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Characterization of organic arsenic compounds in bivalves

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Water-soluble arsenic compounds were extracted with methanol/water (1:1, v/v) from various species of bivalves and also from certified reference materials (NIES No. 6, mussel tissue, and NBS 1566, oyster tissue). The extracts were analyzed with a high-performance liquid chromatograph combined with an inductively coupled argon plasma mass spectrometer serving as an arsenic-specific detector. A certified reference material (NIES No. 6) was used to check the reproducibility of the analysis. The relative standard deviations (RSDs) of the peak area of major arsenic compounds among repeated measurements ($n=6$) on the same extract were less than 3.3%, indicating good reproducibility of the technique. The RSDs of some peaks among measurements of independent extracts, on the other hand, were more than 10%, possibly reflecting the heterogeneity of the sample in terms of the chemical species under the present experimental conditions. In many of the samples analyzed in the present study, two arsenic-containing ribofuranosides were detected in addition to arsenobetaine. A compound bearing a glycerophosphoryl glycerol moiety was dominant in such cases. Interestingly, a bivalve living in an estuary (*Corbicula japonica*) did not contain a detectable amount of arsenobetaine though it had arsenic-containing ribofuranosides. The distribution of arsenic species in the various parts of a clam (*Meretrix lusoria*) and a mussel (*Mytilus coruscum*) was also analyzed.

Keywords: HPLC ICP MS, arsenic speciation, arsenobetaine, arsenic-containing ribofuranosides, tetramethylarsonium, certified reference material, bivalve molluscs

INTRODUCTION

Since marine organisms generally contain fair amounts of arsenic, the chemical forms of arsenic in them have been investigated in order to evaluate the toxicological implications of consuming such organisms as well as elucidating the cycling of the element in the marine ecosystem. After the first report by Edmonds *et al.* in 1977,[1] arsenobetaine (**VIII** in Fig. 1) has been identified in many marine animal samples as the major or sole water-soluble arsenic compound.[2–4] Simple methylated species, viz. the tetramethylarsonium ion (**VI**) and trimethylarsine oxide (**V**), were also detected in some of the samples.[5] Several reports also claimed the presence of arsenocholine (**VII**) in some samples, while other researchers did not find it in the same species.[5] Edmonds and his colleagues, on the other hand, isolated and identified more complicated organoarsenic compounds, termed arsenic-containing ribofuranosides (**X**, **XI**, **XII**), or simply arsenosugars, in the extract of a brown alga.[6,7] Later, various derivatives of arsenosugars (**X–XV**) including the lipid-soluble form (**XVI**), have been identified in many marine algae, and the nature of species-specific distribution of these derivatives has been elucidated.[8,9] All the macroalgae analyzed so far were reported to contain some of the arsenosugars while none of them contained a detectable amount of arsenobetaine which is ubiquitous in marine animals.

Among marine animals, bivalves, which belong to the phylum Mollusca, seem to occupy a specific position with regard to the chemical form of arsenic in them. Squid, cuttlefish[4] and octopus,[10] other members of the Mollusca, were reported to have arsenobetaine as a dominant or sole arsenic species, as in the case of other animal species. On the other hand, Edmonds *et al.* first isolated and identified arsenosugars (**X** and **XIV**) instead of arsenobetaine from the kidney of a giant clam, *Tridacna maxima*.[11] The giant clam was known to contain symbiotic microalgae, and they attributed the source of the arsenosugar derivatives to the

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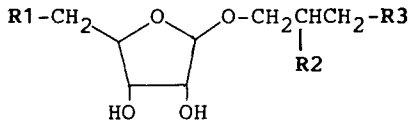
I AsO_4^{3-}
II AsO_3^{3-}
III $CH_3AsO_3^{2-}$
IV $(CH_3)_2AsO_2^-$
V $(CH_3)_3AsO$
VI $(CH_3)_4As^+$
VII $(CH_3)_3As^+CH_2CH_2OH$
VIII $(CH_3)_3As^+CH_2COO^-$
IX $(CH_3)_2As(O)CH_2CH_2OH$

