Studies on the Chemical Structures of Organic Matrices and Their Functions in the Biomineralization Processes of Molluscan Shells

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Abstract
Molluscan shells protect the soft body from predators and the external environment and consist of calcium carbonate in an organic matrix. The interaction between calcium carbonate and the organic matrix forms the microstructure of the molluscan shell. In this review, we discuss several organic molecules that may be important in the formation of the shell microstructure. The iridescent color of pearls is attributed to the characteristic nacreous microstructure of molluscan shells. The Japanese pearl oyster, *Pinctada fucata*, is used in pearl aquaculture in Japan. The shell of *P. fucata* consists of two layers, prismatic and nacreous. Prismalin-14 in the prismatic layer interacts with calcium carbonate and binds to chitin. Pif in the nacreous layer interacts with aragonite crystals and plays important roles in forming the organic framework in a compartment-like structure. In contrast, limpets have a crossed lamellar microstructure in their shells. The organic matrices of limpet shells induce the formation of spindle-like aragonite crystals. Recent studies have increased our understanding of the calcification process of molluscan shells, and the findings can be applied to increase yields of high-quality pearls, lowering the cost and energy of pearl aquaculture.

1. Introduction
Mollusc shells are composed of calcium carbonate (more than 90% by mass) and a small amount of an organic matrix (0.01–5% by mass) (Lowenstam and Weiner 1989). Calcium carbonates exhibit three major crystal polymorphs, calcite, aragonite, and vaterite, in addition to an amorphous form (Lippmann 1973). Calcite is the most stable polymorph of the three under ambient conditions, whereas aragonite is metastable, and vaterite is the most unstable polymorph. Most molluscan shells consist of aragonite and (or) calcite, whereas vaterite is rarely found.

The shell of the Japanese pearl oyster, *Pinctada fucata*, consists of two mineralized layers, nacreous and prismatic (Fig. 1A). Both layers are composed of calcium carbonate and an organic matrix. In the course of shell formation, the periostracum, which is not mineralized and covers the external surface of the shell, is formed first; subsequently, the prismatic layer is formed on the periostracum. Finally, the nacreous layer is formed on the prismatic layer (Checa 2000). The nacreous layer is made of tablets of aragonite single crystals. The aragonite crystal compartment in the nacreous layer is sandwiched between sheets of organic matrix. In contrast, the prismatic layer is composed of columnar calcite surrounded by the organic matrix. The calcite crystals, surrounded by the organic framework, are oriented on the c-axis perpendicular to the shell surface.

The microstructure of the shell of the limpet, *Lottia kogamogai*, consists of five distinct layers stacked in...
the direction of shell thickness. The five layers were termed M+3, M+2, M+1, M, and M-1 from outside to inside, where M means myostracum (Fig. 2A) (MacClintock 1967; Fuchigami and Sasaki 2005; Suzuki et al. 2010). The outermost M+3 layer consists of calcite with a mosaic structure, granular submicron sub-grains with small-angle grain boundaries. The M+2 layer consists of flat prismatic aragonite crystals with a leaf-like cross section. The prismatic crystals are surrounded by dense organic sheets forming a compartment-like structure. The M+1 and M-1 layers have a crossed lamellar microstructure consisting of flat aragonite prisms with a rectangular cross-section (Figs. 2B, C). The M layer has a prismatic structure of aragonite perpendicular to the shell surface, with irregularly shaped cross-sections formed by the adductor muscle.

The shell is in contact with the mantle, which supplies the periostracum and calcified layers with inorganic ions and the organic matrix through the extrapallial fluid (Petit et al. 1980; Waller 1980). The organic matrix of mollusc shells contains lipids, proteins, and polysaccharides, such as chitin. Crystallization in the shell, including that of organic matrices, produces an elaborate microstructure with micro- or nano-scale structures (Boggild 1930; Taylor and Kennedy 1969; Carter 1990; Carter and Hall 1990). Organic-inorganic microstructures reduce the area of the cleavage plane in the crystals and impart high mechanical strength and toughness to the shell (Kamat et al. 2000; Okumura et al. 2012). Previous reports have suggested that the organic matrices in the shell play key roles in regulating crystal nucleation, polymorphy, growth, morphology, and orientation in the calcification process (Lowenstam and Weiner 1989). A previ-

