Functional analyses of chitinolytic enzymes in the formation of calcite prisms in *Pinctada fucata*

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Abstract
Background: The mollusk shells present distinctive microstructures that are formed by small amounts of organic matrices controlling the crystal growth of calcium carbonate. These microstructures show superior mechanical properties such as strength or flexibility. The shell of *Pinctada fucata* has the prismatic layer consisting of prisms of single calcite crystals. These crystals contain small-angle grain boundaries caused by a dense intracrystalline organic matrix network to improve mechanical strength. Previously, we identified chitin and chitinolytic enzymes as components of this intracrystalline organic matrix. In this study, we analyzed the function of those organic matrices in calcium carbonate crystallization by *in vitro* and *in vivo* experiments.

Results: We analyzed calcites synthesized in chitin gel with or without chitinolytic enzymes by using transmission electron microscope (TEM) and atom probe tomography (APT). TEM observations showed that grain boundary was more induced as concentration of chitinolytic enzymes increased and thus, chitin became thinner. In an optimal concentration of chitinolytic enzymes, small-angle grain boundaries were observed. APT analysis showed that ion clusters derived from chitin were detected. In order to clarify the importance of chitinolytic enzymes on the formation of the prismatic layer in vivo, we performed the experiment in which chitinase inhibitor was injected into a living *Pinctada fucata* and then analyzed the change of mechanical properties of the prismatic layer. The hardness and elastic modulus increased after injection of chitinase inhibitor. Electron back scattered diffraction (EBSD) mapping data showed that the spread of crystal orientations in whole single crystal also increased by the effect of inhibitor injections.

Conclusion: Our results suggested that chitinolytic enzymes may function cooperatively with chitin to regulate the crystal growth and mechanical properties of the prismatic layer, and chitinolytic enzymes are essential for the formation of the normal prismatic layer of *P. fucata*.

Full-text
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However, the manuscript can be downloaded and accessed as a PDF.

Figures
Figure 1

Analysis of the organic matrices in a calcite prism of *Pinctada fucata*. a, b, Scanning electron microscopy image of the prismatic layer. a, Surface structure. b, Longitudinal structure. c, Transmission electron microscopy image of the cross-section of a calcite prism. The arrow shows interference fringes of equal inclination (black lines), which are observed in a single crystal, and which are interrupted by small-angle grain boundaries. d, Schematic illustration describing how to extract the organic matrices from calcite prisms. e, Structural formula of chitin, which is comprised of N-acetyl-Dglucosamine. f, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the acid-soluble organic matrices (lane 1) and acid-insoluble detergent-soluble organic matrices (lane 2). The box shows main proteins of the organic network. g, Schematic diagram of PfCN (upper) and PfCB (lower).
Figure 2

Synthesis of calcium carbonate crystals with chitin and chitinolytic enzymes. a, Schematic illustration describing the method of crystallization. b–d, 21 Scanning electron microscopy images of the synthesized calcium carbonate crystals in a chitin gel treated with chitinolytic enzymes at different concentrations or in chitin nanofiber solution. b, 0 mg/mL; c, 0.12 mg/mL; and d, 1.2 mg/mL chitinolytic enzymes; and e, chitin nanofiber solution. Dotted lines show the steps of crystals. The steps increased as chitin became thinner. Increasing steps is likely to make crystal shape round.
Figure 3

Analysis of the synthesized calcium carbonate crystals. a–d, Transmission electron microscopy images of a cross-section of synthesized calcium carbonate crystals in a chitin gel treated with chitinolytic enzymes at different concentrations or in chitin nanofiber solution. Low magnification (left) and high magnification (right). a, 0 mg/mL; b, 0.12 mg/mL; and c, 1.2 mg/mL chitinolytic enzymes; and d, chitin nanofiber solution. White arrows denote interference fringes of equal inclination and red arrows denote chitin fibers. White circles show that interference fringes of equal inclination are interrupted by small angle grain boundaries. e–h, Distribution of ions of the synthesized calcium carbonate crystals analyzed by atom probe tomography from a direction parallel to a longitudinal crystal. e, 0 mg/mL; f, 0.12 mg/mL; and g, 1.2 mg/mL chitinolytic enzymes; and h, chitin nanofiber solution. Clusters of some ions are observed. Red, Ca2+; yellow, COH+; and pink, COH2+. 
Figure 4

Change in mechanical properties following allosamidin injection. a, b, Microdiffraction maps of the prism calcites analyzed by electron backscatter diffraction. a, Non-injection. b, Allosamidin injection. Colors denote the orientation of calcite. Right graphs are line profiles from the origin to the end of arrows described in the maps. Red lines show an absolute value and blue lines show a relative value to the previous point. c, 22 d, Force-displacement curves measured by nanoindentation. c, Non-injection measured at eight points. d, Allosamidin injection measured at six points. A force was loaded up to 100 mN, followed by unloading. Blue denotes the spread of displacement. e, Hardness and reduced elastic modulus calculated from nanoindentation data.

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