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Arsenobetaine as the Major Arsenic Compound in the Muscle of Two Species of Freshwater Fish

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The chemical form of arsenic contained in the muscle of certain freshwater fish was examined using cultured specimens of rainbow trout (*Salmo gairdneri*) and wild specimens of Japanese smelt (*Hypomesus nipponensis*). More than 95% of the total arsenic of both species was extracted with methanol and recovered in the water-soluble fraction. The major arsenic compound of both species was purified by cation-exchange chromatography on Dowex 50, gel filtration on Bio-Gel P-2 and HPLC on Asahipak GS-220H. Behavior in the above purification procedure and analyses of the purified compounds by HPLC–ICP and TLC confirmed that the major arsenic compound of both species was arsenobetaine. Arsenobetaine found in cultured rainbow trout seems to be derived from the commercial assorted feed containing arsenobetaine as the major arsenical. On the other hand, the result with wild Japanese smelt suggested that arsenobetaine is a naturally occurring compound in the freshwater environment.

Keywords: arsenic; arsenobetaine; freshwater fish; rainbow trout; Japanese smelt

INTRODUCTION

Arsenic existing at high levels in marine organisms, especially those contributing to the human diet, has long been of concern as a hazard to human health. Since the toxicity of arsenic depends on the chemical form in which it exists, extensive studies have been conducted to clarify the chemical forms of arsenic in marine organisms. It is now well established that the major or sole arsenic constituent in a variety of marine animals is arsenobetaine, while marine algae lack arsenobetaine and instead contain more complex compounds, viz. arsenosugars, as the major arsenicals.[1] Furthermore, animal experiments reveal that arsenobetaine has no acute toxicity[2] and that it is rapidly excreted in urine even when ingested.[2–4] Arsenosugars were also shown to be readily eliminated from the body not through urine but through feces when orally administered to mice,[5] although their acute toxicity is still unknown. Thus, high levels of arsenic as arsenobetaine in marine animals and arsenosugars in marine algae are unlikely to pose a serious problem to human health.

On the other hand, little information is available on the chemical form of arsenic in freshwater organisms, probably because of their rather low concentrations of arsenic compared with marine organisms. Therefore, no toxicological assessment has been done for the arsenic contained in freshwater organisms. As far as we know, the only work concerning the speciation of arsenic in freshwater organisms is that of Lawrence *et al.*,[6] who examined the chemical form of arsenic in the muscle of several species of Canadian freshwater fish such as pike and bass, together with Canadian marine animals including fish, lobster, shrimp and scallop, and reported that arsenobetaine occurred in all marine animals and arsenocholine only in shrimp, while both arsenicals were absent in the freshwater fish examined. Although the arsenic compound in the freshwater fish was assumed to be more hydrophilic than arsenobetaine, its chemical form remained unsolved. Under these circumstances, the present study was undertaken to identify arsenicals in the muscle of two species of freshwater fish, rainbow trout (*Salmo gairdneri*) and Japanese smelt (*Hypomesus nipponensis*).

MATERIALS AND METHODS

Materials

Cultured specimens of *S. gairdneri* and wild specimens of *H. nipponensis* were purchased at the Tokyo Central Wholesale Market. The muscle

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pooled from 10 specimens of *S. gairdneri* or 102 specimens of *H. nipponensis* was minced using a homogenizer and stored at −20 °C until use. Dowex 50X2 was purchased from Muromachi Kagaku (Tokyo, Japan); Bio-Gel P-2 from Nippon Bio-Rad Laboratories (Tokyo, Japan); pre-packed columns of Asahipak GS-220H and Chemcosorb 7SCX from Asahi Kasei (Tokyo, Japan) and Chemco (Tokyo, Japan), respectively; and pre-coated silica gel plates from Kanto Chemical (Tokyo, Japan). Sodium arsenate and dimethylarsinic acid were purchased from Wako Pure Chemical (Tokyo, Japan), and sodium monomethylarsonic acid from Ventron Corp. (Beverly, MA, USA); synthesized preparations of arsenobetaine, trimethylarsine oxide, arsenocholine and tetramethylarsonium iodide were kindly donated by Dr T. Kaise, Tokyo College of Pharmacy. Two kinds of standard material, albacore tuna (No. 50) and oyster tissue (No. 1566), which were used in justifying the determination method for arsenic, were obtained from the US Department of Commerce, National Bureau of Standards (now NIST) (Washington, DC, USA). Nitric acid (containing 61% HNO_3), perchloric acid (containing 60% $HClO_4$) and sulfuric acid (containing 97% H_2SO_4) used for wet-digestion were of super special grade. The other reagents were of analytical grade.

