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Water-Soluble and Lipid-Soluble Arsenic Compounds in Japanese Flying Squid *Todarodes pacificus*

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Water-soluble and lipid-soluble arsenic compounds in Japanese flying squid *Todarodes pacificus* were analyzed. Regardless of the tissues, the major water-soluble arsenic compound was identified as arsenobetaine by LC/ESI-MS analysis, as reported for a number of marine animals. Lipid-soluble arsenic compounds were found at relatively high levels in liver and testis. LC/ESI-MS analysis of water-soluble arsenic compounds released from liver phospholipids by either chemical hydrolysis or phospholipase D hydrolysis demonstrated that the major arsenolipids are dimethylarsinic acid (DMA)-containing glycerophospholipid (phosphatidyldimethylarsinic acid) and DMA-containing sphingomyelin where the choline moiety of sphingomyelin is replaced by DMA. This is the first work to report the presence of DMA-containing phospholipids in marine invertebrates.

KEYWORDS: Arsenic; arsenolipids; LC/ESI-MS; lipid-soluble arsenic compounds; squid; *Todarodes pacificus*; water-soluble arsenic compounds

INTRODUCTION

Marine organisms naturally contain arsenic, a representative harmful element, at much higher levels (several μg/g or more) than terrestrial organisms, and hence seafood consumption is the main source of arsenic exposure for the general population. In view of the fact that arsenic exhibits toxicological properties depending on its chemical forms, it is essential to speciate and quantify arsenic compounds contained in marine organisms and seafood products. Most work has focused on water-soluble arsenic compounds and established that arsenobetaine (AB) and arsenosugars (dimethylarsinoylribosides) are predominant in marine animals and marine algae, respectively (*1*, *2*). Other methylated water-soluble arsenic compounds, such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), arsenocholine (AC), and tetramethylarsonium ion (TEMA), are also widely found in marine organisms mostly as minor constituents. DMA promoting carcinogenesis in mammals (*3*−*6*) and TEMA showing weak but significant cytotoxicity (*7*, *8*) may need further detailed study to assess their toxicity to human. On the whole, however, the methylated water-soluble arsenic compounds so far identified in marine organisms are of little toxicological importance because they exhibit much less acute toxicity to mammals than inorganic arsenic compounds, arsenate (As(V)) and arsenite, and are not accumulated in mammals even when ingested (*1*).

On the other hand, relatively little is known about the chemical forms and toxicological aspects of lipid-soluble arsenic compounds (arsenolipids) in marine organisms due to their low concentrations and the difficulty of isolating them. A lipid-soluble arsenic compound was first isolated from brown alga *Undaria pinnatifida* and rigorously identified as phosphatidylarsenosugar (*9*). Recently, the major lipid-soluble arsenical in another brown alga *Laminaria digitata* was also shown to be phosphatidylarsenosugar by analyzing water-soluble arsenic compounds released upon phospholipase D hydrolysis of phospholipids (*10*). As for marine animals, arsenolipids have been examined on the basis of the speciation of water-soluble arsenic compounds produced by chemical hydrolysis of lipids. The results indicated that the major arsenolipids are phosphatidylarsenosugar and phosphatidylarsenocholine in western rock lobster *Panulirus cygnus* (*11*), DMA-containing and AC-containing phospholipids in starspotted shark *Mustelus manazo* (*12*, *13*), DMA-containing lipids in fish oil (*14*), and DMA-containing phospholipids in ringed seal *Pusa hispida* (*15*). In addition, some unknown lipid-soluble arsenic compounds have also been detected in starspotted shark (*12*, *13*) and fish oil (*14*).

As described above, the diversity of lipid-soluble arsenicals is recognized even among the limited species of marine organisms so far studied. In this study, we chose Japanese flying

Table 1. Arsenic Concentrations in Five Tissues of Squid

	As concentration (μg of As/g)[a]				
no. of specimens	muscle	liver	testis[b]	ovary[c]	gill
15	3.1–11.0 (4.7 ± 3.7)	3.5–10.0 (6.8 ± 2.0)	2.0–13.8 (4.4 ± 3.9)	3.1–12.7 (5.2 ± 2.9)	2.3–10.2 (3.7 ± 2.9)

[a] Arsenic concentrations are expressed in both range and mean ± SD (in parentheses). [b] Obtained from 10 male specimens. [c] Obtained from 5 female specimens.