Fig. 1. Shell microstructures of P. fucata. (A) The shell of P. fucata. Black arrow indicates the nacreous layer and white arrow indicates the prismatic layer. (B) SEM image of the prismatic layer. (C) Schematic representation of the microstructure in the prismatic layer. (D) SEM image of the nacreous layer. (E) Schematic representation of the microstructure in the nacreous layer.
Fig. 2. Shell microstructures of limpets. (A) The shell of *L. kogamogai*. The left picture shows the inner side of the shell and the right picture showed the side view. The white rectangles of 1, 2, 3 and 4 indicate the positions of M+3, M+2, M+1 and M-1 layers, respectively. Reprinted from *Journal of Structural Biology*, 171, Suzuki M, Kameda I, Sasaki T, Saruwatari K, Nagasawa H, Kogure T. Characterization of the multilayered shell of a limpet, *Lottia kogamogai* (Mollusca: Patellogastropoda), using SEM-EBSD and FIB-TEM techniques, 223–230, Copyright 2010, with permission from Elsevier. (B) SEM image of the crossed lamellar microstructure from *C. rota*. White rectangles show the first-, second-, and third-order lamellae. (C) Schematic representation of the hierarchical organization of the crossed lamellar microstructure. Reprinted with permission from *Crystal Growth & Design*, 11, Suzuki M, Kogure T, Weiner S, Addadi L. Formation of aragonite crystals in the crossed lamellar microstructure of limpet shells, 4850–4859, http://pubs.acs.org/doi/abs/10.1021/cg2010997, 2011. Copyright 2011 American Chemical Society.
ous study reported that organic matrices extracted from the nacreous layer induce the formation of aragonite crystals under ambient conditions (Watabe and Wilbur et al. 1960). Another study showed that organic matrices in the mollusk shell contain many Asp residues (Weiner and Hood 1975). This led to the hypothesis that the carboxyl group of Asp in the β-sheet structure induces the formation of metastable aragonite crystals (Addadi and Weiner 1985; Weiner and Addadi 1991). To be understood the function of the organic matrix, many researchers have examined the organic matrices of mollusk shells and mantle tissue. The rapid development of molecular biology since the mid-twentieth century has assisted in determining the entire sequence of matrix proteins found in the mollusk shell. Exhaustive analyses (e.g., proteome, transcriptome, and whole genome analyses) of mollusk shell formation have only recently begun (Joubert et al. 2010; Berland et al. 2011; Marie et al. 2010, 2011a, 2011b, 2011c). To reveal the mechanism of formation of the shell microstructure, we identified key organic molecules using functional analysis. In this review, we discuss the identification of functional organic molecules from the prismatic and nacreous layers of P. fucata and the organic matrices of the crossed lamellar microstructure of limpet shells.

2. The prismatic layer in the pearl oyster

2-1. Microstructure

The prismatic layer of P. fucata, which is the outermost layer in several bivalves, consists of columnar calcite crystals (Lutts et al. 1960; Mann 2002) (Figs. 1B, C). The columnar calcite crystals are surrounded by a thick organic compartment wall (Gregoire 1961; Kennedy et al. 1969; Wada 1961). The crystallographic c-axis of each crystal is perpendicular to the shell surface (Checa et al. 2005; MacDonald et al. 2010; Olson et al. 2013). Previous studies have indicated that each prism is not a single crystal but consists of subunits (Watabe and Wada 1956; Wada 1961; Dauphin et al. 2003). Sinuous boundaries on the etched surfaces of the calcite crystals were observed in the prismatic layer of P. margaritifera, suggesting that they are organic membranes that divide the calcite prism into domains. Electron microscopic observations have shown that the calcite crystals in the prismatic layer are made of a rounded “cortex” less than 200 nm in size surrounded by organic material. There are several degrees of misorientation between subunits of the calcite crystal (Okumura et al. 2010). The misorientation is mainly rotated around the c-axis and separates sub-grains of a few hundred nanometers divided by small-angle grain boundaries, which are probably the origin of the gradual change in crystal orientation inside the domains.

