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Occurrence of Ceramide Aminoethylphosphonate in Edible Shellfish, *AGEMAKI*, *Sinonovacula constricta*[1]

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ABSTRACT

Ceramide aminoethylphosphonate was isolated from the *AGEMAKI*, *Sinonovacula constricta*. The compound was purified with a combination of mild alkaline hydrolysis of the total lipids, QAE-Sephadex-A25 column chromatography and two dimensional thin layer chromatography. The infrared spectrum showed an absorption band at 1180 cm^{-1} due to C-P bond, and was essentially identical with that of the standard ceramide aminoethylphosphonate. Upon hydrolysis of the substance by strong acid, neither change in the chromatographic behaviors of this substance nor liberation of inorganic phosphate was observed. The stability of the compound to acid hydrolysis suggested the presence of a C-P bond. On comparison with synthetic compound, the aqueous hydrolysis product behaved like 2-aminoethylphosphonic acid on thin layer chromatograms.

The predominant fatty acids were palmitic acid and stearic acid. The results obtained here suggest that a person may ingest and absorb 2-aminoethylphosphonic acid through food chain's such as edible shellfish.

INTRODUCTION

Natural compounds containing a carbon-phosphorus bond were first detected when Horiguchi and Kandatsu[(1)] isolated 2-aminoethylphosphonic acid (ciliatine) from acid hydrolysates of a proteolipid-like substance prepared from ciliate

[1] This report was presented at the General Meetig of the Agricultural Chemical Society of Japan, held in Tokyo, April 3, 1984

protozoa of sheep rumen. Since then this compound has been shown to be present either in the free form or in the bound to proteins or lipids in the protozoan *Tetrahymena pyriformis*, fresh water molluscus, terrestrial mammals, and marine animals in the phyla coelenterate mollusca, echinoderamata.

The distribution of the compound has been reviewed by Quin[2], and notable additional marine sources reported by Hori et al.[3] Since the isolation of 2-aminoethylphosphonic acid, thirteen other aminophosphonic acids structurally related to 2-aminoethylphosphonic acid have been discovered in biological materials.[4]

N-methyl derivative containing the carbon-phosphorus bond are α-amino-β-phosphonopropionic acid isolated by Kittredge and Hughes[5] from Zoanthus sociatus and N-methyl, N, N-dimethyl, and N, N, N-trimethyl aminoethylphosphonic acid isolated by Kittredge et al[6] from Anthopleura xanthogrammica.

So far, nine phosphonate-containing lipids (termed phosphonolipid)[7] have been found as analogs of phosphatidylethanolamine, phosphatidylcholine and sphingoethanolamine from tissue of lower[8] and higher animals.[9]

In addition, much information about the metabolism in lower animals and higher animals has been reported.[4,10]

Rouser et al[11] first isolated a characteristic lipid from the sea anemone by means of column chromatography, and it was assumed tentatively to consist of a ceramide attached to 2-aminoethylphosphonic acid by an ester linkage. Since then this ceramide 2-aminoethylphosphonate has been shown to be widely present in shellfish both in fresh water and sea water,[12] and Fungi.[13]

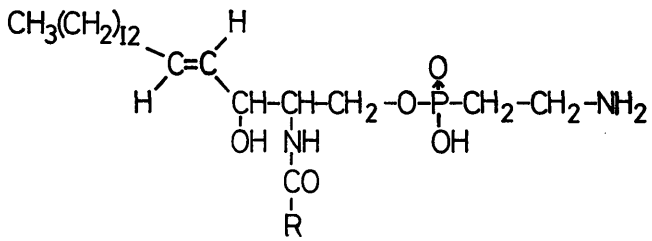

Fig. 1 Structure of Ceramide 2-Aminoethylphosphonate

Recently increasing attention has been paid to fish and shellfish as natural resources.

Therefore, our interest in mammalian metabolism of aminophosphonic acids has led to the present investigation on the detection and isolation of the ceramide aminoethylphosphonate from an edible shellfish, *AGEMAKI*, *Sinonovacula constricta*.