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Table 2. Concentrations of Total Arsenic, Ratios of Water-Soluble and Lipid-Soluble Arsenic to Total Arsenic, and Concentrations of Water-Soluble Arsenic Compounds in Four Tissues of Squid

			ratio to total As (%)		concentration of water-soluble arsenic compounds (μg of As/g)[a]			
specimen	tissue	total As (μg of As/g)	water-soluble As	lipid-soluble As	DMA	AB	TMAO	TEMA
A	muscle	5.4	98.7	0.0	0.25 ± 0.01	5.00 ± 0.16	0.08 ± 0.004	ND[b]
	liver	9.2	89.5	10.1	0.71 ± 0.05	6.77 ± 0.047	0.19 ± 0.015	ND
	testis	6.6	82.9	6.1	ND	5.17 ± 0.59	ND	ND
	gill	6.2	97.5	0.0	0.14 ± 0.02	6.27 ± 0.20	0.07 ± 0.008	0.01 ± 0.001
B	liver	8.6	88.3	9.3				
C	liver	6.2	87.7	10.9				

[a] Data are expressed in mean ± SD (three determinations). [b] ND: not detected.

Table 3. Results of Fractionation of Squid Lipids by Adsorption Column Chromatography on Silicic Acid

			weight ratio (%)			As ratio (%)		
specimen	lipid used (g)	amount of As in lipid used (μg of As)	neutral lipid	glycolipid	phospholipid	neutral lipid	glycolipid	phospholipid
A	9.7	30.7	90.0	4.2	5.8	18.9	0.0	82.1
B	9.3	25.5	91.5	3.5	5.0	8.8	0.0	91.2
C	8.1	18.7	87.8	4.8	7.4	14.4	0.0	85.6

squid *Todarodes pacificus* (simply called squid hereinafter), the cephalopod most abundantly consumed in Japan, as a sample, because mollusks have not been studied for lipid-soluble arsenic compounds. It should be noted that not only muscle but also viscera (especially liver) of the squid are sometimes eaten. Also, fermented squid ("Shiokara" in Japanese), which is widely consumed in Japan, is produced using both muscle and liver. Therefore, we first analyzed water-soluble arsenicals in several tissues of the squid by liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS), a quantification method for arsenic compounds recently developed in our laboratory (*16*). Next, we chemically and enzymatically produced water-soluble arsenicals from phospholipids of squid liver containing significant amounts of lipid-soluble arsenicals and analyzed them by LC/ESI-MS. The present paper deals with the identification of AB as the major water-soluble arsenic compound in squid tissues and DMA-containing phospholipids (phosphatidyldimethylarsinic acid and DMA-containing sphingomyelin) as the major arsenolipids in liver.

MATERIALS AND METHODS

Reagents. As(V) (disodium form) was purchased from Kanto Kagaku (Tokyo, Japan), DMA was from Wako Pure Chemical Industries (Osaka, Japan), and MMA, AB, TMAO, AC, and TEMA (iodide form) were from Trichemical Laboratory (Kitatsuru, Japan). Phospholipase D (EC 3.1.4.4, type IV from cabbage) was purchased from Sigma-Aldrich Corp. (Tokyo, Japan). Deionized water used for all solutions was prepared with an Auto Pure WQ500 system (Millipore, Bedford, MA). Organic solvents (HPLC grade) such as methanol and chloroform and nitric acid (containing 60% HNO_3, super special grade) and sulfuric acid (containing 95% H_2SO_4, super special grade) used for wet-digestion were purchased from Kokusan Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

Squid Sample. Fifteen fresh specimens of squid were purchased at the Tokyo Central Wholesale Market. Muscle, liver, gonad (testis or ovary), and gill were dissected from each specimen and stored at −20 °C until use.

Preparation of Water-Soluble and Lipid-Soluble Arsenic Fractions. One specimen (specimen A) was randomly selected for analysis of water-soluble and lipid-soluble arsenic compounds in this study. Additional two specimens (specimens B and C) were also used for analysis of lipid-soluble arsenicals in liver. According to the procedure described by Hanaoka et al. (*12*, *13*), each tissue sample (3−5 g) was homogenized with 10 volumes of chloroform−methanol (2:1) and centrifuged. The residue was again extracted with the same volume of chloroform−methanol (2:1). Because the use of only organic solvents is suggested to be insufficient to extract water-soluble arsenic compounds, especially inorganic arsenicals (*17*), the residue was further extracted once with 10 volumes of deionized water. All of the extracts were combined, shaken vigorously, and allowed to stand overnight for the complete separation of the two layers. The water−methanol layer was evaporated to dryness in vacuo and dissolved in 5−10 mL of deionized water. This solution was desalted with a micro-acilyzer G1 (Asahi Chemical Industry, Tokyo, Japan) and ultrafiltered using a 5000 Da molecular weight cutoff Vivaspin (Vivascience AG, Hanover, Germany). The filtrate thus obtained was used as a water-soluble arsenic fraction. On the other hand, the chloroform layer was evaporated to dryness in vacuo, and the residue dissolved in 10 mL of chloroform was used as a lipid-soluble arsenic fraction.