Arsenic-containing ribofuranosides



	R^1	R^2	R^3
Water-soluble			
X	$(CH_3)_2As(O)$—	—OH	—OH
XI	$(CH_3)_2As(O)$—	—OH	$-O-P(=O)(O^-)-O-CH_2CHCH_2OH$
XII	$(CH_3)_2As(O)$—	—OH	$-SO_3^-$
XIII	$(CH_3)_2As(O)$—	$-NH_3^+$	$-SO_3^-$
XIV	$(CH_3)_2As(O)$—	—OH	$-SO_4^-$
XV	$(CH_3)_3As^+$—	—OH	$-SO_4^-$
Lipid-soluble			
XVI	$(CH_3)_2As(O)$—	—OH	$-O-P(=O)(O^-)-O-CH_2CH(CH_2OC(O)(CH_2)_{14}CH_3)-OC(O)(CH_2)_{14}CH_3$

Figure 1 Arsenic compounds in marine environment.

algae.[11] We found, however, the presence of two arsenic species indistinguishable from arsenosugar derivatives (**X** and **XI**) in some other bivalves in addition to arsenobetaine, using the combination of high performance liquid chromatograph with inductively coupled argon plasma atomic emission spectrometry.[4]

The purpose of the present study is to confirm the results of previous reports and to obtain more information on the nature of the arsenic compounds in bivalves. A combination of a high-performance liquid chromatograph with an inductively coupled argon plasma mass spectrometer (HPLC ICP MS)[12] was used to identify and quantify arsenic species in the samples. Certified reference materials were used to check the reproducibility of the data obtained by the technique. The distribution of arsenic species in the various parts of clam and mussel was also assessed to obtain more insight into the source and the possible physiological meaning of these organoarsenic compounds.

EXPERIMENTAL

The water-soluble arsenic compounds used as the standards (Fig. 1, **I–XV**) were prepared as reported previously.[12] HPLC ICP MS (a Perkin–Elmer 410 Bio LC system combined with a Yokogawa Electric PMS100 ICP mass spectrometer) analyses were performed as described previously by using Inertsil ODS (4.6 mm × 250 mm; Gasukuro Kogyo, Japan) and Asahipak GS220 (7.6 mm × 500 mm; Asahi Kasei Kogyo Co., Japan) for ion-pair chromatography (for cationic and anionic species) and for gel-permeation chromatography, respectively.[5,9,12] All the samples were filtered before injection by a 0.45 μm membrane filter (Minisart NML, Sartorious, Germany). Quantification of each arsenic species was done by comparing the peak area with that of cacodylate standard (dimethylarsinic acid sodium salt) injected separately.[9] Total arsenic contents were determined by an ICP atomic emission spectrometer (Seiko Electric JY-38) after wet

Table 1 Total and water-soluble arsenic concentrations in the certified reference materials

Sample	Certified value	Concentration (μg As g^{-1} on dry wt basis)		
		Water-soluble	Residue	Total
NIES No. 6	9.2±0.5	6.4±0.3	4.1±0.1	10.5±0.4
DORM-1	17.7±2.1	17.8±0.4	1.1±0.1	18.9±0.5
DOLT-1	10.1±1.4	7.6±0.3	2.0±0.1	9.6±0.4

digestion of the samples by concentrated nitric acid. Fresh specimens of mussel (*Mytilus coruscum*) were collected near Rishiri Island, Hokkaido, Japan, sent to our Institute on ice, and kept at −20 °C until use. All the other fresh bivalves were obtained in a market. Certified (standard) reference materials used in the present study are as follows: NIES No. 6 (mussel tissue, *Mytilus edulis*) from the National Institute for Environmental Studies, Japan; NBS1566 (oyster tissue, *Crassostrea gigas*) from the National Bureau of Standards (now National Institute of Standards and Technology, NIST), USA; DORM-1 (dogfish muscle) and DOLT-1 (dogfish liver) from the National Research Council of Canada.

Each of the reference materials (0.2 g dry weight) was weighed into the centrifuge tube. To each tube was added 5 cm^3 of methanol/water (1:1, v/v), and the tube was sonicated for 10 min. After centrifugation (2000 rpm × 10 min), the extract was removed by a Pasteur pipette. The extraction process was repeated five times for each sample, and the extracts were combined, evaporated to dryness, dissolved in 2 cm^3 of water, and analyzed by HPLC ICP MS. Whole or various parts of the fresh samples were weighed, homogenized with methanol/water [1:1, v/v; about 5:1 (v/w) for each sample], sonicated for 10 min, and centrifuged (2500 rpm × 20 min) to obtain the extract. The extraction process was repeated five times, and the extracts were combined, evaporated to dryness, dissolved in water, and analyzed by HPLC ICP MS. In the case of *Corbicula japonica*, tissues of several specimens were combined together for extraction because of its small size.

