Chapter 35
Functional Analysis on Shelk2 of Pacific Oyster

Jun Takahashi, Chieko Yamashita, Kenji Kanasaki, and Haruhiko Toyohara

Abstract Shelk2, a novel shell matrix protein from the Pacific oyster, *Crassostrea gigas*, is reported to be involved in shell biosynthesis of the prismatic layer. Results of RNAi experiment on *shelk2* showed that Shelk2 has a key role in shell regeneration. When dsRNA of *shelk2* was injected into the adductor muscle of Pacific oyster, the prismatic layer did not grow normally during shell regeneration. Observation of regenerated shell using scanning electron microscopy (SEM) revealed that the size of each column in the prismatic layer was reduced, and the edge of the column top looked rounder. From these results, it was deduced that the columns were less tightly bound with each other than in normally regenerated shells. Furthermore, the surface of the column appeared to be rough. Unexpectedly, the expression level of *shelk2* mRNA was not reduced but remarkably enhanced by the knockdown experiment. Further experiments including gene and protein expression will be necessary for a better understanding of its function and role in oyster shell regeneration.

Keywords Biomineralization · Knockdown · Mollusk · Pacific oyster · Shelk2 · Shell · Silk-like protein

35.1 Introduction

Mollusk is the second largest metazoan taxon with many members possessing mineralized hard tissues formed as a result of biomineralization. The molluscan shell is synthesized and maintained by the epithelial cells of the mantle, which is a specific tissue present only in mollusks. Generally, the molluscan shell is composed of >90% inorganic materials that mainly consist of $CaCO_3$ and <10% organic matrices, including polysaccharides and proteins. Various organic matrices play an important

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role in the crystallization and/or framework formation of the shell, while most of them reported so far do not share identity in their amino acid sequences among species, with the exception of acidic proteins (Takahashi et al. 2013).

The identification of most organic matrix substances, including proteins, so far has been accomplished by the decalcification of shells and subsequent extraction with specific solutions (Marin et al. 2000). This conventional method is suitable for the identification of relatively abundant proteins, but certain vital proteins cannot be obtained because of their low solubility and/or instability in solution.

Instead of the shell itself, we focused on the mantle where the genes involved in shell regeneration are expressed to identify essential proteins involved in shell biosynthesis. We have successfully cloned mantle edge-specific genes from Pacific oyster, *Crassostrea gigas*, by means of a subtractive hybridization method, then found two novel genes, *shelk1* and *shelk2* (Takahashi et al. 2012). The mRNA of *shelk2* was specifically expressed in the outer fold of the mantle edge, suggesting that it is possibly involved in the synthesis of the prismatic structure. *In situ* hybridization revealed gradual increase in *shelk2* mRNA expression during shell regeneration, suggesting the possible involvement of Shelk2 in shell formation (Takahashi et al. 2012).

Deduced amino acid sequences of both proteins were highly homologous to those of arthropod silk fibroins (Hayashi and Lewis 1998; Hinman and Lewis 1992). Interestingly, tandem repeats of poly-alanine (poly-Ala) motifs were identified in the amino acid sequence of Shelk2 of *C. gigas*. Poly-Ala motifs have also been reported in silk fibroins of arthropods (Guerette et al. 1996) and two shell matrix proteins of mollusks, including the MSI60 of Japanese pearl oyster (Sudo et al. 1997) and Shelk2 of *Crassostrea nippona* (Takahashi et al. 2012). However, the function of Shelk2 still remains unknown. Therefore, in this study, we made an attempt to elucidate their function *via* knockdown experiment.

35.2 Materials and Methods

Adult Pacific oysters (shell length, 5–7 cm; shell height, 7–11 cm) were purchased from the market and maintained in artificial seawater for a day before using them for the RNAi experiments.

