

Speciation of Small Molecules and Inorganic Ions in Salmon Egg Cell Cytoplasm by Surfactant-Mediated HPLC/ICP-MS

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The speciation of diverse elements in salmon egg cell cytoplasm was performed by a surfactant-mediated HPLC/ICP-MS hyphenated system. In the present experiment, an ODS column coated with CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), which is a zwitterionic bile acid derivative, was employed as a surfactant-mediated separation column, and ICP-MS was used as an element-selective detector. The present surfactant-mediated HPLC allowed us to separate large and small molecules within 10 min; large molecules, such as proteins, were eluted within 2.5 min, while small molecules were eluted after 2.5 min, but within 10 min. In the present experiment, Fe, Cu, and Zn in egg cell cytoplasm were observed mostly in species with large molecular weights, indicating that these elements are contained as metalloproteins or metalloenzymes in egg cell cytoplasm. On the contrary, it was found that P, S, Mo, and halogens in egg cell cytoplasm were contained as small molecules or inorganic ions. The major species of P in egg cell cytoplasm was identified as the phosphate ion (PO_4^{3-}). Molybdenum, Cl, and Br in egg cell cytoplasm were molybdate (MoO_4^{2-}), chloride (Cl^-), and bromide (Br^-) ions, respectively.

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Introduction

In recent years, many studies on bio-trace elements have been carried out by analyzing various biological samples, such as blood serum, urine, hair, and organs.¹ However, there have only been a few studies on bio-trace elements on the cell basis, which is a fundamental unit of animals and plants. An egg is biologically one cell, in which DNAs, RNAs, and proteins should be synthesized for ontogenesis. It is considered that many elements in egg cells may be engaged in the various biological functions, including the syntheses of DNAs, RNAs, proteins, and other biological substances. Therefore, it may be interesting to investigate the concentrations and distributions of trace elements in egg cell.

In a previous study,² the present authors performed a multielement determination of major-to-ultratrace elements in salmon egg by ICP-AES and ICP-MS. As a result, about 40 elements in whole egg cells and cell cytoplasm could be determined over a concentration range of 9 orders of magnitude. Such studies were carried out with the aim of an all-elements-analysis in one cell, although the study is on a way of the final goal. It was found that P, Fe, Cu, Zn, Co and Ag were significantly enriched in salmon egg cell cytoplasm. This fact suggests that these elements may play important roles in the ontogenesis of salmon egg. In order to elucidate the roles of diverse elements in ontogenesis, however, it is necessary to investigate not only the total concentrations of the elements in egg cell, but also the chemical species of the elements therein. So far, several studies on the speciation of the elements in

animal eggs have been performed; metallothionein,^{3,4} a Zn-binding protein similar to metallothionein,⁵ or a Zn-binding protein distinct from metallothionein,⁶ were detected in sea urchin egg. However, the systematic studies on detection of metalloproteins, metalloenzymes, small molecular organic-metal complexes, or inorganic ions in animal cells have not yet been performed.

In the present authors' laboratory, the CHAPS-coated ODS (octadecylsilica) column was developed as a surfactant-mediated separation column by adsorbing CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), a zwitterionic bile acid derivative, on the ODS column.^{7,8} The surfactant-mediated HPLC allowed rapid separation of large and small molecules based on the mixed-mode separation properties; large molecules were eluted by a size-exclusion mode, and small molecules were separated by electrostatic or hydrophobic interactions. As a result, large molecular species with molecular weights larger than 10000 Da were eluted within 2.5 min, while small molecular species with molecular weights smaller than 10000 Da were eluted after 2.5 min, but within 10 min. The present surfactant-mediated HPLC could be easily coupled with ICP-MS for element-selective detection because a dilute buffer solution at the sub-mM level was used as a mobile phase, and then the surfactant-mediated HPLC/ICP-MS system could be applied to the speciation of trace elements⁹ as well as cisplatin¹⁰ in human blood serum. In the present study, thus, the surfactant-mediated HPLC/ICP-MS system was applied to the speciation of diverse elements in salmon egg, especially to identify some small molecules or inorganic ions.