Table 2 Results of HPLC ICP MS analysis of mussel certified reference material, NIES No. 6 (μg As g^{-1} dry wt)[c]

Arsenic species[a]	Within the same batch of extract ($n=6$)		Among different batches of extracts ($n=6$)	
	Average	RSD (%)	Average	RSD (%)
VIII	1.38±0.03	2.2	1.45±0.05	3.2
IV[b]	0.71±0.01	1.9	0.65±0.09	13.4
X	0.61∓0.02	3.3	0.77±0.10	13.2
XI	0.99∓0.02	2.2	1.07±0.09	8.7

[a] **VIII**, arsenobetaine; **IV**, cacodylate; **X**, **XI**, arsenic-containing ribofuranosides (see Fig. 1). [b] Sum of cacodylate and small unknown peak (see Fig. 2). [c] Quantitation performed by Inertsil ODS (buffer pH 6.8).

RESULTS AND DISCUSSION

Total and extracted arsenic concentrations from the mussel certified reference material, NIES No. 6, are shown in Table 1 together with the data on fish reference materials, DORM-1 (dogfish muscle) and DOLT-1 (dogfish liver). The same extraction procedure was employed in each case. However, the extraction efficiency of mussel is lower than those for the fish reference materials, and is rather comparable to the values of the red algae in the previous report.[9] The residual arsenic could not be extracted by repeating the procedure further. The nature of the arsenic in the residue is not clear.

The quantitative analytical results of the extract of NIES No. 6 are summarized in Table 2. The identification of each chemical species was done by a comparison of its retention time with those of authentic standards under three different column conditions. As shown in the table, the RSD of repeated analysis ($n=6$) of the same extract was less than 3.3%, indicating good reproducibility of the HPLC ICP MS system. The RSD among different batches of the extract ($n=6$), on the other hand, sometime exceeded 10%. Furthermore, a notable difference was detected

in minor constituents in the chromatograms of different extracts, as shown in Fig. 2. These data may suggest deviation of extraction efficiency in each case or occurrence of a heterogeneity problem as to the chemical form of arsenic in the certified reference material. The amount of each sample used in the present study (about 200 mg) was slightly smaller than the recommended value for elemental analysis (>250 mg), but the difference is small and does not seem to be a major factor causing such a significant effect.

As shown in Fig. 2, the reference material produced from blue mussel (*Mytilus edulis*) contains not only arsenobetaine, but also two arsenosugar derivatives (**X** and **XI**) and cacodylate as major water-soluble arsenic species. There are several other minor peaks in the chromatogram, but they are not yet identified rigorously. A notable peak detected in Fig. 2A alone (retention time 6.3 min) does not correspond to any of the standards we have. The two arsenosugar derivatives were also detected in the extract of another standard reference material produced from oyster, *Crassostrea gigas* (NBS1566) (Fig. 3). Again, arsenobetaine and cacodylate were detected in addition to these arsenosugars, but

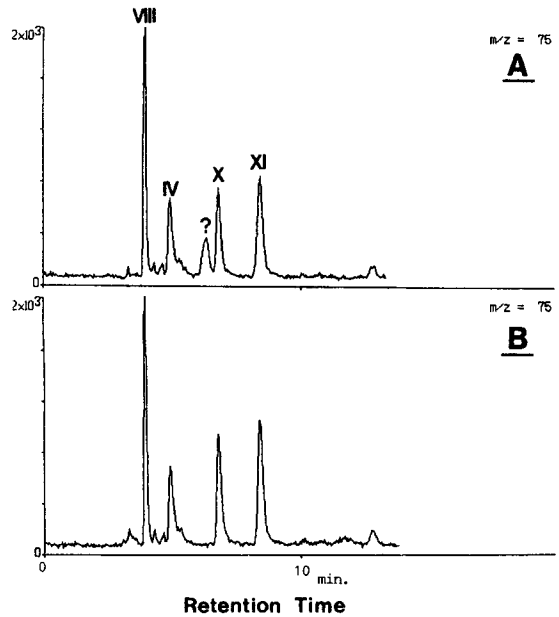


Figure 2 Chromatograms of the extracts of mussel certified reference material, NIES No. 6. A and B are the chromatograms of different batches of extracts. The column was Inertsil ODS; buffer, 10 mmol dm^{-3} tetraethylammonium 4.5 mmol dm^{-3} malonic acid (pH 6.8); flow rate 0.75 cm^3 min^{-1}; 5 µl of each extract was injected.

