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Post-mortem Conversion of Arsenobetaine in Mollusk *Liorophura japonica*[*1]

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Specimens of chitons *Liorophura japonica* were incubated at higher temperatures (25 and 37 ℃) or left in coastal waters to confirm the conversion or degradation of arsenobetaine accumulated in them. While the intact muscle accumulated arsenobetaine as substantially the sole arsenical, inorganic arsenic(Ⅲ) and/or methanearsonic acid was accumulated in the intact viscera besides arsenobetaine. After 30 days, several arsenic compounds were detected as microbial conversion products in the incubated round body, which could not be separated into muscle and viscera because of their autolysis and putrefaction. The compounds were inorganic arsenic(Ⅴ), dimethylarsinic acid, trimethylarsine oxide, tetramethylarsonium salt and unknown compounds. On the other hand, except for the unknown arsenical, the same compounds as those were detected also in the muscle of chitons left in the sea water.

1. Introduction

Arsenobetaine $[(CH_3)_3 As^+ CH_2 COO^-]$ was identified for the first time by Edmonods and Francescony in the tail muscle of western rock lobster.[1] At present, it is known as the organo-arsenical ubiquitously occurring in marine animals.[2-4] Recently, this compound was reported to be contained in terrestrial organisms, three species of mashrooms.[5] This result is interesting to discuss the distribution or circulation of arsenic compounds including arsenobetaine in the biosphere. On the other hand, in this study, we dealt with the degradation of arsenobetaine occurring in marine animal (chitons) to pursue the arsenic circulation in ecosystems which were restricted to marine ones. From the results from several *in vitro* experiments in which arsenobetaine was degraded by the microorganisms occurring in various marine origins, the ubiquitous occurrance of arsenobetaine decomposing microorganisms was obvious.[3,6-12] Furthermore, it was completely degraded to inorganic arsenic when the degradation activity of microorganisms was high.[13] This means that an ar-

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senic cycle is completed within the marine ecosystems when the generally accepted hypothesis that arsenobetaine is biosynthesized from inorganic arsenic in seawater through the food chain is taken into account. That is, a cycle that bigins with the methylation of inorganic arsenic and terminates with the degradation of arsenobetaine to inorganic arsenic.[8,13]

In order to confirm that the degradation behavior shown in our *in vitro* degradation experiments are applicable to the natural environment, we have previously performed *in vivo* degradation experiment in which a shark *Mustelus manazo* were buried in the coastal sands. As a result, a part of arsenobetaine, which was accumulated as substantially sole arsenical in the shark, was degraded to inorganic arsenic.[14,15] In this study, chitons *Liorophura japonica* were used in this degradation experiment. This animal, a hervivore, was chosen because it accumulates arsenobetaine as the majour arsenical in spite of their belonging to lower trophic level[3]. Specimens of chitons were left in the natural environment (in seawater) to confirm the degradation of arsenobetaine accumulated in them. The degradation was also confirmed in them incubated at higher temperatures (25 and 37 ℃).

2. Materials and Methods

2.1 Chitons *Liorophura japonica*

Several specimens of chitons *Liorophura japonica* were collected from the seashore of Yoshimi and killed by stabbing with a heated eyeleteer. They were apportioned into three groups; two groups were incubated at 37℃ for 3, 7 and 30 days (12 individuals each) or at 25 ℃ for 30 days (12 individuals). The other group (18 individuals) was wrapped in a net (ϕ 1 mm) hanging in seawater for 30 days. In the chitons taken up from the seawater after 30 days, all the vicera and part of the muscle were lost: the remaining muscle was used for this study.

2.2 Extraction and purification of arsenic compounds

After removing the valves, the water-soluble arsenic compounds were extracted from the muscle, viscera or round body of the chitons by 20 times their volumes of chloroform-methanol (2:1) as described previously.[3]

The extracts were applied on a cation-exchange resin Dowex 50W-×8 (50-100 mesh, H^+ form) column (2.1 × 60 cm) and eluted with water (600 cm^3), 2 mol dm^{-3} pyridine and 1 mol dm^{-3} HCl(600 cm^3), successively. Arsenic containing fraction was further chromatographed with a cation-exchange resin, Dowex 50W-×2 (200-400 mesh, pyridinium form) column (1 × 50 cm) equilibrated with 0.1 mol dm^{-3} pyridine-formic acid buffer (pH 3.1). Elution was carried out successively with the same buffer (200 ml) and 0.1 mol dm^{-3} pyridine (200 ml).

