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A novel extracellular EF-hand protein involved in the shell formation of pearl oyster

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Abstract

Mollusk shell formation is a complicated and highly controlled calcium metabolism process. Previous studies revealed that several EF-hand calcium-binding proteins actively participate in the regulation of shell mineralization. In this study, we cloned a full-length cDNA encoding a novel extracellular EF-hand calcium-binding protein (named EFCBP) from the pearl oyster, *Pinctada fucata*, according to the EF-hand motifs of calmodulin. Although it shares high similarity with the calmodulin family in its EF-hand signatures, EFCBP just has two EF-hand motifs and belongs to a new separate group from the other EF-hand proteins according to a phylogenetic analysis. EFCBP is specifically expressed in shell mineralization-related tissues, viz. the mantle, the gill, and the hemocytes. Moreover, its expression responds quickly only to the shell damage, but not to the damage of other tissues and the infection of the lipopolysaccharides from *Escherichia coli*. These results suggest that EFCBP might be an important regulator of shell formation. This finding may help better understand the functions of EF-hand proteins on the regulation of mollusk shell formation.

Keywords: EF-hand; Biomineralization; Calcium metabolism; Regulator; EFCBP; *Pinctada fucata*

1. Introduction

The mollusk shell, especially the nacre, is a wonderful masterpiece of nature. Although calcium carbonate accounts for more than 95% of the nacre weight, unlike inorganic calcium carbonate, it is arranged in a highly functional and strictly controlled way under the instructions of many organic macromolecules secreted from the mantle tissues or elsewhere [1–3]. These characteristics endow nacre with excellent mechanical, physical, and chemical properties [3–5], which have attracted the interest of many biologists, as well as material scientists, nanotechnologists and chemists in recent decades.

As for the biologists, unraveling the molecular mechanism under the macrostructure of the shell and the nacre is not enough. It should not be neglected that from the viewpoint of evolution and physiology, the mollusk shell may be seen as a product of calcium metabolism that releases the stress of calcium accumulated in the body of the marine mollusk and also serves as a reservoir of calcium ions. Shell formation involves the sequential processes of the calcium absorption, accumulation, transportation, and incorporation. And more importantly includes the complicated regulation networks which coordinate the cellular activities with the environmental changes [2]. However, these processes, especially the regulation mechanism involved in, have not been well investigated. On the other hand, another biominerallization system in vertebrate, namely the bone formation system, has been extensively studied. Its regulation mechanism may give us some clues to our studies on shell formation. In the bone formation process, calcium-binding proteins play a pivotal role in constructing the extracellular

Abbreviations: CaM, calmodulin; CaLP, calmodulin-like protein; PFMG1, *Pinctada fucata* mantle gene 1; PFMG6, *Pinctada fucata* mantle gene 6; RACE, rapid amplification of cDNA ends; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; DEPC, diethypyrocarbonate; LPS, lipopolysaccharides; BLAST, basic local alignment search tool

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matrix for calcium sedimentation and modulating the physiological activities of osteoblasts and osteoclasts. The former function is carried out by a group of acidic phosphoproteins which are rich in acid amino acids and phosphorylation modification [6,7]; and the latter is usually performed by a number of protein families containing the EF-hand domains, such as osteonectin, calcyclin, S100A4, and calbindin [8–12]. Acidic matrix proteins isolated from the shell act like the phosphoproteins and induce the formation of shell calcium carbonate crystals [13,14], while as to the functions of EF-hand protein families on shell mineralization, only *Pinctada fucata* mantle gene 1 (PFMG1) [15], calmodulin (CaM) [16] and calmodulin-like protein (CaLP) [17,18] cloned from the oyster *P. fucata* and calconectin [19] cloned from the oyster *Pinctada margaritifera* have been studied. As the EF-hand proteins regulate the activities of bone formation-related cells, we postulate that it might function similarly in the mollusk mantle cells. Therefore, we tried to find more EF-hand proteins in the mantle cells of the pearl oyster *P. fucata* and reveal their functions on shell formation.

In the previous work of our group, we cloned *P. fucata* CaM and CaLP, and studied their functions on calcium metabolism and biomineralization [16–18]. Based on these studies, we firstly tried to find some other calmodulin-like proteins. We designed degenerate primers according to the characteristics of calmodulin EF-hand motifs, viz. the first and the third amino acids are aspartic acid, the fourth and the sixth amino acid are glycine, the second amino acid is alanine or lysine, and the fifth amino acid is aspartic acid or asparagine. Via rapid amplification of cDNA ends (RACE), we acquired a full-length cDNA sequence encoding a new kind of extracellular EF-hand calcium-binding protein that was named EFCBP. Using a homology search with the Basic Local Alignment Search Tool (BLAST) program, we found three other proteins with high similarity with EFCBP. A phylogenetic analysis of their EF-hand signatures indicated that they belong to a separate group from the other EF-hand proteins. As described below, EFCBP is expressed in the specific mantle areas, the gill, and the hemolymph. These tissues and cells have been reported previously to take active part in the shell formation. Moreover, a notching in the shell margin could increase the expression of EFCBP rapidly, while either the damage of other tissues or infection with lipopolysaccharides (LPS) from *Escherichia coli* did not alter its expression. Its specific response to shell damage suggested that EFCBP might be an important regulator of shell formation.