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Table 1 Operating conditions for the HPLC/ICP-MS hyphenated system

HPLC	
Column	CHAPS-coated ODS column
Mobile phase	0.2 mM Tris-HCl + 0.2 mM CHAPS (pH 7.4)
Flow rate	0.7 ml min ⁻¹
Injection volume	20 µl
ICP-MS	
Seiko SPQ 8000A	
Plasma conditions	
Rf frequency	27.12 MHz
Rf power	1.3 kW
Gas flow rate	
Coolant gas	Ar 16 l min ⁻¹
Auxiliary gas	Ar 1.0 l min ⁻¹
Carrier gas	Ar 0.8 l min ⁻¹
Sampling conditions	
Sampling depth	10 mm from work coil
Sampling cone	copper, 1.1 mm orifice diameter
Skimmer cone	copper, 0.35 mm orifice diameter
Nebulizer	glass concentric type
Sample uptake rate	0.7 ml min ⁻¹
Data acquisition	
Data point	1 point/peak
Dwell time	100 ms/point
Measurement time	600 s

Experimental

Instrumentation

A chromatographic system was composed of a pump (Model LC-10; Shimadzu, Kyoto), a sample injector (Model 7725; Rheodyne, Cotati, CA, USA) with a 20 µl sample loop, a separation column, and a UV absorption detector (Model UV-970; Jasco, Tokyo). In the present experiment, an ODS column (L-column; 4.6 mm i.d. × 250 mm long; Chemicals Evaluation and Research Institute, Tokyo) coated with CHAPS was employed as the surfactant-mediated separation column. A CHAPS-coated ODS column was prepared by a dynamic coating method in a similar manner to those used in previous studies.^{7,8} The aqueous solution of 2 mM CHAPS was flowed for 2 h to coat the surface of the ODS column, and then rinsed with pure water for *ca.* 1 h. After conditionings of the column by flowing the carrier solution (mobile phase), it was subjected to a separation analysis. The mobile phase used in the present experiment was a 0.2 mM Tris-HCl buffer solution (pH 7.4), into which 0.2 mM CHAPS was added to maintain the long-term stability of the CHAPS-coated ODS separation column.⁹

An ICP-MS instrument (Model SPQ 8000A; Seiko Instruments, Chiba) was used as an element-selective detector for HPLC. The HPLC system was coupled with the ICP-MS instrument *via* Teflon tubing between the outlet of the UV absorption detector and the nebulizer of the ICP-MS. The operating conditions of the HPLC/ICP-MS hyphenated system are summarized in Table 1, which were almost the same as those employed in previous studies.^{9,10}

Chemicals and samples

The hydrochloric acid used was of electronics industry grade (Kanto Chemicals, Tokyo). Tris(hydroxymethyl)aminomethane (Tris buffer) used for the mobile phase was of GR grade (Merck Japan, Tokyo). CHAPS was purchased from Aldrich Japan (Tokyo). Blue dextran and proteins (β -amylase, alcohol dehydrogenase, albumin, and carbonic anhydrase) used for the

Table 2 Retention times for various compounds obtained by the CHAPS-coated ODS column with UV absorption detection or ICP-MS detection^a

Analyte	Molecular weight/Da	Retention time/min
Blue dextran	2000000	2.10
β -Amylase	200000	2.25
Alcohol dehydrogenase	150000	2.25
Albumin	66000	2.30
Carbonic anhydrase	29000	2.38
Caffeine	194.2	6.55
Citric acid	192.1	4.00
Theophylline	180.2	5.31
Theobromine	180.2	4.20
Uric acid	168.1	4.20
I ⁻	126.9	4.42
SO ₄ ²⁻	96.1	3.50
PO ₄ ³⁻	95.0	3.08
Br ⁻	79.9	3.17
Cl ⁻	35.5	3.42

a. Inorganic ions (I⁻, SO₄²⁻, PO₄³⁻, Br⁻, and Cl⁻) were detected by ICP-MS at *m/z* 127, 48 (as ³²S¹⁶O), 31, 79, and 51 (as ³⁵Cl¹⁶O), respectively. Other compounds (proteins, drug compounds, and biomolecules) were detected by UV absorption detection at 254 nm.

molecular-weight calibration of the CHAPS-coated ODS column were obtained from Sigma (St. Louis, MO, USA). Small molecules (aromatic compounds, amino acids, ATP, and inorganic salts), also used for the column calibration or the spike experiment, were purchased from Wako Pure Chemicals (Osaka). Pure water used throughout the present experiment was prepared by a Milli-Q water purification system (Model Milli-Q SP TOC; Nihon Millipore Kogyo, Tokyo).