2.3 High performance liquid chromatography

Each extract was analyzed by high performance liquid chromatography (Tosoh Co., Ltd, CCP 8000-series) using ODS 120T column (4.6 × 250 mm; Tosoh Co., Ltd) with a mobile phase of 11.2 mmol dm^{-3} solution of sodium heptanesulphonate in water/acetonitrile/acetic acid (95/5/6, by vol.; flow rate, 0.8 cm^3 min^{-1}; sample size, 5 mm^3).[16] Twenty or 50 mm^3 of each eluate collected for every 25 s was injected into the graphite furnace atomic absorption spectrometer (GFAA) and analyzed as described previously.[3] The mixture of the authentic arsenic compounds (all with 100 mg as As per kg), which have been detected in the *in vitro* degradation experiments of arsenobetaine so far, were also fractionated (retention times, s: inorganic arsenic(V) [As(V)] 150-225;

inorganic arsenic (Ⅲ) [As(Ⅲ)] 225-300; methanearsonic acid (MMA) 225-300; dimethylarsinic acid (DMA) 325-400: arsenobetaine 525-625; trimethylarsine oxide (TMAO) 725-850; tetramethylarsonium iodide (TMA salt) 1125-1275 s).

2.4 Confirmation of the metabolite

The purified arsenic metabolite was subjected to thin layer chromatography performed on cellulose thin layer (Avicel SF, thickness: 0.1 mm, Funakoshi Yakuhin Co., Ltd.). Iodine vapor was used to indicate the position of the metabolite. $SnCl_2$-KI reagent[17] and Dragendorff reagent was also used to indicate that of the authentic arsenic compounds. The metabolite was also analyzed using a combination of gas chromatographic separation with hydride generation followed by a cold trap technique and selected ion monitor mass spectrometry.[18]

3. Results

3.1 The arsenic compounds accumulated in muscle or vicera of chitons

The extracted arsenic compounds from the muscle and vicera were fractionated by HPLC. Single peak, whose RT agreed with arsenobetaine, was shown in the muscle. On the other hand, two peaks were shown in the vicera: the RT of one peak agreed with that of arsenobetaine and the other with As(Ⅲ) and/or MMA. The relative distribution rate of these peaks was 69.6 % (arsenobetaine) and 30.4 %, respectively.

In order to confirm the absence of own conversion pathway for the arsenicals in chitons, the conversion experiments were preceded by one in which liver homogenates added with toluene (1 %) were incubated at 37 ℃ for 3 days. As the results, no conversion of the accumulated compounds occurred in them, indicating that the conversion shown in this study was attributed to microbial conversion activities.

3.2 Fractionation of arsenic compounds extracted from chitons incubated at 37 or 25 ℃.

The extracted arsenic compounds from the muscle of rotten chitons incubated for 3 days were analyzed by HPLC. Besides arsenobetaine, three arsenic peaks were shown and their RT agreed with those of [MMA and/or As(Ⅲ)], DMA and TMAO. Their ratios to the total were 14.6% [MMA and/or As(Ⅲ)], 40.5% (DMA), 27.6% (arsenobetaine) and 17.3% (TMAO). These conversion products were detected also in the vicera: their ratios were 3.9% [MMA and/or As(Ⅲ)], 42.0% (DMA), 42.2% (arsenobetaine) and 11.8% (TMAO). The decrease of the ratio of [MMA and/or As(Ⅲ)] suggested their methylation.

As for the chitons incubated for 7 or 30 days, whole bodies were subjected to the extraction without dissection, because the muscle and the vicera were no longer separable because of the autolysis and putrefaction of tissues or organs. After 7 days of incubation, As(Ⅴ) appeared other than the compounds detected in the muscle or vicera incubated for 3 days. The ratios of the compounds were 6.8 [As(Ⅴ)], 11.6, [MMA and/or As(Ⅲ)], 27.2 (DMA), 26.5 (arsenobetaine) and 21.6% (TMAO). Two additional metabolites, TMA salt and an unknown arsenic compound, appeared after 30 days of incubation. The RT of the unknown compound (600-675 s) was between RTs of arsenobetaine and TMAO. Their ratios to the total of the detected arsenicals were 6.2 [As(V)], 14.6 [MMA and/or As(Ⅲ)], 19.4 (DMA), 21.9 (arsenobetaine), 14.5 (TMAO), 14.5 (TMA salt) and 9.0% (the unknown arsenical).