MATERIALS AND METHODS

Materials:

QAE-Sephadex-A25 was the product of pharmacia. *AGEMAKI*, *Sinonovacula constricta* was purchased on the local market. Monomethylaminoethylphosphonic acid was purchased from Calbiochem Los Angeles.

Fractionation of phosphorus fractions in Sinonovacula constricta[14]:

The freeze dried material (1.56g) of the *Sinonovacula constricta* was extracted twice with 30 ml of acetone by an efficient magnetic stirrer in a 100 ml beaker at room temperature. The acetone extracts was centrifuged at 3000 rpm for 10 min. The supernatant was evaporated to dryness under reduced presure (A fraction). The residue was extracted three times with 50 ml of chloroform-methanol (2 : 1, v/v) for 60 min. at 40℃, and centrifuged at 3000 rpm for 10 min.

The three combined extracts were evaporated to dryness under reduced pressure (B fraction). The residue was suspended in 50 ml of 5% trichloroacetic acid (TCA) and the suspension was stirred at 30℃ for 30 min. The suspension was then centrifuged for 10 min. at 3000 rpm. The precipitate was re-extracted twice under the same conditions.

The combined supernatant was evaporated to dryness under reduced pressure (C fraction), the precipitate refluxed twice in 50 ml of 5% TCA for 15 min. and centrifuged for 10 min. at 4000 rpm. The supernatant was evaporated to dryness under reduced pressure (D fraction), and the precipitate (E fraction) washed with 30 ml of ether, ethanol and hot ethanol successively.

A portion of each fraction was hydrolyzed with 10 ml of 6 N HCl at 120℃ for 24 hrs. Water was added to the hydrolysate, and the mixture was extracted several times with ether. The aqueous layer was filtered, evaporated to dryness, and redissolved with 10 ml of water for analysis of phosphonate- and total-phosphorus.

Preparation of Lipid Extracts:

The freeze dried material (5 g) of AGEMAKI, *Sinonovacula constricta* was extracted with 50 ml of chloroform-methanol (2 : 1, v/v) at 40℃ for 60 min., and the insoluble residue was re-extracted twice under the same conditions. The three combined extracts were evaporated to dryness under reduced pressure, redissolved in 10 ml of chloroform-methanol (2 : 1, v/v) and washed twice with 70 ml of cold acetone. The precipitate was collected by centrifugation at 3000 rpm for 10 min., redissolved in 10 ml of chloroform-methanol (2 : 1, v/v) and washed three times with 0.2 volume of 0.017 % $MgCl_2$ according to the method described by Folch et al.[15]

The lower phase was evaporated to dryness under reduced pressure. The isolation and purification of the sphingolipid from the total lipid was achieved by three methods; by the mild alkaline hydrolysis according to Dawson[16] and Hori et al.[17], by the QAE-Sephadex column chromatography and by the preparative thin layer chromatography.

Mild Alkaline Hydrolysis:

To a solution of the lipid in 10 ml of chloroform-methanol (2 : 1,v/v), 10 ml of 0.5 N methanolic sodium hydroxide was added. The mixture was stirred at 37℃ for 60 min. and then adjusted with 2.6 N methanolic hydrochloride to pH 1. The mixture was kept at 37℃ for 60 min. The methanol was removed under reduced pressure, and the residual solid was shaken with 1 ml of water containing 0.27g of sodium sulfate and later with 20 ml of water. The lipid was extracted three times with 20 ml of chloroform. After centrifugation, the three combined extracts were evaporated to dryness under reduced pressure. The residual solid was washed twice with cold acetone, the precipitate of sphingolipid was collected by centrifugation at 3000 rpm for 15 min.

