

Title	A novel silk-like shell matrix gene is expressed in the mantle edge of the Pacific oyster prior to shell regeneration.
Author(s)	Takahashi, Jun; Takagi, Masaya; Okihana, Yumiko; Takeo, Kei; Ueda, Takahisa; Touhata, Ken; Maegawa, Shingo; Toyohara, Haruhiko
Citation	Gene (2012), 499(1): 130-134
Issue Date	2012-05-10
URL	<a href="http://hdl.handle.net/2433/155565">http://hdl.handle.net/2433/155565</a>
Right	© 2011 Elsevier B.V.
Type	Journal Article
Textversion	author

1 **A novel silk-like shell matrix gene is expressed in the mantle edge of the Pacific oyster**  
2 **prior to shell regeneration**

3

4 Jun Takahashi <sup>a,b</sup>, Masaya Takagi <sup>a</sup>, Yumiko Okihana <sup>a</sup>, Kei Takeo <sup>a</sup>, Takahisa Ueda <sup>a</sup>,  
5 Ken Touhata <sup>c</sup>, Shingo Maegawa <sup>d</sup>, Haruhiko Toyohara <sup>a,\*</sup>

6

7 a: Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

8 b: Research Fellow of the Japan Society for the Promotion of Science (JSPS), Tokyo  
9 102-8472, Japan

10 c: Biochemistry and Food Technology Division, Fisheries Research Agency of Japan,  
11 Kanagawa 236-8648, Japan

12 d: Graduate School of Informatics, Department of Intelligence Science and Technology,  
13 Kyoto University, Kyoto 606-8501, Japan

14 \*: Corresponding author

15 > E-mail: toyohara@kais.kyoto-u.ac.jp

16 > Tel / Fax: +81-75-753-6446

17 > Present address: Graduate School of Agriculture, Kyoto University,  
18 Kyoto 606-8502, Japan

19

20

21 *Keywords:*

22 Biom mineralization; Calcite; Pacific oyster; Poly-alanine; Spider-silk protein

23

24

**1 Abstract**

2           During shell formation, little is known about the functions of organic matrices,  
3 especially about the biomineralization of shell prismatic layer. We identified a novel gene,  
4 *shelk2*, from the Pacific oyster presumed to be involved in the shell biosynthesis. The  
5 Pacific oyster has multiple copies of *shelk2*. *Shelk2* mRNA is specifically expressed on the  
6 mantle edge and is induced during shell regeneration, thereby suggesting that Shelk2 is  
7 involved in shell biosynthesis. To our surprise, the database search revealed that it encodes  
8 a spider silk-like alanine-rich protein. Interestingly, most of the Shelk2 primary structure is  
9 composed of two kinds of poly-alanine motifs—GXNA<sub>n</sub>(S) and GSA<sub>n</sub>(S)—where X denotes  
10 Gln, Arg or no amino acid. Occurrence of common motifs of Shelk2 and spider silk led us  
11 to the assumption that shell and silk are constructed under similar strategies despite of their  
12 living environments.

13

14

## 1 **1. Introduction**

2 Molluscan shells are known to be made of CaCO<sub>3</sub> crystals, and their shapes differ  
3 according to species. However, the mechanism by which these shells are synthesized is yet  
4 unclear. They are among the most extensively studied biominerals and are composed of  
5 various types of layers, including periostracum, foliate, cross-lamellar, prismatic, and  
6 nacreous (Mann, 2001; Wilt et al., 2003; Marin et al., 2008). Organic matrices such as  
7 polysaccharides and proteins can affect the diversity of shell shapes by arranging the CaCO<sub>3</sub>  
8 crystals in a specific manner. Some proteins such as Aspein (Tsukamoto et al., 2004),  
9 Asprich (Gotliv et al., 2005), Caspartin, Calprismin (Marin et al., 2005), KRMP (Zhang et al.,  
10 2006), MSI31 (Sudo et al., 1997), and Prismarin-14 (Suzuki et al., 2004; Suzuki and  
11 Nagasawa, 2007) are reported to be involved in the synthesis of the prismatic layer, which is  
12 composed of calcite crystalline structures. Most of the organic matrix proteins identified  
13 thus far are associated with the synthesis of prismatic layers in the Japanese pearl oyster  
14 *Pinctada fucata*. These proteins have been mainly isolated by decalcification of shells using  
15 calcium chelators or acids. Further, some soluble as well as insoluble proteins harboring  
16 critical protein-binding domains have been extracted for the analysis of shell  
17 biomineralization. However, because this conventional method is better suited for  
18 identification of abundant proteins, certain vital proteins could not be isolated because of  
19 their low solubility and/or instability in the solutions used for the extraction.