2-2. Organic matrices

To identify the key organic molecules for the formation of the prismatic layer, the acid-insoluble and SDS/DTT-soluble fraction of the prismatic layer was applied to SDS-PAGE (Suzuki et al. 2004). The stains-all staining, which stains acidic proteins blue, revealed a blue band around 14 kDa that represents the specific protein in the prismatic layer. The 14 kDa protein was purified using reverse-phase HPLC and named prismalin-14. N-Terminal amino acid sequence analysis of prismalin-14 did not show any amino acid residues, suggesting that the N-terminus was blocked. We then treated prismalin-14 with pyroglutamate aminopeptidase. The mass of the resulting product after digestion decreased from 11890.8 to 11784.2. The difference in mass values and the substrate specificity of the enzyme indicated that the N-terminal amino acid residue was pyroglutamate. N-Terminal amino acid sequence analysis of the digested product unambiguously identified 34 amino acid residues. To determine the amino acid sequence of the remaining part of prismalin-14, it was digested separately with trypsin, thermolysin, and pepsin. The entire amino acid sequence of prismalin-14 was determined by the amino acid sequencing of digested fragment peptides. Thus, prismalin-14 is composed of 105 amino acid residues and contains only 11 types of amino acid. There are many hydrophobic residues in the central part and many hydrophilic residues in both termini. Prismalin-14 has pyroglutamate at the N-terminus, four tandem PIYR repeats from Pro32 to Arg48, a Gly/Tyr (GY)-rich region from Tyr51 to Gly97, and two Asp-rich regions in the N- and C-terminal sections (Fig. 3A). To determine the interaction between calcium carbonate and prismalin-14, we measured the inhibition of the precipitation of calcium carbonate. The formation of calcium carbonate precipitates was monitored by recording the turbidity of a solution containing 100 μl of 20 mM NaHCO₃ (pH 8.7) and 10 μl of sample solution after the addition of 100 μl of 20 mM CaCl₂ to the solution. Changes in the turbidity of the solution were measured every 1 min for 5 min by absorbance at 570 nm using a spectrophotometer. Prismalin-14 inhibited calcium carbonate precipitation in a dose-dependent manner, with complete inhibition at a concentration of 2.0 μM.

To reveal the structure-function relationship of prismalin-14, we prepared five recombinant peptides: recombinant prismalin-14 (rprismalin-14), ΔN, ΔNAC, PIYR, and GY (Suzuki and Nagasawa 2007). Rprismalin-14 has a sequence consisting of an N-terminal alanine and the full sequence of natural prismalin-14 containing all four domains. ΔN has a natural prismalin-14 sequence from positions 29 to 105 containing the PIYR repeats and GY-rich and C-ter-
minal regions. ANAC has an N-terminal alanine and natural prismalin-14 sequence from positions 20 to 97 containing the PIYR repeats and GY-rich regions. PIYR has an N-terminal alanine and natural prismalin-14 sequence from positions 1 to 50 containing the N-terminal and PIYR repeat regions. GY has an N-terminal alanine and natural prismalin-14 sequence from positions 51 to 105 containing the GY-rich and C-terminal regions. The inhibitory activity of these peptides on calcium carbonate precipitation and chitin-binding activity were measured (Figs. 3B, C). The measurements of inhibitory activity on calcium carbonate precipitation showed that AN showed lower activity and ANAC showed almost no inhibitory activity at the same concentration. These results suggest that both the N- and C-terminal Asp-rich regions are related to inhibitory activity on calcium carbonate precipitation in vitro. To measure the chitin-binding activity, the sample solution was mixed with chitin powder. After incubation for binding, the mixture of the sample solution and chitin powder were successively washed with distilled water, 0.2 M NaCl, 1.0 M acetic acid and 2% SDS in