Fractionation by Column Chromatography:

The dried residue of the sphingolipid was dissolved in a small amount of chloroform-methanol (2 : 1,v/v) and applied to a 1.2 × 17 cm column of QAE-Sephadex A-25 (prewashed with chloroform-methanol-water (2 : 4 : 1,v/v)). The column was first eluted with 100 ml of chloroform-methanol-water (2:4:1, v/v), and then with 500 ml of chloroform-methanol-1M sodium acetate (2:4:1, v/v). The eluate was collected in 1.4 ml fractions and examined by thin layer chromatography on kiesel gel 60 F_{254} (Merck, 0.25 mm, 20 × 20 cm) using chloroform-methanol -28% aqueous ammonia (65:35:5,v/v). The spots were detected with I_2 vapor and ninhydrine.

Fraction No. 86 of the eluate was collected and evaporated to dryness under reduced pressure. The dried residue was dissolved in a small amount of chloroform-methanol-1M sodium acetate (2 : 4 : 1,v/v) and further applied to a 1.2 × 17 cm column of QAE-Sephadex A-25 as described above. The column was eluted with 200 ml of chloroform-methanol-1M sodium acetate (2:4:1,v/v). The eluate was collected in 1.4 ml fractions and examined by thin layer chromatography as described above.

Fractions No. 7 - 9 of the eluate were collected and evaporated to dryness under reduced pressure. As will be described below, this dried material (134 mg) was used as crude phosphonolipids.

Isolation of Ceramide Aminoethylphosphonate by Preparative Thin Layer Chromatography:

The dried residue was dissolved in 1 ml of chloroform-methanol (2 : 1,v/v) and applied to 5 thin layer plates of Kiesel gel 60 F_{254} (Merck, 2 mm, 20 × 20 cm).

Chromatography was carried out at 20℃ using chloroform-methanol-acetic acid-water (100 : 20 : 12 : 5 v/v). The spots were detected with I_2 vapor. To isolate ceramide aminoethylphosphonate, the desired area of the thin layer plates was scraped, packed into a column (1.0 × 10 cm) and the material was eluted from the scraped Kiesel gel with chloroform-methanol-water (10 : 10 : 1,v/v). The eluate was evaporated to dryness under reduced pressure and crystallized by dissolving in ethyl acetate-methanol (3 : 2).

Infrared Analysis:

The infrared absorption spectrum was determined on a pellet of potassium bromide using a Nihonbunko IR-S Spectrophotometer.

Qualitative Analysis:

The ceramide aminoethylphosphonate was hydrolyzed with 6N HCl at 120℃ for 24 hrs. The liberated fatty acids were extracted with light petroleum and the water soluble compounds were extracted by Folch partition. The aqueous phase was evaporated to dryness under reduced pressure.

The presence of aminoethylphosphonate in the acid hydrolysate was demonstrated by thin layer chromatography (Kiesel gel 60 F_{254}, 20 × 20 cm, 0.25 mm) using 99% ethanol-7% aqueous ammonia (1:2,v/v).[13,18]

Authentic 2-aminoethylphosphonic acid prepared by the method of Kosolapoff[19] was co-chromatographed and the spots were detected by Rosenberg's method.[20]

Quantitative Analysis:

Fatty acid esters were obtained from the ceramide aminoethylphosphonate by methanolysis (5% HCl in methanol at 100℃ for 4 hrs) subjected to gas chromatography. The sphingosine content of the sample was determined by the method of Lauter et al[21] which depends on the colorimetric estimation of the complex formed between sphingosine and methyl orange. Phosphonate phosphorus was estimated by the method of Tamari et al.[22] Total phosphorus was estimated by the method of Chen et al.[23] Analysis of carbon, hydrogen, and nitrogen were carried out with a Perkin-Elmer 240 Microanalysis Apparatus.

Gas Liquid Chromatographic Analysis of Fatty Acid Composition:

The fatty acid methyl esters were separated on a Shimadzu GC-9A apparatus equipped with a flame ionization detector.

The column was a 3mm × 2m glass column packed with 5% Shinchron E71 on 80-100 mesh Chimalite, and the instrument was operated at 200℃. The peaks were identified by comparison with those of standard methyl esters on the basis of individual retention time.