20 To identify other essential proteins involved in shell biosynthesis, we focused on the  
21 mantle instead of the shell. Miyamoto et al. (1996) showed that the mantle edge is known to  
22 synthesize the calcite of the prismatic layer. We cloned certain mantle edge-specific genes  
23 from the Pacific oyster *Crassostrea gigas* by using a subtractive hybridization method. We  
24 succeeded in identifying two novel genes specifically expressed on the mantle edge.  
25 Unexpectedly, the deduced amino acid sequences of both the resulting proteins were found to

1 be highly homologous to those of spider silk proteins. Thus, we termed these oyster genes  
2 “*shelk*,” meaning silk-in-the-shell, and designated these proteins as Shelk1 and Shelk2. Our  
3 attempts to characterize both these genes have shown that they are indeed expressed in the  
4 mantle edge.

5

## 6 **2. Materials and Methods**

### 7 *2.1. Materials*

8 All procedures were performed according to published molecular cloning protocols  
9 or according to the manufacturers’ instructions. The Advantage 2 PCR Enzyme System  
10 (Clontech) or TaKaRa Ex Taq (TAKARA) was used for polymerase chain reactions (PCRs).  
11 PCR primers were commercially synthesized (Hokkaido System Science). In a thermal  
12 cycler T-Gradient Thermoblock (Biometra), the standard PCR reactions were performed  
13 under the following conditions: 94 °C for 3 min; 30–40 cycles of 98 °C for 30 s, 55–60 °C for  
14 30 s, and 72 °C for 60–75 s. PCR products were cloned into the pGEM-T Easy vector  
15 (Promega) before sequencing (Fasmac). All restriction enzymes (TOYOBO) were used  
16 under the recommended buffer conditions. Commercially available adult Pacific oysters  
17 and the Iwagaki oyster *Crassostrea nippona* were maintained in artificial seawater without  
18 feed for 1 week before using them for the experiments. *Meretrix lusoria* (clam),  
19 *Mizuhopecten yessoensis* (Japanese scallop), *Mytilus galloprovincialis* (mussel),  
20 *Hemicentrotus pulcherrimus* (sea urchin), and *Halichondria japonica* (sponge) were collected  
21 from the sea around Japan.

22

### 23 *2.2. Subtractive cloning of shelk2 cDNA*

24 The mantle edge and mantle pallial were collected from a Pacific oyster. The  
25 mRNA was extracted using the MicroPoly(A)Purist Kit (Ambion). Briefly, suppression

1 subtractive hybridization was performed between the mantle edge and mantle pallial by using  
2 the PCR-Select cDNA Subtraction kit (Clontech). We identified several genes specifically  
3 expressed in the mantle edge (unpublished data). Full-length *shelk2* cDNA was cloned by 5'  
4 and 3' RACE-PCR using the SMART RACE cDNA Amplification Kit (Clontech). PCR  
5 reactions were performed under the standard PCR program using the primers provided in the  
6 kit and the gene-specific primers for 5' and 3' RACE-PCRs (5'-CTA ATG GTC CAT ACG  
7 GTT TGT GAT AAT AG-3', 5'-CGT CAT ACT TGG AAT AGT GAC TAT AAG TG-3',  
8 5'-GAT CAC CCG ACC AAG TCC AGT GAC AC-3', and 3'-GTT CTA TAA AAA CCA  
9 AGC AAA AGA CGA C-5').

10

### 11 2.3. Genome walking of the *shelk2* gene

12 Genomic DNA of the Pacific oyster and other marine organisms was isolated from  
13 fresh tissues (Asahida et al., 1996). Primers for genomic DNA cloning were designed on the  
14 basis of the Pacific oyster *shelk2* cDNA sequence (5'-ATG CTG AAG CTT GTC TCC ATC  
15 GTT TGC CTT-3' and 5'-TTA ATA GGT CTT TTT ATG TCT GAT GCC ACC-3'), with the  
16 standard PCR program. The GenomeWalker Universal Kit (Clontech) was used to obtain  
17 the *shelk2* gene using 250 ng of genomic DNA as a template.

18

### 19 2.4. Observation of shell regeneration and *in situ* hybridization

20 The Pacific oyster shells were cut on the ventral side (the opposite side of the umbo)  
21 into approximately 30-mm wide and 10-mm thick pieces by using a pair of nippers and used  
22 for time-dependent observations and *in situ* hybridization experiments during the shell  
23 biosynthesis process.

