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Formation of toxic arsenical in roasted muscles of marine animals

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Arsenobetaine, an organo-arsenic compound known to be non-toxic, occurs ubiquitously in marine animals. To elucidate the food hygiene safety of the degradation products of arsenobetaine formed on cooking, arsenicals generated by roasting the muscles of the starspotted shark *Mustelus manazo* and of the red crayfish *Panulirus longipes femoristriga* were investigated. As a result, both muscle types were found to contain the tetramethylarsonium ion, which is reported to show a higher acute toxicity than dimethylarsinic acid (cacodylic acid) or methanearsonic acid. As a minor compound, arsenate was also detected in the muscle of *M. manazo*.

Keywords: arsenic; arsenobetaine; roasting (cooking); tetramethylarsonium ion; toxicity; marine animal

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INTRODUCTION

Marine organisms concentrate various elements in their tissues, including arsenic, a well-known poison since ancient times. Great attention has been paid to arsenic in marine organisms after organic compounds of arsenic were identified as the major arsenic compounds in these organisms.[1,2] The first organic arsenic compound to be isolated and identified was arsenobetaine $(CH_3)_3As^+CH_2$-COOH, which was obtained from the western rock lobster *Panulirus longipes cygnus*.[3] Since then this compound has been shown to occur in various marine animals including, for example, blue shark (Chondrichthyes),[4] school whiting (Osteichthyes),[5] sea cucumber (Mollusca)[6] and various kinds of zooplankton.[7] In other words, arsenobetaine is present in marine animals independently of their feeding habits and trophic levels.[8] The isolation of arsenobetaine led to further studies that detected arsenocholine $(CH_3)_3As^+CH_2CH_2OH$ and the tetramethylarsonium ion $(CH_3)_4As^+$ in various animals, and arsenosugars in various algae and phytoplankton.[9–13]

The toxicities of ubiquitous arsenobetaine in marine animals and arsenosugars in algae or phytoplankton are reported to be considerably lower than inorganic arsenicals.[14–16] Arsenobetaine is non-toxic (LD_{50} in mice was >10 g kg^{-1}).[14] The conversion of arsenobetaine to unidentified compound(s) was reported to have occurred in canned or pickled seafood products.[17] Although arsenobetaine itself is non-toxic, the degradation products of arsenobetaine formed on cooking could be toxic. We investigated the degradation products of arsenobetaine generated by roasting the muscles of the starspotted shark *Mustelus manazo* and of the red crayfish *Panulirus longipes femoristriga*. As a result, after exposure to the heating process, both muscle types were found to contain the tetramethylarsonium ion, which is reported to show a higher acute toxicity than dimethylarsinic acid (cacodylic acid) $(CH_3)_2AsOOH$ or methanearsonic acid $CH_3AsO(OH)_2$.[18]

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MATERIALS AND METHODS

Authentic arsenic compounds

Arsenobetaine, arsenocholine, trimethylarsine oxide $(CH_3)_3AsO$, and tetramethylarsonium iodide were purchased from Trichemical Co., dimethylarsinic acid was from Nakarai Chemical Co., methanearsonic acid was from Ventron Co., disodium arsenate $Na_2HAsO_4{\cdot}7H_2O$ was from Wako Pure Chemical Co. and arsenic trioxide was from Mallinckrodt Co.

Roasting of muscles of *M. manazo* and *P. longipes femoristriga*

Fresh *M. manazo* and frozen *P. longipes femoristriga* were purchased. A preliminary investigation was performed with the muscle of *M. manazo* because arsenobetaine was abundant (30–50 μg g^{-1}) and is practically the only arsenic compound in this shark.[19,20] The white muscle of *M. manazo* was roasted over a fire standing at about 690 °C on a stainless steel grill or in a stainless steel pan. The tail muscles from two *P. longipes femoristriga* were cut into two equal pieces. One half from each specimen was normally roasted ($n = 2$) and the other was burned ($n = 2$) on the grill. An entire tail muscle was completely burned ($n = 1$).