RESULTS

The results of phosphonate content of the acetone soluble fraction, lipid fraction cold TCA soluble fraction, hot TCA soluble fraction and protein fraction of the AGEMAKI, *Sinonovacula constricta* are shown in Table 1. The amount of phosphonate in the acetone fraction, the lipid fraction, the cold TCA soluble fraction and the protein fraction were 3.25, 164.60, 41.35, 68.46 and 35.57 μg per g of sample, respectively.

Table 1. Phosphorus Analysis of Phosphorus Fractions Obtained from AGEMAKI, *Sinonovacula constricta*.
The details of the procedure are described in the text.

Fraction	C-P(μg/g)	T-P(μg/g)	C-P/T-P(%)
A	3.25(1.0)	9.70	33.5
B	164.60(52.5)	1145.52	14.4
C	41.35(13.2)	3165.89	1.3
D	68.46(21.9)	2173.67	3.1
E	35.57(11.4)	367.36	9.7

C-P : Phosphonate-phosphorus, T-P : Total-phosphorus,
( ) ; as % of Total C-P

The lipid fraction contained 52.5% of the phosphonate in the *Sinonovacula constricta* powder, and the amount of phosphonate of the lipid fraction was 3.9, 2.4 and 4.6 times higher than in the cold TCA fraction, in the hot TCA fraction and in the protein fraction, respectively.

Lipid extraction yielded 1.1 g purified total lipids (11.0% of the dry weight). Phospholipid content of the total lipid was about 14.6% by wt. Phosphorus content of the total phospholipid amounted to 3.9%. About 28% of the total phosphorus was found as phosphonate, and it was identified as 2-aminoethylphosphonic acid.

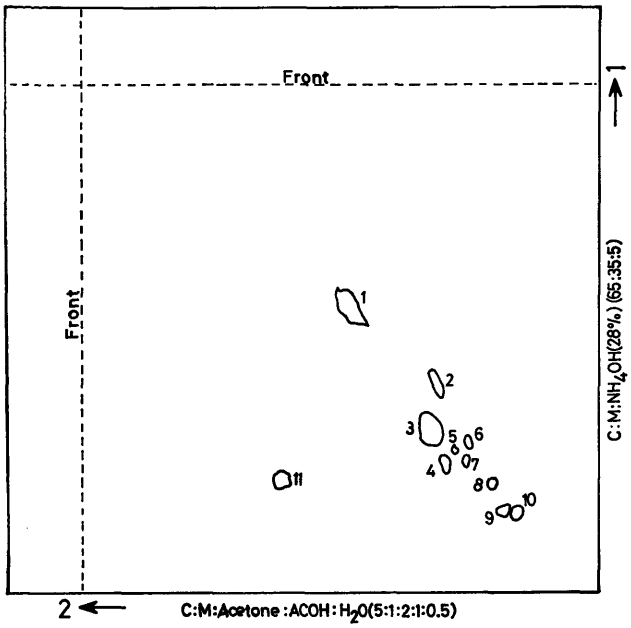

Fig. 2 Two Dimensional Thin Layer Chromatogram of the Phospholipids of *Sinonovacula constricta*.

The chromatogram was developed in chloroform-methanol -28% aqueous ammonia (65 : 35 : 5, v/v) in the upper direction, then chloroform-methanol-acetone-acetic acid-water (5:1:2:1:0.5, v/v) toward the left. Spot are : 1, phosphatidylethanolamine ; 2, phosphatidylcholine ; 3, ceramide aminoethylphosphonate ; 4, phosphatidylserine ; 5, phosphatidylinositol ; 6-10, unidentified lipids ; 11, phosphatidic acid.

A typical thin layer chromatographic separation of phospholipid classes is depicted in Figure 2, while the quantitative phospholipid distribution determined by phosphorus analysis of the spots shown in Figure 2 is presented in Table 2.

With minor exceptions, the polar lipid composition of *Sinonovacula constricta* was identical to that of other invertebrates. [24-29]

Table 2. Phospholipid Composition of the *AGEMAKI*, *Sinonovacula constricta*.

