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Arsenic compounds in the edible red alga, *Porphyra tenera*, and in *nori* and *yakinori*, food items produced from red algae

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Arsenic compounds were extracted with methanol/water (1:1, v/v) from freeze-dried samples of the edible red alga, *Porphyra tenera* (*asakusanori* in Japanese), and also from *nori* and *yakinori*, food items produced from red algae. The extracts were analyzed by high-performance liquid chromatography (HPLC) with an inductively coupled plasma mass spectrometer (ICPMS) serving as an arsenic-specific detector. Two arsenic-containing ribofuranosides were identified in all samples tigated, and they accounted for almost all the extractable arsenic (8.7–21 mg As kg^{-1} dry sample) in each sample. A compound bearing a glycerophosphoryl glycerol moiety was dominant in every sample. Arsenite, arsenate, methylarsonate, and dimethylarsinate were quantified by hydride generation and atomic absorption spectrometry. Arsenate and dimethylarsinate were detected in all samples at low levels (around 0.15 and 0.05 mg As kg^{-1} dry sample, respectively). Arsenite was detected only in the unprocessed *Porphyra tenera* at 0.005 mg As kg^{-1} dry sample. Methylarsonate was not found in any of the samples.

Keywords: Arsenic-containing ribofuranosides, arsenosugars, high-performance liquid chromatography, inductively coupled plasma mass spectrometry, red alga, *Porphyra tenera*, *nori*, *yakinori*, hydride generation—atomic absorption spectrometry, methylarsenate compounds

INTRODUCTION

Information on the chemical forms of arsenic in marine organisms is necessary to evaluate the toxicological implication of eating them as well as to elucidate the cycling of the element in the marine environment. After the discovery of arsenobetaine in the tail muscle of the western rock lobster,[1] much work was done to elucidate the major arsenic species in marine animals. Arsenobetaine [$(CH_3)_3As^+CH_2COO^-$] was found to be the major arsenic species[2–4] in all animal samples investigated. On the other hand, information on arsenic species in seaweeds is still limited.

Edmonds and Francesconi isolated and identified arsenic-containing ribofuranosides (so-called 'arsenosugars': **X** and **XII**,[5] or **XI** and **XII**[6] in Fig. 1) from the extract of a brown alga, *Ecklonia radiata*, and also found the presence of similar compounds (**X** and **XIV**) in a giant clam, *Tridacna maxima*,[7] which is known to have symbiotic algae in its tissue. Subsequently arsenosugars were identified in the brown alga, *Laminaria japonica* (*Makonbu* in Japanese) (**X**, **XI**, **XII**,[8] *Hizikia fusiforme* (*hijiki*) (**XI**, **XII**, **XIV** and **I**),[9] *Sargassum thunbergii* (*umitoranoo* (**XIV**, **XV**),[10] *Sphaerotrichia divaricata* (*ishimozuku*) (**X**, **XI**, **XII**, **XIII**),[11] *Undaria pinnatifida* (*wakame*) (**X**, **XI**, **XII**),[12] and in the green alga, *Codium fragile* (*miru*) (**X**, **XI** and **IV**).[13] A phospholipid derivative of the arsenosugar (**L–I**) was identified as the major lipid-soluble arsenic compound in *U. pinnatifida*.[14] Several investigators[15–18] looked for [inorganic arsenate (**I**) and arsenite (**II**) in Fig. 1] and simple methylated arsenic species [methylarsonate (**III**) and dimethylarsinate (**IV**)] in algae. The results of these investigations suggest that algae generally accumulate arsenic in forms other than inorganic or simple methylated species except for the brown algae in the family Sargassaceae which were found to contain considerable concentrations of arsenate.