Arsenic content

The arsenic contents were determined after digesting the samples with 2.6 cm^3 of 61% nitric, 0.5 cm^3 of 95% sulfuric and 1.0 cm^3 of 60% perchloric acids. To the residue in the digestion beaker were added 1.0 cm^3 of 25% diammonium hydrogen citrate, 2.0 cm^3 of 37% hydrochloric acid, 0.2 cm^3 of 1 mol dm^{-3} potassium iodide and 0.2 cm^3 of 0.4 mol dm^{-3} stannous chloride; this was diluted with water to a volume of 10 cm^3 to give an arsenic concentration of 10–80 μg dm^{-3}, which was determined by arsine (AsH_3) evolution–electrothermal atomic absorption spectrometry (Nippon Jarrel Ash, model AA 845).[8] The detection limit for arsenic was 1 ng.

Extraction of the water-soluble arsenic compounds

Samples were subjected to extraction twice with ten times its volume of chloroform/methanol (2:1). To each extract water was added to reach a ratio of water/chloroform–methanol ratio of 1:4, shaken for 2 min and stored overnight.[21] The arsenicals extracted in the upper phase were termed water-soluble arsenic compounds and those in the lower phase were termed lipid-soluble.

High-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP-MS)

A Hewlett Packard 1050 solvent delivery unit and a 100 μl injection loop of a Rheodyne, six-port injection valve were used. The arsenic compounds (arsenobetaine, trimethylarsine oxide, arsenocholine and tetramethylarsonium ion) were separated at a flow rate of 1.5 cm^3 min^{-1} on a Supelcosil LC-SCX cation-exchange column (250 mm × 4.6 mm i.d.) with a 0.01 mol dm^{-3} pyridine–formic acid buffer (pH 5.0). The exit of the column was connected to a Bobinton-type nebulizer via a 50 cm PEEK capillary tubing (0.13 mm i.d.). An HP 4500 (Hewlett Packard, Waldbronr, Germany) inductively coupled plasma mass spectrometer (ICP-MS) served as an arsenic-specific detector.

High-performance liquid chromatography–graphite furnace atomic absorption (HPLC–GFAA) spectrometry

The water-soluble arsenic fraction was fractionated with a high performance liquid chromatograph, CCPD 8000 series (Tosoh Co. Ltd), using a Nucleosil 10SA column [4.6 × 250 mm, Wako–Junyaku–Kogyo Co.; mobile phase, 0.1 mol dm^{-3} pyridine–formic acid buffer (pH 3.1); flow rate, 1.0 cm^3 min^{-1}]. A portion of each eluate fraction was analyzed by a graphite furnace atomic absorption spectrometer serving as the arsenic-specific detector as described before.[8]

Thin layer chromatography

The thin layer chromatography (TLC) was performed on a cellulose thin layer (Avicel SF, thickness: 0.1 mm, Funakoshi Yakuhin Co., Ltd). In order to confirm the position of the fractionated arsenic compound, the cellulose thin layer was removed at 5 mm intervals. Each of the samples removed was added to a portion of 20% ethanol, mixed with a vortex mixer for 20 s and analyzed with GFAA spectrometry. Dragendorff reagent[22] was used to authenticate tetramethylarsonium ion.

RESULTS

Formation of tetramethylarsonium ion in the burned muscle

In the preliminary experiment using *M. manazo*, the white muscle was completely burned on the grill or in the pan for several minutes until at least all surfaces of the muscle were completely burned, having lost 68% or 74% mass respectively. The arsenic compounds in the burned muscles were identified and quantified with HPLC–ICP-MS. Approximately 56% of the arsenobetaine was converted to the tetramethylarsonium ion by roasting on the grill and 41% in the pan, clearly showing the degradation of arsenobetaine by roasting. The conversion occurred even if the muscle had not been directly roasted by a fire. Besides tetramethylarsonium ion, inorganic arsenic(V) was detected as a minor degradation product in the muscle roasted on the grill. In the subsequent experiments with *P. longipes femoristriga*, the muscle was roasted on a grill.

Formation of tetramethylarsonium ion in the roasted lobster muscle

The muscles of *P. longipes femoristriga* were roasted until they had lost 15% (normal), 37% (burned) or 84% (almost completely burned) of their mass. The total, water-soluble and lipid-soluble arsenic concentrations in unroasted and roasted muscles are shown in Table 1. The water-soluble arsenic compound fractions were identified by HPLC–ICP-MS.

Almost all arsenic in the unroasted muscle was arsenobetaine; tetramethylarsonium ion was not detected in these tissues (Fig. 1). Arsenobetaine and tetramethylarsonium ion were present in the roasted muscles (Fig. 1). The tetramethylarsonium ion accounted for 0.6% of the total arsenic in the normally roasted muscles, for 15% in the burned muscles, and for 44% in the completely burned muscles.

