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Biomineralization: Functions of calmodulin-like protein in the shell formation of pearl oyster

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Abstract

Calmodulin-like protein (CaLP) was believed to be involved in the shell formation of pearl oyster. However, no further study of this protein was ever performed. In this study, the in vitro crystallization experiment showed that CaLP can modify the morphology of calcite. In addition, aragonite crystals can be induced in the mixture of CaLP and a nacre protein (at 16 kDa), which was detected and purified from the EDTA-soluble matrix of nacre. These results agreed with that of immunohistological staining in which CaLP was detected not only in the organic layer sandwiched between nacre (aragonite) and the prismatic layer (calcite), but also around the prisms of the prismatic layer. Take together, we concluded that (1) CaLP, as a component of the organic layer, can induce the nucleation of aragonite through binding with the 16-kDa protein, and (2) CaLP may regulate the growth of calcite in the prismatic layer.

Keywords: Calmodulin-like protein; Biomineralization; Organic layer; Pearl oyster; *Pinctada fucata*

1. Introduction

Biominerals, such as bone and shell, are complex composites of inorganic minerals and organic macromolecules that together exhibit unusual toughness, strength and hardness. The mechanical design of biominerals is so intriguing that scientists have been exploring it for many years [1]. The pearl oyster shell is a biomineralization product of $CaCO_3$ crystals, matrix proteins and other biopolymers, and it consists mainly of two mineralized layers, the inner nacreous layer (nacre) and the outer prismatic layer.

There are three non-hydrated crystalline polymorphs of calcium carbonate: calcite, aragonite and vaterite. Of these minerals, calcite has the most thermodynamically stable structure, while vaterite is the least stable [2] and it can be transformed into aragonite in aqueous media. During the course of shell formation, the prismatic layer (calcite) is first deposited and then the nacre (aragonite) is added as the shell grows in thickness. The remarkable switch between the $CaCO_3$ polymorphs (calcite and aragonite) is controlled by macromolecules that are secreted by the outer mantle epithelium. However, the nature of this process is not yet known [1]. The mechanism of polymorphic transformation has represented a major challenge in the field of biomineralization research [3].

The shell organic matrix is so important that many works have been focused on its fractionation and characterization [4], Heretofore, some matrix proteins have been identified from the nacre or the prismatic layer of molluscan shell [5]. In addition, an organic layer [6,7] observed between the prismatic layer and the nacreous layer was believed to play an important role in the layer transition, i.e. polymorphic transition (from calcite to aragonite). However, the organic layer is so thin that it has not been researched very much due to the difficulty of obtaining material from this layer.

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Recently, we cloned calmodulin and calmodulin-like protein (CaLP) genes from *Pinctada fucata* and the results suggested that they are both involved in shell formation of the pearl oyster [8,9]. It was noted that the expression of CaLP mRNA in various tissues of the pearl oyster is different with the highest level in the mantle, a crucial organ participating in the course of shell formation, while the expression of calmodulin has no significant differences [9]. Perhaps this indicates that CaLP is more important than calmodulin in the shell formation of the pearl oyster.

In this experiment, we have expressed and purified recombinant *P. fucata* CaLP and subsequently detected a CaLP-binding protein (at 16 kDa) in the EDTA-soluble matrix (ESM) of nacre by protein blotting. We found that, in vitro, CaLP not only can modify calcite singly, but also can nucleate aragonite when combined with the 16-kDa protein. These results are consistent with those of in situ immunohistological staining. This is the first report that presents direct evidence about the participation of CaLP in the shell formation.

2. Materials and methods

2.1. Expression and purification of oyster CaLP in Escherichia coli

The coding region of the oyster CaLP was amplified as described by Li et al. [9]. The purified PCR products were digested and inserted into pET-15b (Novagen). The recombinant plasmid was confirmed and transformed into *E. coli* BL21 (DE3, Novagen). After induction, bacterial cells were harvested by centrifugation. For the purpose of protein purification, the bacterial pellet was resuspended and sonicated on ice. Then the lysate was heated for 10 min at 90 °C. The supernatant was collected and loaded onto a DEAE-Sepharose Fast Flow (Amersham) column previously equilibrated with 20 mM Tris–HCl, pH 7.5. Then, the column was washed with the same buffer and eluted with a linear gradient of 0–0.8 M NaCl in the same buffer. Fractions were analyzed by SDS-PAGE. CaLP-containing fractions were collected and dialyzed against Milli-Q water at 4 °C. The protein yields were measured by BCA assay kit (Pierce).