Fractionation of Lipids. The lipid-soluble arsenic fraction (10.7 g of lipids containing 33.8 μg of As for specimen A, 10.3 g of lipids containing 28.2 μg of As for specimen B, and 9.1 g of lipids containing 21.0 μg of As for specimen C) was prepared from 36.4 g (specimen A), 33.0 g (specimen B), or 28.7 g (specimen C) of liver. To separate into three groups of lipids, 9.7 g (specimen A), 9.3 g (specimen B), or 8.1 g (specimen C) of the liver lipids was applied to adsorption chromatography on a silicic acid column (2 × 40 cm, Wakogel C-300, Wako Pure Chemical Industries). The column was eluted successively with 500 mL each of chloroform, acetone, and methanol to obtain neutral lipids, glycolipids, and phospholipids, respectively.

Chemical Hydrolysis of Phospholipids. Chemical hydrolysis of phospholipids was performed according to the method of Hanaoka et al. (*13*). In brief, the liver phospholipids (150 mg containing 6.72 μg of As) from specimen A were treated first with 0.027 M NaOH at 37 °C for 20 min to prepare alkali-labile and alkali-stable fractions. Next, the alkali-labile fraction was hydrolyzed with 6 M HCl in a boiling water bath for 1 h, and the alkali-stable fraction was hydrolyzed with saturated $Ba(OH)_2$ under reflux for 5 h. Water-soluble arsenicals derived from the alkali-labile and alkali-stable fractions were analyzed by LC/ESI-MS.

Enzymatic Hydrolysis of Phospholipids. Hydrolysis of phospholipids with phospholipase D was performed according to the method of Wurster and Copenhaver (*18*). The liver phospholipids (278 mg