24 Non-radioactive *in situ* hybridization was performed using paraffin sections from the  
25 mantle of Pacific oysters (Sakamoto et al., 2008). Digoxigenin UTP-labeled antisense and

1 sense RNA probes were transcribed from *shelk2* partial cDNA (349 bp) by using a DIG RNA  
2 labeling mix (Roche Diagnostics) under the standard PCR program. The mantles of the  
3 Pacific oysters were fixed in Bouin's fixative. Fixed tissues were dehydrated in an ethanol  
4 series and embedded in paraffin wax. Thin sections of approximately 8- $\mu$ m thickness were  
5 prepared using a microtome PR-50 (Yamato Koki).

6

### 7 2.5. Tissue-specific RT-PCR of *shelk2*

8 Total RNA was extracted from liquid nitrogen frozen adult Pacific oyster  
9 tissues—mantle edge, mantle pallial, gill, intestine, and adductor muscle—by using  
10 Sepasol-RNA I (Nacalai Tesque). The RNA was purified using RNase-Free DNase  
11 (Promega) and Oligotex-dT30 (TAKARA). ReverTra Ace (TOYOBO) was used for  
12 reverse transcription and cDNA construction. PCR was performed with gene-specific  
13 primers (5'-TTG GAG GAC TCG TCG GTC TGG GTG GTG-3' and 5'-GAA TCC ACT  
14 TGC AGA TGC AGC AGC AGC G-3') with the standard PCR program, based on the  
15 reverse-transcribed cDNA template.

16

### 17 2.6. Southern blotting of the *shelk2* gene

18 Digoxigenin UTP-labeled DNA probes were designed from *shelk2* partial cDNA  
19 (231 bp) by standard PCR using alkali-labile DIG-11-dUTP (Roche) and primers (5'-GTC  
20 ATT GGA GGA CTC GTC GG-3' and 5'-TGG GAC TGA TCC GAA TCC AC-3'). The  
21 Pacific oyster genomic DNA was partially digested using the restriction enzymes *Bam*HI,  
22 *Eco*RI, and *Hind*III, and used for hybridization. DNA transfer, fixation to the membrane  
23 (Biodyne Plus 0.45  $\mu$ m (PALL)), and hybridization were performed with the DIG High Prime  
24 DNA Labeling and Detection Starter Kit I (Roche). CDP-star (GE Healthcare UK),  
25 Hi-RENDOL (FUJIFILM), and Hi-RENFIX (FUJIFILM) were used for immunological

1 detection.

2

### 3 **3. Results**

#### 4 *3.1. Identification of shelk2*

5 First, we partially excised the edge of the prismatic layer of the oyster shell to  
6 observe shell regeneration (Fig. 1). After 24 h, the mantle edge appeared at the dissected  
7 area of the shell for regeneration and began to construct a film-like shell framework structure,  
8 which is considered a part of the new shell. This fresh shell framework structure grew with  
9 time, and had covered the broken area within 5 days of cutting.

10 In the present study, we report the structure of Shelk2, while we are currently  
11 pursuing the structural analysis of Shelk1. The full-length cDNA sequence of *shelk2*  
12 (GenBank ID: AB474183) was obtained from mRNA specifically expressed at the mantle  
13 edge of the Pacific oyster. We obtained an 894-bp fragment with a coding sequence (CDS)  
14 corresponding to 297 amino acids of the deduced protein sequence (Fig. 2). From the  
15 results of 5' and 3' RACE-PCR and genome walking, the CDS of *shelk2* was mapped into a  
16 single exon (exon 2). A sequence of 16 amino acids at the N-terminus is thought to  
17 represent a putative signal peptide on the basis of the results obtained using the SignalP 4.0  
18 (<http://www.cbs.dtu.dk/services/SignalP/>). The deduced amino acid sequence has 12  
19 poly-alanine (poly-Ala) repeat motifs, 3 repeats of which are accompanied by PYYGFNLGG  
20 (Fig. 3a). Each poly-Ala motif has a series of 6–10 Ala residues, and sometimes, a Ser at  
21 the C-terminus or middle of the motif. These poly-Ala motifs do not exist as a single motif  
22 in Shelk2.

23 The results of Protein BLAST (blastp) homology searches of Shelk2 against the  
24 protein databases of NCBI (<http://www.ncbi.nlm.nih.gov/>) and Compagen  
25 (<http://compagen.zoologie.uni-kiel.de/>) revealed no existing or putative homologs under



1 default searching conditions at the expect threshold ( $E \leq 10$ ). However, typical poly-Ala  
2 motifs have been reported in the silk proteins of spiders and some insects. For example, a  
3 spider dragline silk fibroin, Spidroin 2 of *Nephila clavipes* (GenBank ID: M92913), has  
4 almost the same length of a series of poly-Ala motifs (Fig. 3b).