Fig. 3. Structural and functional analyses of prismalin-14. Amino acid sequences of natural prismalin-14 and its related peptides are shown in (A). The nucleotide sequence data reported was registered to the DDBJ Nucleotide Sequence Databases under the accession number AB159512. rprismalin-14: N-terminal alanine and 105 amino acids of natural prismalin-14 containing all the four domains. AN: natural prismalin-14 from position 29–105 containing the PIYR, GY-rich and C-terminal regions. ANAC: N-terminal alanine and natural prismalin-14 from position 20–97 containing the PIYR and GY-rich regions. PIYR: N-terminal alanine and natural prismalin-14 from position 1–50 containing the N-terminal and PIYR regions. GY: N-terminal alanine and natural prismalin-14 from position 51–105 containing the GY-rich and C-terminal regions. PQ represents pyroglutamic acid. (B) Inhibitory activity on calcium carbonate precipitation assay for each truncated peptide. Changes in the turbidity of the assayed solutions are investigated. Diamond: 0.05% NH4HCO3 alone; cross: 2.0 μM ANAC; triangle: 2.0 μM AN; and square: 2.0 μM rP-14. Results are expressed as means ± S.E.M. (n = 3). (C) Chitin-binding assay for each fragmentary peptide. Lane 1, water-washings; lane 2, 0.2 M NaCl-washings; lane 3, 1.0 M acetic acid-washings; lane 4, extract with SDS/2-mercaptoethanol. BSA was used as a negative control. Reprinted with permission from FEBS Journal, 274, Suzuki M, Nagasawa H. The structure-function relationship analysis of Prismalin-14 from the prismatic layer of the Japanese pearl oyster, Pinctada fucata, 5158–5166, 2007. Copyright 2007 FEBS.

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Chitin is a polysaccharide consisting of N-acetyl-D-glucosamine (Fig. 4A), and it is used as the body scaffold in many invertebrates. Although chitin-binding activity in the GY-rich region of prismalin-14 was demonstrated, chitin had not yet been identified in the prismatic layer of *P. fucata*. We identified chitin in the prismatic layer of *P. fucata* using a combination of Calcofluor White staining with IR and NMR spectral analyses (Figs. 4B, C) (Suzuki et al. 2007).

We also identified a chitin synthase gene (PfCHS1) from the mantle tissue of *P. fucata* (Suzuki et al. 2007). The PfCHS1 cDNA consisted of 7636 bp, comprising a 5′-UTR (238 bp), an ORF (6,828 bp), a stop codon (TAG), and a 3′-UTR (567 bp) (Fig. 5). The deduced amino acid sequence of PfCHS1 was analyzed using computer programs. The calculated molecular mass and pI of the protein were about 264 kDa and 6.39, respectively. In general, chitin synthase consists of three domains. The N-terminal region, or A domain, has eight transmembrane segments. The central region, or B domain, generally has a catalytic function transferring N-acetyl-D-glucosamine to the chitin chain. The C-terminal region, or C domain, has seven transmembrane segments and two coiled-coil formations. These three domains were conserved in PfCHS1. In addition to these three domains, a homology search indicated that the N-terminal domain (1–758 a.a.) of PfCHS1 has high similarity with the myosin head domain of ScunM, unconventional myosin obtained from scallop mantle tissue, and ArCHS1 identified from *Atrina rigida*, a chitin synthase gene. This myosin head domain is thought to interact with actin in cytoskeletal function (Fujiiwara et al. 1997; Park et al. 1999; Takeshita et al. 2005).

3. The nacreous layer in the pearl oyster

3-1. Microstructure

The nacreous layer in molluscan shells demonstrates pearly optics and marvelous mechanical stiffness,
which originate from the regular alternation of organic matrix sheets and platy aragonite crystals (Figs. 1D, E). The framework of the organic sheet consists of chitin and proteins. According to TEM observations and XRD measurements, the platy aragonite crystals are composed of single crystal aragonite tablets with the c-axes perpendicular to the shell surface (Chateigner et al. 2000). A previous study used an epitaxial nucleation model of the organic sheet to align the crystal orientation (Weiner and Traub 1984). In this model, acidic matrix proteins on the organic sheet interact with amorphous calcium carbonate (ACC) and induce development of aragonite tablets (Addadi et al. 2006). However, it is difficult to understand the mechanism of formation of aragonite tablets using only this model. Previous reports showed many pores in the organic sheets. Pores called mineral bridges connect the upper and lower aragonite tablets separated by organic sheets. A previous study showed that the mineral bridge in gastropods penetrates the center of aragonite tablets (Checa et al. 2009), whereas the mineral bridge in bivalves is formed from the contact point of two aragonite tablets (Checa et al. 2011). An earlier study showed that nano-sized aragonite crystals nucleate in random crystallographic orientation inside dimples on the surface of the organic matrix that covers the outer prismatic calcite of the prismatic layer (Saruwatari et al. 2009). The dimples are filled with horn-like aragonite crystals that grow and become the initial nacreous layer, indicating that geometric selection and mineral bridges regulate the crystal orientation of the initial aragonite tablets.

