

Chapter 40

Chitin Degraded by Chitinolytic Enzymes Induces Crystal Defects of Calcites



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Abstract Mollusk shells have unique microstructures and mechanical properties such as hardness and flexibility. Calcite in the prismatic layer of *P. fucata* is extremely tough due to small crystal defects and localized organic networks inside calcites. Electron microscopic observations have suggested that such crystal defects are caused by the organic networks during calcite formation. Our previous work reported that the chitin which is the main component of organic networks and chitinolytic enzymes that bind to chitin were identified. In this article, to investigate the effects of chitin and chitinolytic enzymes on the formation of calcites, calcites were synthesized in chitin gel after treatment with chitinolytic enzymes. Chitin fibers seemed to become smooth and loosened after degradation. The crystal defects became larger as the chitin fibers became more degraded by chitinolytic enzymes in a dose-dependent manner. These results suggest that the shape of chitin fiber, which is regulated by the degradation of chitinolytic enzymes, contributes to the formation of small crystal defects.

Keywords Biomineralization · Prismatic layer · Chitinase · Chitin · *Pinctada fucata*

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40.1 Introduction

Biominerals are biogenic mineralized tissues containing a small amount of organic matrices which regulate crystal nucleation, orientation, polymorphism, and morphology of inorganic substances (Belcher et al. 1996; Chen et al. 2008; Falni et al. 1996; Weiner and Addadi 1997). The shell of *Pinctada fucata*, Japanese pearl oyster, has two layers: prismatic and nacreous layer. The prismatic layer focused on in this study is composed of calcite prisms that are surrounded by thick polygonal organic frameworks as intercrystalline organic matrices. Each prism in the prismatic layer of *P. fucata* consists of several small calcites. Scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses have revealed that each calcite contains subgrain units of several hundred nanometers divided by crystal lattice distortions known as small-angle grain boundaries (Okumura et al. 2012, 2013); however, the crystal structures of adjacent grains were continuous. Calcites that contain such crystal defects are tougher and stiffer than single calcites of another type of shell that contain no crystal defects because the defects inhibit the propagation of cracks (Olson et al. 2013). TEM observations have also revealed that organic matrices inside the small calcite of *P. fucata* prisms are localized in networks (Okumura et al. 2012). The location of the organic network correlates with the location of the crystal defects, indicating that the organic network affects the formation of the crystal defects. Our previous report clarified chitin and chitinolytic enzyme as the components of this organic network (Kintsu et al. 2017), in which we indicated that the chitin treated by chitinolytic enzymes may induce the formation of crystal defects.

In this article, we performed calcium carbonate crystallization using chitin hydrogel and chitinolytic enzymes *in vitro* to assess the effects of such organic matrices on lattice distortion inside the synthesized crystals.

40.2 Materials and Methods

Calcium carbonate crystallization in the chitin hydrogel. Chitin hydrogel was prepared according to the previous method (Tamura et al. 2006). The prepared chitin hydrogel was incubated with Yatalase (an enzyme complex containing chitinase and chitinase activities from *Corynebacterium* sp. OZ-21; TaKaRa) as chitinolytic enzyme for 24 h. After incubation, chitin hydrogel was filtered and washed with 10 mM calcium chloride to remove Yatalase. The chitin hydrogel was spread on a plate and put into desiccator filled with the gas of 5 g of ammonium carbonate (Kanto Chemical) to crystallize calcium carbonate in the chitin hydrogel for 24 h. Chitin hydrogel was dissolved in 50% sodium hypochlorite to collect crystals.

Calcium carbonate crystallization using chitin nanofiber. A chitin nanofiber solution (1.1% (w/v)) was prepared according to the previous report (Ifuku et al. 2010). A solution of 10 mM calcium chlorite was added to the chitin nanofiber

solution, followed by calcium carbonate crystallization in the chitin nanofiber solution using the same method described above.

Fixation of chitin gel. Chitin gel was fixed by using the method of cell fixation with glutaraldehyde, osmium tetroxide, and potassium permanganate for reference with some modifications (Gunning 1965). Chitin gel was incubated sequentially in glutaraldehyde, osmium tetroxide, and potassium permanganate solution for 1 h. After fixation, the sample was dehydrated by ethanol and embedded in Spurr resin (Polysciences, Inc., USA). Sections prepared by ultramicrotome using a diamond knife.

Variance of lattice spacing determined from XRD spectra. The variance of lattice spacing in calcite crystal was estimated from peak broadening of XRD spectra using a RINT-Ultima⁺ diffractometer (Rigaku) with graphite-monochromated Cu K α radiation emitted at 40 kV and 20 mA according to the method reported previously (Okumura et al. 2012). The variance of lattice spacing ($\Delta d/d$) of the samples was calculated using Williamson-Hall plot (Williamson and Hall 1953) as follows:

$$\frac{\Delta(2\theta)\cos\theta}{\lambda} = \frac{(\Delta d/d)2\sin\theta}{\lambda} + \frac{0.94}{L}$$

40.3 Results and Discussion

40.3.1 Observation of Chitin Fibers by TEM

In an initial phase of prism formation, after the organic frameworks in the prismatic layer are constructed, the space surrounded by the organic framework is filled with organic gel solution composed mainly of chitin and matrix proteins; calcium carbonate is then crystallized with organic gel solution in the space. The organic gel solution such as chitin and chitinolytic enzymes we identified can develop into organic networks during calcite crystallization and affect the crystallization to form small-angle grain boundaries. Therefore, we performed an in vitro calcium carbonate crystallization experiment using chitin hydrogel and chitinolytic enzymes.