The salmon egg samples were purchased as a whole ovary (sujiko) in a fish market without any treatment. Eggs were collected gently from the ovary, and washing with pure water; the cell cytoplasm and cell membrane were then separately collected from whole egg cells with Teflon tweezers and a Teflon needle. Cell cytoplasm (intracellular fluid) was subjected to an analysis after 10-fold dilution with Tris buffer.

Results and Discussion

Separation characteristics of CHAPS-coated ODS column

As previously reported,^{8,10} the CHAPS-coated ODS column has excellent characteristics to separate large and small molecules simultaneously in a short time (within 10 min). In the present experiment, in order to apply the CHAPS-coated ODS column to the speciation of small molecules and inorganic ions in salmon egg cell cytoplasm, the retention behaviors of various kinds of molecules in the surfactant-mediated HPLC column were re-examined by UV absorption detection or ICP-MS detection. The retention times obtained for various compounds are given in Table 2, together with their molecular weights. As can be seen, large molecules such as β -amylase, alcohol dehydrogenase, albumin, and carbonic anhydrase, whose molecular weights are larger than 10000 Da, were eluted within 2.5 min. On the other hand, small molecules, such as drug compounds (caffeine, theophylline and theobromine), biomolecules (citric acid and uric acid), and some inorganic ions in aqueous solution, were eluted after 2.5 min. From these results, it was found that the CHAPS-coated ODS column prepared in the present experiment could be applied to the

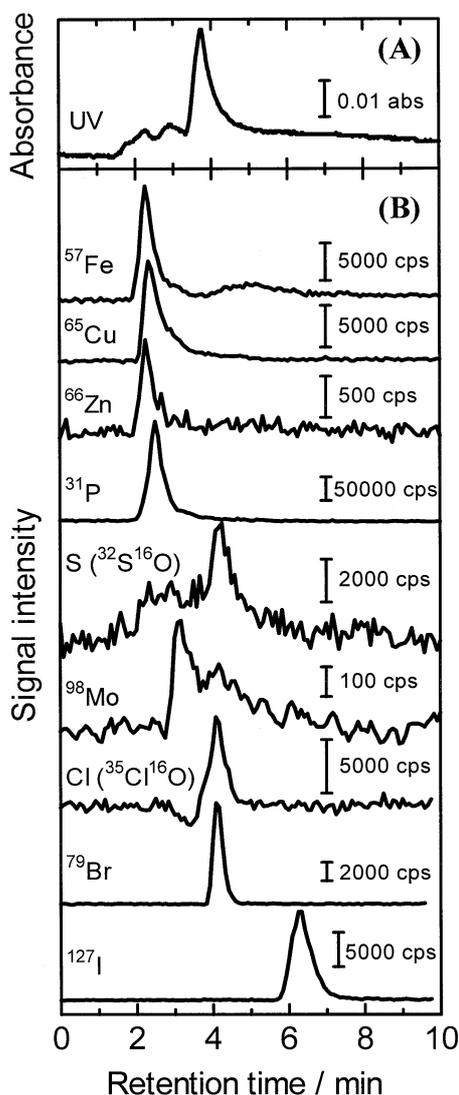


Fig. 1 Chromatograms for diverse elements in egg cell cytoplasm obtained by the surfactant-mediated HPLC/ICP-MS hyphenated system. (A) UV absorption detection at 254 nm, (B) ICP-MS detection at each m/z . Column, CHAPS-coated ODS column; mobile phase, 0.2 mM Tris-HCl buffer + 0.2 mM CHAPS (pH 7.4); flow rate, 0.7 ml min^{-1} ; sample injection volume, 20 μl .

speciation of small molecules and inorganic ions, while separating them from large molecules. It should be stressed here that the salmon egg cell cytoplasm sample was directly injected into the CHAPS-coated ODS column after diluting by 5 - 10 times with the Tris buffer solution (pH 7.4) because proteins and other large molecules were quickly eluted from the present column without any adsorption.