Purification and confirmation of the degradation product

In order to purify the degradation product from arsenobetaine, the water-soluble arsenic fraction extracted from almost completely burned muscle was analyzed with HPLC–GFAA using a Nucleosil 10SA column. As a result, two arsenic peaks were detected: one peak with a shoter retention time agreed with that of arsenobetaine, and the other, with a longer retention time, with tetramethylarsonium ion. Because the occurrence of arsenobetaine has already been proved in the tail muscle of *P. longipes cygnus*, which belongs to the same species as *P. longipes femoristriga* used in this study, only the arsenical species formed by roasting was analyzed by TLC as follows. The arsenic fraction that showed the retention time agreeing with tetramethylarsonium ion was concentrated and chromatographed on a cellulose thin layer together with authentic tetramethylarsonium ion. As shown in Table 2, the Rf value of the compound agreed with that of authentic tetramethylarsonium ion in five solvent systems and thus the compound was confirmed as tetramethylarsonium ion.

DISCUSSION

The conversion products from arsenobetaine by roasting, tetramethylarsonium ion and arsenate, were detected for the first time by HPLC–ICP-MS in the muscle of marine animals. The toxicity of arsenate is much higher than that of tetramethyl-

Table 1 Total, water-soluble, lipid-soluble, and residual arsenic concentrations in unroasted and roasted muscles of *P. longipes femoristriga*

	Mean arsenic concentration ($\mu g\ g^{-1}$ mass)			
Muscle	Total	Water soluble	Lipid soluble	Residual
Unroasted[1] (wet mass)	25.5	25.1	0.2	0.1
Normally roasted[1] (15% mass lost)	24.9	21.7	0.4	0.4
Burned[1] (37% mass lost)	29.8	26.7	0.7	0.9
Completely burned[2] (84% mass lost)	48.9	38.7	1.7	2.4

[1] $n = 2$.
[2] $n = 1$.