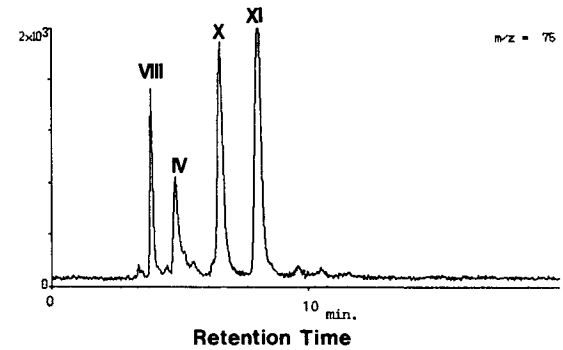


Figure 3 Chromatogram of the extract of oyster standard reference material, NBS1566. See the caption of Fig. 2 for the elution conditions.

the proportion of arsenosugars was even higher than that in the case of mussel. The presence of several minor arsenic compounds was also evident. These certified reference materials are not intended for organic analysis, and the present data may not reflect the real concentration of these arsenic compounds in the original material (for example, **XI** decomposes to **X** rather easily). The present data, however, clearly indicate that mussel and oyster contain not only arsenobetaine but also arsenosugar derivatives as major arsenic species.

The extracts of various bivalves were analyzed in the same manner as above, and the results are summarized in Table 3. A clam (*Meretrix lusoria*) and a hard-shelled mussel (*Mytilus coruscum*) were dissected into several parts, and the extract of each part was also analyzed. Except for *Corbicula japonica*, all the samples contained arsenobetaine as a major water-soluble arsenic compound. The concentration, however, varies from around 0.2 to more than 2 µg As g^{-1} fresh tissue. In both clam and hard-shelled mussel, the adductor muscle contained the highest concentration of arsenobetaine. Arsenobetaine was not detected in the extracts of *Corbicula japonica*, a small bivalve living in estuaries (detection limit is less than 0.01 µg As g^{-1} fresh tissue). On the other hand, arsenosugar derivatives—one containing the glycerophosphoryl glycerol moiety (**XI**) and another (**X**) which is probably a precursor or a degradation product of **XI**—were detected in almost all samples, including *Corbicula japonica* (Table 3). Other arsenosugars, especially **XIV** which was first isolated from the kidney of the giant clam *Tridacna maxima*,[11] were not detected in any of the samples. In contrast to arsenobetaine, the adductor muscles of clam and

Table 3 Arsenic species in bivalves

Sample (Japanese name)	Arsenic concentration (μg As g^{-1} fresh tissue)[a]				
	Arsenobetaine (VIII)	Tetramethylarsonium (VI)	Arsenosugar (XI)	Arsenosugar (X)	Others
Meretrix lusoria (Hamaguri)					
Whole 1	0.78	0.17	0.92	0.1	—
Whole 2	0.33	0.25	0.17	0.03	0.57
Adductor muscle	2.06	0.24	0.57	—	0.34
Foot	1.82	0.46	0.65	—	0.16
Digestive gland[b]	1.39	2.07	1.58	—	—
Mantle	1.03	1.35	1.12	0.1	—
Mantle edge	0.26	1.34	1.14	0.1	—
Gill	0.14	6.07	2.44	—	—
Tapes japonica (Asari)					
Whole 1	0.63	—	0.67	0.07	0.57
Whole 2	0.75	—	0.70	0.07	0.59
Whole 3	0.49	—	0.46	0.05	0.48
Whole 4	0.73	—	1.48	0.15	0.73
Corbicula japonica (Yamatosijimi)					
Mix 1[c]	—	—	0.54	0.14	0.68
Mix 2[d]	—	—	0.53	0.22	0.06
Anadara broughtonii (Akagai)					
Whole	1.03	—	0.11	—	0.05
Tresus keenae (Mirukui)					
Whole	0.57	0.03	0.16	—	0.26
Spisula sachalinensis (Hokkigai)					
Whole	0.66	—	—	—	0.15
Mytilus coruscum (Igai)					
Adductor muscle	2.57	—	0.01	—	0.02
Foot	0.81	—	0.05	—	0.11
Digestive gland	1.35	0.06	0.13	0.03	0.18
Remaining part of the body	1.41	0.02	0.06	—	0.12
Mantle	0.90	—	0.03	—	0.15
Mantle edge	1.36	0.04	0.03	—	0.09
Gill	0.93	0.06	0.08	—	0.05