Chromatograms of diverse elements in salmon egg cell cytoplasm obtained by the surfactant-mediated HPLC/ICP-MS system

According to our previous work,² P, Zn, Fe, Cu, and Mo were enriched in salmon egg cell cytoplasm, and their total concentrations were 4250 $\mu\text{g g}^{-1}$, 17.9 $\mu\text{g g}^{-1}$, 14.5 $\mu\text{g g}^{-1}$, 6.93 $\mu\text{g g}^{-1}$, and 0.012 $\mu\text{g g}^{-1}$, respectively, on the fresh weight basis; P was the most abundant element among the elements contained in cell cytoplasm, except for H, C, N, and O. This salmon egg cell cytoplasm sample was subjected to the following speciation analysis.

The chromatograms for diverse elements in salmon egg cell cytoplasm with ICP-MS detection are shown in Fig. 1, together with the chromatogram obtained by UV absorption detection at 254 nm. It is noted here that clear peaks were detected in the UV absorption-detected chromatogram at around 2.2, 3.0 and 3.8 min, although large absorptions were observed in the retention time range over 1.5 - 10 min. It can be said from the results in Table 2 that the peak around 2.2 min may correspond to large molecules (proteins), while the peaks around 3.0 and 3.8 min may be corresponding to some small molecules or inorganic ions.

In the chromatograms detected by ICP-MS, shown in Fig. 1, Fe, Cu, and Zn provided their main peaks at around 2.2 min, respectively. The retention times for these peaks almost agreed with those for proteins obtained by UV absorption detection. Thus, the peaks observed around 2.2 min may be attributed to those elements binding with proteins in egg cell cytoplasm. These results suggest that Fe, Cu, and Zn are contained mostly as metalloproteins in egg cell cytoplasm. It was reported that metallothionein was detected in sea urchin egg.^{3,4} Thus, metallothionein may be one of the metalloproteins possibly contained in salmon egg as a Zn-binding protein, although metallothionein could not be identified only from the present experimental results. Further research is now in progress by size-exclusion chromatography to assure the existence of metallothionein.

As for P, the retention time for the observed peak was 2.5 min, which was slightly different from those of Fe, Cu, and Zn. Since it was speculated that ATP (adenosine triphosphate) or phosphate ion (PO_4^{3-}) might be a small molecule or inorganic ion of P, a further experiment was performed (described in the next section) to identify the peak of P in Fig. 1. The chromatogram of S in salmon egg cytoplasm is also shown in Fig. 1. In the present experiment, S was detected at m/z 48 (as $^{32}\text{S}^{16}\text{O}$) by ICP-MS because ^{32}S was interfered with the polyatomic ion of $^{16}\text{O}^{16}\text{O}$. As can be seen in the chromatogram of S (Fig. 1), several peaks were observed in the large and small molecular regions, although the chromatogram was rather complicated with a poor signal intensity. The peaks of S in the small molecular region were observed around 3.0 min and 4.2 min. It is noted here that the small peak of S was observed at a retention time of around 2.1 min. This fact may indicate that S is partly contained as S-containing amino acids (cysteine and methionine) in some proteins.

In addition, a chromatogram of Mo in egg cell cytoplasm was obtained by ICP-MS. In the chromatogram for Mo, a large peak and a small peak were detected at 3.1 min and 4.1 min, respectively. These results suggest that, at least, two chemical species of Mo may exist as the small molecules or inorganic ions in salmon egg cell cytoplasm, not metalloproteins or metalloenzymes. In chromatograms for halogens, the retention times of Cl, Br, and I were 4.0 min, 4.1 min, and 6.3 min, respectively. Thus, halogens are likely to be contained as halide ions in egg cell cytoplasm.

Speciation of some small molecular species and inorganic ions in egg cell cytoplasm

In order to identify the chemical forms of some small molecules and inorganic ions in egg cell cytoplasm, spike experiments were carried out, where some possible compounds were spiked in the sample solution. The unspiked and spiked sample solutions were analyzed by the surfactant-mediated HPLC/ICP-MS system. In the case of P, ATP and phosphate ion (PO_4^{3-}) were spiked into egg cell cytoplasm. The chromatograms obtained by UV absorption detection at 254 nm