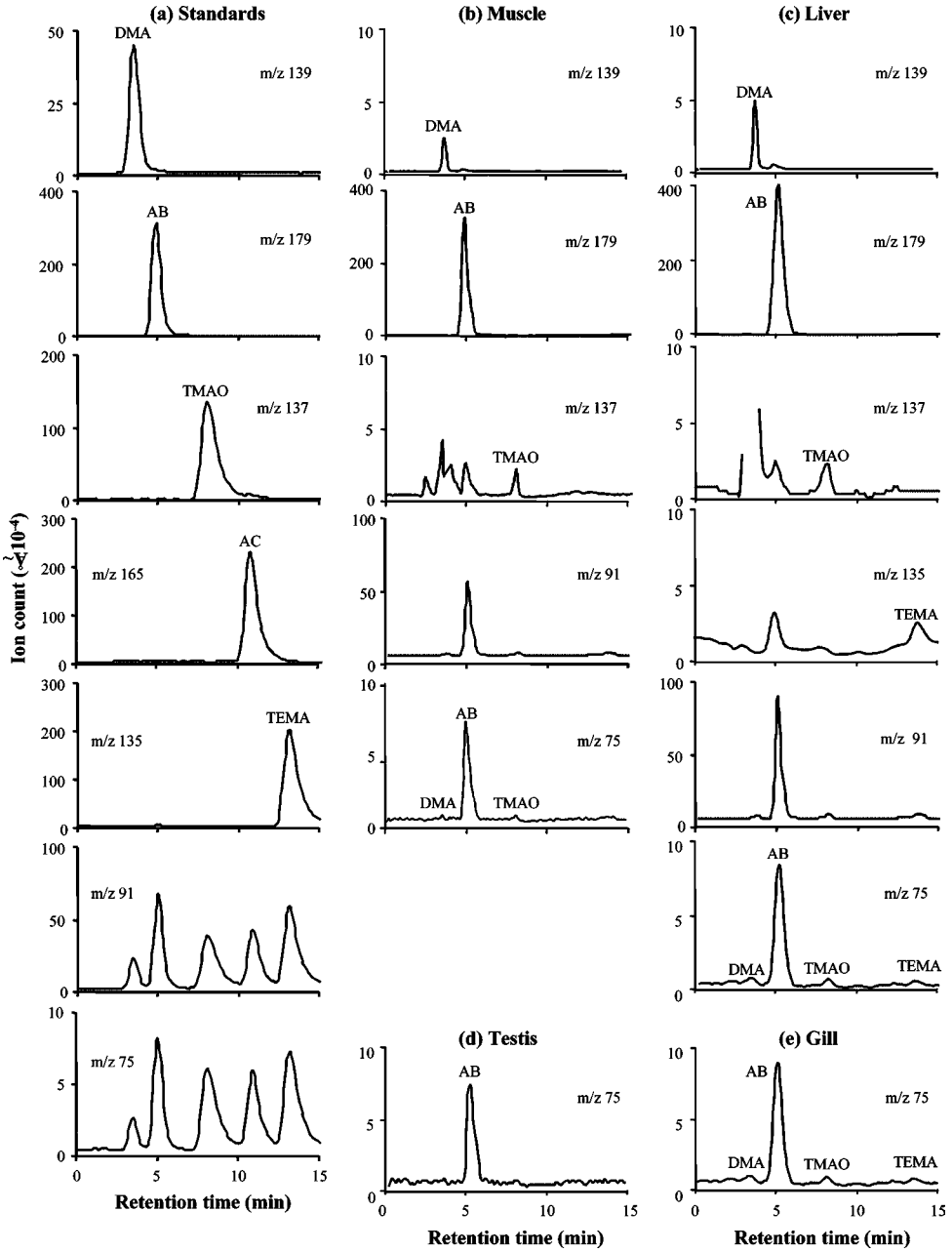

Figure 1. Cation-exchange LC/positive-ion mode ESI-MS chromatograms of standard arsenic compounds (50 ng of As each) (**a**) and water-soluble arsenic compounds from squid muscle (**b**), liver (**c**), testis (**d**), and gill (**e**). Injection volume was 10 μL. Column, Nucleosil 100-10SA (0.4 × 25 cm); solvent, 0.03 M pyridine–formic acid buffer (pH 3.1) containing 20% methanol; flow rate, 1 mL/min. The postcolumn eluate was splitted at a ratio of 3:7 to introduce 30% of the eluate into the ESI-MS system. Combinations of monitored ion and cone voltage: $[M + H]^+$ (*m*/*z* 139 for DMA, *m*/*z* 179 for AB, and *m*/*z* 137 for TMAO) at 45 V; M^+ (*m*/*z* 165 for AC and *m*/*z* 135 for TEMA) at 45 V; *m*/*z* 91 (CH_3As^+H and/or $As^+{=}O$) at 150 V; *m*/*z* 75 (As^+) at 240 V. In the case of muscle (**b**) and liver (**c**), mass chromatograms are not shown for molecular ions of undetectable arsenic compounds (AC and TEMA for muscle and AC for liver). For testis (**d**) and gill (**e**), only the mass chromatogram at *m*/*z* 75 is illustrated.

containing 12.5 μg of As for specimen A, 300 mg containing 8.0 μg of As for specimen B, and 235 mg containing 11.7 μg of As for specimen C) were suspended in 25 mL of sodium acetate buffer (pH 5.6) containing 0.25 mM $CaCl_2$ and added with 0.1 mL of phospholipase D solution (containing 30 units). After reaction at 37 °C for 6 h with vigorous shaking, the suspension was shaken twice with 50 mL of chloroform−methanol (2:1), to remove lipid-soluble compounds such as resulting phosphatidic acid and unreacted phospholipids. The aqueous layer was then evaporated in vacuo and dissolved in 5 mL of distilled water. The solution was desalted and ultrafiltered as described above and subjected to arsenic speciation analysis by LC/ESI-MS. A control experiment was similarly carried out without addition of the enzyme to the phospholipid suspension.

Determination of Arsenic. Each sample (about 1 g for tissues and 0.1−1.0 g for lipids in the lipid-soluble arsenic fraction and the fractions separated by silicic acid column chromatography) was weighed and digested with 10 mL of nitric acid and 5 mL of sulfuric acid using an automatic microwave digester A301 (Prolabo, Fontenay-Sous-Bois, France) according to the manufacturer's instructions. The digest was made up to 10 mL with deionized water, passed through a 0.8 μm filter, and determined for arsenic on an inductively coupled argon plasma atomic emission spectrometer (ICAP-757V; Nippon Jarrell-Ash, Kyoto, Japan) under the following conditions: wavelength, 193.7 nm; rf power, 1.4 kW; integration time, 10 s. Calibration of the ICAP was carried out using deionized water and As(V) solution (10 μg of As/mL). Aqueous samples were directly analyzed for arsenic concentration