Phosphlipid class	Percentage
Phosphatidylethanolamine	25.4
Phosphatidylcholine	9.5
Ceramide aminoethylphosphonate	23.1
Phosphatidylserine	8.0
Phosphatidylinositol	3.0
Unidentified lipid	20.2
Phosphatidic acid	10.8

Phosphatidylethanolamine (spot 1, 25.4%) appears to be present in large amounts together with small amounts of phosphatidylcholine (spot 2. 9.5%) and phosphatidylserine (spot 4, 8.0%). Ceramide aminoethylphosphonate (spot 3) comprised 23.1% of the lipid phosphorus. The phosphonate analog of the phosphatidylethanolamine was not detected in the phospholipid of *Sinonovacula constricta*.

Figure 3 shows the thin layer chromatogram of the lipid obtained by the mild alkaline hydrolysis method.

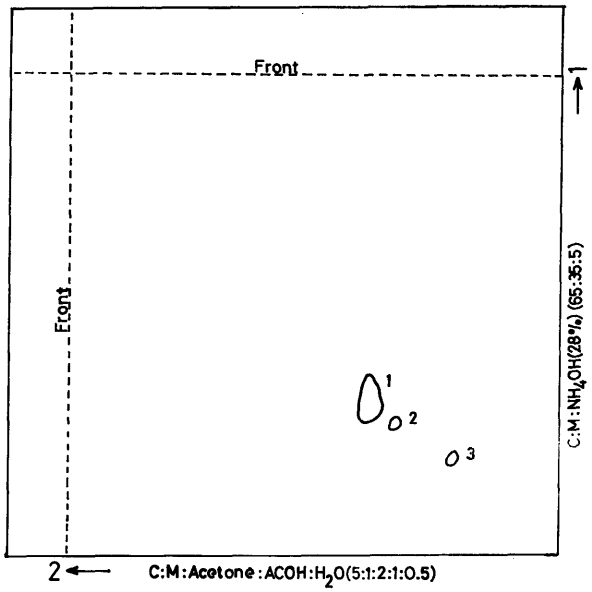

Fig. 3 Two Dimensional Thin Layer Chromatogram of the Alkaline Stable Lipids Obtained by the Mild Alkaline Hydrolysis as Described in the Text.
The chromatogram was developed as in Fig. 2. Spot are : 1, ceramide aminoethylphosphonate (75.8 %) ; 2, probably hydroxy fatty acid containing ceramide aminoethylphosphonate (12.3%) ; 3, sphingomyelin (11.9%).

Ceramide aminoethylphosphonate (spot 1), and probably what is hydroxy fatty acid containing ceramide aminoethylphosphonate (spot 2) and sphingomyelin (spot 3), consisted of 75.8, 12.3 and 11.9% of the sphingolipid phosphorus, respectively.

The result obtained here indicates that ceramide aminoethylphosphonate is the major sphingolipid component of the lipid moiety of the *Sinonovacula constricta*.

Ceramide aminoethylphosphonate was purified by QAE- Sephadex column chromatography from the sphingolipid fraction obtained by mild alkaline hydro-

lysis of the phospholipid fraction, and pure ceramide aminoethylphosphonate was then obtained by preparative thin layer chromatography.

18mg of ceramide aminoethylphosphonate was finally obtained from the sphingolipid (0.1g) of *Sinonovacula constricta* (5g dry weight).

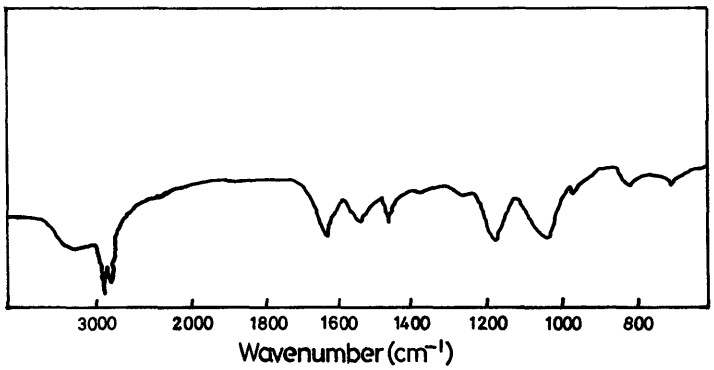

Fig. 4 Infrared Spectrum of the Ceramide Aminoethylphosphonate Purified by a Combination of the Mild Alkaline Hydrolysis, the Column Chromatography and the Preparative Thin Layer Chromatography as Described in the Text.

