Biochimica et Biophysica Acta, 1042 (1990) 280–286
Elsevier

BBALIP 53310

Determination of the presence of ceramide aminoethylphosphonate and ceramide *N*-methylaminoethylphosphonate in marine animals by fast atom bombardment mass spectrometry

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(Received 23 June 1989)
(Revised manuscript received 19 September 1989)

Key words: Ceramide aminoethylphosponate; Ceramide *N*-methylaminoethylphosphonate; Phosphonosphingolipid distribution; FAB-MS; (Marine animal)

Phosphonosphingolipids from 15 kinds of shellfish were analyzed by fast atom bombardment mass spectrometry to determine the contents of ceramide aminoethylphosphonate (CAEPn) and ceramide *N*-methylaminoethylphosphonate (CMAEPn). Two pairs of ions, at *m/z* 126 and 140 in the positive ion mode and at *m/z* 124 and 138 in the negative ion mode, were used to distinguish between aminoethylphosphonic acid and *N*-methylaminoethylphosphonic acid in CAEPn and CMAEPn. Interestingly, mollusca in the early stage of evolution have both CAEPn and CMAEPn, while most in the middle stage have only CMAEPn and those in the highest stage have only CAEPn.

Introduction

Two phosphonosphingolipids (PnSL) have been shown to be naturally occurring sphingolipids containing aminoalkylphosphonic acid. One is a ceramide aminoethylphosphonate (CAEPn) which has aminoethylphosphonic acid (AEPn) as a water-soluble component. This was isolated from sea anemone, *Anthopleura elegantissima*, in 1963 by Rouser et al. [1]. AEPn was first discovered in nature as a lipid component. This discovery was soon followed by the finding of CAEPn from bivalves, *Corbicula sandai* [2,3] and *Ostrea gigas* [4]. Recently, CAEPn was reported in jellyfish *Pelagia noctiluca* [5]. Another PnSL is an *N*-methyl derivative of CAEPn, ceramide *N*-methylaminoethylphosphonate (CMAEPn), which was isolated from a marine snail, *Turbo cornutus*, in 1969 by Hayashi et al. [6], and then found in some snails, *Monodonta labio* [7,8] and *Chlorostoma argyrostoma turbinatum* (unpublished data). These findings made it clear that these two PnSLs are widely distributed in marine animals, especially those in the lower part of the phylogenetic tree [9]. However, it is difficult to determine, using intact lipids, which animals contain which PnSL. Since each PnSL has a complex molecular species in the ceramide moiety (combination of fatty acids and long chain bases) in addition to differences in AEPn and MAEPn, complete separation of closely located PnSLs into individual units is almost impossible by column chromatography using silicic acid or Iatrobeads. Tedious chemical techniques are required, such as hydrolysis of lipids, separation of water-soluble materials and analysis of C-P compounds by paper chromatography or gas chromatography-mass spectrometry. To solve this problem, we recently developed a convenient method for distinguishing between CAEPn and CMAEPn using characteristic ions for C-P compounds in FAB-MS spectra [10] and also established the conditions for high performance liquid chromatography (HPLC) for effective separation of the molecular species of PnSLs.

In this report, we describe how the new method can be used to examine the distribution of CAEPn and CMAEPn in some marine animals and to identify these

Abbreviations: PnSL, phosphonosphingolipid; CAEPn, ceramide aminoethylphosphonate; CMAEPn, ceramide *N*-methylaminoethylphosphonate; AEPn, aminoethylphosphonic acid; MAEPn, *N*-methylaminoethylphosphonic acid; HPLC, high-performance liquid chromatography; FAB-MS, Fast atom bombardment mass spectrometry.

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PnSLs from their molecular species after separation by HPLC.