Chitin hydrogel was prepared by dissolving chitin powder in methanol saturated with calcium chloride dehydrate. To compare the differences of chitin fibers between before and after treatment with chitinolytic enzymes at concentration of 1.2 mg/mL, the chitin gels were observed by using TEM. Chitin gel was fixed according to a chemical fixation procedure for physiological tissues in order to observe in natural condition. TEM images of the chitin gels showed that a lot of chitin fibers of a few dozen of nanometer were observed in both conditions and no apparent differences of thickness and length could be seen (Fig. 40.1). However, while chitin fibers without treatment of chitinolytic enzymes became entangled with each fiber (Fig. 40.1a, b), chitin fibers with treatment of chitinolytic enzymes became smooth and loosened, not entangled (Fig. 40.1c, d), indicating that the chitinolytic enzymes may

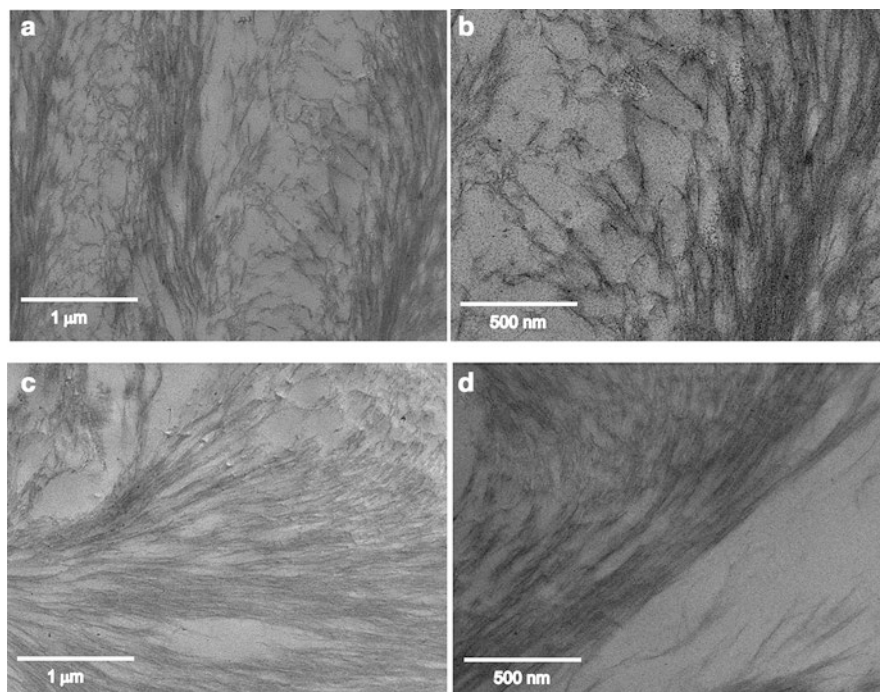


Fig. 40.1 TEM images of chitin gel. (a) Chitin gel before treatment with chitinolytic enzymes and (b) its high magnification. (c) Chitin gel after treatment with chitinolytic enzymes at the concentration of 1.2 mg/mL and (d) high magnification

degrade branched molecular chains of chitin which was getting entangled with each other. We suggest from this result that chitin fibers are loosened by chitinolytic enzymes and become such organic network as observed in the prism of *P. fucata*.

40.3.2 *Synthesis of Calcium Carbonate Crystals in Chitin Hydrogel Treated with Chitinolytic Enzymes*

To investigate how chitin and chitinolytic enzymes have effects on the formation of the small crystal defects in single calcites, calcium carbonate was crystallized in the chitin hydrogel with or without Yatalase which is a commercially available chitinolytic enzyme produced by a *Streptomyces* strain. After crystallization in the chitin hydrogel, calcium carbonate crystals were collected and observed by SEM. The normal shape of calcium carbonate crystals (a typical rhombohedral calcite) was formed in the chitin hydrogel without Yatalase treatment (Fig. 40.2a). In contrast, in the chitin hydrogel treated with 1.2 mg/mL chitinolytic enzyme, the shape of the crystal was completely changed and appeared to be round (Fig. 40.2b). As the concentration of chitinolytic enzymes increased, the chitin fiber became thinner, and

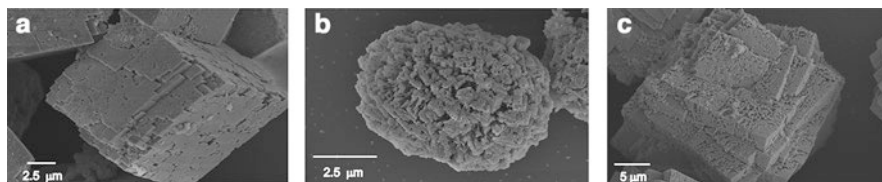


Fig. 40.2 The calcite crystals synthesized under different conditions. Calcite crystal formed in the chitin hydrogel treated with chitinolytic enzymes at concentrations of (a) 0 mg/mL, and (b) 1.2 mg/mL, and (c) in the chitin nanofiber solution

the surface microstructure of the calcite crystals in the chitin hydrogel changed. These results enabled us to guess that the thinness of chitin is a key factor to induce crystal defects.

