lee[14] indicated the alcohol moieties of wax esters from plankton are metabolized into plasmalogens of salmon species.

In view of the facts mentioned above, it can not be ruled out the possibility that the striking difference in the aldehyde composition between the snails, sea-weed feeder, and the bivalves, plankton feeder may be ascribed to their feed.

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