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Structural and functional analyses of calcium ion response factors in the mantle of *Pinctada fucata*

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ABSTRACT

The pearl oyster, *Pinctada fucata*, is cultured for pearl production in Japan. The shell of the pearl oyster consists of calcium carbonate and a small amount of organic matrix. Despite many studies of the shell matrix proteins, the mechanism by which calcium elements are transported from the mantle to the shell remains unclear. Investigating the molecular mechanism of calcium transportation, we prepared artificial seawater with a high concentration of calcium ions (10ASW) to induce calcification in the pearl oyster. When pearl oysters were cultured in 10ASW, unusual nanoparticles were precipitated on the surface of the nacreous layer. SDS-PAGE and 2D-PAGE analyses revealed that some calcium-sensing proteins (Sarcoplasmic Ca-binding Protein (Pf-SCP) and Pf-filamin A) might be related to the synthesis of these nanoparticles. The recombinant proteins of Pf-SCP can bind to calcium ions and accumulate nanoparticles of calcium carbonate crystals. However, transcriptomic analysis of the pearl oysters grown in 10ASW showed that the matrix protein genes in the shell did not differ before and after treatment with 10ASW. These results suggest that, despite increasing calcium transportation to the shell, treatment with a high concentration of calcium ions does not induce formation of the organic framework in the shell microstructure. These findings offer meaningful insights into the transportation of calcium elements from the mantle to the shell.

1. Introduction

The shell of *Pinctada fucata* is more than 90% calcium carbonate, along with a small amount of organic matrix (Akira, 1995). The shell of *P. fucata* contains two layers, the nacreous and the prismatic, with completely different microstructures. They are made up of organic matrices that interact with inorganic substances to precisely regulate crystal morphology and orientation (Belcher et al., 1996; Weiner and Addadi, 1997). In the nacreous layer, thin aragonite tablets are stacked on thin organic membranes, whereas each calcite prism in the prismatic layer is surrounded by an intercrystalline organic framework in a honeycomb structure (Bevelander and Nakahara, 1969; Tong et al., 2002). The components of these layers, including inorganic and organic substances, are secreted from mantle epithelial cells (Marie et al., 2012). Between the shell and the mantle epithelial cells is a space filled with extrapallial fluid, which is separate from the external seawater but has a salinity almost identical to that of seawater (Koji, 1978). The inorganic and organic shell materials are secreted into the extrapallial fluid, and the organic matrix induces precipitation of the inorganic substances to form the shell. Many researchers have identified various organic compounds related to shell calcification and described their functions; for example, prismalin-14 (Suzuki et al., 2004), aspein (Isowa et al., 2012; Tsukamoto et al., 2004), and shematrin (Yano et al., 2006) are from the prismatic layer; nacrein (Miyamoto et al., 2005, Miyamoto et al., 1996), MSI60 (Asakura et al., 2006; Sudo et al., 1997), N16 (Metzler et al., 2010; Samata et al., 1999), N19 (Yano et al., 2007; Zhang and He, 2011), and Pif (Bahn et al., 2015; Suzuki et al., 2009) are from the nacreous layer.

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Furthermore, proteomic analysis of shell proteins, transcriptomic analysis of the mantle, and genomic sequencing of *P. fucata* have been performed. Proteomic analysis allowed identification of novel proteins, resulting in 9 non-specific proteins isolated from both the prismatic and the nacreous layers, 10 nacreous-specific proteins, and 16 prismatic-specific proteins (Liu et al., 2015). A transcriptomic and genomic sequence database indicated that *P. fucata* has more than 23,000 genes (Takeuchi et al., 2012). In addition, 700 proteins were manually annotated so far (Kawashima et al., 2013). Although many proteins and genes related to shell calcification have been analyzed, the mechanism and regulation of calcium response factors, such as calcium transportation from the mantle to the shell, remain unclear.

Previous reports have shown that calcium ions secreted from the mantle are derived from the external medium, whereas most of the carbon sources for bicarbonate ions and the organic matrices are derived from metabolic carbon dioxide (Robertoson, 1941). Research has also demonstrated that low-salinity seawater improves the quality of pearls, which have the same microstructure as nacre (Hayashi, 2008). Additionally, it has been reported that gene expression changes depending on the pH of the external seawater (Liu et al., 2012). Therefore, seawater conditions, especially the concentration of calcium ions, have major effects on shell calcification. We suggest that calcium absorption from the shell occurs when the external medium contains a low calcium level, whereas calcium emission onto the shell occurs when the calcium level is high, maintaining a constant blood level of calcium through regulation of calcium response factors related to shell calcification.

In this study, we reared *P. fucata* in artificial seawater containing a high calcium concentration to identify the calcium response factors. Rearing oysters in artificial seawater with a high calcium concentration has not been attempted previously because the calcium concentration in natural seawater (NSW) is already supersaturated, with calcium carbonate and calcium sulfate precipitating at higher calcium concentrations. Therefore, we decreased the concentrations of both bicarbonate and sulfate ions to prevent precipitation. Using a combination of SDS-PAGE and LC-MS/MS, we identified two proteins that respond to changes in the calcium concentration, whereas variations in the gene expression levels of *P. fucata* were comprehensively analyzed via next-generation sequence analysis.