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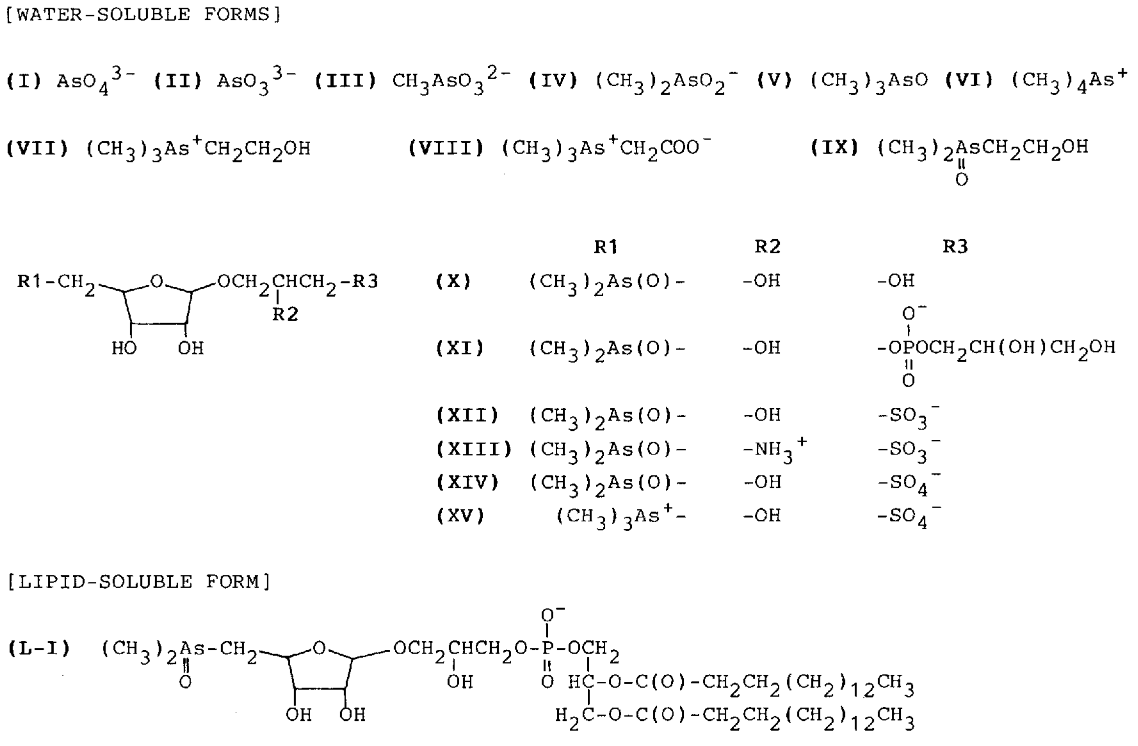

Figure 1 Formulae for water- and lipid-soluble arsenic compounds. The 15 water-soluble arsenic compounds (**I–XV**) were used as standards in the present study.

The red alga, *Porphyra tenera* (*asakusanori*), and other *Porphyra* species are cultivated and harvested in Japan in large quantities. They are dried and processed to a sheet-like foodstuff, named '*nori*' in Japanese, and are sometimes baked further to be '*yakinori*'. Information on the chemical form of arsenic in these samples is necessary for the evaluation of any risk accompanying their consumption. A comparison between the fresh alga and the products will provide information on the conversion of arsenic species during processing and cooking. Organic arsenic compounds have not yet been identified in red algae.

The present paper reports the chemical forms of water-soluble arsenic compounds in the fresh red alga. *P. tenera*, and in *nori* samples. A combination of high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC ICP MS)[19] was applied to identify major arsenic species. The quantities of toxic inorganic and simple methylated arsenic species (**I–IV**) were determined further by hydride generation–atomic absorption spectrometry.

EXPERIMENTAL

The water-soluble arsenic compounds used as the standards (Fig. 1, **I–XV**) were prepared as reported previously.[4,19] HPLC ICP MS[19] (a Perkin–Elmer 410 Bio LC pump combined with a Yokogawa Electric PMS100 ICP mass spectrometer) and HGAAS[18] (Varian AA-175) analyses were performed essentially under the conditions reported previously, except that Inertsil ODS (4.6 mm × 250 mm; Gasukuro Kogyo, Tokyo, Japan), instead of Inertsil ODS-2, was used for ion-pair chromatography for the separation of cationic and anionic compounds.[20] Total arsenic concentrations in the digested samples were determined using an ICP atomic emission spectrometer (ICP AES; Seiko Denshi JY-38) by monitoring the emission line at 193.7 nm. The fresh alga was obtained from a market, and was stored at −20°C until use. The *nori* samples, including four *nori* sheets from different districts of production and grades, and one *yakinori*, were kindly supplied by Mr Y. Ichihi of the National

Table 1 Concentrations of total arsenic, water-soluble arsenic and arsenic species in the fresh red alga, *P. tenera*, and the products *nori* and *yakinori*

