Arsenic Speciation and Distribution in the Extracts from Salmon Egg Cell Cytoplasm and Cell Membrane by HPLC/ICP-MS

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Abstract
Speciation of arsenic in the extracts from salmon egg cell cytoplasm and cell membrane was carried out by an HPLC/ICP-MS hyphenated system. The extraction of arsenic species from salmon egg cells was performed by using methanol/water (1:1, v/v) with the aid of sonication. The extracts were evaporated to dryness, and the residues were dissolved with the HPLC mobile phase solution. In the 75As-detected chromatograms, arsenobetaine (AB), trimethylarsine oxide (TMAO), dimethylarsinic acid (DMA), and arsenate were found for egg cell cytoplasm, while arsenate, AB, DMA, and monomethylarsonic acid (MMA) were for egg cell membrane. The concentration of each arsenic species was determined by using the arsenic standard compounds. As a result, it was found that the total concentrations of arsenic species in the extracts from cell cytoplasm and cell membrane were 22.7 ng g⁻¹ and 44.2 ng g⁻¹, respectively, which corresponded to ca. 12% and 35% of the total amounts of arsenic in cell cytoplasm and cell membrane, respectively. The percentage of methylated arsenic species (AB, DMA, and TMAO) in the extract from cell cytoplasm was 93% of the total extracted arsenic species, and that of inorganic arsenic (arsenate) was only 7%, in which AB was relatively the most abundant (ca. 80%). On the contrary, the abundance of arsenate in the extract from egg cell membrane was 35%, which was more abundant than that in egg cell cytoplasm.

Keywords: arsenic, speciation, egg cell, cell cytoplasm, cell membrane

Introduction
Arsenic (As) has been receiving great attention in environmental or biological chemistry because arsenic species provide the toxicity and bio-essentiality, depending on its chemical forms. Thus, many studies on arsenic speciation have been carried out in terms of environmental or biological samples, such as water, soil, sediment, marine organisms, human blood, human urine, and so on [1-4]. It is known that As is significantly accumulated in marine fishes, and major species of As in marine fishes is nontoxic arsenobetaine [3], even though that in seawater is toxic arsenate (AsO₄³⁻). The abundant presence of arsenobetaine in marine fishes suggests that the biological transformation of arsenic species occurs in them; toxic arsenate accumulated from seawater is converted to nontoxic arsenobetaine through monomethylarsonic acid and dimethylarsinic acid [5]. Thus, speciation of arsenic in marine fishes is the interesting research subject to elucidate its biological functions.

An egg is biologically one cell, which is the origin of the living organism. In egg cell, DNAs, RNAs, and proteins should be synthesized for ontogenesis. It is considered that many elements in egg cell may play important roles in the various biological functions including syntheses of genes, proteins, and other biological substances. In the previous study [6], the present authors performed the multielement determination of major-to-ultratrace elements in salmon egg by ICP-AES and ICP-MS. As a result, 40 elements in salmon egg could be determined over the concentration range in 9 orders of magnitude [6], and there-
after another 12 elements were detected in egg cell. Such studies were carried out to establish all-elements analysis in one biological cell under the research program on the Extended All Present Theory of the Elements proposed by one of the authors (H. H.) [7]. It was found that P, Fe, Cu, Zn, Co, and Ag were significantly enriched in salmon egg. For example, the concentration of Zn in egg was about 18 $\mu$g g$^{-1}$ on the wet-weight basis, which corresponds to the bio-accumulation factor of ca. 50000, compared to its concentration in seawater. It is also known that metalloproteins or metalloenzymes of Zn play important roles in gene and protein syntheses. Therefore, the high concentration of Zn in egg cell suggests some essential roles of Zn in enzymatic activities. In addition, the existence of metalloproteins of Zn including metallothionein has been known in the case of sea urchin egg [8-11]. In our preliminary work [6], As was also detected in salmon egg cell, where the total concentrations of As in cell cytoplasm and cell membrane of salmon egg were 192 ng g$^{-1}$ and 128 ng g$^{-1}$ on the wet-weight basis, respectively. The weights of cell cytoplasm and cell membrane of one salmon egg were 0.12 g and 0.03 g, respectively, and thus, the total concentration of As in whole egg cell was 179 ng g$^{-1}$ on the wet-weight basis. Several studies on arsenic speciation in bird eggs have been carried out from the viewpoint of toxicity and biomonitoring of As [12, 13], but speciation of arsenic compounds in fish eggs has not been performed yet. In the present study, hence, arsenic speciation in salmon egg was performed by ion-pair formation HPLC/ICP-MS to elucidate arsenic species and their distributions in salmon egg cell cytoplasm and membrane.

### Materials and Methods

#### Instrumentation

A chromatographic system was composed of a pump (Model LC-6AD; Shimadzu, Kyoto, Japan), a sample injector (Model 7125; Rheodyne, Cotati, CA, USA), and an ODS column (CAPCELL PAK C$_{18}$MG, 4.6 mm i.d. $\times$ 250 mm long; Shiseido, Tokyo, Japan). The mobile phase in the present experiment was 4 mM malonic acid in 0.05% methanol (pH 3.0), into which 4 mM tetramethylammonium hydroxide (TMAH) and 10 mM 1-butane sulfonic acid sodium salt were added as the ion pairing reagents [14].