2. Materials and methods

2.1. Culture of living P. fucata in the artificial seawater

The living pearl oysters, *P. fucata*, (the size is 5–6 cm) were transported from the Ago Bay (Mie Prefecture, Japan) by the Fisheries Research Division, Mie Prefectural Science and Technology Promotion Center, Japan. The living pearl oysters were cultured in the aquarium containing the normal artificial sea-water for one day at 20 °C. Then, 4–6 living pearl oysters were cultured in the 10 L aquarium containing the experimental artificial seawater with various concentrations of calcium ion for one week at 20 °C. The components of the experimental artificial seawater were shown in Table 1. After culture for one week, the shells were collected and washed by distilled water. On the other hand, live tissues were separated into each part and stored at −80 °C. The tissues were crushed and powdered in liquid nitrogen to extract the RNA or protein samples.

Only the shells from the pearl oyster were also incubated in the experimental artificial seawater for one week at 20 °C. After culture for one week, the shells were collected and washed by distilled water.

Table 1
Components of each seawater formula.

	NSW	1ASW	0ASW	10ASW
Cl^-	0.54	0.54	0.54	0.54
Na^+	0.46	0.46	0.46	0.25
SO_4^{2-}	0.03	0.03	0.03	0.01
Mg^{2+}	0.05	0.05	0.05	0.05
Ca^{2+}	0.01	0.01	**0.00**	**0.10**
K^+	0.01	0.01	0.01	0.01
				(M)

2.2. Observations of the shells by electron microscope

The washed shells from each condition were dried and attached on aluminum stages using a carbon tape. The microstructures of shell were observed with secondary electron images (SEI) at the acceleration voltage of 2–15 kV using the scanning electron microscope (SEM) (Hitachi S-4800). The samples were coated with Pt–Pd for high-resolution SEI recording. The cross sections of shell microstructure were prepared using Hitachi FB-2100 FIB system. Transmission Electron Microscope (TEM) observations were performed using a JEOL JEM-2010 TEM operated at 200 kV. All images and selected-area electron diffraction patterns were also recorded by the CCD camera (Gatan ESW-500W, Pleasanton, CA, U.S.A.).

2.3. Extraction of proteins from the mantle

The frozen mantle tissues from each condition were homogenized in the buffer (50 mM HEPES-KOH (pH 7.8)/420 mM KCl/0.1 mM EDTA/5 mM $MgCl_2$/20% glycerol) containing cOmplete mini, EDTA free (1 tablet/10 mL), and the buffer extract was collected. After concentration and desalting by ultrafiltration tube (M. W. 10,000 cut off), the protein concentration of each extract was measured and applied to SDS-PAGE and two dimensional-PAGE.

2.4. Identification of proteins

The protein extract solution was distilled using swelling buffer (8 M urea/4% CHAPS/18 mM dithiothreitol (DTT)/0.002% Coomassie Brilliant Blue (CBB)). Prior to isoelectric focusing, IPG strips (pH 4–7, 11 cm, GE Healthcare) were passively rehydrated with 200 μL of protein solution (1 mg/ml of protein concentration) in wells for 12 h. Isoelectric focusing was conducted using the following protocol: 500 V for 2 h, gradient voltage increase to 1000 V for 1 h, and gradient voltage increase to 6000 V for 2.5 h, 6000 V for 40 min, and IEF Parameters at 50 μA/strip (Ettan IPGphor, GE Healthcare). To prepare for the second dimension SDS-PAGE, strips were incubated in equilibration buffer (50 mM Tris–HCl (pH 8.8)/6 M urea/30% glycerol/1% SDS) for 15 min at two times, first with 0.25% DTT and second with 4.5% iodoacetamide. IPG strips were then placed on top of 12.5% polyacrylamide gels at 30 mA. Gels were subsequently stained with CBB or silver staining (Invitrogen, silver quest).

After visualization of protein bands, the intensely stained portion was excised and analyzed by LC-MS/MS. The gel bands or spots were cut into pieces and transferred into 1.5 mL tube. The pieces were vortexed with 50 μL of 50% acetonitrile in 100 mM ammonium bicarbonate to decoloration and washed with 100 mM ammonium bicarbonate. Then 100 μL of acetonitrile was added and kept stand for 15 min, tapping every 5 min. After discarding supernatant and drying pieces, the pieces were incubated with 50 μL of 10 mM DTT/100 mM ammonium bicarbonate at 56 °C for 60 min to be reduced. The supernatant was discarded and 50 μL of 55 mM iodoacetamide/100 mM ammonium bicarbonate for 45 min at room temperature to be alkylated. The pieces were washed by being incubated with 100 mM ammonium bicarbonate for 15 min and then 100% of acetonitrile for 15 min respectively twice. After discarding the supernatant and drying pieces, the pieces were incubated with 250 ng Trypsin Gold (Promega) and 10 μL of 50 mM ammonium bicarbonate for 6 h to digest the proteins. The supernatant was transferred to a new tube. 20 μL of 5% formic acid/50% acetonitrile was added to the pieces, followed by vortex for 20 min, and then the supernatant was transferred to the previous same tube. This