	Concentration[a] (mg elemental arsenic kg^{-1} dry sample)					
		Extracts				
Sample	Dry powder	MeOH/H_2O[b]	HCl[b]	XI[c]	X[c]	Others[c]
Nori-1	17.0 ± 0.7	11.6 ± 0.2	0.6	11.8 ± 0.6	0.2 ± 0.01	n.d.[d]
Nori-2	27.8 ± 0.6	21.0 ± 0.6	0.5	22.1 ± 1.4	0.4 ± 0.05	n.d.
Nori-3	27.3 ± 0.6	19.9 ± 0.5	0.5	20.1 ± 0.8	0.4 ± .03	n.d.
Nori-4	17.5 ± 0.2	12.1 ± 0.1	0.3	11.8 ± 0.2	0.3 ± 0.02	n.d.
Yakinori	23.7 ± 0.2	15.2 ± 0.4	1.2	15.3 ± 0.2	0.2 ± 0.02	0.2 ± 0.01[e]
Fresh	12.8 ± 0.3	8.7 ± 0.3	0.3	6.8 ± 0.4	2.6 ± 0.1	n.d.

[a] Average ± standard deviation from four analyses. The HCl extracts were analyzed only once. [b] Extractant: methanol/water (1:1, v/v) or 0.1 M aqueous HCl. [c] Determined by HPLC ICP MS. [d] Not detected; detection limit was 0.1–0.2 mg As kg^{-1} dry sample. [e] Unidentified compound.

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Each sample was freeze-dried for a week, and was powdered; 2 g of the sample was extracted in an ultrasonic bath (10 min at room temperature) with 20–30 cm^3 of methanol/water solution (1:1, v/v). The extraction was repeated five times, and the extracts were combined, evaporated at a temperature (less than 40°C), and dissolved in water (to 5 cm^3 in a volumetric flask). Inorganic and simple methylated arsenic species (**I–IV**) in this solution were quantified using HG AAS as reported previously.[18] The composition of the reaction mixture was as follows. For the determination of inorganic (**I, II**), methylarsonate (**III**) and dimethylarsinate (**IV**): 0.2 M HCl (10 cm^3) containing 4×10^{-3} M EDTA, the extract (0.1 cm^3), 5% $NaBH_4$ dissolved in 0.25% NaOH (1 cm^3). For the determination of arsenite (**II**): 4% citric acid (pH 6.0 adjusted by NaOH) (10 cm^3), the extract (0.1 cm^3), 1% $NaBH_4$ (1 cm^3). Total arsenic concentration of each solution (1 cm^3) was determined separately by ICP AES after wet digestion with nitric acid (2 cm^3)/perchloric acid (0.5 cm^3) in a Pyrex glass tube heated in a heating block (made of aluminium). After heating to almost dryness, 1 cm^3 of 0.1 M HCl was added to each tube and the arsenic concentration was determined. A portion of each extracted solution was diluted 10 times further, and an aliquot (5 μl for ion-pair chromatography and 20 μl for gel-permeation chromatography) was injected into the HPLC ICP MS system. In addition to mass number 75 (for ^{75}As), mass number 77, which corresponds to the molecular ion $(^{40}Ar^{37}Cl)^+$, was monitored simultaneously to

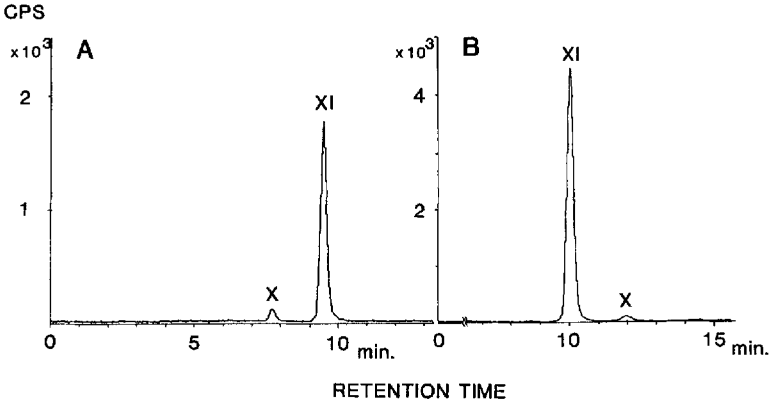

Figure 2 Chromatograms of the extract of *Nori*-2 on HPLC ICP MS. A, column = Inertsil ODS, buffer = 10 mM tetraethylammonium hydroxide/4.5 mM malonic acid (pH 6.8), 0.75 $cm^3 min^{-1}$. B, column = Asahipak GS-220, buffer = 25 mM tetramethylammonium hydroxide/25 mM malonic acid (pH 6.8), 1 $cm^3 min.^{-1}$

