

Assay of Alkaline Phosphatase in Salmon Egg Cell Cytoplasm with Fluorescence Detection of Enzymatic Activity and Zinc Detection by ICP-MS in Relation to Metallomics Research

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Assay of alkaline phosphatase (ALP) in salmon egg cell cytoplasm was performed by size exclusion chromatography (SEC) with fluorescence detection of enzymatic activity and element-selective detection of zinc by ICP-MS, where 4-methylumbelliferyl phosphate (4-MUP) was used as the substrate for the enzymatic reaction of ALP. Two peaks were observed at retention times of ca. 21 and 27 min in the fluorescence-detected SEC chromatogram for salmon egg cell cytoplasm diluted 10-fold with 0.1 M Tris-HNO_3 buffer solution, corresponding to the molecular weights (MWs) of $\geqq$300 and 100 kDa, respectively. On the other hand, in the ^{66}Zn-detected chromatogram for the same sample, a broad peak with a shoulder on the side of longer retention time was observed at ca. 21 min (MW $\geqq$ 300 kDa). When salmon egg cell cytoplasm was diluted 10-fold with pure water or 0.01 M Tris-HNO_3 buffer solution, precipitation occurred. The supernatant obtained after filtration produced only one clear peak at a retention time of ca. 27 min in the fluorescence-detected chromatogram, at which the peak corresponding to zinc was also observed in the Zn-detected chromatogram. Therefore, the peak near 27 min was assigned to hydrophilic ALP in the salmon egg cell cytoplasm. Furthermore, the concentration of hydrophilic ALP (MW ca. 100 kDa) in the salmon egg cell cytoplasm was about 350 ng g^{-1}, which was determined as an *E. coli* ALP-equivalent concentration by fluorescence intensity measurements.

Recently, "metallomics" was proposed as a new research field for biometal-assisted function science,[1,2] which should be in symbiosis with genomics and proteomics to understand a biological system comprehensively. In metallomics, metal-binding biomolecules and bioactive metal ions are defined as "metallomes." In order to promote metallomics research, therefore, the chemical speciation of the major-to-ultratrace elements, as well as their elemental concentrations and distributions in the biological systems, are especially important to elucidate their biological or physiological functions of the elements.[1] In last few years, we have performed multielement determination of the elements in salmon egg cells by using ICP-AES (inductively coupled plasma atomic emission spectrometry) and ICP-MS (inductively coupled plasma mass spectrometry),[2–4] especially with the goal of all-elements analysis of one biological cell. So far, 67 elements among 78 stable isotope elements, which excludes radioactive and rare gas elements, in the major-to-ultratrace concentration range have been determined and/or detected in salmon egg cells, and their bio-accumulation factors were estimated by comparing with the concentrations of the elements in open seawater.[2]

In addition, the methods for the identification, or assay, of metalloproteins and metalloenzymes as well as for elucidation of their biological functions must be exploited in metallomics research.[1] In the previous papers,[4,5] it was found that various metallic elements (e.g., Fe, Cu, Zn, Co, Ni, Hg, etc.) in salmon egg cell cytoplasm existed as protein-binding species, by using HPLC with a CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)-coated ODS (octadecylsilica) column in combination with UV absorption and ICP-MS. The CHAPS-coated ODS column was useful for separation of large molecules (proteins) and small molecules/ions, but it could not separate various proteins with different molecular weights (MW).[6,7] However, it was demonstrated that proteins in mercury speciation analysis could be separated by using a SEC (size exclusion chromatography) column in combination with a CHAPS-coated ODS column.[8]

Herein, we report an assay of ALP in salmon egg cell cytoplasm, which was carried out by measuring the enzymatic activity of ALP as well as by detecting zinc contained in ALP in order to examine its existence as one of zinc proteins (enzymes). ALP is a zinc enzyme that hydrolyzes phosphate monoesters in biological substances, for example, ATP (adenosine triphosphate), and it is ubiquitously distributed in prokaryotes (microorganisms) as well as in eukaryotes (animals).[9,10]