Determination of arsenic

For the determination of total arsenic, about 5 g of the minced muscle was accurately weighed into a flat-bottomed 100 cm^3 flask and then digested with a mixture of nitric acid (25 cm^3), perchloric acid (5 cm^3) and sulfuric acid (0.5 cm^3) at about 200 °C. After wet-digestion and evaporation of the acids, the flask was washed with distilled water and the washings made up to 5 cm^3 in a volumetric flask. This solution was passed through a filter paper and the filtrate determined for arsenic with an inductively coupled argon plasma emission spectrometer (ICP; Jarrell–Ash AtomComp Series 800) under the following conditions: argon flow rate (nebulizer) 0.45 dm^3 min^{-1}, (auxiliary) 0.3 dm^3 min^{-1}, (plasma) 17 dm^3 min^{-1}; wavelength 193.7 nm; radio-frequency power, 1.25 kW; observation height 16 mm; and integration time 20 s. Before use, the ICP was calibrated using distilled water and sodium arsenate solution (10 μg As cm^{-3}) made in distilled water. The above determination method for total arsenic, comprising wet-digestion and analysis by ICP, was confirmed to be reliable when applied to the standard materials (albacore tuna and oyster tissue); the arsenic content estimated three times for each standard material was in each case within the range of certified values.

The arsenic concentrations of aqueous samples obtained via extraction and chromatographic procedures were estimated directly on the ICP without wet-digestion.

Extraction

One kilogram of the minced muscle was extracted three times with 3 dm^3 of methanol and the methanolic extract evaporated to dryness *in vacuo*. The residue was suspended in 400 cm^3 of distilled water and shaken three times with an equal volume of ether to remove lipids. The aqueous phase was used as the water-soluble fraction.

Column chromatography

Each water-soluble fraction was applied to a Dowex 50X2 column (5 × 60 cm, H^+ form), which was eluted with 1.5 dm^3 of distilled water (unadsorbed fraction), followed by 1.5 dm^3 of 1 mol dm^{-3} NH_4OH (NH_4OH fraction). The NH_4OH fraction was then subjected to gel filtration on a Bio-Gel P-2 column (2.5 cm × 95 cm) with 0.1 mol dm^{-3} NH_4HCO_3. Fractions of 8 cm^3 were collected at a flow rate of about 30 cm^3 h^{-1} and determined for arsenic. Arsenic-containing fractions were combined, concentrated *in vacuo* and further purified by high-performance liquid chromatography (HPLC) on an Asahipak GS-220H column (0.76 cm × 50 cm). The column was eluted with 0.1 mol dm^{-3} formate at a flow rate of 1 cm^3 min^{-1} and fractions of 0.5 cm^3 were collected manually and measured for arsenic. Fractions enriched in arsenic were combined and analyzed by the HPLC–ICP system, essentially according to the method of Shiomi *et al.*[7] In brief, a Chemcosorb 7SCX column (0.46 cm × 25 cm) was used with 0.05 mol dm^{-3} pyridine–formate buffer (pH 3.1). The eluate was continuously introduced into the nebulizer of the ICP and monitored for arsenic under the same conditions as described above; the exception was that the integration time was shortened to 5 s. Since continuous monitoring of arsenic was not achieved with our ICP, arsenic concentrations were recorded at 10 s intervals.

For comparison, seven standard arsenic com-

Table 1 Amount of arsenic in the water-soluble fraction and the unadsorbed and NH$_4$OH fractions obtained by Dowex 50 column chromatography

	Amount of arsenic (µg)		
		Dowex 50	
Fish[a]	Water-soluble fraction	Unadsorbed fraction	NH$_4$OH fraction
S. gairdneri	1420 (97.3)[b]	152 (10.4)	1230 (84.2)
H. nipponensis	1030 (95.4)	162 (15.0)	629 (58.2)

[a] One kilogram of the *S. gairdneri* muscle (1.46 µgAs g^{-1}) or the *H. nipponensis* muscle (1.08 µg As g^{-1}) was used as the starting material.

[b] Values in parentheses represent percentages relative to total arsenic.

pounds (arsenate, monomethylarsonic acid, dimethylarsinic acid, arsenobetaine, trimethylarsine oxide, arsenocholine and tetramethylarsonium iodide) were used in gel filtration on Bio-Gel P-2 and two arsenic compounds (arsenobetaine and trimethylarsine oxide) in analysis by HPLC–ICP.

Thin-layer chromatography (TLC)

The material purified by column chromatography on Dowex-50, Bio-Gel P-2 and Asahipak GS-220H was analyzed by TLC on a pre-coated silica gel plate (5 cm × 20 cm) with ethanol–acetic acid–water (65:1:25, by vol.). After development, spots were visualized with iodine vapor. Arsenobetaine was used as the reference.