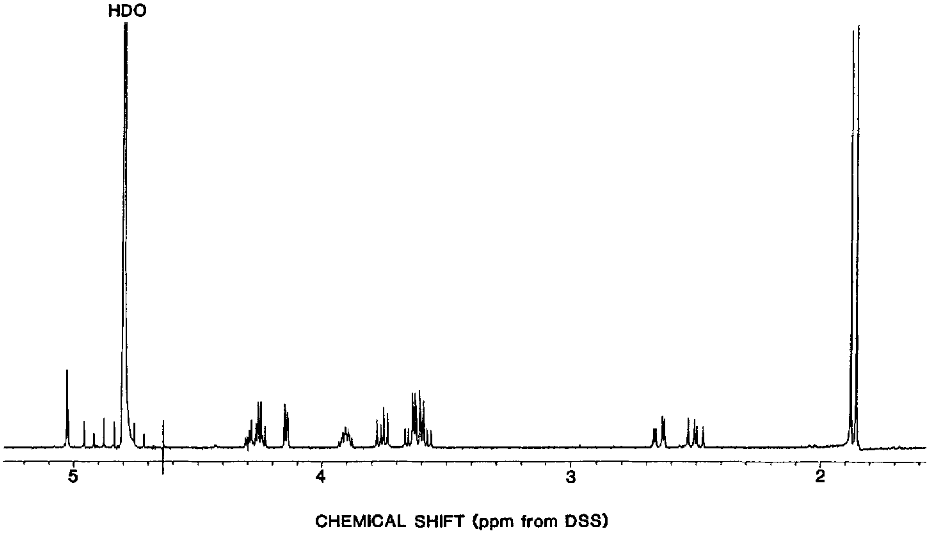

Figure 3 400-MHz ^{1}H NMR spectrum of the arsenosugar **X** in D_2O purified from the extract of the fresh alga, *P. tenera*. The chemical shift values were identical to the reported values.[5,8]

discriminate the interference caused by the molecular ion $(^{40}Ar^{35}Cl)^+$. Identification of each compound was done by comparison of the retention time with those of the 15 arsenic standards. The procedure was carried out under three different column conditions to confirm the identification. Quantitation was done on the gel-permeation column, Asahipak GS220 (7.6×500 mm; Asahi Kasei Kogyo, Japan), by comparing the peak area with that of a known amount (1.5 ng as As) of dimethylarsinate injected alternately. In our system, the peak response was found to be proportional to the quantity of arsenic regardless of the chemical form.[19] The residues after extraction with methanol/water were extracted further with 0.1 M HCl (3×20 cm^3, extracted in an ultrasonic bath for 10 min each). The extracts were combined and evaporated, and arsenic analysis was performed in the same manner as above. Total arsenic concentrations of the fresh alga and *nori* products were determined separately by digesting 0.1 g of each dried sample with nitric acid/perchloric acid.

Purification of compound **X** from the fresh alga was performed as described previously.[8] The ^{1}H NMR spectrum was recorded on a JEOL JNM GX-400 NMR spectrometer. DSS (sodium 2,2-dimethyl-2-silapentane sulphonate) was used as an external standard.

RESULTS AND DISCUSSION

Total and water-soluble arsenic concentrations are listed in Table 1 together with the quantities of each arsenic species determined by HPLC ICP

Table 2 Concentrations of arsenate, arsenite, methylarsonate, and dimethylarsinate in dry samples of *nori*, *yakinori* and *P. tenera*

		Concentration (mg elemental arsenic kg^{-1} dry sample)		
Sample	Arsenite	Arsenate	Methylarsonate	Dimethylarsinate
Nori-1	n.d.[a]	0.13	n.d.	0.028
Nori-2	n.d.	0.16	n.d.	0.080
Nori-3	n.d.	0.17	n.d.	0.058
Nori-4	n.d.	0.16	n.d.	0.028
Yakinori	n.d.	0.33	n.d.	0.038
P. tenera	0.005	0.14	n.d.	0.021

[a] Not detected; detection limit was 0.0025 mg As kg^{-1} dry sample.

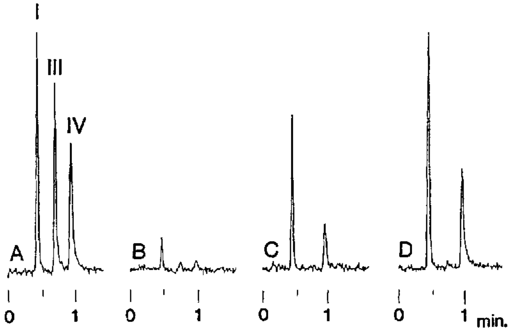

Figure 4 Chromatograms of HG AAS. A, chromatogram of a standard (5 ng As each) containing arsenate (**I**), methylarsonate (**III**), and dimethylarsinate (**IV**). B, chromatogram of a blank. C, chromatogram of the etract from *Nori*-2. D, chromatogram of the extract from *Nori*-2 spiked with 2.5 ng As each of arsenate (**I**) and dimethylarsinate (**IV**).