[a] Column, Asahipak GS220; buffer, 25 mmol dm^{-3} tetramethylammonium 25 mmol dm^{-3} malonic acid (pH 6.8 adjusted by NH_4OH). [b] —, Not detected (detection limit is less than 0.01 μg As g^{-1} fresh tissue for any species). [c] Including soft tissues surrounding digestive gland. [d] Including whole tissues of three bivalves. [e] Including whole tissues of five bivalves.

mussel accumulated lower concentrations of arsenosugars compared with other parts. The tetramethylarsonium ion was detected in all tissues of *Meretrix lusoria*, and also in some other bivalves in smaller amounts. In accordance with the report by Shiomi *et al.*,[13] the gill of the clam was found to accumulate the highest amount of the tetramethylarsonium ion. A smaller clam contained less tetramethylarsonium ion than a larger one.

The present study confirmed our previous finding[4] that some bivalves, namely *Meretrix lusoria*, *Tapes japonica* and *Tresus keenae*, contain not only arsenobetaine but also the arsenosugar derivative **XI** (designated as **XII** in the previous report[4]). Furthermore, some other bivalves, namely *Mytilus edulis*, *Mytilus coruscum*, *Crassostrea gigas*, *Anadara broughtonii* and *Corbicula japonica*, were found to contain either or both of two arsenosugars, **X** and **XI**. These data strongly suggest that arsenosugars **XI** and **X** are among the usual major arsenic species in bivalves. In addition to bivalves, some of the gastropod molluscs, such as *Notohaliotis gigantea*

(abalone)[4] and *Depressiscala auritum* (Shibata, Y. and Morita, M., unpublished work), were found to contain arsenosugars in addition to arsenobetaine. On the other hand, cephalopod molluscs such as squid, cuttlefish and octopus were reported to contain no detectable arsenosugars, as in the case of fish and crustaceans. To our knowledge, there is no report that these bivalves and gastropod molluscs contain symbiotic algae as in the case of giant clam, indicating that these arsenosugar derivatives may be derived from sources other than the algae in the bivalves or gastropods.

One possible other source of the arsenosugars is food.[4] These bivalves are plankton-feeders, and abalone is known to eat seaweed. The results of the analysis of each dissected part of the clams, however, showed that **XI** is distributed everywhere in the body. The variation of its concentration was not so great compared with arsenobetaine and the tetramethylarsonium ion. Interestingly, **XI** showed a similar distribution pattern to tetramethylarsonium ion, i.e. it is highest in the gill and lowest in the adductor muscle, the reverse pattern to that of arsenobetaine. Hard-shelled mussels also gave essentially a similar distribution pattern, although the quantities of arsenosugar **XI** and tetramethylarsonium ion were much lower than those in the clams. On the other hand, there are apparently no such clear relationships between the quantities of arsenobetaine and arsenosugar **XI** or tetramethylarsonium ion among different bivalve species. The physiological meaning of these findings is not clear at this stage, but the specific distribution of each chemical species among different tissues may cause a heterogeneity problem, as discussed above.

Another interesting finding is the absence of arsenobetaine in the tissues of *Corbicula japonica*. *Corbicula japonica* lives in estuaries, i.e. in low-salinity regions. Marine animals are known to use organic small molecules including glycinebetaine for osmo-regulation.[14] A possible explanation of the ubiquity of arsenobetaine among marine animals is that it is erroneously accumulated in the body because of the similarity of its chemical properties to glycinebetaine. Francesconi *et al.* found that mussels[15] and fish[16] accumulate arsenobetaine efficiently from sea water and foods, respectively. In the case of mussels, the concentration accumulated in the tissue correlated well to the concentration in the ambient seawater.[15] Based on these data, it may be possible to speculate that the absence of arsenobetaine in *Corbicula japonica* either reflects the lack of availability of arsenobetaine through food or from ambient water, or reflects the lower amount of osmo-regulators necessary within the body. Interestingly, this bivalve accumulates a fair amount of arsenosugar derivatives **XI** and **X**, and the total arsenic concentration is still comparable with that of other bivalves.

As shown above, bivalves in general contain not only arsenobetaine but also arsenic-containing ribofuranosides as quite common water-soluble arsenic species. The quantities of these, however, seem to vary widely even within the same bivalve species. More information related to the season, location, size, etc., will be necessary to clarify the source and possible physiological implication of these arsenic compounds.

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