Experimental

Instrumentation. A schematic diagram for the enzymatic activity measurement by the fluorescence detection and for zinc detection by ICP-MS is shown in Fig. 1. The HPLC system for the measurement of ALP enzymatic activity consisted of an HPLC pump (model PU-980, Jasco, Tokyo, Japan), a sample injector (model 7725, Rheodyne, Cotani, CA, U.S.A.) with a 500 μL sample loop, a SEC column (Superose 12 10/300 GL, Amersham Bioscience, Piscataway, NJ, U.S.A.) with a molecular permeation

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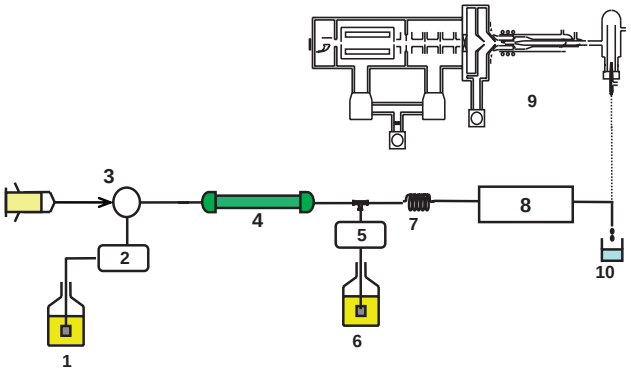

Fig. 1. A schematic diagram of the enzymatic activity measurement system. 1. Mobile phase (0.1 M Tris-HNO_3 buffer solution, pH 8.5); 2. HPLC pump (flow rate: 0.5 mL min^{-1}); 3. sample injector with a 500 µL loop; 4. SEC column (Superose 12 10/300 GL); 5. HPLC pump (flow rate: 0.1 mL min^{-1}); 6. substrate (0.5 mM 4-MUP in 0.1 M Tris-HNO_3 buffer solution, pH 8.5); 7. reaction coil (0.5 mm i.d. × 5 m long); 8. fluorescence detector at 430 nm; 9. ICP-MS; 10. waste.

range of 1–300 kDa, a reaction coil (0.5 mm i.d. × 5 m long), and a fluorescence detector (model 921-FP, Jasco). The substrate for enzymatic activity measurement was supplied to the sample flow after the SEC column by another HPLC pump (model PU-980). In addition, an ICP-MS instrument (model SPQ 8000A, SII Nanotechnologies, Chiba, Japan) was used to detect zinc in ALP, where the eluent from the fluorescence detector was supplied to the ICP-MS nebulizer through Teflon tubing.

Samples and Chemicals. Salmon egg cells were bought in a fish market, and they were washed with pure water repeatedly in the laboratory. Salmon egg cell cytoplasm was extracted from cells with a Teflon needle and a Teflon tweezer, and then, it was diluted 10-fold with 0.1 M Tris-HNO_3 buffer solution (pH 8.5) or with pure water, which was used for the enzymatic activity measurements involving ALP and for the detection of zinc by ICP-MS. The substrate, 4-methylumbelliferyl phosphate (4-MUP), was purchased from Sigma (St. Louis, Mo, U.S.A.). Standard proteins, such as β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa), which were used for molecular weight calibration of the SEC column, were also purchased from Sigma.

Enzymatic Activity Measurement for ALP. The enzymatic activity of ALP was measured using 4-MUP as a substrate.[5,6] The enzymatic reaction of ALP with 4-MUP is illustrated in Fig. 2, where the substrate (4-MUP) is converted to the product (4-methylumbelliferon, 4-MU). In the measurement of enzymatic activity of ALP in salmon egg cell cytoplasm, the cytoplasm solution that was diluted 10-fold with 0.1 M Tris-HNO_3 buffer solution (pH 8.5), or with pure water, was injected into the SEC column, and the eluent that passed through the SEC column was mixed with the substrate solution in the reaction coil at room temperature (ca. 25 °C). After the reaction coil, the fluorescence of 4-MU was detected at 430 nm (excitation wavelength: 360 nm). Furthermore, the concentration of water-soluble hydrophilic ALP in salmon egg cell cytoplasm was determined as an *E. coli* ALP-equivalent concentration by measuring the fluorescence intensity of *E. coli* ALP, in a similar manner to the method described above.