Materials and Methods

Preparation of phosphonosphingolipids

Fresh tissues (50–100 g) of each shellfish were extracted with acetone two times to remove water and neutral lipids. The residue was extracted with chloroform/methanol (2 : 1, v/v) three times and with chloroform/methanol (1 : 3, v/v) once. The chloroform/methanol extracts were combined and subjected to Folch's partition. The lipid partitioned into the lower phase was treated with mild alkali to remove glycerolipids. As the alkali-stable lipid which is obtained contains glycosphingolipids, phosphonosphingolipids and phosphonoglycosphingolipids, these were separated by Iatrobeads column chromatography. The column (1.5 × 15 cm) was eluted successively with 100 ml of chloroform/methanol (98 : 2, v/v), 300 ml of acetone/methanol (9 : 1, v/v), 100 ml of acetone/methanol (4 : 1, v/v), 100 ml of chloroform/methanol (9 : 1, v/v), 300 ml of chloroform/methanol (4 : 1, v/v), 300 ml of chloroform/methanol (3 : 2, v/v) and finally 200 ml of methanol. Phosphonosphingolipids were eluted with chloroform/methanol (4 : 1, v/v). This fraction was rechromatographed once more using an Iatrobeads column. The purified PnSL gave a slightly broad spot on thin-layer chromatography, but further purification was not attempted to avoid separation into the individual molecular species.

Conditions of FAB-MS

Mass spectrometry was carried out with a JEOL JMS-HX100 double-focusing mass spectrometer fitted with a high-field magnet and FAB ion source. The mass spectrometer was operated at an accelerating voltage of 5 kV and equipped with a post-accelerating detector. Samples were dissolved in chloroform/methanol (2 : 1 or 1 : 1, v/v; 1–10 μg/μl) and 1 μl of the solution was added to the matrix (glycerol for the positive ion mode and triethanolamine for the negative ion mode) on the stainless-steel FAB probe target. The sample was bombarded with a 6 kV xenon atom beam. The exact mass measurement by FAB-MS was carried out using cluster ions of glycerol that were used as a mass calibrant.

HPLC of phosphonosphingolipids

HPLC was performed with a Shimadzu LC-6A solvent delivery system equipped with a model SPD-6A UV spectrometric detector. PnSL (25 μg) was separated into its molecular species on a 4.6 × 150 mm column packed with Cosmosil $5C_{18}$ (Nakarai Tesque, Kyoto). Methanol was used as the mobile phase at a flow-rate of 0.5 ml/min. For preparative purposes, 2 mg of PnSL dissolved in 10 μl of chloroform/methanol (2 : 1, v/v) was applied to the column (8.0 × 250 mm) which was packed also with Cosmosil $5C_{18}$ and eluted with methanol at a flow-rate of 2.0 ml/min. The elution profiles of PnSL were monitored by the ultraviolet absorbance at 205 nm.

Results

Positive and negative ion FAB mass spectra of abalone PnSL are shown in Fig. 1. In addition to the $[M + H]^+$ ions, those indicating ceramide and long-chain base [10] in the positive ion mode and the $[M - H]^-$ ion in the negative ion mode, there are also two pairs of ions, at *m/z* 126 and 140 in the positive ion mode and at *m/z* 124 and 138 in the negative ion mode. These ions have been assigned to C-P compounds, AEPn and MAEPn contained in PnSL, by the FAB-MS/MS method as described previously [10]. In the present work, the exact mass numbers of these ions were measured to corroborate this assignment. The exact masses of ions at *m/z* 126 and 140 in the positive ion mode and *m/z* 124 and 138 in the negative ion mode are shown in Table I. The possible elemental formulas calculated based on the exact masses are also listed in Table I. Among the four elemental formulas in the positive ion mode, $C_2H_9NO_3P$ for 126.0313 and $C_3H_{11}NO_3P$ for 140.0466 were chosen as the most suitable formulas. They correspond to two ion structures, $[HO\text{-}P(O)(OH)CH_2CH_2NH_2 + H]^+$ and $[HO\text{-}P(O)(OH)CH_2CH_2NH(CH_3) + H]^+$, respectively. In the negative ion mode, $C_2H_7NO_3P$ for 124.0168 and $C_3H_9NO_3P$ for 138.0317 seem to show $[HO\text{-}P(O)(O)\text{-}CH_2CH_2NH_2]^-$ and $[HO\text{-}P(O)(O)\text{-}CH_2CH_2NH(CH_3)]^-$, respectively. These results show that the ions at *m/z* 126 and 124 originated from AEPn which was derived from CAEPn and the ions at *m/z* 140 and 138 MAEPn from CMAEPn. Therefore, the intensities of these ions can be used to determine whether the PnSLs obtained from some animals are CAEPn and/or CMAEPn. Next, the percent intensities (i.e., the intensity of the characteristic ion of CAEPn or CMAEPn divided by the sum of the intensities of both characteristic ions multiplied by 100) of each ion pair (*m/z* 138 and 124, and *m/z* 140 and 126) were measured using a series of mixtures with molar ratios of 4 : 1, 3 : 2, 1 : 1, 2 : 3 and 1 : 4 of pure CAEPn [11] and CMAEPn [6], as shown in Fig. 2. In the negative ion mode, a linear relationship was found between the percent intensities of *m/z* 138 and 124 and the concentration of CAEPn and CMAEPn. However, in the positive ion mode, the relationship was not linear, although the intensities of *m/z* 140 and 126 were proportional to concentration of CAEPn and CMAEPn.