For the synthesis of *shelk2* dsRNA, we used T7 RiboMAX Express RNAi System (Promega, Madison, WI, USA) following the manufacturer's instructions. The dsDNA templates of *shelk2* and *EF-1α* for both RNA syntheses were cloned into pTAC-2 plasmid (BioDynamics Laboratory, Tokyo, Japan), prepared using TaKaRa Ex Taq DNA polymerase (TaKaRa, Shiga, Japan) or PrimeSTAR GXL DNA polymerase (TaKaRa) with PCR primers shown in Table 35.1. These primers were designed on the basis of *C. gigas shelk2* sequence (GenBank ID: AB474183) and *EGFP* sequence. Thermal cycler T-Gradient Thermoblock (Biometra, Goettingen, Germany) was used for the amplification according to the conventional reaction program.

Table 35.1 Primers for dsRNA syntheses and qPCR analyses

Name	Sequence (5' -> 3')
dsRNA synthesis	
S2-101 Fw	ATGCTGAAGCTTGTCTCCATCGTTTGCCTT
S2-102 Rv	TTAATAGGTCTTTTTATGTCTGATGCCACC
T7 S2-117 Fw	GGATCCTAATACGACTCACTATAGGATGCTGAAGCTTGTCTCC
T7 S2-121 Rv	GGATCCTAATACGACTCACTATAGGTTAATAGGTCTTTTTATGTCTGATGCC
EGFP-903 Fw	ATGGTGAGCAAGGGCGAGGAGCTG
EGFP-904 Rv	TTACTTGTACAGCTCGTCCATGCC
T7 EGFP-901 Fw	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAG
T7 EGFP-902 Rv	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTC
qPCR analysis	
Cg_EF1a-802 Fw	AAGTCTTGGAAGAGGCACCA
Cg_EF1a-803 Rv	CAGCCTTCTCAACCTCCTTG
S2-126 Fw	CTCCATCGTTTGCCTTTTTG
S2-127 Rv	AGTCCTCCAATGACACCACC

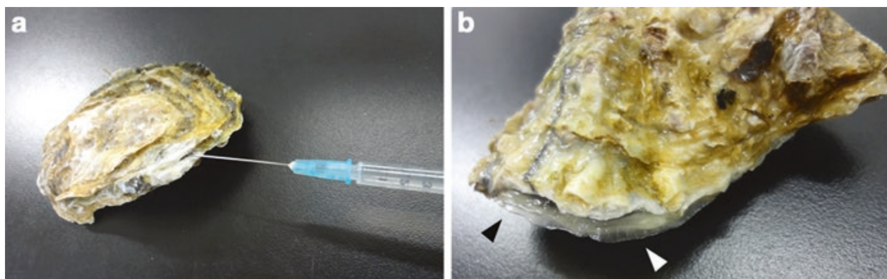

Fig. 35.1 Knockdown experiment and shell regeneration. (**a**) Shell surrounding the adductor muscle was excised by a pair of nippers within 3 cm, and dsRNA was injected into the adductor muscle. A constant volume (200 μl) of PBS solution containing 30 μg of *EGFP* dsRNA and 10 μg or 30 μg of *shelk2* dsRNA was injected into each group (n = 5). (**b**) Plastic-like structure of new shell was regenerated after a day of injection, and it was more clearly observed after the next 2 days (arrowheads). We collected the structure and the mantle edges after 7 days of injection

The Pacific oyster shells were cut on the ventral side near the adductor muscle into approximately 3-cm wide portions using a pair of nippers. To knock down the *shelk2*, the designed *shelk2* dsRNA (10 μg or 30 μg in 200 μL PBS) or *EGFP* dsRNA (30 μg in 200 μL PBS, for control) was injected into the adductor muscle of each oyster (Suzuki et al. 2009; Funabara et al. 2014; see Fig. 35.1a). The oysters were then kept in artificial seawater for 7 days without feeding. Then their mantles and the newly regenerated prismatic layers (Fig. 35.1b) were collected for qPCR experiments and SEM observation, respectively.

For SEM observation of the regenerated shell, Miniscope TM3000 (Hitachi High-Technologies, Tokyo, Japan) was used at two magnifications (×500 and ×2000).