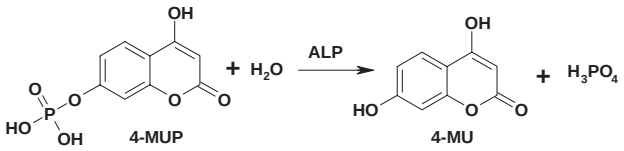

Fig. 2. The scheme of enzymatic reaction of alkaline phosphatase with 4-MUP. 4-MUP (4-methylumbelliferyl phosphate): substrate; 4-MU (4-methylumbelliferon): product.

In addition, a CHAPS-coated ODS column was used to collect the fraction of the hydrophilic proteins in salmon egg cell cytoplasm. The HPLC system using a CHAPS-coated ODS column was almost the same as that used in the previous work.[3–5] The separation conditions of the HPLC experiments with the CHAPS-coated ODS column were as follows: mobile phase, 0.1 M Tris-HNO_3 buffer + 0.2 mM CHAPS (pH 7.4); flow rate, 0.7 mL min^{-1}; sample injection volume, 500 µL.

Results and Discussion

Assay of *E. coli* Alkaline Phosphatase with Fluorescence Detection of Enzymatic Activity. The applicability of the present fluorescence detection system to the enzymatic activity measurement for ALP was first examined using *E. coli* ALP (MW 89 kDa). A peak was observed at a retention time of ca. 30 min in the fluorescence-detected chromatogram for ALP. According to the molecular weight calibration of the SEC column using several standard proteins, the retention time of the peak for *E. coli* ALP in the fluorescence-detected chromatogram corresponded to a MW of ca. 90 kDa, which is consistent with the molecular weight of *E. coli* ALP. Zinc was also detected at the same peak position for enzymatic activity of ALP. Therefore, the present fluorescence detection system can be used to assay the ALP in salmon egg cell cytoplasm.

Assay of ALP in Salmon Egg Cell Cytoplasm by Detections of Enzymatic Activity and Zinc. A typical fluorescence-detected chromatogram for salmon egg cell cytoplasm that was diluted 10-fold with 0.1 M Tris-HNO_3 buffer is shown in Fig. 3a. In Fig. 3a, two peaks were clearly observed at retention times of ca. 21 and 27 min, corresponding to MW $\geqq$ 300 and 100 kDa, respectively. These results suggest that at least two kinds of ALPs may exist in salmon egg cell cytoplasm.

In addition, the ^{66}Zn-detected SEC chromatogram for salmon egg cell cytoplasm is shown in Fig. 3b. A large broad peak was observed at a retention time of ca. 21 min, corresponding to the first peak in the fluorescence-detected chromatogram in Fig. 3a, while the second peak near 27 min in fluorescence-detected chromatogram (Fig. 3a) was not observed in the zinc-detected chromatogram. However, it is noticeable in Fig. 3b, that the broad Zn peak showed some peak tailing with a shoulder peak on the side of longer retention time. The broad Zn peak in Fig. 3b corresponded to the total zinc concentration in various zinc proteins including ALP in salmon egg cell cytoplasm.

From our previous work,[8] when cytoplasm was diluted more than 5-fold with pure water, or 0.01 M Tris buffer solution, proteins with larger MW (corresponding to the first peak at ca. 21 min in Fig. 3a) precipitated from the solution. This fact suggests that larger MW proteins have a large hydrophobicity,