The analytical results of the C-P components using the above four ions by FAB-MS are summarized in

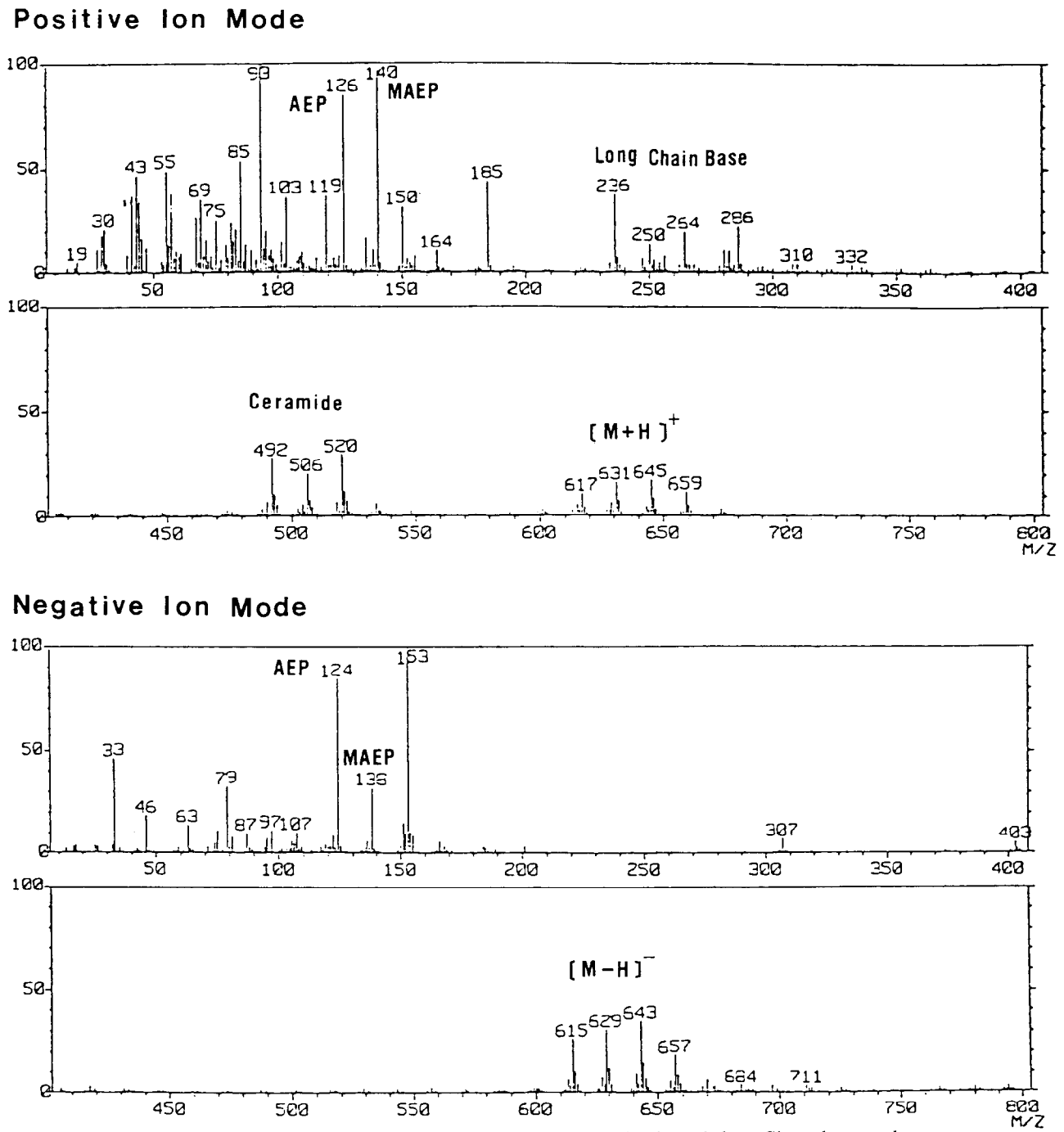

Fig. 1. Positive and negative ion mode FAB mass spectra of the PnSL fraction from abalone. Glycerol was used as a matrix.

Table II. PnSLs obtained from 15 kinds of mollusca were analyzed. Marine mollusca, except Helix, are listed in the order of evolutionary development, from top to bottom in Table II. An early mollusc, chiton, *Liolophura Japonica* which belongs to polyplacophora contained both CAEPn and CMAEPn. The ratio of the two ions at *m/z* 126 and 140 in the positive ion mode was about 65 : 35, but in the negative ion mode, the ions at *m/z* 124 and 138, it was about 85 : 15. Why such a difference arises between the positive and negative ion modes is not known. Among the seven kinds of gatropoda, abalone (*H. japonica*) is the only one having both CAEPn and CMAEPn. In muscle part, the ratio of CAEPn and CMAEPn was about 53 : 46 in the positive ion mode and 73 : 27 in the negative ion mode. On the other hand, these ratios were 25 : 75 and 35 : 65 in visceral tissue. Five kinds of snail belonging to archaeogastropoda, *T. cornutus, M. labio, C. arghyrostoma turbinatum, Chlorostoma xanthostigma*, and *Nerita albicilla*, have almost only CMAEPn. Another snail,