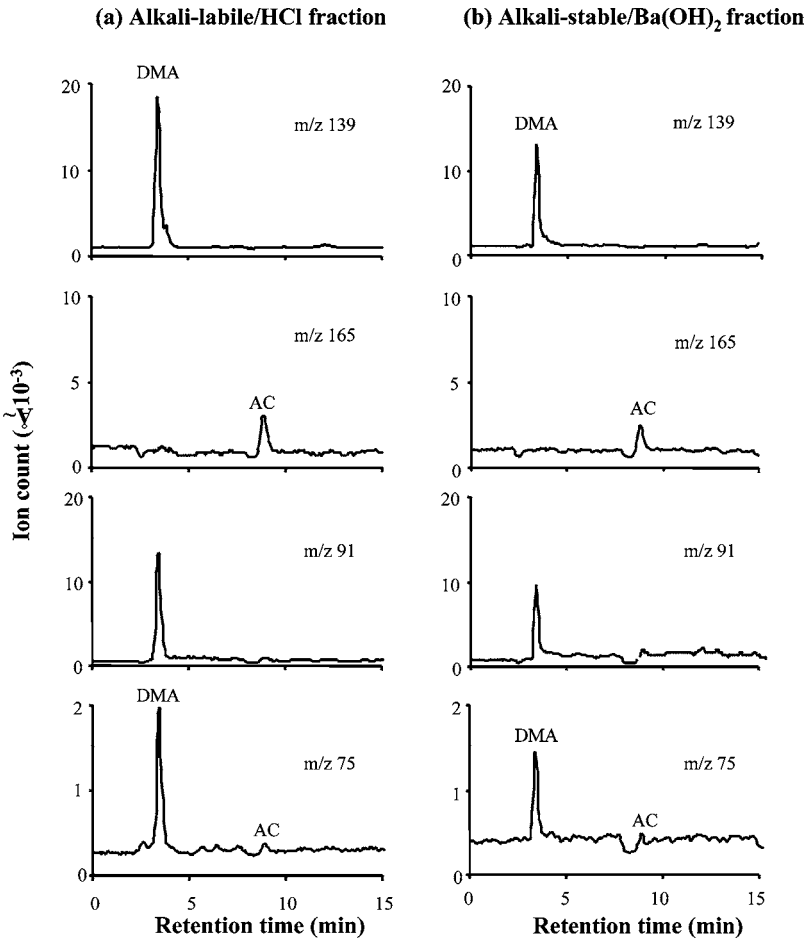

Figure 2. Cation-exchange LC/positive-ion mode ESI-MS chromatograms of water-soluble arsenic compounds in the alkali-labile/HCl fraction (**a**) and alkali-stable/$Ba(OH)_2$ fraction (**b**) prepared from the squid liver phospholipids. Injection volume was 10 μL. Refer to the legend of **Figure 1** for the analytical conditions.

Table 4. Determination of Water-Soluble Arsenic Compounds Released by Chemical Hydrolysis or Enzymatic (Phospholipase D) Hydrolysis of Squid Liver Phospholipids

				amount of water-soluble arsenic compounds released (μg of As)[a]	
specimen	hydrolysis	amount of As in phospholipid used (μg of As)	fraction	DMA	AC
A	chemical	6.72	alkali-labile/HCl	4.43 ± 0.20 (65.9)[b]	0.04 ± 0.005 (0.7)
			alkali-stable/$Ba(OH)_2$	1.12 ± 0.05 (16.7)	0.02 ± 0.005 (0.3)
	enzymatic	12.5	hydrolyzate	1.08 ± 0.04 (8.6)	ND[c]
B	enzymatic	11.7	hydrolyzate	0.90 ± 0.04 (7.7)	ND
C	enzymatic	8.0	hydrolyzate	0.94 ± 0.04 (11.8)	ND

[a] Data are expressed in mean ± SD (three determinations). [b] The mean ratio (%) to the arsenic in the phospholipids used is indicated in parentheses. [c] ND: not detected.

on the ICAP without wet-digestion. The accuracy of our determinations was supported by experiments using the dogfish certified reference material DORM-2 (NRC-CNRC, Ottawa, Canada) as reported previously (*16*).