RESULTS

Arsenic concentrations in *S. gairdneri* and *H. nipponensis* muscles were determined to be 1.46 and 1.08 µg g^{-1}, respectively. As shown in Table 1, more than 95% of the total arsenic of both species was extracted with methanol and recovered in the water-soluble fraction. Results for Dowex 50 column chromatography of the water-soluble fraction are also included in Table 1. The major arsenic compound(s) of both species, constituting 84.2% of total arsenic for *S. gairdneri* and 58.2% for *H. nipponensis*, was found in the NH$_4$OH fraction. The unadsorbed fraction from both species also contained appreciable amounts of arsenic (around 10% of the total arsenic) but its arsenicals were not further examined in this study. In gel filtration on Bio-Gel P-2, the seven standard arsenic compounds used were separated into three peaks; arsenate, monomethylarsonic acid and dimethylarsinic acid appeared in peak 1, arsenobetaine and trimethylarsine oxide in peak 2, and arsenocholine and tetramethylarsonium iodide in peak 3 (Fig. 1A). On the other hand, the NH$_4$OH fraction of both species afforded a single arsenic peak at the position corresponding to peak 2 (Figs 1B and C), suggesting the presence of arsenobetaine and/or trimethylarsine oxide. When the arsenic fraction obtained by gel filtration was subjected to HPLC on Asahipak GS-220H, a single arsenic peak was observed at fraction 27 in both species (Fig. 2).

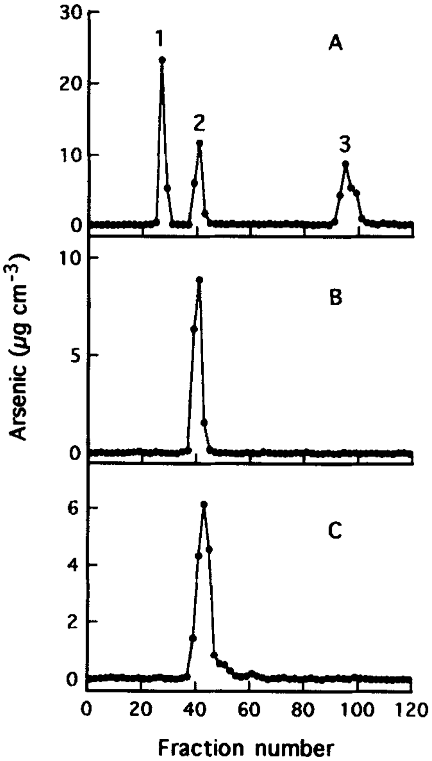

Figure 1 Gel filtration on Bio-Gel P-2 of standard arsenic compounds (A) and arsenic compounds of *S. gairdneri* (B) and *H. nipponensis* (C). Column size, 2.5 cm × 95 cm; solvent, 0.1 mol dm^{-3} NH$_4$HCO$_3$; flow rate, about 30 cm^3 h^{-1}; volume per fraction, 8 cm^3. Seven standard arsenic compounds were used; arsenate, monomethylarsonic acid and dimethylarsinic acid were eluted in peak 1, arsenobetaine and trimethylarsine oxide in peak 2 and arsenocholine and tetramethylarsonium iodide in peak 3. For *S. gairdneri* and *H. nipponensis*, the NH$_4$OH fraction obtained by Dowex 50 column chromatography was used.

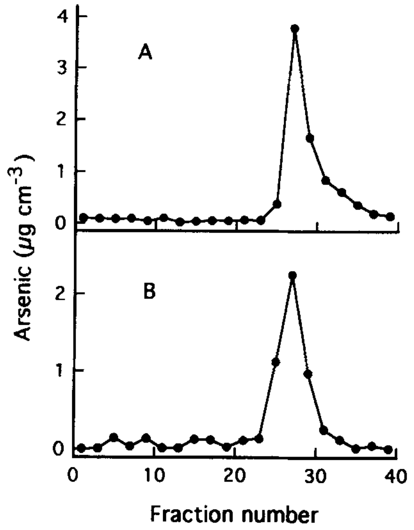

Figure 2 HPLC of the arsenic fraction obtained by gel filtration on Bio-Gel P-2. A, *S. gairdneri*; B, *H. nipponensis*. Column, Asahipak GS-220H, (0.76 cm × 50 cm); solvent, 0.1 mol dm^{-3} formate; flow rate, 1 cm^3 min^{-1}; volume per fraction, 0.5 cm^3.

The arsenical in the purified material from both species exhibited the same behavior as arsenobetaine in HPLC–ICP but was apparently distinguishable from trimethylarsine oxide (Fig. 3). In TLC, the purified material of both species gave a major spot at R_f 0.38, together with a few minor spots. The R_f value of the major spot agreed well with that of arsenobetaine. Following visualization by iodine vapor, each portion of the silica-gel plate corresponding to the major and minor spots was scraped off, homogenized in 2 cm^3 of distilled water and centrifuged. Arsenic was detected only in the supernatant prepared from a portion of the major spot.