TABLE I

Determination of the exact mass of ions at m/z 126, 140, 124 and 138

Exact mass	Elemental formula	Ion structure
126.0313	C_9H_4N $C_6H_6O_3$ C_6H_7OP $C_2H_9NO_3P$	$[HO-\overset{\overset{O}{\|}}{P}(OH)-CH_2CH_2NH_2+H]^+$
140.0466	$C_{10}H_6N$ $C_7H_8O_3$ C_7H_9OP $C_3H_{11}NO_3P$	$[HO-\overset{\overset{O}{\|}}{P}(OH)-CH_2CH_2N(H)CH_3+H]^+$
124.0168	C_9H_2N $C_6H_4O_3$ $C_2H_6NO_5$ C_6H_5OP $C_2H_7NO_3P$	$[HO-\overset{\overset{O}{\|}}{P}(O)-CH_2CH_2NH_2]^-$
138.0317	$C_{10}H_4N$ $C_7H_6O_3$ $C_3H_8NO_3$ C_7H_7OP $C_3H_9NO_3P$	$[HO-\overset{\overset{O}{\|}}{P}(O)-CH_2CH_2N(H)CH_3]^-$

Rapana thomasiana, belonging to neogastropoda, has only CAEPn. Two kinds of clam, *O. gigas* and *Tapes japonica*, and two kinds of sea hare, *Aplysia kurodai* and *Dolabella auricularia*, also have only CAEPn. Cephalopoda, which are the most elaborately evolved molluscs, cuttlefish, *Ommastrephes sloani pacificus*, and octopus, *Octopus vulgaris*, contain only CAEPn. These findings suggest that the more evolutionarily advanced mollusca tend to have a simple C-P component and a simple molecule.