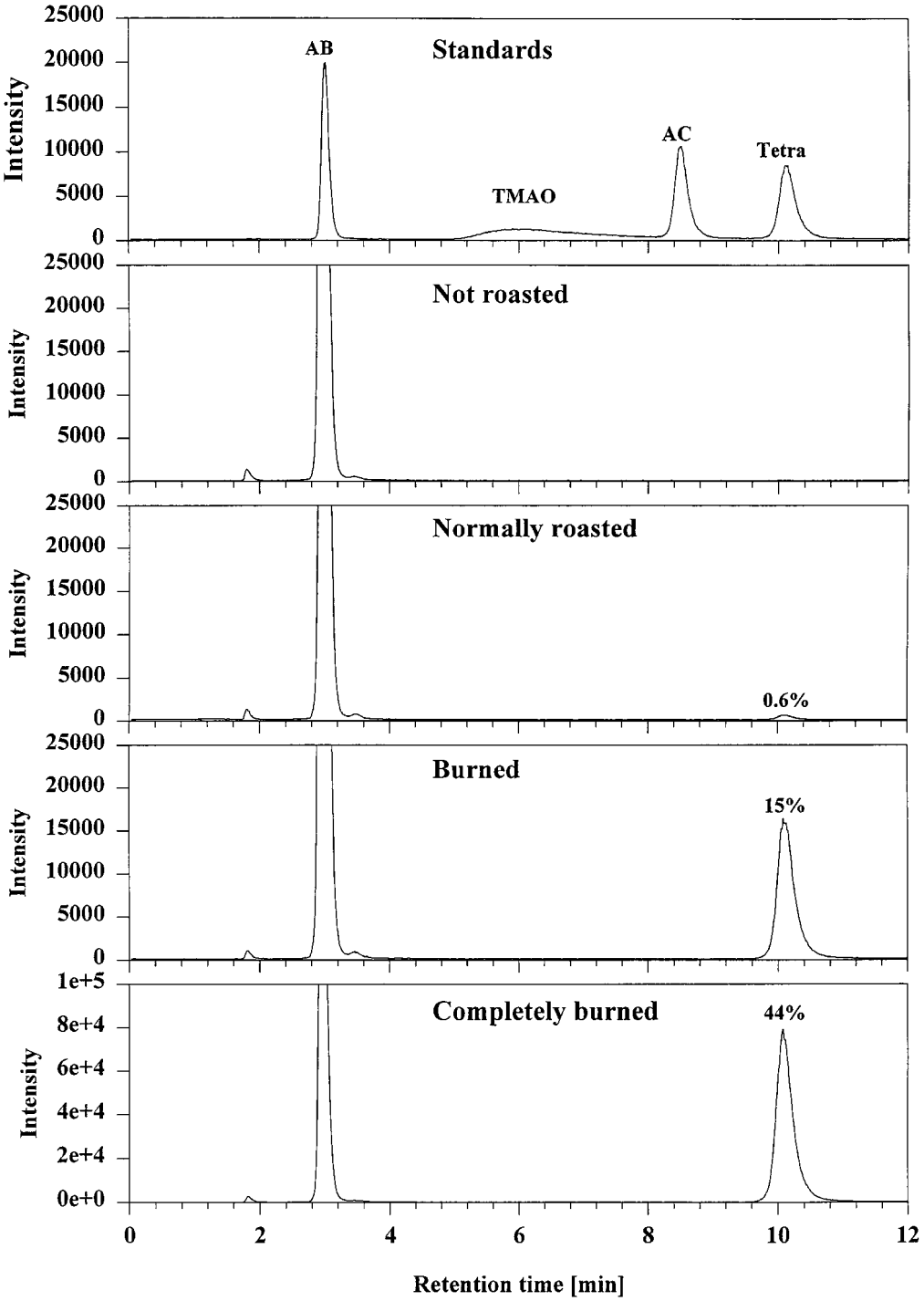

Figure 1 HPLC–ICP-MS cation-exchange chromatograms of a mixture of authentic arsenic compounds (AB, arsenobetaine; TMAO, trimethylarsine oxide; AC, arsenocholine; Tetra, tetramethylarsonium ion) and of aqueous extracts prepared from unroasted, normally roasted, burned and completely burned tail muscles of *P. longipes femoristriga*.

Table 2 Rf values of the purified arsenic compound formed in the roasted lobster muscle

Solvent systems	Purified compound	Tetramethylarsonium ion
Ethylacetate/acetic acid/water (3:2:1)	0.91	0.91
Chloroform/methanol/28% aq. ammonia (3:2:1)	0.92	0.93
1-Butanol/acetone/formic acid/water (10:10:2:5)	0.72	0.72
1-Butanol/acetone/28% aq. ammonia/water (10:10:2:5)	0.60	0.61
1-Butanol/acetic acid/water (4:2:1)	0.88	0.89

arsonium ion as stated. However, the formation of the small amount of arsenate was shown only in the roasted shark muscle, not in the roasted lobster muscles, and was insufficient to be confirmed by TLC; the discussion below will only concern the formation of tetramethylarsonium ion.

The tetramethylarsonium ion was very likely formed via decarboxylation of arsenobetaine. This compound, which was first identified by Shiomi et al. in the gills of the clam *Meretrix lusoria*[23] and subsequently detected in other marine animals,[9–12] shows higher acute toxicity to mice (oral LD_{50} 890 mg As kg^{-1} for the iodide, 580 mg As kg^{-1} for the chloride) than dimethylarsinic acid (1200 mg As kg^{-1}) or methanearsonic acid (oral LD_{50} 1800 mg As kg^{-1}).[24] On the other hand, Kaise et al.[25] found that the growth of cells was largely unaffected by the tetramethylarsonium ion (IC_{50} 8 mg As cm^{-3}). Few chromosomal aberrations were induced in the concentration range of 2–10 mg As cm^{-3}. These results show that the tetramethylarsonium ion has a much lower cytotoxicity than arsenite (IC_{50} 0.0007 mg cm^{-3}, chromosomal aberrations 0.001 mg cm^{-3}) or arsenate (IC_{50} 0.006 mg cm^{-3}, chromosomal aberrations 0.02 mg cm^{-3}). Hence, not only because of its relatively high acute toxicity, but also from the fact that mass balances for orally administered and urinary excreted tetramethylarsonium ion indicate that mice may accumulate this compound in their tissues,[18] the consumption of burnt tissues of marine animals should be made with some care until the effects of the tetramethylarsonium ion on humans are ascertained.

The elucidation of the forms and safety of the conversion product(s) with cooking arising from arsenicals in marine organisms should follow the striking advances so far on the study of forms[8–12] and safety[13–15] of highly accumulated arsenicals in marine organisms. Especially for arsenobetaine as the major arsenical accumulated in animals and for arsenosugars as the major ones in algae, this elucidation is essential.

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