論文の内容の要旨

Proteomic analysis of shell matrix proteins in the pond snail *Lymnaea stagnalis*
(軟体動物 *Lymnaea stagnalis* の貝殻プロテオーム解析)

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Biomineralization is an essential process for the development of hard body parts, which are integral to many organisms. The acquisition of biominerals such as bones, teeth, and shells brought about great diversification of morphology and ecology among animals. Furthermore, biominerals, which often remain as fossils, provide important records of Earth history to understand the interactions, and the histories thereof, between the global environments and life. In order to clarify those interactions and histories, it would be necessary to understand the molecular mechanisms of biomineral formation. In molluscs, their shells have been intensively studied from the viewpoints of morphology, taxonomy, phylogeny, and biomineralization because they show a considerable diversity on the present Earth and have left a rich and continuous fossil record throughout the Phanerozoic. However, detailed molecular mechanisms of molluscan shell biomineralization have not been clarified yet. Molluscan shells consist of inorganic crystals of $CaCO_3$ and organic matters such as polysaccharides and proteins, collectively known as shell organic matrices, which are secreted from the mantle tissue. Recent advances of analytical techniques brought even more drastic changes in our understanding of shell matrix proteins (SMPs). Proteomic analyses combined with genomic and transcriptomic analyses made it possible to almost comprehensively characterize protein sequences contained in biominerals, but a new question has arisen: do all those literally hundreds of SMPs contained in one kind of biomineral have a function in

biominerarization?

In Chapter 2 of this study, I identified potentially important SMPs, among other SMPs, by exploiting the asymmetric shell growth of snails. Formation of an asymmetric shell would require laterally asymmetric expression of SMP genes in the mantle tissue. I examined the expression levels of the 35,951 transcripts expressed in the left and right sides of the mantle tissue in the dextral pond snail, *Lymnaea stagnalis*. This transcriptome dataset was used to identify 207 SMPs by LC-MS/MS. A total of 32 out of the 207 SMP genes show asymmetric expression patterns in the transcriptome data, and they were further verified using quantitative PCR analysis, resulting in identification of four asymmetric genes out of those 32 SMP genes. Among the asymmetrically expressed SMPs in dextral snails in the transcriptome analysis or in the combined transcriptome and qPCR analysis, those that are more highly expressed in the left side than in the right side are three times more numerous than those that are more highly expressed in the right than in the left, suggesting importance of inhibitory roles of SMPs in shell formation. This observation was unexpected because it was assumed that a dextrally coiled shell is produced by a greater shell precipitation on the right than the left side of the mantle, and that more shell precipitation-promoting SMPs would be expressed in the right than in the left.

The 32 SMPs identified have distinctive features, such as conserved domains and low complexity regions, which may be essential in biomineralization. One of the SMPs that showed higher expression in the left than in the right, in both transcriptomic and qPCR analyses (Ls-SMP-88), showed a significant sequence similarity to Pif, an SMP originally isolated from the pearl oyster *Pinctada fucata*. Ls-SMP-88 contains two chitin-binding domains (ChtBD2) and an extracellular domain (Laminin_G) as in Pif, but, it has no von Willebrand factor type A domain (VWA), which is involved in protein binding, and is always found in Pif. Phylogenetic analysis of ChtBD2 and Laminin_G domain sequences indicated that Ls-SMP-88 is closely related to Pif of bivalvia, and that it originated as Pif, but lost the VWA domain subsequently. In pearl oysters, Pif binds aragonite crystals and promotes nacre formation. Although functions of Ls-SMP-88 have yet to be clarified, one possibility is that the loss of the VWA domain led to loss of shell formation-promoting roles, and acquisition of inhibitory roles instead. These results suggest that a dextrally coiled shell is produced by inhibition of shell precipitation on the left. Inhibitory roles of SMPs have long been recognized, and they could be at work in the process of coiled shell formation.

In Chapter 3, focusing on the laterality of the coiling direction of the snail, comparative proteomic analyses between the dextral and sinistral strains of *L. stagnalis* have been performed. *L. stagnalis* shows two types of shell coiling, namely, dextral (wild type) and sinistral (mutant type), and they have a mirror-symmetrical relationship with each other. In order to minimize the differences in genetic background between the dextral and sinistral

strains, those two strains were crossed, and then each of the dextral and sinistral strains was established once again before being subjected to proteomic analyses. In the proteomic analyses, about 100 individuals each of the dextral and sinistral shells were sampled and provided for the comparative analysis. As a result, a total of 443 SMPs have been identified. The 443 SMPs include all the 207 SMPs identified in the dextral strain in Chapter 2.

The comparisons of protein repertoires between the dextral and sinistral shells indicated no difference between them, but relative abundance of the proteins contained in the shell was different for some proteins: the most abundant SMP in the dextral shells, comp88734_c0_seq1, is 3.13 times more abundant in the dextral than in the sinistral shells, while the most abundant SMP in the sinistral shells, comp88616_c0_seq1, is 2.88 times more abundant in the sinistral than in the dextral snail. These results suggest that the abundance profiles of SMPs in each of the dextral and sinistral shells are not simple mirror images. This would mean that the lateral asymmetry in *L. stagnalis* is not a simple matter of asymmetry determination at the very early developmental stage, but involves some "maintenance mechanisms", which result in different expression profiles of SMP encoding genes in the mantle between dextral and sinistral strains in late developmental stages. The fact that the shell shapes are not exact mirror images between dextral and sinistral shells is consistent with this hypothesis. The exact mechanisms, including the gene regulatory pathways of SMP expression need to be elucidated by further analysis.

Furthermore, the expression levels of the 39,069 mantle transcripts have been compared between the right and left sides of mantle tissues in the sinistral strain of *L. stagnalis*. Contrary to expectation, the expression patterns of SMP genes in the sinistral strain did not mirror the asymmetric pattern observed in the dextral strain shown in Chapter 2. Only one SMP (comp153562_c0_seq1) gene indicated a statistically significant difference in expression level between the right and left sides of the mantle, with higher expression in the left (outer side of the sinistral shell) than in the right (axial side of the sinistral shell). The SMP comp153562_c0_seq1 has two EFh (EF-hand) domains which can interact with cations. This SMP was identified in the dextral shells, but was found to show no significant difference in the expression levels of the transcripts between the right and left sides in the dextral snails. Instead, Ls-SMP-61 and Ls-SMP-62, which are distinct from comp153562_c0_seq1, but possess components of conserved domains similar to those of comp153562_c0_seq1, have been identified in the 32 asymmetric SMPs in the dextral strain discussed in Chapter 2, and are expressed higher in the left than in the right in the dextral snails. These observations suggest that a similar (but not the same) set of SMPs control some aspects of shell formation in dextral and sinistral strains. It is notable that, although the dextral strain and sinistral strains have almost the same genome background, they indicate distinctly different expression profiles of SMP genes.

In order to understand the functions of SMPs in the future, it is necessary to analyze the *in vivo* functions of SMPs using genome editing techniques such as CRISPR/Cas9. The SMPs which have been narrowed down as potentially important in this study provide the first candidates to be analyzed in those *in vivo* functional analyses. In this context, investigations of not only the expression profiles of SMP genes but also the arrangements of SMPs in the shell and the relationships between the SMPs and the shell microstructures will be essential. In future, evolution of the molluscan shell morphology may be understood as a history of changes in the developmental programs of the shell. To this end, the lateral asymmetry of shell morphology, on which this study put a focus, provides a unique foothold for elucidating the molecular mechanisms of shell formation. It is hoped that further studies focusing on this aspect help understand the nature of hard tissue formation and evolution.