2.2. Isolation of ESM from nacre of P. fucata

The outer prismatic layer of the shell from *P. fucata* was removed and the inner nacreous layer was crushed to a fine powder. The powdered nacre was decalcified with Tris-buffered 0.5 M EDTA (pH 8.0) at 4 °C for three days with constant stirring. The ESM was obtained by centrifugation at 16,000 rpm for 30 min, followed by exhaustive dialysis against Tris-buffered Milli-Q water. The dialyzed solution was concentrated and analyzed by reduced and unreduced SDS-PAGE.

2.3. Preparation of biotinylated CaLP

Biotinylation of CaLP was performed as described by Billingsley et al. [10]. The efficiency of biotinylated CaLP was proven by dot-blots [11] using CaLP-binding proteins extracted from mantle of the pearl oyster using affinity chromatography [9]. BSA, in equivalent amounts, was used as a negative control.

2.4. Protein blotting

Protein blotting was conducted with the method presented in [10] with slight modifications. Proteins in the gel (reduced PAGE) were electroblotted to nitrocellulose (NC) membrane using the Multiphor II Western blotting system (Amersham). After incubation for 1 h in blocking solution (5% nonfat dry milk/20 mM Tris–HCl, pH 7.4/150 mM NaCl/2 mM $CaCl_2$), the blot was incubated with biotinylated CaLP (20 μg/ml) for 2 h in blocking solution containing either 2 mM $CaCl_2$ or, for control studies, 5 mM EGTA. Thereafter, the blot reacted with alkaline phosphatase (AP)-avidin (Calbiochem/Invitrogen) dissolved in blocking solution for 1 h, followed by color development with NBT/BCIP (Invitrogen).

2.5. Purification of the 16-kDa protein by preparative electrophoresis

The 16-kDa protein was purified by preparative electrophoresis as described in [12] with slight modifications. In summary, after SDS-PAGE, the band of the 16-kDa protein was excised from the gel and cut to pieces. The pieces were ground up and subsequently resuspended in 20 mM Tris–HCl buffer, pH 7.5. After incubation for 3 h at 4 °C, the supernatant was collected by centrifugation. Then the supernatant was extensively dialyzed against Milli-Q water at 4 °C and concentrated by ultrafiltration (Millipore; cut-off, 5 kDa).

2.6. In vitro crystallization experiments

The calcitic crystallization solution was prepared according to [13]. Crystallization experiments were carried out by adding samples to the freshly prepared crystallization solution on a slide at 20 °C. After 24 h, the crystallization solution was removed and the crystals were characterized.

2.7. Crystal characterization

For morphological observation and identification of the induced crystals, Raman spectra, scanning electron microscopy (SEM) and optical microscopy were used. The Raman spectra of the crystals were recorded with a Renishaw RM2000 spectrometer. The samples were positioned on a slide under a microscope (×50) that can focus the laser beam on the sample while collecting the backscattered light. The Raman spectra showed a line with a wavelength of 514 nm provided by an argon laser limited to 4.6 mW power. Spectra were recorded from 100 to 1500 cm^{-1}. The scanning electron micrographs were obtained using a SIRION 200 scanning electron microscope (PEI). After crystallization experiments, the cover glass was coated with gold before imaging. For some larger crystals, an optical microscope (Leica DMIRB) was used.

2.8. Immunohistological staining

Immunohistological staining was performed according to [14] with minor modifications. A transversal section of the shell was incubated in 4% formaldehyde containing solution of 0.5 M EDTA, pH 8, at room temperature with gentle shaking for 7 days for demineralization. After demineralization, the samples were washed with Milli-Q water and incubated in rabbit serum of anti-recombinant CaLP (prepared and donated by Dr. Zi Fang, unpublished data). The serum was diluted 1:20 in TBS and incubated for 1 h with 5% nonfat dry milk. After washing with TBS, the secondary antibody, AP-conjugated goat-anti-rabbit (1:500, Sigma) was applied to the samples for 1 h. Then, the samples were washed with TBS and the color was developed with NBT/BCIP (Invitrogen). The negative control sample was prepared with preimmune serum.

3. Results

3.1. Expression and purification of recombinant CaLP

As shown in Fig. 1, the expressed recombinant CaLP reached approximately 20% of the total bacterial soluble proteins. After heating at 90 °C for 10 min, most bacterial soluble proteins were denatured while the recombinant CaLP demonstrated high heat stability and was purified by anion exchange chromatography. After purification, only a single band was observed on SDS-PAGE, and in the presence of Ca^{2+}, its relative molecular mass was 17 kDa which was consistent with the predicted molecular mass.

3.2. Isolation of ESM from nacre

The analysis of ESM by SDS-PAGE is shown in Fig. 2. Many protein bands can be seen under the reduced conditions,