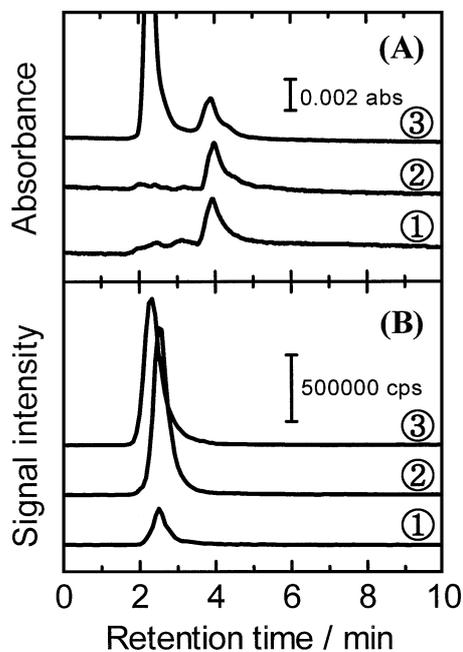


Fig. 2 HPLC chromatograms for P in egg cell cytoplasm obtained by (A) UV absorption detection at 254 nm, and (B) by ICP-MS detection at m/z 31. ① Egg cell cytoplasm, ② egg cell cytoplasm + PO_4^{3-} , ③ egg cell cytoplasm + ATP. Added amounts: 2.50 mg as PO_4^{3-} or 5.00 mg as ATP in 5 g sample.

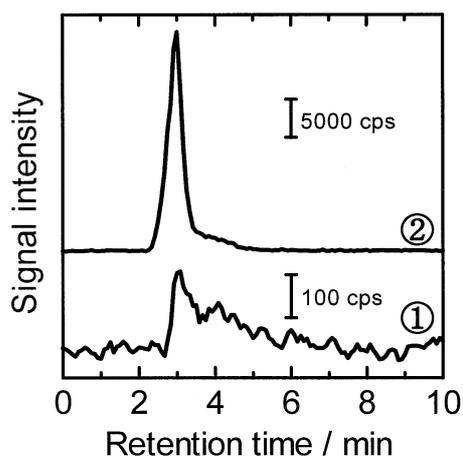


Fig. 3 HPLC chromatograms for Mo in egg cell cytoplasm obtained by ICP-MS detection at m/z 98. ① Egg cell cytoplasm, ② egg cell cytoplasm + MoO_4^{2-} . Added amounts: 330 ng as MoO_4^{2-} in 5 g sample.

and by ICP-MS detection at m/z 31 are shown in Figs. 2(A) and 2(B), respectively. In Figs. 2(A) and 2(B), the chromatograms for the egg cell cytoplasm sample, itself, a PO_4^{3-} -spiked sample and an ATP-spiked sample are shown in each figure. In the UV absorption-detected chromatogram for the ATP-spiked sample (Fig. 2(A)③), a large peak was additionally observed at 2.3 min. On the other hand, in chromatograms obtained by ICP-MS detection, shown in Fig. 2(B), the retention time (2.5 min) for P in the PO_4^{3-} -spiked sample was in rather good agreement with that in egg cell cytoplasm, which was different from the retention time (2.3 min) for P in the ATP-spiked sample. Therefore, small molecules for P in the egg cell cytoplasm may

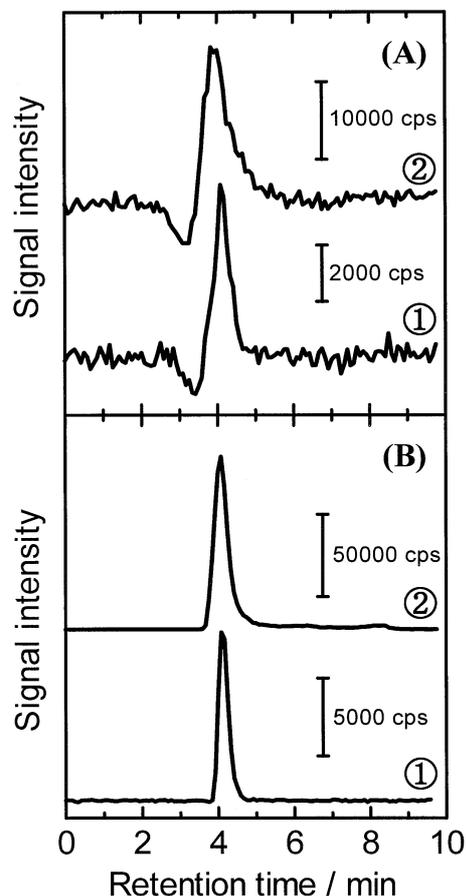


Fig. 4 HPLC chromatograms for (A) Cl and (B) Br in egg cell cytoplasm obtained by ICP-MS detection at m/z 51 for Cl (as $^{35}\text{Cl}^{16}\text{O}$) and 79 for Br, respectively. ① Egg cell cytoplasm, ② egg cell cytoplasm + Cl⁻ or Br⁻. Added amounts: 500 μg as Cl⁻ or 50 μg as Br⁻ in 5 g sample.

be identified as PO_4^{3-} .