3-2. Organic matrices

A previous study showed that organic matrices extracted from the nacreous layer induced aragonite crystal formation in vitro (Watabe and Wilbur 1960). The predicted transmembrane segments are underlined with a solid line. The catalytic site (QRRRW) of the chitin synthase domain is boxed in black. The deduced amino acid sequence of PfCHS1. Conserved motifs of the myosin head domain are boxed with a solid line. The predicted transmembrane segments are underlined with a solid line. The potential coiled-coil structures are underlined with a dotted line. The catalytic site (QRRRW) of the chitin synthase domain is boxed in black. The conserved domains in the myosin head domain are boxed. The nucleotide sequence data was registered to the DDBJ Nucleotide Sequence Databases under the accession number AB290881 (from Suzuki et al. 2007).
residues in the matrix proteins interact with calcium atoms in calcium carbonate. Other studies have implied that some macromolecules in the nacreous layer play important roles in the formation of aragonite crystals (Belcher et al. 1996; Falini et al. 1996; Gotliv et al. 2003; Addadi et al. 2006). Although a number of matrix proteins have been identified from various molluscan shells (Marin et al. 2008; Suzuki and Nagasawa 2013), Asp-rich acidic macromolecules had not been identified from the nacreous layer. To identify the aragonite-inducing factor in the nacreous layer, we searched for an aragonite-specific binding protein from the nacre using a calcium carbonate-binding assay. The calcium carbonate-binding assay showed that an 80 kDa protein (Pif 80) isolated using SDS-PAGE specifically bound to aragonite crystals (Suzuki et al. 2009). cDNA cloning of Pif 80 revealed that the upstream region of the cDNA sequence encoded another unknown 97 kDa protein (Pif 97) in SDS-PAGE (Figs. 6A, B). Both Pif 80 and Pif 97 are acidic proteins with calculated isoelectric point (pI) values of 4.99 and 4.65, respectively. Pif 97 consists of 525 amino acid residues and has two conserved domains, a von Willebrand type A (VWA) domain for protein-protein interaction (Tuckwell 1999; Whittaker and Hynes 2002) and a chitin-binding domain similar to that of Peritrophin A (Elvin et al. 1996; Shen and Jacobs-Lorena 1998; Suetake et al. 2000). Pif 80 contains many charged amino acid residues: Asp (14.9%), Glu (6.5%), Lys (11.1%), and Arg (5.0%). Pif 80 has more charged amino acid residues, Asp (28.5%), Glu (4.1%), Lys (18.7%), and Arg (10.9%), than Pif 97. Pif 80 has many repeat motifs consisting of four amino acid residues, Asp-Asp-Arg (Lys)-Lys (Arg), scattered throughout its sequence. Pif 80 and Pif 97 are sepa-

![Fig. 6. Amino acid sequence analysis of Pif. (A) The complete deduced amino acid sequence of Pif. Pif 97 consists of 525 amino acids (from 23 to 547 a.a.). Pif 80 consists of 460 amino acids (from 548 to 1007 a.a.). Orange box is a signal peptide (from 1 to 22 a.a.). Blue box indicates the VWA domain. Purple box is RMKR (a Kex2-like proteinase cleavage site). Green box is the aragonite-binding protein (Pif 80). Red letters indicate acidic amino acid residues. Blue letters indicate basic amino acid residues. Thin red underline indicates the sequence of Asp-Asp-Arg (Lys)-Lys (Arg), and the thick one indicates the cluster of acidic amino acid residues. The nucleotide sequence data was registered to the DDBJ Nucleotide Sequence Databases under the accession number AB236929. (B) Schematic representation of the deduced amino acid sequence of Pif. The orange box is a signal peptide. The blue box indicates the VWA domain. The yellow box is the Peritrophin A-type chitin-binding domain. RMKR is a Kex2-like proteinase cleavage site. The green box is the aragonite-binding protein (Pif 80). Scale bar showed 100 amino acids. From Science, 325, Suzuki M, Saruwatari K, Kogure T, Yamamoto Y, Nishimura T, Kato T, Nagasawa H. An acidic matrix protein, Pif, is a key macromolecule for nacre formation, 1388–1390, 2009. Reprinted with permission from AAAS.](image-url)
rated at the dibasic site of the C-terminal end of Pif 97 cleavable by a Kex2-like proteinase.