5

### 6 *3.2. shelk2 expression is coupled with shell regeneration in the mantle edge.*

7 To elucidate whether *shelk2* is involved in shell biosynthesis, we examined mantle  
8 tissue by *in situ* hybridization for the expression of *shelk2* mRNA during shell biosynthesis.  
9 As a result, we determined that *shelk2* mRNA was specifically expressed in the outer fold of  
10 the mantle edge (Fig. 4), an area known to express genes involved in the biosynthesis of the  
11 prismatic layer of the shell (Miyamoto et al., 1996). Furthermore, the expression of *shelk2*  
12 mRNA gradually increased during the shell biosynthesis process, before the shell framework  
13 structure covered the dissected shell area (Figs. 1 and 4). mRNA expression level at 12 h  
14 was higher than those at 0 h, and further increased until 72 h. Taken together, we assume  
15 that Shelk2 is involved in the biosynthesis of prismatic layers in the oyster shells.

16

### 17 *3.3. Structural analysis of the shelk2 gene*

18 To verify the presence of *shelk2* in the oyster genome, we performed Southern  
19 blotting and genome walking. At least 8 bands were obtained in the genomic Southern  
20 blotting (Fig. 5), thereby suggesting the presence of multiple gene structures of *shelk2*. As a  
21 result of genome waling, we actually cloned 7 other distinct *shelk2*-like genes with 1–12 base  
22 nucleotide displacement (GenBank ID: AB526832–AB526838).

23

### 24 *3.4. Homologous gene searching of shelk2*

25 To verify the distribution of *shelk2* among marine organisms, we performed PCR

1 analysis on the following marine organisms containing biominerals: Iwagaki oyster (a species  
2 related to the Pacific oyster), clam, Japanese scallop, sea urchin, and sponge. As a result,  
3 we obtained a 987-bp fragment encoding the CDS from Iwagaki oyster corresponding to 328  
4 amino acids of deduced protein sequence (GenBank ID: AB474184). Because of high  
5 amino acid sequence identity (67.4%) with the Pacific oyster *Shelk2*, and conservation of the  
6 poly-Ala motifs and 3 repeats of the characteristic PYYGFNLGG motif, it is strongly  
7 suggested that the cloned gene from Iwagaki oyster is an ortholog of *shelk2* from the Pacific  
8 oyster (Fig. 3a). However, we have failed to clone any additional orthologs from sea urchin,  
9 sponge, or other bivalves.

10

#### 11 **4. Discussion**

12 We identified a novel gene that encodes a spider silk-like protein termed *Shelk2* that  
13 harbors 2 unique types of poly-Ala repeat motifs: a  $GSA_n(S)$  and  $GXNA_n(S)$  motifs, where X  
14 indicates Gln, Arg, or no amino acid. Each type consists of a series of 6–10 Ala residues,  
15 some of which are interspersed with Thr and/or Ser residues. In addition, we identified 3  
16 repeats of the novel motif PYYGFNLGG, followed by the  $GSA_n(S)$  type poly-Ala motif in  
17 *Shelk2*. Importantly, both poly-Ala motifs do not exist as a single motif in *Shelk2* (Fig. 3a).

18 The  $GSA_n(S)$  type motif was identified in various spider dragline silk proteins such  
19 as Spidroin 2 of *N. clavipes* (GenBank ID: M92913) (Hinman and Lewis, 1992), and  
20 fibroin-4 of *Araneus diadematus* (GenBank ID: U47856) (Guerette et al., 1996). It was also  
21 found in the molluscan protein MSI60 of the pearl oyster *P. fucata* (GenBank ID: D86074)  
22 (Sudo et al., 1997). Thus, *Shelk2* and MSI60 could be classified as poly-Ala  
23 sequence-containing proteins, although they differ in the following 3 characteristics: (i) The  
24 molecular weight of MSI60 is 60 kDa, while that of *Shelk2* is 25.6 kDa; (ii) MSI60 has  
25 Asp-rich regions that may enable  $Ca^{2+}$  binding, whereas *Shelk2* lacks regions rich in anionic

1 amino acids; and (iii) Shelk2 has unique PYYGPLNGG motifs, whereas MSI60 does not.  
2 These findings suggest that Shelk2 and MSI60 share similar but distinct functions in the  
3 process of shell biosynthesis. The results of RT-PCR (data not shown) and *in situ*  
4 hybridization (Fig. 4) revealed that both Shelk2 and MSI60 mRNAs are specifically  
5 expressed on the mantle edge (Sudo et al., 1997), which is involved in the formation of  
6 prismatic layers in molluscan shells (Miyamoto et al., 1996).