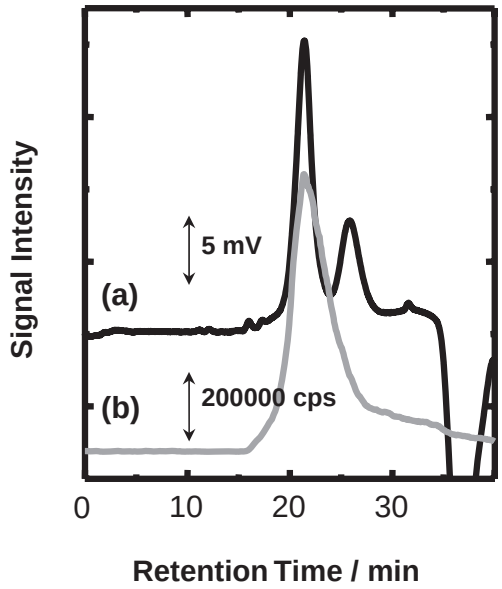

Fig. 3. SEC chromatograms for salmon egg cell cytoplasm with fluorescence- and ^{66}Zn-detection. (a) Fluorescence detection, (b) ICP-MS detection. Salmon egg cell cytoplasm was diluted 10-fold with 0.1 M Tris-HNO_3 buffer solution (pH 8.5). The separation and detection conditions for SEC were the same as in Fig. 1.

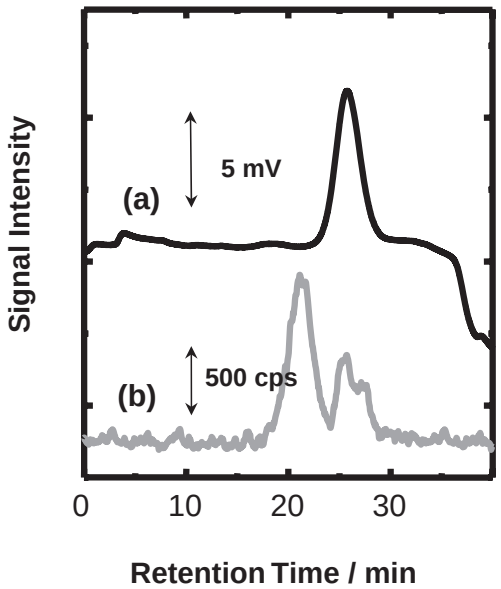

Fig. 4. SEC chromatograms for the protein fraction of water-diluted salmon egg cell cytoplasm with fluorescence- and ^{66}Zn-detection, after passing through a CHAPS-coated column. (a) Fluorescence detection, (b) ICP-MS detection. The separation and detection conditions for SEC chromatogram were the same as in Fig. 1. The protein fraction of cytoplasm used here was obtained as follows. Salmon egg cell cytoplasm was diluted by 10-fold with pure water, and filtered with a membrane filter (pore size 0.45 μm). Then, the filtrate was injected into the CHAPS-coated ODS column and only the water-soluble hydrophilic protein fraction was collected.

which causes them to precipitate during dilution with pure water, or dilute Tris-HNO_3 buffer solution (0.01 M). Thus, in the present experiment, the salmon egg cell cytoplasm was diluted 10-fold with pure water to eliminate the larger MW proteins, and a white precipitate that appeared in solution was eliminated by filtering with a membrane filter (pore size 0.45 μm). Furthermore, the filtrate was evaporated to one-tenth of the original volume.

As reported previously,[5–8] the CHAPS-coated ODS column can be used to separate large molecules, such as proteins, and small molecules/ions. Salmon egg cell cytoplasm contains large quantities of salts,[2,3] which often decrease the separation capability of the SEC column. The separation of proteins from inorganic ions by a CHAPS-coated ODS column improved the separation efficiency of SEC because of desalting.[5,8] In the present experiment, only the protein fraction from the concentrated filtrate from the cytoplasm was collected after separation with the CHAPS-coated ODS column, and it was analyzed using SEC.

In Fig. 4, the SEC chromatograms for the water-soluble protein fraction of salmon egg cell cytoplasm, which were obtained by (a) fluorescence detection and (b) ICP-MS detection, are shown. In the fluorescence-detected SEC chromatogram shown in Fig. 4a, the large peak observed at ca. 21 min in Fig. 3 disappeared, and only one peak, which had almost the same peak intensity as that at ca. 27 min in Fig. 3a, was observed at a retention time of ca. 27 min corresponding to a MW of ca. 100 kDa. In other words, the larger MW proteins with a large hydrophobicity were eliminated from the cytoplasm solution by water dilution. However, two peaks were still observed at retention times of ca. 21 and ca. 27 min in the ^{66}Zn-detected chromatogram in Fig. 4b, although the peak intensity at ca. 21 min was significantly smaller, compared to that in Fig. 3b. In Fig. 4, the second peak in the Zn-detected chromatogram was consistent with that in the fluorescence-detected chromatogram. Thus, the peak near 27 min was assigned to hydrophilic ALP. The first peak in Fig. 4b may be due to other zinc proteins, except for ALP, that remained even after water dilution, because they did not fluoresce.