An ICP-MS instrument (Model Agilent 7500c; Yokogawa Analytical Systems, Tokyo, Japan) was used for the on-line detection of As at $m/z$ 75. The ICP-MS instrument was coupled with the HPLC system via a Teflon tubing between the outlet of the separation column and the nebulizer of the ICP-MS.

#### Chemicals and samples

Malonic acid, 1-buntane sulfonic acid sodium salt, and methanol used for the mobile phase were purchased from Wako Pure Chemicals (Osaka, Japan). Tetramethylammonium hydroxide (TMAH) was obtained from Tama Chemicals (Tokyo, Japan). All the standard arsenic compounds were purchased from Tri Chemical Laboratories (Yamanashi, Japan). The mixed solutions of these arsenic standards with the concentrations of 5, 10, and 20 ng of As per ml for each standard were analyzed for standardization of the arsenic peaks observed in the HPLC chromatograms.

The salmon egg samples were purchased as a whole ovary (sujiko) from a fish market without any treatment. Eggs were collected gently from the ovary, and after washing with pure water, cell cytoplasm (intracellular fluid) and cell membrane were separately collected from whole egg cells with Teflon tweezers and a Teflon needle.

#### Extraction of arsenic species from salmon egg cell

The extraction of arsenic species from egg cell cytoplasm and membrane was performed as follows. Approximately 1 g of cell cytoplasm or membrane sample was taken in a centrifugation tube, and 10 ml of 50% methanol was added. The sample was sonicated for 30 min and then centrifuged at 4000 rpm for 5 min. The supernatant was collected as the analysis sample. This extraction procedure was repeated again. The supernatants collected two times were put together and evaporated almost to dryness at 40°C using a rotary evaporator. The residue was dissolved with 1 ml of the HPLC mobile phase solution. The residual solution was then filtered with a membrane filter (pore size: 0.45 $\mu$m) and subjected to the HPLC/ICP-MS measurement.

In the extraction from egg cell membrane, 0.5 ml of TMAH was added as an alkali reagent into the sample before the extraction procedure, in order to dissolve some arsenic species from lipids or proteins in cell membrane.

#### Results

First, in order to ensure the detectability and speciation ability of the present HPLC/ICP-MS system, some standard arsenic compounds were analyzed under the experimental conditions shown in Figure 1. The following arsenic compounds were used as the standards: arsenate, arsenite, monomethylarsonic acid (MMA), dimethyl-
larsinic acid (DMA), trimethylarsine oxide (TMAO), arsenobetaine (AB), arsenocholine (AC), and tetramethylalarsonium ion (TeMA). The HPLC chromatogram of the arsenic standards (10 ng ml$^{-1}$ arsenic for each compound) detected by ICP-MS at m/z 75 is shown in Figure 1. As can be seen in Figure 1, the standard arsenic compounds were clearly separated from each other within 750 s. Thus, the retention times for standard arsenic compounds observed in Figure 1 could be used for the identification of arsenic species in salmon egg cell sample. The chromatograms for As in salmon egg cell cytoplasm and membrane are shown in Figures 2 (A) and (B), respectively. In Figure 2 (A), one large peak was observed around 300 s and three small peaks were around 165 s, 270 s, and 580 s. According to the retention times of the standards in Figure 1, the large peak could be identified as AB, and another three small peaks as arsenate, DMA, and TMAO. The peaks for arsenic species observed in the chromatogram were quantified by using the calibration graph for each standard compound, which was made by the chromatographic measurement similar to that in Figure 1. The results are summarized in Table 1.

As can be seen in Table 1, the concentrations of AB, TMAO, DMA, and arsenate in egg cell cytoplasm were 17.6, 3.0, 0.4, and 1.7 ng g$^{-1}$ as As on the wet-weight basis, respectively. The sum of them was 22.7 ng g$^{-1}$. The total concentration of arsenic in the extract from egg cell cytoplasm was separately determined by ICP-MS, and it was 22.6 ng g$^{-1}$, which was in good agreement with the result in Table 1. The chromatogram for As in salmon egg cell membrane is shown in Figure 2 (B), where several peaks were observed at the retention times of 165 s, 220 s, 270 s, and 300 s, corresponding to arsenate, MMA, DMA, and AB, respectively. In addition, one small peak was detected at 240 s, whose chemical form could not be identified in the present experiment. The peak of arsenate was the largest among all arsenic species found in egg cell membrane. The concentrations of arsenate, MMA, DMA, and AB in egg cell membrane are also summarized in Table 1, and they were estimated as 15.4, 8.1, 12.3, and 8.4 ng g$^{-1}$ as As on the wet-weight basis, respectively.

**Discussion**

The concentrations of arsenic species in the extracts from cytoplasm and membrane of salmon egg cell are summarized in Table 1, where the relative abundance (%) of each arsenic species in the extract is also shown in parenthesis. As can be seen in Table 1, the abundance of AB was ca. 80% of all the arsenic species in the extract from egg cell cytoplasm. It is noticed here that organic
arsenic species found in egg cell cytoplasm were trimethylated (AB and TMAO) and dimethylated (DMA) arsenic species, and monomethylated (MMA) was not found.