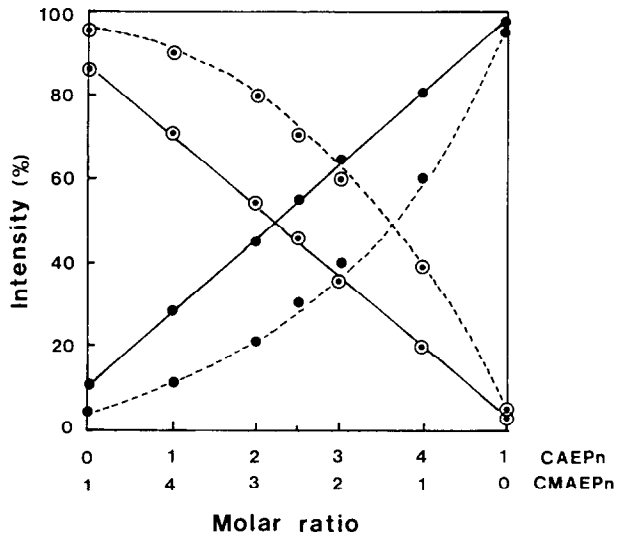

Fig. 2. Relationships between ion intensities and CAEPn and CMAEPn. o------o, ion at m/z 140; o———o, at m/z 138; ●------●, at m/z 126; ●———●, at m/z 124.

TABLE II

Distribution of CAEPn and CMAEPn in marine mollusca (%)

Mollusc		Positive mode		Negative mode	
		m/z 126	m/z 140	m/z 124	m/z 138
Liolophura japonica		64.6	35.4	86.3	13.7
Haliotis japonica	muscle	53.3	46.2	72.5	27.5
	viscera	24.8	75.2	34.8	65.2
Turbo cornutus	muscle	4.7	95.3	12.6	87.4
	viscera	5.2	94.8	13.6	86.4
Monodonta labio		8.2	91.8	19.3	80.7
Chlorostoma argyrostoma turbinatum		8.4	91.6	32.6	67.4
Chlorostoma xanthostigma		3.0	96.7	20.0	80.0
Nerita albicilla		5.8	94.2	12.2	87.8
Rapana thomasiana	muscle	94.4	5.6	96.7	3.3
	viscera	90.1	9.9	96.3	3.7
Ostrea gigas	adductor	95.1	4.9	95.3	4.7
	gills	92.7	7.3	98.1	1.9
	mantle	95.3	4.7	97.6	2.4
	viscera	90.4	9.6	95.7	4.3
Tapes japonica		87.7	12.3	96.9	3.1
Aplysia kurodai		97.0	3.0	98.7	1.3
Dolabella auricularia		90.8	9.2	93.5	6.5
Ommastrephes sloani pacificus		80.2	19.8	91.2	8.6
Octopus vulgaris		81.0	19.0	91.2	8.8
Euhadra congenita hickonis [a]		54.8	45.2	73.3	26.7

[a] Land snail.

Land snail, *Euhadra congenita hickonis*, has CAEPn and CMAEPn at 55 : 45 in the positive ion mode and 73 : 27 in the negative ion mode. This ratio is similar to that in abalone muscle.

As abalone PnSL was a mixture of CAEPn and CMAEPn, we tried separating it into CAEPn and CMAEPn at the molecular species level by HPLC. Fig. 2 shows the resulting chromatograms. Each peak was collected separately and analyzed by positive and negative ion FAB-MS. FAB mass spectra of the positive ion mode are also shown in Fig. 3. Peaks 1 and 2 have the same ceramide ion at m/z 492 and different $[M+H]^+$ ions at m/z 617 and 631. Similarly, peak 3 and peak 4 have the same ceramide ion at m/z 506 and different $[M+H]^+$ ions at 631 and 645. Peak 5 and peak 6 also have the same ceramide ion at m/z 520 and different $[M+H]^+$ ions at m/z 645 and 659. These differences arise from the difference in the C-P component. For peaks 1, 3 and 5, the ion at m/z 126 was detected with strong intensity, but for peaks 2, 4 and 6 the ion at m/z 140 was detected strongly and the ion at m/z 126 not as strongly. Therefore, peaks 1, 3 and 5 represent various molecular species of CAEPn. Peaks 2, 4 and 6 containing CMAEPn do not represent single components. These peaks may contain CAEPn as a minor component. Based on these data, the percent distribution of the C-P component in the eight peaks, as determined by FAB-MS, is summarized in Table III. In peaks 1, 3 and 5,