LC/ESI-MS Analysis of Arsenic Compounds. Speciation and quantification of arsenic compounds in each sample were performed on LC/ESI-MS as described in our previous paper (*16*). Briefly, an Alliance 2695 Separation Module (Waters, Milford, MA) was used as an LC system and a micromass ZQ4000 single quadrupole MS (Waters) equipped with an electrospray ionization LC−MS interface as an ESI-MS system. As(V) and MMA were separated by anion-exchange LC on a SAX-0253-N column (0.4 × 25 cm; Senshu Pak, Tokyo, Japan) and analyzed by ESI-MS in the negative-ion mode. For DMA, AB, TMAO, AC, and TEMA, separation was achieved by cation-exchange LC on a Nucleosil 100-10SA column (0.4 × 25 cm; Macherey-Nagel, Düren, Germany) and analysis by ESI-MS in the positive-ion mode. The ESI-MS was operated under the following conditions: capillary voltage, 3500 V (negative-ion mode) or 1000 V (positive-ion mode); desolvation temperature, 300 °C; source temperature, 100 °C; cone gas (N_2) flow rate, 60 L/h; desolvation gas (N_2) flow rate, 358 L/h; inter channel delay, 0.1 s; dwell time, 0.02 s. Selected ion monitoring was carried out under various combinations of *m/z* and cone voltage suitable for each arsenic compound. All analyses and data acquisitions on LC/ESI-MS were automated via MaxLynx operation software (version 3.5). The limits of quantification (S/N = 10) were 40, 50, 10, 1.5, 1.5, 1.5, and 1.5 ng for As(V), MMA, DMA, AB, TMAO, AC, and TEMA, respectively. Because some instrumental parameters (low molecular weight resolution, high molecular weight resolution, ion energy, and

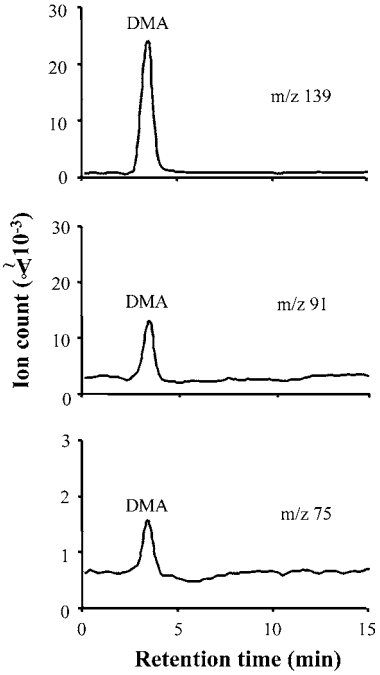

Figure 3. Cation-exchange LC/positive-ion mode ESI-MS chromatograms of water-soluble arsenic compounds cleaved from the squid liver phospholipids by phospholipase D hydrolysis. Injection volume was 10 μL. Refer to the legend of **Figure 1** for the analytical conditions.

multiplier) influencing the significant intensity were recently optimized (details of the optimization will be reported elsewhere), the sensitivity was 3−20 times increased as compared to the previously reported values (*16*).

RESULTS

Concentrations of Total, Water-Soluble, and Lipid-Soluble Arsenic in Squid Tissues. Fifteen specimens (10 male and 5 female specimens) of squid were analyzed for arsenic concentrations in five tissues (muscle, liver, testis, ovary, and gill). As shown in **Table 1**, arsenic concentrations of squid tissues were mostly less than 10 μg of As/g; there was no marked difference in arsenic concentration among the five tissues.

Water-soluble and lipid-soluble arsenic fractions were prepared from four tissues (muscle, liver, testis, and gill) of specimen A and livers of specimens B and C. Regardless of the tissues, the water-soluble arsenic occupied the majority (more than 83%) of the total arsenic (**Table 2**). Noticeable amounts of the lipid-soluble arsenic were found in two tissues (liver and testis) of specimen A; the lipid-soluble arsenic accounted for 10.1% and 6.1% of the total arsenic in liver and testis, respectively. High amounts of the lipid-soluble arsenic were also recognized in livers of specimens B and C.

Speciation and Quantification of Water-Soluble Arsenic Compounds in Squid Tissues. Water-soluble arsenic compounds in four tissues of specimen A were analyzed by LC/ESI-MS. When analyzed by anion-exchange/negative-ion mode ESI-MS, neither As(V) nor MMA was found in any of the water-soluble arsenic fractions from the four tissues (data not shown). On cation-exchange LC/positive-ion mode ESI-MS, a prominent peak was observed at the same retention time as standard AB, as monitored by *m*/*z* 179 at 45 V, *m*/*z* 91 at 150 V, and *m*/*z* 75 at 240 V (**Figure 1**). This peak was definitely assigned to AB. While AB was the sole arsenical in testis, arsenicals other than AB were also detected as the minor constituents in the other three tissues as follows: DMA and TMAO in muscle and DMA, TMAO, and TEMA in liver and gill. AC was contained in none of the tissues. Concentrations of DMA, AB, TMAO, and TEMA in the four tissues are summarized in **Table 2**. AB constituted more than 73% of the total arsenic and more than 82% of the water-soluble arsenic in all four tissues examined.