MS. Total arsenic concentrations range from 13 to 28 mg As kg^{-1} dry weight, similar to the arsenic concentrations reported for other red algae.[16–18] Methanol/water (1:1, v/v) extracted 64–76% of the total arsenic. Only a small fraction of residual arsenic was extracted further with 0.1 M HCl, suggesting that a large part of the residual arsenic is other than the water-soluble forms discussed below.

Chromatograms of an extract on the HPLC ICP MS system are shown in Fig. 2, and the results are summarized in Table 1. Two arsenic peaks appeared in each chromatogram, and they were identified as the arsenic-containing ribofuranosides (arsenosugars), **X** and **XI**, by comparison with the standards. The arsenosugar **XI** is the dominant arsenic compound in all the samples (Table 1). **XI** was identified in every algal species so far examined,[5,6,8–13] and its apparent ubiquitous nature was noted[11] because it occupies a key position in the proposed metabolic pathway of lipid- and water-soluble arsenic compounds.[21] In fact, **XI** was shown to be the polar head group of a lipid-soluble arsenic isolated from the brown alga, *U. pinnatifida.*[14] The other arsenosugar, **X**, present in low concentrations (Table 1), could be a metabolic precursor or degradation product of compound **XI**. Compound **X** was identified in many seaweeds.[5,8,11–13] The presence of compound **X** was confirmed further by taking the ^{1}H NMR spectrum of the compound purified from fresh alga (Fig. 3).

The fresh alga had the lowest concentration of compound **XI** (6.8 mg As kg^{-1}) but the highest concentration of compound **X** (2.6 mg As kg^{-1}) in comparison with the processed products. Recently, the major raw materials of *nori* have not been *P. tenera* but other *Porphyra* species, especially *P. yezoensis* (*susabinori*).[22] The above difference might therefore be attributable to such species differences. It is difficult, however, to determine the real concentration of **X** in each alga, for compound **XI**, the major arsenic compound in this organism, is rather labile and tends to be decomposed to **X**.[6] In our experience, even purified **XI** dissolved in water sometimes decomposed slowly to **X** during storage at −20 °C. Such a decomposition may be caused by an accidental contamination of some hydrolytic enzymes. Probably some part of **X** detected in the extract of the fresh alga may be produced from **XI** during storage or extraction procedure. Relevant enzymes may be inactivated quickly during the processing to make *nori* products, and thus the concentration of compound **X** in these products may be low. The other arsenosugars, such as **XII** and **XIV**, which are major arsenic species in brown algae, and arsenobetaine (**VIII**), ubiquitous in marine animals, are not present in the red algae, *Porphyra* species. In the *yakinori* sample an arsenic compound of unknown structure was detected at low concentration (0.2 mg As kg^{-1}). This compound may have been formed during the baking process. There were no sign of the conversion of **XI** to **X** during the process. The arsenosugars **X** and **XI** have no detectable cytotoxicity and mutagenecity.[23]

Arsenate (**I**), arsenite (**II**), methylarsonate (**III**), and dimethylarsinate (**IV**) are noticeable because of their toxicities.[24,25] These arsenic species were not detected by HPLC ICP MS (detection limit 0.1–0.2 mg As kg^{-1} dry sample under the present experimental conditions). However, arsenate and dimethylarsenate were detected in all the samples (Table 2) with the hydride generation–atomic absorption technique that has a detection limit of 0.0025 mg As kg^{-1}. Typical chromatograms are shown in Fig. 4. Arsenate (0.13–0.33 mg As kg^{-1}) accounts for 1% of the total arsenic in the samples, and dimethylarsinate for a few tenths of 1% (Table 2). Arsenite and methylarsonate are not detected in the samples except for the fresh alga which has a trace amount of arsenite (0.005 mg As kg^{-1}). Based on the reported toxicities of arsenate, arsenite, methylarsonate, and dimethylarsinate[24] and the low concentrations of these arsenic compounds in *nori* and *yakinori*, these foods do not pose any risk to the consumer. The processing of the red algae does not appear to alter the arsenic compounds in any significant way.

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