DISCUSSION

The major arsenic compound in the muscle of two species of freshwater fish, rainbow trout (*S. gairdneri*) and Japanese smelt (*H. nipponensis*), was identified as arsenobtetaine by several chromatographic techniques. This result conformed well with the previous finding[1] on the chemical form of arsenic contained in marine fish. In Dowex 50 column chromatography, the major arsenic compound of both species was recovered in the NH_4OH fraction. We have previously observed that weakly basic arsenicals (dimethylarsinic acid and arsenobetaine) appear in the NH_4OH fraction after Dowex 50 column chromatography, being separable from acidic arsenicals (arsenate, arsenite and monomethylarsonic acid) and strongly basic arsenicals (arsenocholine and tetramethylarsonium iodide).[7,8] Trimethylarsine oxide is also obtainable in the NH_4OH fraction (unpublished data). Among the possible three arsenic compounds in the NH_4OH fraction, the absence of dimethylarsinic acid was evidenced by gel filtration on Bio-Gel P-2 (Fig. 1) and that of trimethylarsine oxide by HPLC–ICP (Fig. 3). In addition, no arsenic compounds differing from arsenobetaine were observed in any of the procedures—gel filtration, HPLC on Asahipak GS-220H, HPLC-ICP and TLC. Thus, arsenobetaine is apparently the only arsenic compound in the NH_4OH fraction, accounting for more than

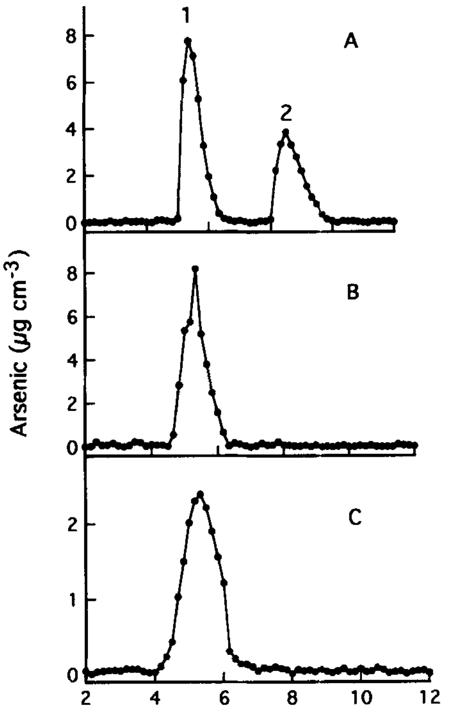

Figure 3 HPLC–ICP of standard arsenic compounds (A) and arsenic compounds from *S. gairdneri* (B) and *H. nipponensis* (C). Column, Chemcosorb 7SCX (0.46 cm × 25 cm); solvent, 0.05 mol dm^{-3} pyridine–formate buffer (pH 3.1); flow rate, 1 cm^3 min^{-1}. The eluate from the column was monitored for arsenic at 10 s intervals by ICP. Two standard arsenic compounds were used; arsenobetaine and trimethylarsine oxide corresponded to peaks 1 and 2, respectively. For *S. gairdneri* and *H. nipponensis*, the arsenic fraction obtained by HPLC on Asahipak GS-220H was used.

80% and about 60% of the total arsenic in the muscle of *S. gairdneri* and *H. nipponensis*, respectively. It can be considered, therefore, that at least these two species of freshwater fish are not a hazard to human health with respect to arsenic.

Cultured specimens were used for *S. gairdneri* in this study. The chemical form of arsenic in the commercial assorted feed for *S. gairdneri* was examined initially in the same manner as was used for the fish muscle in this study and the major form was suggested to be arsenobetaine. Therefore, arsenobetaine in the muscle of cultured *S. gairdneri* appears to be mostly derived from the assorted feed. On the other hand, the result with the muscle of *H. nipponensis*, for which wild specimens were used, suggests that arsenobetaine is a naturally occurring arsenical in the freshwater environment. In the marine environment, arsenobetaine in the fish is assumed to come from arsenosugars in algae, being the end-product in the arsenic cycle through the food chain.[9] The same, or a similar, arsenic cycle is likely to exist in the freshwater environment.

It should be pointed out that our results are inconsistent with those of Lawrence *et al.*,[6] who failed to detect arsenobetaine in the muscle of some Canadian freshwater fish. The arsenic speies in Canadian freshwater fish, though not identified, was eluted unretained through a reversed-phase HPLC column and hence was assumed to be more hydrophilic than arsenobetaine. At present we have no reasonable explanation for the discrepancy between our results and those of Lawrence *et al.* Further speciation studies on arsenic in many freshwater organisms, including fish, are needed to solve the discrepancy. Such studies will also aid in assessing the toxicity of arsenic in freshwater organisms and clarifying the arsenic cycle in the freshwater environment.

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