In order to identify the species of Mo, molybdate ion (MoO_4^{2-}) was spiked as a possible species in egg cell cytoplasm. The chromatograms for the unspiked and spiked samples obtained by ICP-MS detection at m/z 98 are shown in Fig. 3. As can be seen in Fig. 3, the peak of Mo in egg cell cytoplasm at a retention time of around *ca.* 3 min was consistent with that of MoO_4^{2-} . Thus, it is concluded from the result in Fig. 3 that one of species of Mo in egg cell cytoplasm may be MoO_4^{2-} , but the species observed at the retention time of 4.1 min could not be identified in the present experiment.

In the cases of halogens, chloride (Cl⁻) and bromide (Br⁻) ions were spiked into the egg cell cytoplasm sample. Chromatograms for Cl and Br were obtained by ICP-MS detection at m/z 51 for Cl (as $^{35}\text{Cl}^{16}\text{O}$) and 79 for Br. The results are shown in Figs. 4(A) and 4(B), respectively. The retention times for Cl and Br in both spiked and unspiked samples were almost the same, which were 4.0 min for Cl and 4.1 min for Br, respectively. These results suggest that Cl and Br mostly exist as free ions in the egg cell cytoplasm.

In the case of I, iodide (I⁻) and iodate (IO_3^-) ions were spiked into the egg cell cytoplasm. One additional peak was detected at around 4.5 min in the I⁻-spiked sample. On the other hand, two additional peaks were observed at retention times of around 2.7 min and 4.5 min in the IO_3^- -spiked sample. The retention time for I in the egg cell cytoplasm, itself, was 6.3 min, as can

be seen in Fig. 1, which was quite different from those in both I⁻-spiked and IO₃⁻-spiked samples. Thus, the species of I in egg cell cytoplasm could not be identified in the present experiment.

In a spike experiment for the species of S, cysteine and sulfate ion (SO₄²⁻) were spiked into the egg cell cytoplasm. The retention times of S in the cysteine- and SO₄²⁻-spiked samples were 3.1 min and 3.7 min, respectively. As can be seen in the chromatogram for S detected by ICP-MS, shown in Fig. 1, the signal intensities of S were observed over a wide retention time range, which suggests that various S-containing species are contained in salmon egg cell cytoplasm. It is clear that some signals are observed around the retention times for S in the cysteine- and SO₄²⁻-spiked samples. However, it was difficult to identify the species of S only from the results in the present experiment because the chromatogram for S in salmon egg cell cytoplasm was so complicated, even with element-selective detection by ICP-MS.

Conclusion

Chemical speciation of diverse elements in salmon egg cell cytoplasm was carried out by the surfactant-mediated HPLC/ICP-MS hyphenated system, where the CHAPS-coated ODS column was used. Since the CHAPS-coated ODS column provided the separation characteristics to separate large and small molecules simultaneously within 10 min, the elements in the chemical forms of small molecules or inorganic ions could be separated from those binding with proteins. Among such small molecules or inorganic ions, the main species of P, Mo, Cl, and Br were identified as PO₄³⁻, MoO₄²⁻, Cl⁻, and Br⁻, respectively. It is known that ATP is very important as an energy-stock material; the biochemical energy obtained by the hydrolysis of ATP is used for various chemical reactions which occur in biological systems. Thus, it was expected that ATP might be contained in egg cell cytoplasm. In the present work, however, ATP could not be detected in egg cell before

fertilization. Thus, it is speculated that ATP might be synthesized in mitochondria after fertilization, and that PO₄³⁻ stored as a raw material in unfertilized egg may be used to synthesize ATP as well as DNA and RNA after fertilization. Furthermore, although metalloproteins binding with Fe, Cu, and Zn could be detected, they could not be identified from the results in the present experiment. Thus, a study on such metalloproteins, including metallothionein, is now in progress with the aid of size-exclusion chromatography and electrophoresis.

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