The first peak in Fig. 3a observed near 21 min in the fluorescence-detected chromatogram was not present in Fig. 4a after water dilution of cytoplasm, while the second peak near 27 min in Fig. 3a was still observed in fluorescence detection in Fig. 4a. These experimental results indicate that the peak detected near 27 min in the present SEC chromatogram (Fig. 4) indicates the existence of hydrophilic ALP, because it was detected even after water dilution. On the other hand, some of ALP in salmon egg cell cytoplasm might exist as hydrophobic conjugated proteins, such as lipoproteins or glycoproteins, so that they were eliminated in dilution with pure water. ALP exists as conjugated proteins about 3-times more than hydrophilic ALP in salmon egg cell cytoplasm, which was estimated from comparison of the peak areas in Fig. 3a. Based on the reference,[11] the molecular weight of ALP from eukaryotes was estimated as 300 kDa from the SEC chromatogram, but it is not known whether this protein is hydrophilic or hydrophobic. However, the fact that ALP enzymatically hydrolyzes phosphate monoester as conjugated proteins is interesting, and further research to identify the structures of conjugated proteins of ALP is desirable.

The zinc peak at the retention time of about 27 min in Fig. 4b was split, which was repeatedly observed in the duplicate experiments. These results suggested that peak overlap between hydrophilic ALP (MW ca. 100 kDa) and other zinc proteins with MWs smaller than 100 kDa in salmon egg cytoplasm may occur. However, no other zinc proteins could be identified in the present experimental system because of poor further separation.

Concentration of Hydrophilic ALP in Salmon Egg Cell Cytoplasm. The concentration of hydrophilic ALP in salmon egg cell cytoplasm detected in Fig. 4a was determined by measuring the fluorescence intensity for *E. coli* ALP. Since the identity of the ALP in salmon egg cell cytoplasm was not available, *E. coli* ALP was used as the standard in the present experiment. Standard solutions of 0, 350, 700, and 1400 ng g^{-1} of *E. coli* ALP were prepared and analyzed by using the fluorescence detection system in Fig. 1. As a result, a linear calibration curve was obtained, and the concentration of hydrophilic ALP in salmon egg cell cytoplasm was estimated to be ca. 350 ng g^{-1} based on the fresh weight. As described previously, *E. coli* ALP and the ALP found in the present experiment were not the same proteins, because they have different retention times (ca. 30 and 27 min, respectively). Thus, the concentration of hydrophilic ALP determined in the above experiment should be considered to be an *E. coli* ALP-equivalent concentration.

Conclusion

In the present experiment, the existence of hydrophilic ALP with a MW of ca. 100 kDa and hydrophobic conjugated proteins of ALP with a MW $\geqq$ 300 kDa was confirmed by the enzymatic activity measurements together with Zn detection by ICP-MS. Here, since the MW of hydrophilic ALP found in salmon egg cell cytoplasm was ca. 100 kDa, 350 ng g^{-1} of the ALP corresponded to a concentration of 3.5×10^{-9} M, where the specific gravity of the cytoplasm solution that was diluted 10-fold with pure water was assumed to be 1.0 g mL^{-1}. Common ALP generally contains four Zn atoms per molecule, and therefore, the Zn content in the hydrophilic ALP in the cytoplasm was estimated to be 91.5 ng g^{-1}. Based on our previous work,[3] in which the concentration of Zn in salmon egg cytoplasm was 17900 ng g^{-1}, only 0.0052% of the Zn in salmon egg cytoplasm was contained in the hydrophilic ALP. This estimation is supported by comparing the small Zn peak in Fig. 4b with the large Zn peak in Fig. 3b. From these results, the Zn content in the hydrophilic ALP is extremely low, and so many other zinc proteins or enzymes, including conjugated ALP, exist in salmon egg cytoplasm.

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