7         However, although 4 repeats of GSA<sub>n</sub>(S) were identified in MSI60, the GXNA<sub>n</sub>(S)  
8 type poly-Ala motif of Shelk2 has not been identified in any expressed animal protein.  
9 Unlike the poly-Ala motifs in spider silk proteins, the GXNA<sub>n</sub>(S)-type poly-Ala motifs in  
10 Shelk2 often contains Gln at the X position, which may enable Ca<sup>2+</sup> binding for shell  
11 formation. The interaction between Ca<sup>2+</sup> and Gln and/or Asn was suggested on the basis of  
12 a study that calculated the predictive potential energy of protein binding (Dudev et al., 2003).  
13 The poly-Ala motifs preceded by Gln and/or Asn are thought to function not only as a  
14 framework unit, but also as elements promoting Ca<sup>2+</sup> capture for the effective formation of  
15 CaCO<sub>3</sub> crystals in the shell.

16         It is conceivable that Shelk2 of Iwagaki oysters also has the same two types of  
17 poly-Ala motifs. Interestingly, Iwagaki oyster Shelk2 has 14 poly-Ala motifs, whereas the  
18 Pacific oyster has 12 (Fig. 3a). This may be because of the difference in gene duplication of  
19 poly-Ala motifs between both the oyster species after evolutionary divergence. We have  
20 failed to detect *shelk2* homologous genes in clam, Japanese scallop, sea urchin, and sponge,  
21 most likely due to mismatching of PCR primers.

22         In contrast, we have found that the purple sea urchin *Strongylocentrotus purpuratus*  
23 has some putative proteins that have poly-Ala motifs, as determined from the NCBI BLAST  
24 database (invertebrates/echinoderms). However, we could not identify poly-Ala motifs that  
25 included GQN or GRN. In addition, we also have found that a coral species, *Acropora*

1 *digitifera*, for which the whole genome has been sequenced (Shinzato et al., 2011), has  
2 putative proteins harboring GQN(A)<sub>3</sub>, GRN(A)<sub>3</sub>, GS(A)<sub>4</sub>, and (A)<sub>5</sub>SG. This suggests that  
3 poly-Ala containing proteins may be involved in the process of CaCO<sub>3</sub> biomineralization in  
4 marine organisms, including shells, sea urchins, and corals. Further studies on the whole  
5 genome structure of biomineral-making animals will facilitate the elucidation of the  
6 distribution and role of poly-Ala motifs in biomineralization.

7

## 8 **Acknowledgements**

9 This work was financially supported by a Grant-in-Aid for JSPS Fellows, and  
10 Scientific Research (B) (Grant no. 18380124) from the Japan Society for the Promotion of  
11 Science (JSPS) and also supported by the Sekisui Chemical Grant Program for Research on  
12 Manufacturing Based on Learning from Nature for the fiscal year 2005.

13

## 14 **References**

- 15 Asahida, T., Kobayashi, T., Saitoh, K., Nakayama, I., 1996. Tissue preservation and total  
16 DNA extraction from fish stored at ambient temperature using buffers containing high  
17 concentration of urea. *Fish. Sci.* 62, 727–730.
- 18 Dudev, T., Lin, Y., Dudev, M., Lim, C., 2003. First-second shell interactions in metal binding  
19 sites in proteins: a PDB survey and DFT/CDM calculations. *J. Am. Chem. Soc.* 125,  
20 3168–3180.
- 21 Gotliv, B.A., Kessler, N., Sumerel, J.L., Morse, D.E., Tuross, N., Addadi, L., Weiner, S., 2005.  
22 Asprich; a novel aspartic acid-rich protein family from the prismatic shell matrix of the  
23 bivalve *Atrina rigida*. *ChemBioChem* 6, 304–314.
- 24 Guerette, P.A., Ginzinger, D.G., Weber, B.H.F., Gosline, J.M., 1996. Silk properties  
25 determined by gland-specific expression of a spider fibroin gene family. *Science* 272,