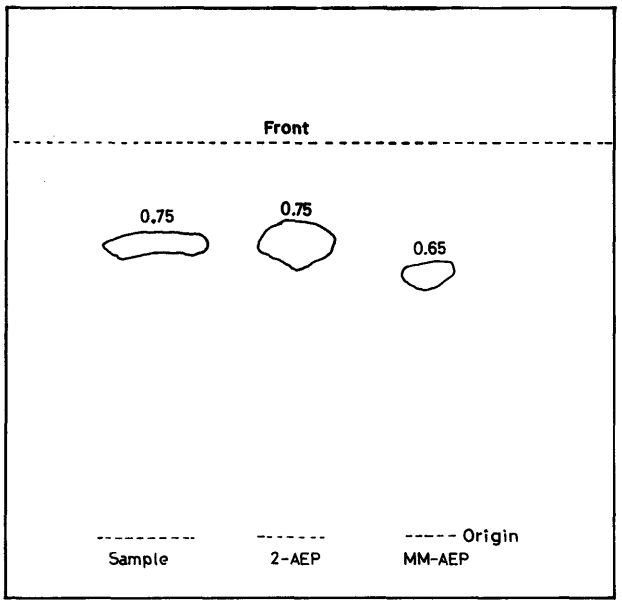

Fig. 5 Chromatographic Identification of the Aminophosphonates in the Acid Hydrolysate of the Ceramide Aminoethylphosphonate.
Authentic 2-aminoethylphosphonic acid (2-AEP), N-monomethylaminoethylphosphonic acid (MM-AEP) and acid hydrolysate were spotted at the origin. Chromatography was carried out on a Kiesel gel 60 F_{254} plate with 99% ethanol-7%aqueous ammonia (1 : 2, v/v).[17] The spots were detected with ninhydrine and Rosenberg reagent.[20]

Figure 4 shows the infrared spectrum of the ceramide aminoethylphosphonate obtained by the alkaline hydrolysis method. The infrared spectrum lacked a band at 1730-1750 cm^{-1} indicating the absence of ester bonds. A band was present at 1180 cm^{-1}, indicating the presence of carbon-phosphorus bond, and was essentially identical with that of ceramide aminoethylphosphonate, obtained by Rouser et al,[11] Hayashi et al[30] and Hori et al.[16,31]

Figure 5 shows the thin layer chromatogram of the carbon-phosphorus compound in the acid hydrolysate of the ceramide aminoethylphosphonate from the *Sinonovacula constricta*.

The carbon-phosphorus compound in the hydrolysate of the ceramide aminoethylphosphonate obtained by acid hydrolysis as described earlier in the text were found to be 2-aminoethylphosphonic acid by thin layer chromatography.

The Rf (0.75) of the spot agreed with that of authentic 2-aminoethylphosphonic acid synthesized by Kosolapoff's method.[19] Inorganic phosphorus, and N-methyl derivatives of 2-aminoethlyphosphonic acid were not detected in the hydrolysate.

Table 3. Analytical Data of the Ceramide Aminoethylphosphonate Purified by the Mild Alkaline Hydrolysis from the *AGEMAKI, Sinonovacula constricta*

	Found	Calcd. for $C_{37}H_{75}O_5N_2P$
N	4.21	4.25
Amino N	1.91	2.13
Long chain base N	1.86	2.13
Sphingosine	41.20	45.40
P	4.62	4.70
N : P (molar ratio)	2.02	2.00
C	66.72	67.43
H	11.31	11.48
Fatty acid	40.20	41.10

Table 3 shows chemical data on the ceramide aminoethylphosphonate isolated from the *Sinonovacula constricta*.