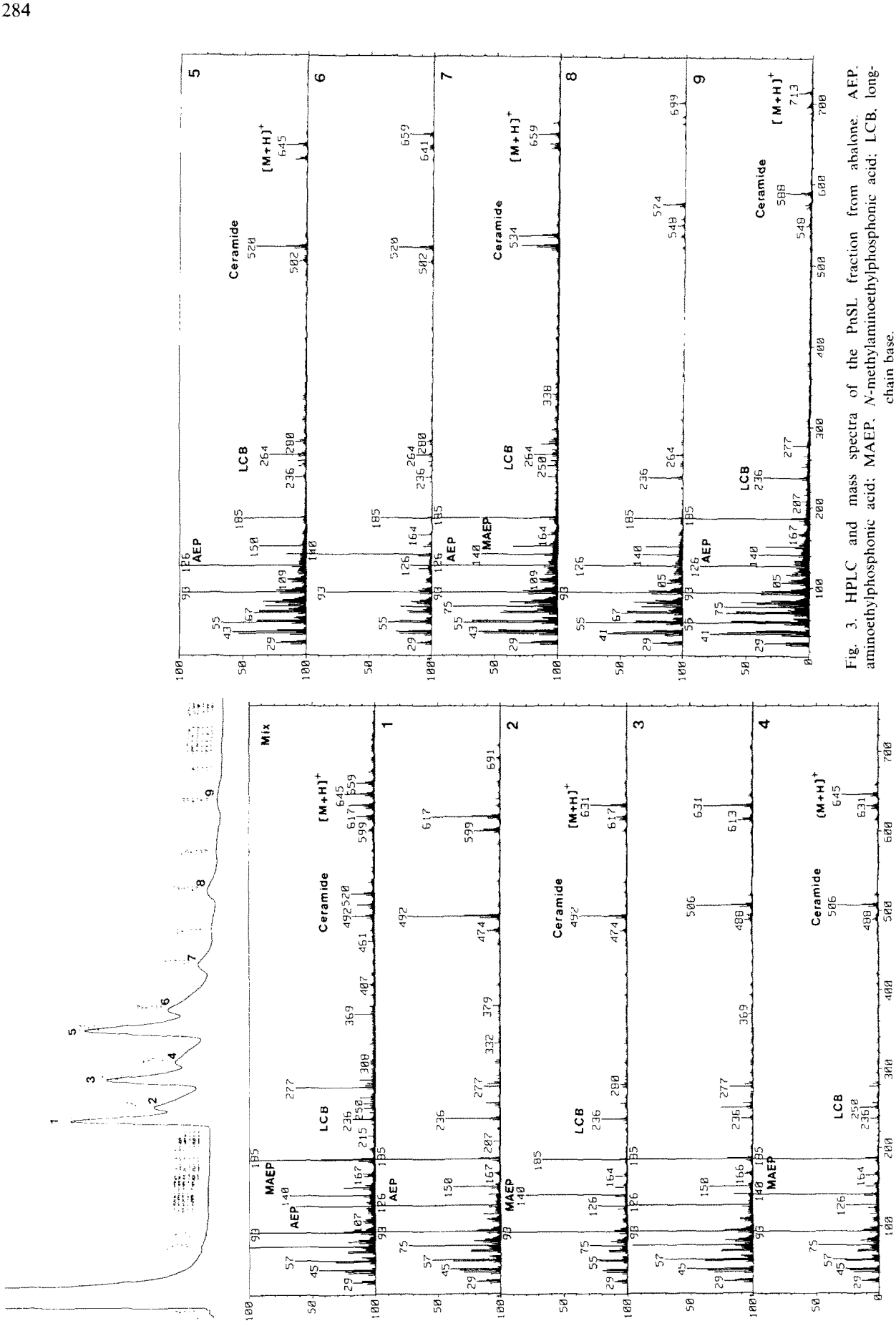

Fig. 3. HPLC and mass spectra of the PnSL fraction from abalone. AEP, aminoethylphosphonic acid; MAEP, *N*-methylaminoethylphosphonic acid; LCB, long-chain base.