The results from the conversion experiment performed at 25℃ were metabolically analogous

to those at 37℃ except for the absence of TMA salt. The ratios of the compounds were 10.1 [As(Ⅴ)], 16.1 [MMA and/or As(Ⅲ)], 46.0 (DMA), 7.3 (arsenobetaine), 16.7 (TMAO) and 9.0% (the unknown arsenical).

3.3 Fractionation of arsenic compounds extracted from chitons left in seawater

Arsenic species which were detected in the chitons left in seawater were similar to those observed in the chitons incubated at higher temperatures. Their ratios to the total were 9.8 [As(Ⅴ)], 11.8 [MMA and/or As(Ⅲ)], 12.0 (DMA), 17.1 (arsenobetaine), 25.3 (TMAO) and 24.0% (TMA).

3.4 Isolation and confirmation of TMAO

Because many metabolites were derived from a rather small amount of chitons, the amount of each metabolite was insufficient to be subjected to purification with column chlomatography except for TMAO. The purification of this compound is rather simple to be accomplished. Therefore, the confirmation of the structure was performed only on the metabolite, whose RT agreed with TMAO, detected in the extract from the round bodies incubated at 37℃ for 30 days. This compound was eluted with pyridine solution from both Dowex 50W-×8 (H^+ form) and Dowex 50W-×2 (pyridinium form) column. Arsenic-containing fraction was lyophilized and subjected to the following analyses.

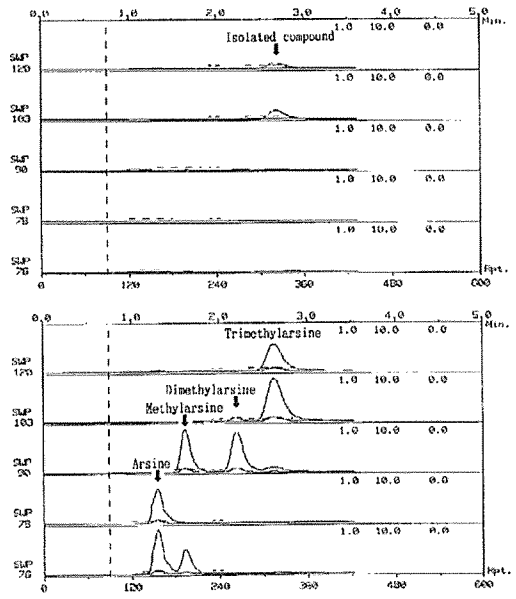

Fig.1 The SIM chromatograms of the arsenic compound isolated from the chitons incubated at 37 ℃ for 30 days (upper figure) and those of arsine, methylarsine, dimethylarsine and trimethylarsine volatalized from each srandard arsenicals (the lower figure).

Table 1. Rf values in TLC of the arsenic compound isolated from the chitons incubated at 37 ℃ for 30 days and reference arsenic compounds

Sample	Rf value				
	Solvent system				
	1	2	3	4	5
Isolated arsenic compound	0.86	0.86	0.71	0.46	0.73
Trymethylarsine oxide	0.85	0.86	0.71	0.47	0.74
Inorganic arsenic (Ⅴ)	0.31	0.00	0.22	0.01	0.21
Inorganic arsenic (Ⅲ)	0.22	0.30	0.40	0.07	0.35
Methanearsonic acid	0.55	0.22	0.50	0.02	0.50
Dimethylarsinic acid	0.80	0.78	0.61	0.23	0.71
Arsenocholine	0.53	0.87	0.54	0.56	0.62
Arsenobetaine	0.73	0.84	0.49	0.35	0.55

Solvent systems: 1,ethyl acetate/acetic acid/water (3:2:1); 2, chloroform/methanol/aq.ammonia (28%) (3:2:1); 3,1-butanol/acetone/formic acid (85%)/water (10:10:2:5); 4,1-butanol/acetone/aq.ammonia (28%)/water (10:10:2:5); 5,1-butanol/acetic acid/water (4:2:1)