As shown in the table, analytical values estimated for the ceramide aminoethylphosphonate agreed closely with those calculated values for ceramide 2-aminoethylphosphonate.

The results of gas chromatographic analysis of fatty acid composition are shown in Table 4. The fatty acid composition was very simple. The fatty

Table 4. Fatty acid Composition of Ceramide Aminoethylphosphonate from the *AGEMAKI*, *Sinonovacula constricta*

Fatty acid	%
Lauric acid	0.30
Myristic acid	0.96
Pentadecanoic acid	2.09
Palmitic acid	59.98
Margaric acid	8.58
Stearic acid	20.29
Linoleic acid	0.03
Linolenic acid	0.24
Arachidic acid	2.28
Eicosapentaenoic acid	0.61
Behenic acid	0.43
Unknown	4.22
Average carbon number	17.3

acids of the ceramide aminoethylphosphonate were characterized by relatively high percentages for palmitic acid and stearic acid, which conforms to fatty acid composition of the ceramide aminoethylphosphonate in other invertebrates.[24-28] The sum of these two acids comprised about 80% of the total fatty acids.

Pentadecanoic acid (2.1%), margaric acid (8.6%) and arachidic acid (2.3%) were also found as minor components.

From the above results, the isolated phosphonolipid was identified as the ceramide 2-aminoethylphosphonate.

DISCUSSION

The distribution of phosphonolipids in nature are primarily limited to lower animals such as mollusca coelenterates and protozoa, although the lipids have also been found in small quantities in mammalian tissues.[32-34] In the phosphonolipids, it has been established that glycero-aminoethylphosphonate (GP_nL) occurs as the major phospholipid of the ciliary membranes of protozoan species.[35,36] To date, there is still no report on the detection of the GP_nL in shellfish and sea anemones.

On the other hand, ceramide aminoethylphosphonate consists of 2-aminoethylphosphonic acid substituted on the primary hydroxyl group of a characteristic sphingosine base.

Ceramide aminoethylphosphonate are found in high concentration in mollusca,

coelenterates and shellfish such as *Turbo cornutus*, *Monodonta labio* and *Tegula lischkei*, while the concentration is low in *Tetrahymena*. Matsubara[37] found that the oyster adductor muscle contained the highest concentration (45% of the total sphingolipids) of ceramide aminoethylphosphonate. Komai et al[38] found that ceramide aminoethylphosphonate occurs in approxymately 11% of the phospholipids in the nervous system of *Aplysia kurodai* a marine gastropod. Moreover, Komai et al speculate that ceramide aminoethylphosphonate may be indispensible in shellfish for neuronal function.

Matsubara[39] and Itasaka et al[40] reported that ceramide aminoethylphosphonate and ceramide monomethylaminoethylphosphonate occurs in some shellfish and that the concentration ratio of the former and the latter were 89 : 11 in *Sinotaia histrica*, 68 : 32 in *Semisulcospira bensoni*, 69 : 31 in *M. labio* and 85 : 15 in the land snail, *Helix pomatia*.

While this sphingolipid is unigue to the invertebrates, its exact function is unknown but their resistance to endogenous hydrolytic enzymes is highly suggestive of a protective function.

Kittredge et al[41] indicate that the presence of a covalent carbon-phosphorus linkage in the anemone has been postulated as functionally analogous to a fixation process.

Mason[29] indicates the possibility that the presence of a highly ionic lipid such as ceramide aminoethylphosphonate could play a direct role in facilitating the transport of small ions from the aqueous environment into the intercellular space of the anemone.

On the other hand, the occurrence of 2-aminoethylphosphonic acid in the human tissues has been reported.[42]

These results suggest that 2-aminoethylphosphonic acid originating from marine products may be found in many different type tissue of human, and that a person could ingest this compound through fishery products as well as dairy products.[43]

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