TABLE III

Percent distribution of C-P component in nine peaks of PnSL separated by HPLC

Peak	$[M+H]^+$	Ceramide	Positive mode (%)		$[M-H]^-$	Negative mode (%)		Main molecular species
			m/z 126	*m/z* 140		*m/z* 124	*m/z* 138	
1	617	492	91.0	9.0	615	96.3	3.7	$C_{16:0}$-$d_{16:1}$-AEP
2	631	492	22.5	77.5	629	40.9	59.1	$C_{16:0}$-$d_{16:1}$-MAEP
3	631	506	86.4	13.6	629	92.0	8.0	$C_{16:0}$-$d_{17:1}$-AEP
4	645	506	20.9	79.1	643	40.6	59.4	$C_{16:0}$-$d_{17:1}$-MAEP
5	645	520	86.0	13.4	643	93.0	7.0	$C_{16:0}$-$d_{18:1}$-AEP
6	659	520	14.9	85.1	657	40.0	60.0	$C_{16:0}$-$d_{18:1}$-MAEP
7	659	534	62.2	37.8	657	80.9	19.1	$C_{17:0}$-$d_{18:1}$-AEP
8	699	574	70.2	29.8	697	85.9	14.1	$C_{22:1}$-$d_{16:1}$-AEP
9	713	588	68.3	31.7	711	83.6	16.3	$C_{23:1}$-$d_{16:1}$-AEP

AEPn was about 90% and the values obtained in the positive and negative modes showed good agreement. For peaks 2, 4 and 6, such agreement was not found. There was also a difference in the percent distribution between the positive and negative ion modes. This difference was larger for a mixture of CAEPn and CMAEPn than for only CAEPn or CMAEPn, as shown in Tables II and III. The limitations of this new method are discussed below.

Discussion

Many methods have been proposed to distinguish between CAEPn and CMAEPn. For example, water-soluble components obtained by hydrolysis of PnSL have been analyzed by paper chromatography using Hanes-Isherwood reagent [12] to detect phosphorus, but this method can only be used for qualitative purposes [8]. Gas chromatography-mass spectrometry has also been used to analyze C-P compounds after their trimethylsilylation under various conditions [13]. As *N*-silylation is difficult, multiple and nonreproducible peaks of trimethylsilyl derivatives appear owing to incomplete silylation [13]. Moreover, the *N*-trimethylsilyl derivatives are very unstable and easily decompose on the column [14,15]. For the same reason, gas chromatography is also unsuitable for quantitative determination of C-P compounds. An immunochemical method, i.e., ELISA (enzyme linked immunoadsorbent assay), has been applied to the determination of C-P compounds. Antibodies against CAEPn and CMAEPn are prepared by immunizing rabbits with CAEPn and CMAEPn separately and their cross-reactions to pure CAEPn and CMAEPn are examined. The anti-CAEPn antibody also cross-reacts with CMAEPn at the level of a few percent. In the same manner, the anti-CMAEPn antibody cross-reacts with CAEPn at a level of a few percent. Although exact determination can not be attained, this method is better than those using by paper chromatography and gas chromatography.

The mass spectrometric method we propose here is more convenient than the methods described above, since the ion pairs *m/z* 126 and 140 and *m/z* 124 and 138 were easily obtained from FAB-MS with intact lipids without degradation or derivatization. This eliminates the tedious preparation of antibodies. However, some problems still remain with this mass spectrometric method. For qualitative analysis of PnSL, the positive ion mode is better than the negative ion mode, as ions for ceramide and long-chain base appear more clearly. However, ions indicating the C-P compounds display different intensities between the positive and negative ion modes. As described in the Results, the negative ion mode gave a linear relationship, while the positive one did not. The negative ion mode results showed better agreement with those from chemical analyses [8].

An inexplicable phenomenon was the few percent displacement at the origin for the ion at *m/z* 140 in the positive ion mode and *m/z* 138 in the negative ion mode when pure CAEPn was used. Pure CMAEPn also gave a few percent displacement from the 100% level of the ion at *m/z* 126 in the positive ion mode and *m/z* 124 in the negative ion mode. Such displacement may arise due to the influence of the matrix on the intensity of the background at such a low mass range. Further study is needed to resolve this problem. However, overall our results show that this method is a promising one and offers results showing differences in CAEPn and CMAEPn distribution with evolution in mollusca.

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