To clarify the function of Pif \textit{in vivo}, knockdown of Pif gene expression by RNA interference (RNAi) was performed. The expression level of the group injected with 30 mg of Pif dsRNA was suppressed to approximately 40% that of the phosphate-buffered saline (PBS)- or green fluorescent protein (GFP) dsRNA-injected group. The surface structure of the nacreous layer in each injection group was observed using SEM. The normal orderly structure of the nacreous layer was observed in the PBS and GFP dsRNA-injected groups (Fig. 7A), whereas disordered growth of the nacreous layer was observed in the Pif dsRNA-injected group, indicating that Pif plays important roles in forming the microstructure of aragonite tablets in the nacreous layer (Fig. 7B). In cross-sections of Pif dsRNA-injected groups, elongated aragonite crystals were observed on the nacre surface, suggesting that Pif is an essential component of the organic framework of the nacreous layer.

To reveal the mechanisms of Pif association with calcium carbonate crystallization, an \textit{in vitro} calcium carbonate crystallization experiment was performed. The acid-insoluble and SDS-soluble organic matrices from the nacreous layer were separated into four fractions using gel filtration HPLC. The fraction containing Pif 80, Pif 97, and the N16 complex was applied to a chitin-coated glass plate and incubated in a calcium carbonate-supersaturated solution. Aragonite and vaterite crystals were formed from this solution. A thin cross-section of one of the aragonite crystals was prepared with a focused ion beam (FIB) system. TEM observation of the inner structure showed that the crystal was formed between the chitin membrane and the glass plate and that the single crystal had a c-axis per-

Fig. 7. Functional analyses of Pif. (A) and (B) show the SEM images of the nacreous surface from knockdown experiments of the Pif gene using RNAi. PBS injection of negative control (A) and 30 \( \mu g \) of Pif dsRNA injection (B). (C) A cross section of calcium carbonate crystal, that was formed with nacre matrix proteins complex including Pif, was observed with TEM. The crystal was formed in the space between the chitin membrane and the glass plate. C indicated chitin and G indicate glass plate. (D) The electron diffraction pattern of the gray circle area in (C), indicating a single aragonite crystal and its c-axis perpendicular to the glass plate. From \textit{Science}, \textbf{325}, Suzuki M, Saruwatari K, Kogure T, Yamamoto Y, Nishimura T, Kato T, Nagasawa H. An acidic matrix protein, Pif, is a key macromolecule for nacre formation, 1388–1390, 2009. Reprinted with permission from AAAS.
Fig. 8. Pif molecules in various molluscan species. Schematic representation of each Pif molecule from various species. White box, signal peptide; bright red box, VWA domain; blue box, chitin-binding domain; black box, aragonite-binding protein; purple box, aragonite and calcite-binding protein; gray box, the homologous sequence with aragonite-binding protein; green box, former laminin G domain; red box, latter laminin G domain; asterisk, dibasic cleavage site. Pif, Pif from *P. fucata*; pmPif, Pif homologue from *P. margaritifera*; pmxPif, Pif homologue from *P. maxima*. ppPif, Pif homologue from *P. penguin*. BMSP (Blue Mussel Shell Protein), Pif homologue from *M. galloprovincialis*. LG233460, Pif homologue from the genome database of *L. gigantea*.

Perpendicular to the glass plate (Figs. 7C, D). A possible formation mechanism of such an aragonite crystal is as follows: first, the Pif complex binds to chitin and accumulates calcium carbonate inside the chitin membrane. Then, similar to the mineral bridges, the crystal orientation of the calcium carbonate precipitated in the chitin membrane is aligned in one direction perpendicular to the glass plate.