Speciation of Lipid-Soluble Arsenic Compounds in Squid Liver. Considerable amounts of arsenolipids were found in liver and testis. In this study, speciation of the lipid-soluble arsenic was performed with liver, which is a much larger tissue than testis. As shown in **Table 3**, neutral lipids, glycolipids, and phospholipids, which were separated from the liver lipids by adsorption column chromatography on silicic acid, occupied 87.8−91.5%, 3.5−4.8%, and 5.0−7.4% of the total lipid mass, respectively. Arsenic determination by ICAP showed that 82.1−91.2% of the lipid-soluble arsenic in liver existed in the phospholipid fraction; the remaining small amounts of lipid-soluble arsenic were contained only in the neutral lipid fraction. These results implied that the major lipid-soluble arsenic compounds are arsenic-containing phospholipids, similar to the previous studies on arsenolipids in marine organisms (*9*−*13*, *15*).

The phospholipids of squid liver (specimen A) were first subjected to sequential chemical hydrolysis according to the method of Hanaoka et al. (*13*), to release water-soluble arsenic compounds that can be readily analyzed by LC/ESI-MS. Following mild alkaline hydrolysis using 0.027 M NaOH, water-soluble and lipid-soluble compounds were recovered in the alkali-labile and alkali-stable fractions, respectively. Water-soluble arsenic compounds produced by severe hydrolysis of the alkali-labile fraction with 6 M HCl and the alkali-stable fraction with saturated $Ba(OH)_2$ were analyzed by LC/ESI-MS. Analysis by anion-exchange LC/negative-ion mode ESI-MS revealed the absence of As(V) and MMA in the hydrolysates of both alkali-labile and alkali-stable fractions (data not shown), while DMA, together with AC as a minor arsenical, was predominantly found in both alkali-labile/HCl and alkali-stable/$Ba(OH)_2$ fractions as analyzed by cation-exchange/positive-ion mode ESI-MS (**Figure 2**). As shown in **Table 4**, DMA in the alkali-labile/HCl and alkali-stable/$Ba(OH)_2$ fractions accounted for 65.9% and 16.7% of the arsenic in the liver phospholipids.

In the next step, the liver phospholipids from specimens A−C were subjected to enzymatic hydrolysis with phospholipase D. As analyzed by cation-exchange LC/positive-ion mode ESI-MS, DMA was detected as the sole arsenic compound in the hydrolysate (**Figure 3**), while As(V) and MMA were not by anion-exchange LC/negative-ion mode ESI-MS (data not shown). In a control experiment without phospholipase D, no arsenicals including DMA were found, supporting that DMA was certainly cleaved from phospholipids by enzymatic hydrolysis. However, the estimated amounts of DMA accounted for only 7.7−11.8% of the arsenic contained in the phospholipids used (**Table 4**). This low recovery of DMA seemed to be attributable to the low efficiency of enzymatic reaction. The result that AC, which was found as the minor arsenical in the chemical hydrolysate, was below the detection limit in the enzymatic hydrolysate was also explainable by the low efficiency of enzymatic reaction.

DISCUSSION

This study demonstrated that the water-soluble arsenic occupies the majority of the total arsenic in four tissues of squid and that the major water-soluble arsenic compound is AB, regardless of the tissues. These results agree well with those so far obtained for a number of marine animals (*1*, *2*). More

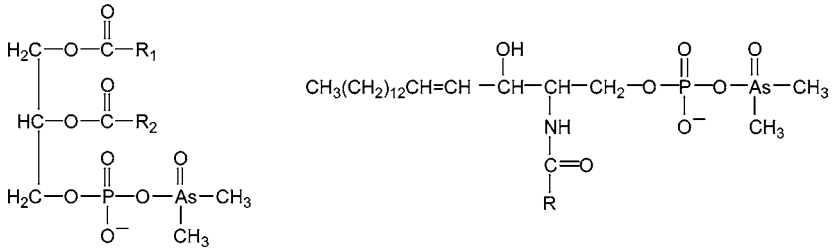

Figure 4. Structures of phosphatidyldimethylarsinic acid (left) and DMA-containing sphingomyelin (right) contained as the major arsenolipids in squid liver.

importantly, arsenolipids contained at relatively high levels in liver were systematically analyzed by a combination of chemical hydrolysis and enzymatic hydrolysis techniques. As discussed below in detail, our results provide evidence that the major arsenolipids are phosphatidyldimethylarsinic acid and DMA-containing sphingomyelin where the choline moiety of sphingomyelin is replaced by DMA (**Figure 4**).