- 1 112–115.
- 2 Hinman, M.B., Lewis, R.V., 1992. Isolation of a clone encoding a second dragline silk fibroin,  
3 J. Biol. Chem. 267, 19320–19324.
- 4 Mann, S., 2001. Biomineralization: Principles and Concepts in Bioinorganic Materials  
5 Chemistry, Oxford University Press, New York.
- 6 Marin, F., Amons, R., Guichard, N., Stigter, M., Hecker, A., Luquet, G., Layrolle, P., Alcaraz,  
7 G., Riondet, C., Westbroek, P., 2005. Caspartin and calprismin, two new proteins of the  
8 shell calcitic prisms of the Mediterranean fan mussel *Pinna nobilis*. J. Biol. Chem. 280,  
9 33895–33908.
- 10 Marin, F., Luquet, G., Marie, B., Medakovic, D., 2008. Molluscan shell proteins: primary  
11 structure, origin, and evolution. Curr. Top. Dev. Biol. 80, 209–276.
- 12 Miyamoto, H., Miyashita, T., Okushima, M., Nakano, S., Morita, T., Matsushiro, A., 1996. A  
13 carbonic anhydrase from the nacreous layer in oyster pearls. Proc. Natl. Acad. Sci. USA  
14 93, 9657–9660.
- 15 Sakamoto, K., Uji, S., Kurokawa, T., Toyohara, H., 2008. Immunohistochemical, in situ  
16 hybridization and biochemical studies on endogenous cellulase of *Corbicula japonica*.  
17 Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 150, 216–221.
- 18 Shinzato, C., Shoguchi, E., Kawashima, T., Hamada, M., Hisata, K., Tanaka, M., Fujie, M.,  
19 Fujiwara, M., Koyanagi, R., Ikuta, T., Fujiyama, A., Miller, D.J., Satoh, N., 2011. Using  
20 the *Acropora digitifera* genome to understand coral responses to environmental change,  
21 Nature 476, 320–323.
- 22 Sudo, S., Fujikawa, T., Nagakura, T., Ohkubo, T., Sakaguchi, K., Tanaka, M., Nakashima, K.,  
23 Takahashi, T., 1997. Structures of mollusc shell framework proteins. Nature 387,  
24 563–564.
- 25 Suzuki, M., Murayama, E., Inoue, H., Ozaki, N., Tohse, H., Kogure, T., Nagasawa, H., 2004.

- 1 Characterization of prismaticin-14, a novel matrix protein from the prismatic layer of the  
2 Japanese pearl oyster (*Pinctada fucata*). *Biochem. J.* 382, 205–213.
- 3 Suzuki, M., Nagasawa, H., 2007. The structure–function relationship analysis of Prismaticin-14  
4 from the prismatic layer of the Japanese pearl oyster, *Pinctada fucata*, *FEBS J.*, 274,  
5 5158–5166.
- 6 Tsukamoto, D., Sarashina, I., Endo, K., 2004. Structure and expression of an unusually acidic  
7 matrix protein of pearl oyster shells. *Biochem. Biophys. Res. Commun.* 320,  
8 1175–1180.
- 9 Wilt, F.H., Killian, C.E., Livingston, B.T., 2003. Development of calcareous skeletal elements  
10 in invertebrates. *Differentiation* 71, 237–250.
- 11 Zhang, C., Xie, L., Huang, J., Liu, X., Zhang, R., 2006. A novel matrix protein family  
12 participating in the prismatic layer framework formation of pearl oyster, *Pinctada*  
13 *fucata*. *Biochem. Biophys. Res. Commun.* 344, 735–740.
- 14
- 15

1 **Figure captions**

2 **Fig. 1.** Time course of shell regeneration process beginning with the shell cut. The bar  
3 indicates 1 mm. The mantle edge of the oyster appears at the edge of cut shell after 12 h.  
4 The newly fresh shell framework (arrow heads) formed by the mantle edge can be observed  
5 after 24 h, and gradually increased up to 5 days.

6  
7 **Fig. 2.** The cDNA sequence of *shelk2* (GenBank ID: AB474183) and deduced amino acid  
8 sequence of Shelk2 protein. The putative signal peptide is indicated in italics. The stop  
9 codon is indicated with an asterisk. The nucleotides used for *in situ* hybridization are  
10 shaded. Arrows indicate the binding sites of the primers used for genomic Southern  
11 blotting.

12  
13 **Fig. 3.** (a) Schematic representation of the Shelk2 protein of the Pacific oyster and Iwagaki  
14 oyster. A sequence of 16 amino acids at the N-terminal is thought to represent a putative  
15 signal peptide. The diamonds indicate poly-Ala motifs consisting of  $GSA_n(S)$  or  $GXNA_n(S)$   
16 sequences, where X denotes Gln, Arg or no amino acid. The ellipses represent the 3 repeats  
17 of PYYGFNLGG motif followed by the  $GSA_n(S)$  type poly-Ala motifs. (b) The alignment  
18 of Shelk2 and a spider dragline silk protein Spidron2 of *N. clavipes* (GenBank ID: M92913).

19  
20 **Fig. 4.** (a) The overhead view of adductor muscle (AM), gill (Gi), gonad (Go), mantle edge  
21 (ME) and mantle pallial (MP) of the Pacific oyster with shell. (b) The cross-section diagram  
22 of the Pacific oyster mantle and shell layers. The heavy line in the outer fold (OF) of the  
23 mantle edge indicates the expression area of *shelk2* mRNA. (c) *In situ* hybridization during  
24 shell biosynthesis reveals the *shelk2* mRNA expressed in OF, represented by stained cellular  
25 areas (arrow heads). The mRNA expression levels gradually increased up to 72 h of

1 biosynthesis. The bar indicates 200  $\mu$ m. The negative control section stained with the  
2 sense probe showed no signals (data not shown).