The purified arsenical was analyzed using hydride generation/cold trap/GC MS/SIM without previous hydrolysis with sodium hydroxide. As shown in Fig.1, only trimethylarsine [As(CH_3)$_3$] was detected. The generation of trimethylarsine in this analysis without the hydrolysis indicates the purified compound to be TMAO. Further, the thin layer chromatography of this compound produced only a single spot positive to iodine vapor, and each Rf value was the same as that of synthetic TMAO in five solvent systems (Table 1).

From the results from HPLC, hydride generation/cold trap/GC MS/SIM analysis and thin layer chromatography, the purified compound was comfirmed to be TMAO.

Discussion

Various arsenic compounds were able to be detected by HPLC analysis despite the rather small amount of muscle and viscera of chitons used. Arsenobetaine was the majour arsenic compound in the muscle or vicera in fresh chitons notwithstanding their belonging to the lower trophic level. This result was the same as that we previously reported[3] except for the presence of [MMA and/or As(Ⅲ)] in viscera in this study. Although the presence or absence of [MMA and/or As(Ⅲ)] in vicera may depend on such factors as temperature, season or age, little is known about the reason for this phenomenon at the present stage.

The presence of [MMA and/or As (Ⅲ)] in the viscera was unfortunate for this study. It was not understandable whether the formation of such methylated arsenic as DMA and TMAO was attributed to the degradation of arsenobetaine or methylation of [MMA and/or As (Ⅲ)]. This means that the discussion on the compounds derived from arsenobetaine should be narrowed down to those detected in the extracts from chiton muscle incubated for 3 days {[MMA and/or As(Ⅲ)], DMA and TMAO}, and chiton muscle, which had lost the viscera, left in the seawater for 30 days {As(V), [MMA and/or As(Ⅲ)], DMA, TMAO and TMA salt}. The metabolites which have been commonly derived in our degradation experiments *in vitro* of arsenobetaine were also detected in their extracts. Those were TMAO, DMA and inorganic arsenic(V).

Besides these compounds, [MMA and/or As (Ⅲ)] and TMA salt were also detected. The formation of these compounds may suggest the difference in the degradation behavior between *in vitro* and *in vivo* degradation : MMA formed also in the shark left in the coastal sands.[14,15] Although the confirmation of the structure was accomplished only in TMAO in this study, other compounds will have to be confirmed to pursue the microbial conversion or degradation behavior of organic arsenic in the future experiments using many more chitons.

Regardless of round body (incubated for 25 or 37 ℃ for 30 days) or muscle only (left in seawater for 30 days), analogous metabolites were detected as conversion products despite the presence of [MMA and/or As(Ⅲ)] in the former. This may suggest that there is no great difference in the diversity of the conversion products whether a marine animal accumulates arsenobetaine as the only arsenic compound or not. The appearance, however, of the unknown compound was noticeable in the round chitons incubated for 30 days. Although there is a possibility that the appearance is in relation to the presence of [MMA and/or As(Ⅲ)] in the viscera, It can not be concluded from the results only in this study.

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ヒザラガイ *Liorophura japonica* に蓄積されたアルセノベタインの微生物による変換

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ヒザラガイ *Liorophura japonica* に蓄積されているアルセノベタインの分解あるいは変換を確認するため，これらをインキュベート（25および37℃）あるいは海水中に放置した。死直後においては，筋肉中にはアルセノベタインのみが，内臓中にはアルセノベタインの他に3価の無機ヒ素またはメタンアルソン酸が蓄積されていた。30日間インキュベートしたものでは，自己消化および腐敗のため筋肉と内臓を分離することができず，丸のままからヒ素化合物の抽出・分析を行なった。その結果，5価の無機ヒ素，ジメチルアルシン酸，トリメチルアルシンオキシド，テトラメチルアルソニウム塩および未知のヒ素化合物が検出された。一方，海水中に放置したものでは，内臓のすべておよび筋肉の一部が消失していたため，残っていた筋肉のみから抽出を行なった。その結果，未知の化合物を除いて同様のヒ素化合物が検出された。

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