For qPCR analyses, total RNA was extracted from the collected mantles using Sepasol-RNA I Super G (Nacalai tesque, Kyoto, Japan), while using Handy Sonic UR-20P (Tomy Seiko, Tokyo, Japan) for mantle homogenization. We used

PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa) for RT-PCR and first strand cDNA synthesis. Primers for qPCR were also designed on the basis of *C. gigas shelk2* sequence and *C. gigas EF-1α* sequence (GenBank ID: AB122066). For the qPCR reaction, KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) was used in StepOnePlus Real-Time PCR System (Life Technologies Japan, Tokyo, Japan) employing the comparative C_T ($\Delta\Delta C_T$) method.

35.3 Results and Discussion

35.3.1 Regeneration of Shell Prismatic Layer Observed by SEM

Figure 35.2 shows the SEM results (top view) of the newly generated plastic-like structure in the prismatic layer. In general, the prismatic layer gradually grew from the lower left to the upper right direction during the natural regeneration of a cracked shell, as shown in Fig. 35.2a. During this process, it is assumed that gap among the columns is filled densely. When dsRNA of *EGFP* was injected as the control experiment, the prismatic layer grew in a similar manner (Fig. 35.2c, d).

In contrast, when dsRNA of *shelk2* was injected, the prismatic layer did not grow normally (Figs. 35.2e–h). In particular, the size of each column was reduced, and the reduction was more remarkable by the 30-μg injection than by the 10-μg injection (Fig. 35.2g, h). In addition, the edge of the column top looked rounder; resultantly the columns were not tightly bound to each other compared with the control experiment as well as the natural regeneration. Furthermore, the surface of the column top looked rough, whereas those of the control experiment and the natural regeneration were smooth.

35.3.2 Real-Time PCR

To determine the effect of *shelk2* knockdown by RNAi, the expression of *shelk2* mRNA was evaluated (Fig. 35.3). Unexpectedly, the expression level of *shelk2* mRNA was considerably higher than that in the control experiment, in which *EGFP* dsRNA (Fig. 35.3) or PBS (data not shown) was injected. Generally, target gene expression is reduced in the knockdown experiments. Actually in the experiments of shells, the expression of *Pinctada fucata* genes including Pif and Nacrein were reduced by the previous knockdown experiment (Suzuki et al. 2009; Funabara et al. 2014), although their expressions were examined 7 or 8 days after injection similar to our experiments. In fact, reduction was observed in the knockdown experiment of another oyster silk-like gene, *shelk1*, in our experiment (data not shown).