3

4 **Fig. 5.** Southern blotting of *shelk2* genomic DNA digested with the restriction enzymes;  
5 *Bam*HI (lane 1), *Eco*RI (lane 2), and *Hind*III (lane 3) shows the oyster has multiple  
6 *shlek2*-like genes in its genome. 1% agarose gel was used for the analysis.



Fig. 1.

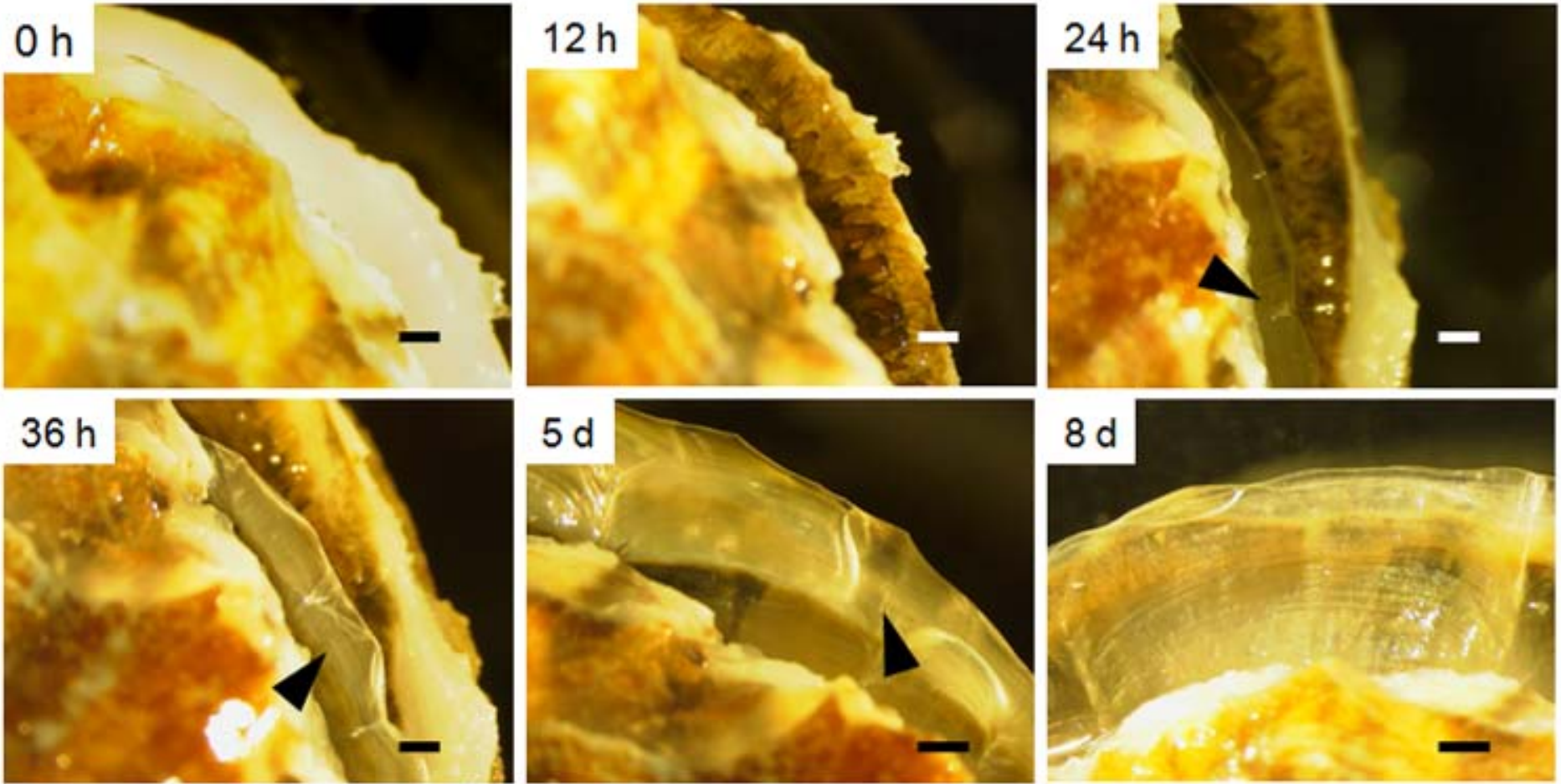








Fig. 4.