As for arsenic species in egg cell membrane, inorganic arsenate was the most abundant species in cell membrane, and its abundance was 35%, while those of MMA, DMA, and AB were 18%, 28%, and 19%, respectively. It was reported that the addition of alkali reagent increased the extraction of alkali-labile arsenolipid fraction [15, 16]. Thus, in the present experiment for egg cell membrane, small amount of TMAH was added to the sample before the extraction of arsenic species, as described earlier. As can be seen in Table 1, arsenic species were more extracted from cell membrane than from cell cytoplasm. This may be due to the addition of TMAH in cell membrane, although such experiment was not done for cell cytoplasm. It is noted here that the addition of TMAH may increase the extraction of MMA, DMA, and AB from the arsenolipid fractions in egg cell membrane, but the addition of TMAH may not be effective for the extraction of arsenate from egg cell membrane, because arsenate is contained as a water-soluble compound.

The experimental results shown in Table 1 indicate that the concentrations of arsenic species in the extracts from egg cell cytoplasm and membrane were 22.7 ng g⁻¹ and 44.2 ng g⁻¹ as As, respectively. As described earlier, the total concentrations of As in egg cell cytoplasm and membrane, which were determined by ICP-MS after acid digestion, were 192 ng g⁻¹ and 128 ng g⁻¹, respectively, on the wet-weight basis. Thus, the amounts of arsenic species found in the extracts were 12% and 35% of the totals in cell cytoplasm and cell membrane, respectively. These results suggest that most of arsenic species are contained as lipid-soluble species (arsenolipid compounds) in salmon egg cell.

It is also seen from Table 1 that, the content of organic arsenic species (AB, DMA, and TMAO) in the extract from cell cytoplasm was 93% of the total As extracted, while that of inorganic arsenic (arsenate) was only 7%. On the contrary, the content of inorganic arsenic (arsenate) in the extract from egg cell membrane was 35% of the total As extracted. The concentrations of inorganic arsenate in cell cytoplasm (1.7 ng g⁻¹) and in cell membrane (15.4 ng g⁻¹) were 0.9% and 12% of the total arsenic (192 ng g⁻¹ in cell cytoplasm and 128 ng g⁻¹ in cell membrane), respectively. Thus, arsenate in cell membrane was more abundant than that in cell membrane. It is known that arsenate is an inhibitor of the enzymatic reactions, which may result in the toxicity of As in the biological system. Thus, the fact that arsenate is much less abundant inside egg cell suggest the occurrence of some molecular conversion of arsenate to methylated organic species in cell membrane to restrain the transportation of arsenate into egg cell cytoplasm.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration as As / ng g⁻¹</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>arsenate</td>
<td>1.7 (7%)</td>
<td>15.4 (35%)</td>
</tr>
<tr>
<td>arsenite</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>MMA</td>
<td>n.d.</td>
<td>8.1 (18%)</td>
</tr>
<tr>
<td>DMA</td>
<td>0.4 (2%)</td>
<td>12.3 (28%)</td>
</tr>
<tr>
<td>TMAO</td>
<td>3.0 (13%)</td>
<td>n.d.</td>
</tr>
<tr>
<td>AB</td>
<td>17.6 (78%)</td>
<td>8.4 (19%)</td>
</tr>
<tr>
<td>AC</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>TeMA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Total: 22.7 44.2

a) MMA: monomethylarsonic acid, DMA: dimethylarsinic acid, TMAO: trimethylarsine oxide, AB: arsenobetaine, AC: arsenocholine, TeMA: tetramethylarsonium ion.

b) The concentration of each species was estimated on the wet-weight basis. The value in parenthesis was the relative abundance (%) of each species in the extract from cytoplasm or membrane.

c) n.d.: not detected.

Table 1 The concentrations of arsenic species extracted from cytoplasm and membrane of salmon egg cell.
Conclusion

In the present study, arsenic speciation in the extracts from salmon egg cell cytoplasm and cell membrane was performed to elucidate the distributions of arsenic species in them. As a result, it was found that major arsenic species in egg cell cytoplasm was arsenobetaine, while that in egg cell membrane was arsenate. The less abundant distribution of arsenate in egg cell cytoplasm may indicate its conversion to organic arsenic species in cell membrane to reduce the toxicity of arsenate in egg cell cytoplasm. The different distributions of arsenic species in egg cell cytoplasm and egg cell membrane may also be interesting to elucidate the physiological functions of arsenic species in egg cell. As described earlier, however, arsenic species in the extract from egg cell cytoplasm was only 12% of the total. In the present experiment, egg cell cytoplasm was not treated with TMAH, and thus, arsenic species in the alkali-labile arsenolipid fractions might not be extracted here. Thus, further studies on lipid-soluble arsenic species should be performed to elucidate the whole features of arsenic species in salmon egg cell, in relation to the species distributions of other metallic elements including zinc proteins.

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