If Pif were a key organic molecule in the formation of the nacreous microstructure, Pif molecules would have to be conserved in various molluscan species. We used a homology cloning and calcium carbonate-binding assay to identify homologs of Pif from *Pinctada margaritifera*, *Pinctada maxima*, *Pteria penguin*, *Mytilus galloprovincialis*, and *Lottia gigantea* (Suzuki et al. 2011a, 2013). *P. margaritifera* and *P. maxima* are used for pearl aquaculture in some tropical countries and are often included in the same genus as *P. fucata*. *Pteria penguin* is also used for pearl aquaculture in tropical countries and is closely related to *P. fucata*. *M. galloprovincialis* has a disordered nacreous layer and is closely related to *P. fucata*. *L. gigantea*, a gastropod, is phylogenetically distant from bivalves (*Pinctada*, *Pteria*, and *Mytilus*). We found Pif homologs in all five species. The VWA and chitin-binding domains are conserved in all Pif homologs, whereas the amino acid sequences of the Pif 80 regions vary widely among species. Sequence alignment of all Pif homologs revealed the presence of a novel, significantly conserved sequence between the chitin-binding domain and the C-terminus of Pif 97. This conserved sequence contained the N-terminal region of the laminin G domain (Talts et al. 1998; Talts and Timpl 1999; Smirnov et al. 2002). Further examination of the Pif 80 regions revealed that the C-terminal parts of the laminin G domains in the genus *Pinctada* and *P. penguin* are located at the center of the Pif 80 sequences (Fig. 8). The laminin G domain is a calcium ion-mediated receptor for steroids, integrins, heparin, and α-dystroglycan and is involved in interactions with extracellular matrix proteins (Tryggvason 1993; Yurchenco et al. 1993; Yu and Talts 2003). The calcium-binding activity of the laminin G domain may be responsible for the calcium carbonate-binding activity of Pif 80.

### 4. The crossed lamellar microstructure in limpets

#### 4.1. Microstructure

The crossed lamellar microstructure has a complex hierarchical structure with first-, second-, and third-order lamellae (Wilmot et al. 1992; Kobayashi and Akai 1994; Su et al. 2004; Suzuki et al. 2010) (Fig. 2C). The third-order lamellae are composed of aragonite crystals up to several micrometers in length and about 100–200 nm in cross-section. The thin prisms of the third-order lamellae are aligned in parallel to form plates (second-order lamellae) inclined to the shell surface. The inclined plates are, in turn, stacked to form...
thick blocks, which may be hundreds of micrometers long (first-order lamellae). These blocks are arranged in layers where the planes of the lamellae are rotated in orientation. According to recent observations, much smaller building blocks within the crystal (100 nm thick, 200–300 nm wide, and 100–200 nm long), possibly created by frequent (110) twinning, are a further subdivision of the third lamellar crystals into fourth-order lamellae (Dauphin and Denis 2000; Pokroy and Zolotoyabko 2003). Geological aragonite crystals typically grow as elongated needles along the fast-growing c-axis, whereas aragonite crystals in the crossed lamellar microstructure are variably orientated in different species.

We investigated the shell microstructure of the limpet *L. kogamogai*. The shell of *L. kogamogai* has five layers M+3, M+2, M+1, M, and M-1, with a crossed lamellar microstructure consisting of aragonite flat prisms with rectangular cross-sections. The boundaries of the alternating blocks are parallel to the circumference of the shell in the M+1 layer and to the radius of the shell in the M-1 layer. TEM observation and selected area electron diffraction (SAED) patterns showed that twin boundaries are along the lamellar prism and parallel to the shorter side of the rectangular cross-section. In contrast, the c-axis is roughly perpendicular to the longer side of the prism but is inclined by ca. 15° toward the lamellar prism axis. Crystal growth along the prism in the crossed lamellar structure may also be accelerated by the re-entrant corner effect of the {1 1 0} twins.

We observed the growth front of the crossed lamellar microstructure in fresh *Cellana rota* shells using cryo-SEM (Fig. 9A) (Suzuki et al. 2011b). Cryo-SEM images showed that a granular layer was present in the central part of the growth surface, overlying the typi-
cal prisms of the crossed lamellar microstructure (Figs. 9C, D). The granular layer had the same contrast as the crossed lamellar microstructure in the backscattered image, suggesting that the granular layer consists of amorphous calcium carbonate (ACC) (Fig. 9B).