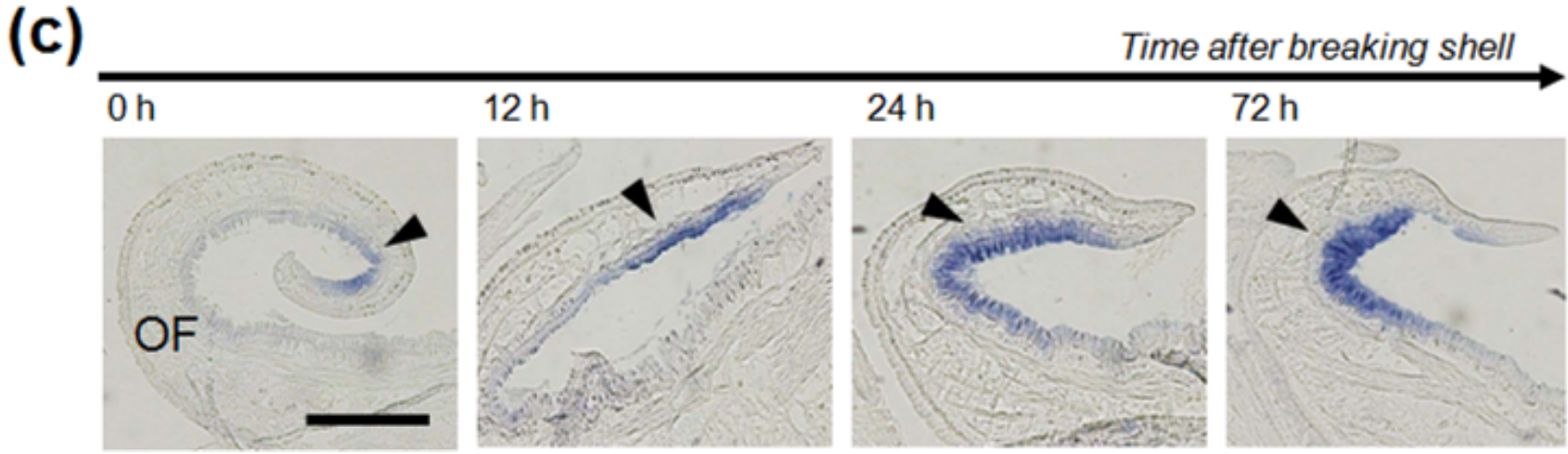
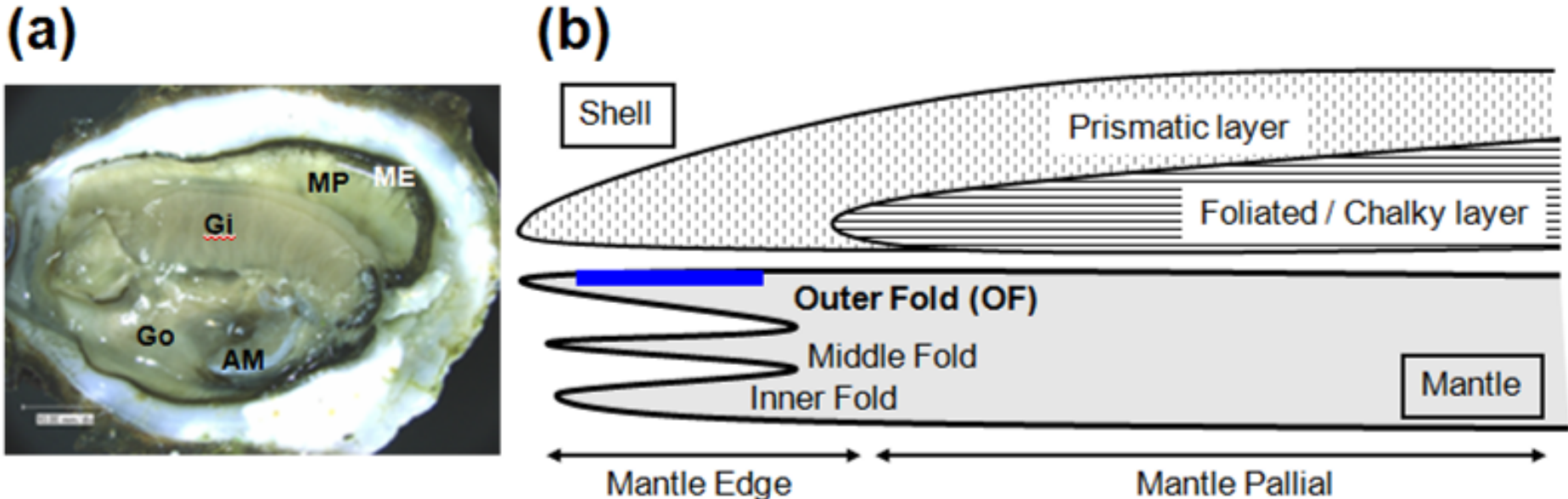


Fig. 5.