Although it is indicated that thinness and length of chitin fiber are important, it is difficult to achieve a uniform thickness and length of chitin fibers using chitinolytic enzymes. Chitin nanofibers prepared by mechanical cleavage were used because the chitin nanofiber has a uniform thickness of several dozens of nanometers. Calcium carbonate was precipitated in the chitin nanofiber solution using the same method as described above without chitinolytic enzymes. SEM image of formed calcium carbonate crystals showed that chitin nanofiber can also affect crystallization and the surface microstructure became polygonal (Fig. 40.2c).

40.3.3 X-Ray Diffraction (XRD) Analyses of Calcite Crystals

To estimate the reason for the crystal defects among synthesized calcite crystals, XRD spectra of calcite samples were analyzed to calculate the variance of lattice spacing ($\Delta d/d$) as crystal defects. Figure 40.3 shows the ratio of $\Delta d/d$ gained from Williamson-Hall plots (data not shown) of each crystal synthesized in the presence of chitin pretreated with 0, 1.2 mg/mL chitinolytic enzymes, chitin nanofibers, and *P. fucata* prisms. At concentrations of 0, the $\Delta d/d$ value was very low and showed few crystal defects. In contrast, at a concentration of 1.2 mg/mL, the $\Delta d/d$ value was high, and the $\Delta d/d$ value in the chitin nanofiber was nearly equal to that observed at a concentration of 1.2 mg/mL. However, the $\Delta d/d$ value in the *P. fucata* prisms was approximately 1.7-fold higher than that observed at a concentration of 1.2 mg/mL.

These results showed that lattice distortion became larger as the chitin fiber became thinner. Although the data are not shown, TEM observation of the cross section of the calcite clarified that thinner chitin fibers were more easily embedded in calcium carbonate crystals. However, the lattice distortion ratio of synthesized calcite was still much smaller than that of prism calcite. This is perhaps because there are many acidic matrix proteins identified from the prismatic layer (Gotliv et al. 2005; Suzuki et al. 2004). Such proteins may also contribute to the increase in the lattice distortion ratio in calcite prisms.

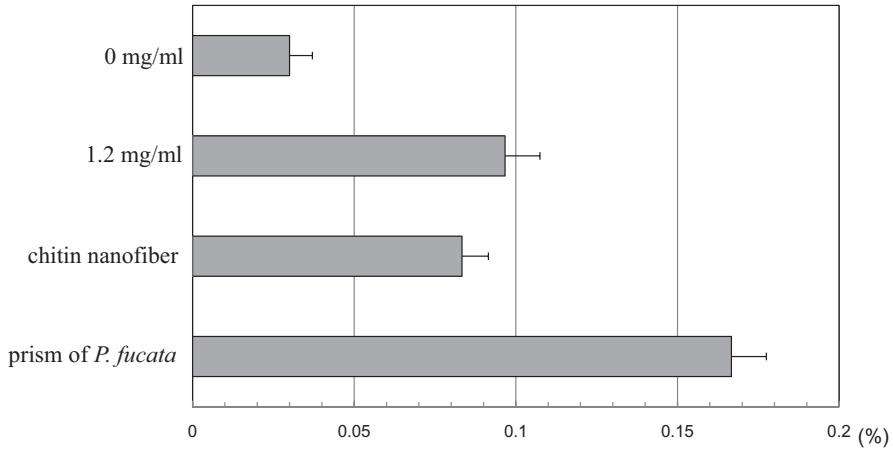


Fig. 40.3 Variance of lattice spacing calculated from Williamson-Hall plots. The synthesized calcites in the chitin hydrogel treated with chitinolytic enzymes at the concentrations of 0, 1.2 mg/mL, and in the chitin nanofiber solution, and prisms of the prismatic layer. Error bars indicate the standard deviation of the measurements ($n = 3$)

Taken together, chitin degradation plays an important role in the formation of calcite prisms in the prismatic layer. Crystal defects became larger as the chitin fibers were degraded by chitinolytic enzymes, because the increasing surface area of chitin fibers strengthens the physical and/or chemical interaction between calcium carbonate and the chitin fiber. This strong interaction may allow chitin fibers to attach to the crystal growth front in a random manner, which prevents calcium or carbonate ions from being well-oriented around crystal growth planes, leading to the crystal lattice distortion. Therefore, in the prismatic layer of *P. fucata*, the thickness of chitin fiber may be precisely regulated by chitinolytic enzyme to induce the small-angle grain boundaries. This novel mechanism may provide useful insights to the fields of biomineralization and biomimetic material engineering.

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