4-2. Organic matrices

Recently, the complete genome sequence of the limpet, Lottia gigantea, was analyzed (Joint Genome Institute, <http://genome.jgi.doe.gov/Lotgi1/Lotgi1.home.html> (Grigoriev et al. 2012)). The genome sequence information will be useful in elucidating the role of organic matrices in shell formation. Proteome analyses of the shell of L. gigantea were performed using the genome database (Mann et al. 2012). The amino acid sequences of some proteins were similar to the shell matrix proteins previously identified in different bivalve or gastropod shells, such as BMSP, dermatopontin, nacrein, perlustrin, perlucin, and Pif. In addition to homologous proteins, many uncharacterized proteins were discovered. These proteins contain repeated short linear motifs or homorepeats. Such proteins may play roles in the formation of the crossed lamellar microstructure. However, the detailed functions of these proteins in the shell formation of the limpet remain to be elucidated.

Fig. 10. The spindle-like aragonite crystal observed by electron microscopes. (A) SEM image of the precipitate obtained after 24 h, in the presence of the soluble organic matrices extracted from the crossed lamellar microstructure of L. gigantea. The white rectangle indicates the position of the cross-section that was cut out by FIB, to obtain the specimen for TEM observation. (B) Bright field TEM image of the cross section from (A). (C) A magnified image of the region marked with a white rectangle in (B). The white arrows delimit the plate (viewed edge-on) on which electron diffraction was performed (D) Electron diffraction pattern from the area of (C) marked with a black circle. The diffraction pattern corresponds to that of aragonite. The direction of the white arrow is perpendicular to the plate in (C) and corresponds to the (110) plane vector in (D), indicating that the crystal plates in the precipitate are delimited by large (110) faces. Reprinted with permission from Crystal Growth & Design, 11, Suzuki M, Kogure T, Weiner S, Addadi L. Formation of aragonite crystals in the crossed lamellar microstructure of limpet shells, 4850–4859, http://pubs.acs.org/doi/abs/10.1021/cg2010997, 2011. Copyright 2011 American Chemical Society.
formation of L. gigantea are unknown.

We analyzed the function of organic matrices in the crossed lamellar microstructure of limpets. The extracted acid soluble organic matrices were used in in vitro calcium carbonate crystallization experiments (Suzuki et al. 2011b). TEM and SEM observations showed that spindle-like aragonite crystals were formed in the presence of the extracted organic matrices (Fig. 10A). A thin cross-section of these spindle-like aragonite crystals was prepared to observe the inner microstructure (Fig. 10B). The SAED patterns of the edge plate of the spindle-like aragonite crystal showed that the plate surfaces of the single, thin lamellar crystals correspond to the (110) plane (Figs. 10C, D). The morphology and the crystallographic orientation of the synthetic aragonite crystals induced by the organic matrix extracted from the crossed lamellar microstructure are thus similar to those of the biogenic crystals of third-order lamellae. The samples were allowed to stand for a few days to confirm that the crossed lamellar organic macromolecules stabilize the aragonite crystals, as in the early stages of the experiment. These results suggest that the organic matrices in the crossed lamellar microstructure of limpets function in the stabilization of aragonite crystals and induce the elongation of the (110) plane to form third-order lamellae. To identify the key organic molecule(s) from the crossed lamellar microstructure, further research will be needed.

5. Conclusion

Many studies have revealed various organic matrices in molluscan shell microstructures. Although the primary sequences and chemical structures of organic matrices have been identified, functional analyses of these organic matrices were not sufficient to reveal the complete mechanism of molluscan shell biomineralization. We reviewed the function of organic matrices in relation to the three typical molluscan shell structures, the prismatic layer, nacreous layer, and crossed lamellar structure, as well as to the structure-function relationships of these organic molecules. Prismatic-14, a matrix protein specifically present in the prismatic layer, has both calcium carbonate- and chitin-binding activities. Pif, a matrix protein specifically present in the nacreous layer, has a chitin-binding activity. Prismalin-14, a matrix protein specifically present in the prismatic layer, has both calcium carbonate- and crystals: stereochemical requirements in biomineralization. Proc. Natl. Acad. Sci. USA 1985; 82: 4110–4114.

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