At present, it is very difficult to analyze arsenolipids directly by LC-based methods such as HPLC-ICPMS and LC/ESI-MS, although a promising method for direct analysis of them by HPLC-ICPMS has recently been reported (*19*). Therefore, speciation of arsenolipids in marine organisms has so far been conducted mostly by analyzing water-soluble arsenic compounds derived from their chemical hydrolysis (*11*−*15*). In our chemical hydrolysis experiments, DMA was identified as the major water-soluble arsenical in both alkali-labile/HCl and alkali-stable/Ba(OH)$_2$ fractions. Of the two classes of phospholipids (glycerophospholipids and sphingolipids), glycerophospholipids are known to be sensitive to mild alkaline hydrolysis, and hence glycerylphosphoryl groups, which are formed by the liberation of two fatty acids from glycerophospholipids, must exist in the alkali-labile fraction. On the other hand, sphingolipids that are resistant to mild alkaline hydrolysis must be contained in the alkali-stable fraction. However, our chemical hydrolysis data do not simply imply that the major arsenolipids of squid liver are DMA-containing glycerophospholipids and DMA-containing sphingolipids, since it cannot be excluded that DMA is a byproduct derived from other arsenicals bound to phospholipids during severe hydrolysis. For example, arsenosugars, which are known to be constituents of arsenophospholipids in brown algae (*9*, *10*) and western rock lobster (*11*), are possibly degraded to DMA during sever hydrolysis.

To confirm whether DMA itself or its precursors are bound to phospholipids, an enzymatic hydrolysis technique with phospholipase D, which specifically cleaves the ester bond between phosphate and a polar head group (e.g., choline) of phospholipids, is considered to be useful. This technique gives no information as to whether water-soluble arsenic compounds are derived from glycerophospholipids or sphingolipids, because phospholipase D acts on both types of phospholipids. However, enzymatic hydrolysis can be performed under much milder conditions than chemical hydrolysis, making it possible to analyze only intact water-soluble arsenicals cleaved from phospholipids. Because of this advantage, the enzymatic hydrolysis with phospholipase D was recently used in the study of Devalla and Feldmann (*10*) who analyzed lipid-soluble arsenicals in brown alga and those in tissues and faces of seaweed-eating sheep. In this study, DMA was identified as the water-soluble arsenical cleaved from the liver phospholipids by phospholipase D, demonstrating that DMA itself is certainly contained in the phospholipids.

On the basis of the results obtained by both chemical hydrolysis and enzymatic hydrolysis techniques, we can conclude that the major arsenolipids of squid liver are phosphatidyldimethylarsinic acid and DMA-containing sphingomyelin. In previous studies, either one of the chemical hydrolysis or enzymatic hydrolysis techniques has been employed to analyze arsenolipids (*10*−*15*). As described above, however, intact water-soluble arsenicals are not always produced from phospholipids by the chemical hydrolysis technique, and the type of phospholipids to which water-soluble arsenicals are bound is not deduced by the enzymatic hydrolysis technique. We thus consider that a combination of the chemical hydrolysis and enzymatic hydrolysis techniques could be used to elucidate the structures of arsenolipids in marine organisms until the spread of their direct analysis by LC-based methods such as HPLC-ICPMS.

DMA-containing phospholipids have so far been reported in starspotted shark (*12*, *13*), ringed seal (*15*), and seaweed-eating sheep (*10*). DMA-containing lipids found in fish oil (*14*) are also likely to be DMA-containing phospholipids. Despite the possible wide distribution of DMA-containing phospholipids in animals, the digestive gland of western rock lobster, the only marine invertebrate so far studied for lipid-soluble arsenicals, is devoid of DMA-containing phospholipids and instead contains phosphatidylarsenosugar and phosphatidylarsenocholine (*11*). Therefore, this study is the first to report the presence of DMA-containing phospholipids in marine invertebrates.

To clarify the structures of lipid-soluble arsenicals in many marine organisms, further speciation study should be continued. It should be also noted that the metabolism of arsenolipids in mammals is little understood. Very recently, the metabolism of cod liver arsenolipids in human was studied, but unfortunately the arsenolipids used were not elucidated (*20*). Therefore, future study on the metabolism of arsenolipids with known structures in mammals is needed to accurately assess the safety of arsenic contained in seafood products.

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