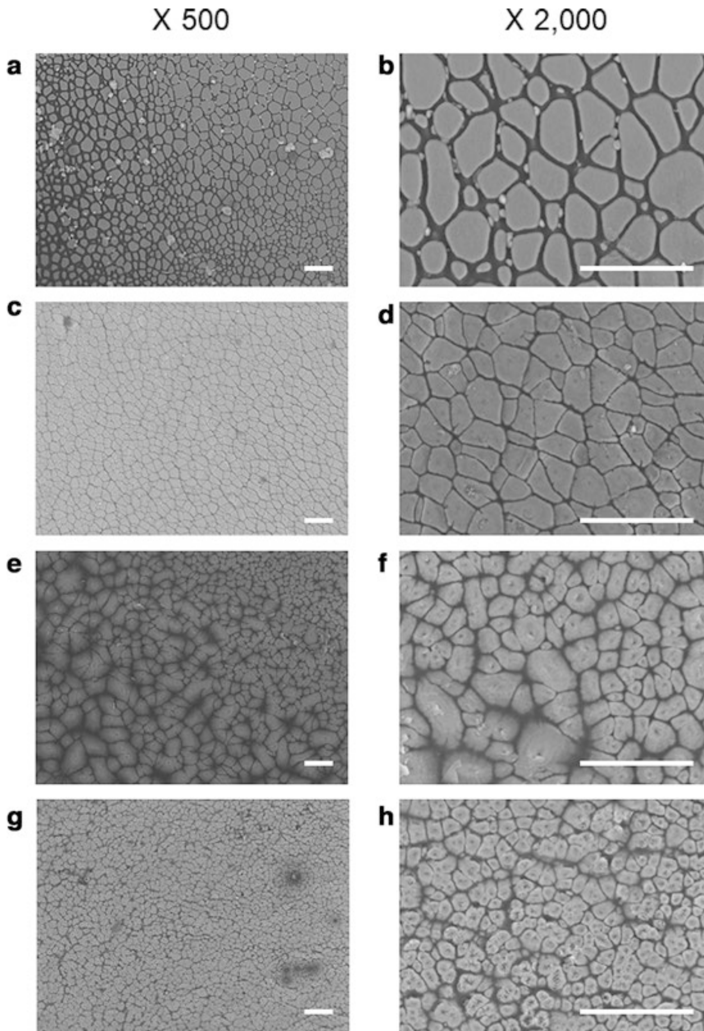

Fig. 35.2 SEM observation of the regenerated prismatic layers at two magnifications (×500 and ×2000). The bar indicates 30 μm. (**a**, **b**) Shell was excised, but no operation was performed. (**c**, **d**) dsRNA of *EGFP* was injected (control). (**e**, **f**) *Shelk2* dsRNA (10 μg) was injected. (**g**, **h**) *Shelk2* dsRNA (30 μg) was injected

As a result of *shelk2* knockdown, *shelk2* mRNA was expressed remarkably during shell regeneration, suggesting that Shelk2 would increase. Then the increase in the amount of the protein would induce the reduction in the column size of the prismatic layer. However, detailed studies on the change in expression levels of *shelk2* mRNA after injection are required for the full understanding of its remarkable expression.

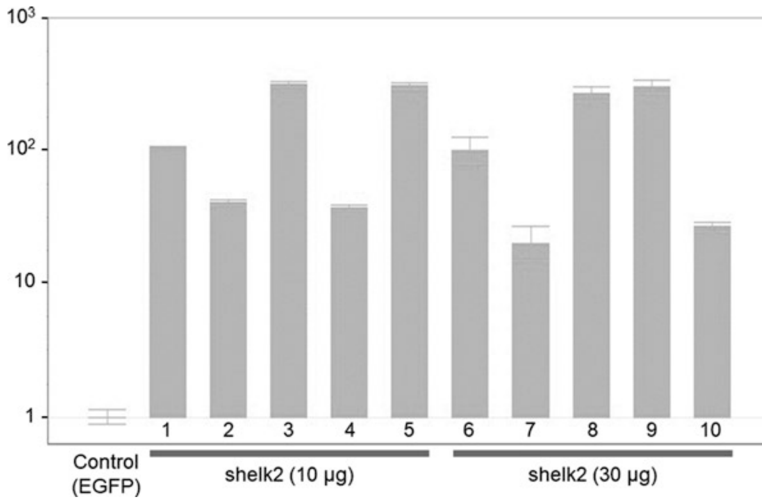

Fig. 35.3 Knockdown of *shelk2* by means of RNAi. The expression levels of *shelk2* mRNA in the mantle, which are normalized to those of *EF-1α*, were determined with real-time quantitative PCR. Five oysters were used in each experiment group. The graph bar shows the *shelk2* mRNA expression level of 7 days after injection with dsRNAs against 10 μg (bar number: 1–5) or 30 μg (6–10) of *shelk2*, and those of the *EGFP* group (average of 5 oysters) is attributed a relative value of 1.0. Unexpectedly, the *shelk2* expression levels increased more than ten times

35.3.3 Plan for Subsequent Studies

We have unexpectedly detected the remarkable expression of *shelk2* mRNA by real-time PCR analysis, but no information was available on the expression level of Shelk2. We are now trying to raise an antibody against Shelk2 for the detection of its expression and subsequent observation using SEM and western blotting during regeneration following knockdown experiments.

Since *shelk2* has multiple copies (Takahashi et al. 2012), the reactionary excess expression of the genes at multiple sites would be due to the temporal *shelk2* mRNA suppression caused by the RNAi. To validate the speculation, we attempt to identify the overexpressed gene after RNAi experiment. Further studies on the molecular mechanism of oyster shell synthesis, especially on the remarkably rapid regenerating process, would lead to the application in medical and cosmetic fields.

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