2. Materials and methods

2.1. RNA preparation and RACE

Live individuals of adult oyster *P. fucata* were obtained from the Guofa Pearl Farm in Beihai, Guangxi Province, China. Total RNA was extracted separately from the mantle, viscus, gill, hemocytes, and adductor muscle tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of the RNA was determined by electrophoresis on a 1.2% formaldehyde-denatured agarose gel stained with ethidium bromide. The quantity of RNA was determined by measuring OD_{260} with an Ultrospec 3000 UV/Visible Spectrophotometer (Amersham, Piscataway, NJ, USA).

5′-RACE and 3′-RACE was performed by using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) and Advantage 2 cDNA Polymerase Mix (Clontech) following the manufacturer's instructions. Single-stranded cDNA for all RACE reactions was prepared from the mantle total RNA, using PowerScript™ (Clontech). Two degenerate primers, EFP5 (5′-GAY GCN GAY GGN RAY GG-3′) and EFP5′ (5′-GAY AAR GAY GGN RAY GG-3′) were designed for 3′-RACE. The total volume of a 3′-RACE reaction was 20 μl. The reaction system contained 1 μl cDNA template, 2 μl 10× BD Advantage 2 PCR Buffer, 2 μl 10× UPM (Universal Primer A Mix), 1 μl 50 μM EFP5 or EFP5′, 0.4 μl 10 mM dNTP Mix, 0.4 μl 50× BD Advantage 2 Polymerase Mix, and 13.2 μl PCR-Grade Water. Touchdown PCR program was adopted, namely within the first 10 cycles, the annealing temperature decreased by 1 °C every PCR cycle till to the calculated Tm values of the degenerate primer, and then the annealing temperature was fixed at (Tm-2) °C for another 25 cycles. The amplified product was cloned into pGEM-T Easy Vector (Promega) and sequenced. Then according to the cDNA sequence acquired above, a gene-specific primer EFP3 (5′-TTC TGA CAG GCG TCT ATC AAC G-3′) was synthesized and used for the 5′-RACE. The PCR conditions were similar with 3′-RACE except that the concentration of EFP3 was 20 μM. The PCR product was then cloned into pGEM-T Easy Vector (Promega) and sequenced.

To confirm the accuracy of the cDNA sequence, the full-length cDNA was amplified with the primer pair of EFgsp5 (5′-CAA AGG AAA AGC TCA ATT AGG AG-3′) and EFgsp3 (5′-ATA CAG AGA TAA GAT CTG CTT CC-3′), using the mantle cDNA library of *P. fucata* as a template. The PCR product was then cloned into pGEM-T Easy Vector (Promega) and sequenced.

2.2. Analysis of the deduced protein sequence

The presence and location of the signal peptide was analyzed with the SignalP v3.0 server (CBS prediction servers; http://www.cbs.dtu.dk/). Protein domains were determined using the PROSITE database (http://www.expasy.org/prosite/). The secondary structure prediction was carried out according to the method of McGuffin et al., using the PSIPRED Protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). Protein sequence similarity searches were performed with the BLAST program in GenBank (http://www.ncbi.nlm.nih.gov/). Protein multiple alignments and phylogenetic analysis were performed by the ClustalX program.

2.3. Gene expression analysis by RT-PCR

Total RNA was extracted from different tissues as described above. Equal quantities (2 μg) of the total RNA were reverse-transcribed into cDNA with Superscript III RNase H^- Reverse Transcriptase and oligo(dT) primers in 20 μl reaction mixtures (Invitrogen, CA, USA). GAPDH was adopted as the positive control for cDNA preparation and was amplified with the primer pair of GAPDH5 (5′-GCC GAG TAT GTG GTA GAA TC-3′) and GAPDH3 (5′-CAC TGT TTT CTG GGT AGC TG-3′). EFCBP was amplified with the primer pair of EFgsp5 and EFgsp3. Negative control was the RT-PCR in the absence of any cDNA template. First, GAPDH was amplified, and according to the quantities of the PCR products of GAPDH, the amounts of the cDNA templates were adjusted to be the same. Then EFCBP was amplified. The PCR process of GAPDH and EFCBP consisted of 95 °C for 5 min, 28 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, and finally 72 °C for 10 min. The PCR products were subcloned as described above and verified by sequencing. The experiments were repeated using different individuals and the results were consistent.

2.4. In situ hybridization

The mantle was separated from the adult *P. fucata* and immediately fixed in 4% paraformaldehyde and 0.1% DEPC solution overnight. *In situ* hybridization of EFCBP mRNA was carried out on frozen sections of the mantle (10 μm thick). Digoxigenin-labeled RNA probes were generated from the pGEM-T Easy Vector with the EFCBP insertion in the multiple cloning sites, using a DIG