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Research article

Molecular characterization of *CHST11* and its potential role in nacre formation in pearl oyster *Pinctada fucata martensii*

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ABSTRACT

Background: C4ST-1 catalyzes the transfer of sulfate groups in the sulfonation of chondroitin during chondroitin sulfate synthesis. Chondroitin sulfate consists of numerous copies of negatively charged sulfonic acid groups that participate in the nucleation process of biomineralization. In the present study, we obtained two *CHST11* genes (*PmCHST11a* and *PmCHST11b*) which encoded the C4ST-1 and explored the functions of these genes in the synthesis of chondroitin sulfate and in the formation of the nacreous layer of shells.
Results: Both *PmCHST11a* and *PmCHST11b* had a sulfotransferase-2 domain, a signal peptide and a transmembrane domain. These properties indicated that these genes localize in the Golgi apparatus. Real-time PCR revealed that both *PmCHST11a* and *PmCHST11b* were highly expressed in the central zone of the mantle tissue. Inhibiting *PmCHST11a* and *PmCHST11b* via RNA interference significantly decreased the expression levels of these genes in the central zone of the mantle tissue and the concentration of chondroitin sulfate in extrapallial fluid. Moreover, shell nacre crystallized irregularly with a rough surface after RNA interference.
Conclusions: This study indicated that *PmCHST11a* and *PmCHST11b* are involved in the nacre formation of *Pinctada fucata martensii* through participating in the synthesis of chondroitin sulfate.

1. Introduction

Chondroitin sulfate proteoglycans are common in animal tissues. These proteoglycans are implicated in the development of human bone and joint diseases [1,2,3]. When negatively charged sulfonic acid groups are abundant, chondroitin sulfate participates in crystal nucleation and metal ion enrichment in biomineralization [4]. Chondroitin sulfate has a protective role in cartilage [5] by inhibiting cartilage arthritis [6]. Chondroitin-4-sulfate and chondroitin-6-sulfate exist in the organic matrix of nacre from the shell of *Haliotis rufescens* and the location of these proteoglycans indicates that chondroitin sulfate is associated with biomineralization in molluscs [7].

Carbohydrate sulfotransferase 11 (*CHST11*)/chondroitin-4-sulfotransferase-1 (C4ST-1) catalyzes the transfer of sulfate to the C-4 position of chondroitin sulfate disaccharides [8] during the synthesis of chondroitin-4-sulfate (C4S) and is involved in several biological processes [9,10,11,12]. Karlsson et al. [13] and Reynard et al. [14] reported that *CHST11* is associated with osteoarthritis and with chondrogenesis. The potential functions of acid glycosaminoglycan (GAGs) in nacre formation have been documented recently [15]. However, the role of *CHST11* in the synthesis of sulfate chondroitin and its influences on shell nacre formation are unknown.

The pearl oyster *Pinctada fucata martensii* is cultured in China and Japan for the production of nucleated pearls. In addition, *P. f. martensii* is the model species for the study of nacre formation [16]. We found that the organic matrix extracted from nacreous shells of *P. f. martensii* contain more acidic GAGs than those extracted from prismatic layers. Furthermore, the organic matrix extracted from the nacreous shells of *P. f. martensii* contained more acidic GAGs than that extracted from the shells of *Crassostrea gigas*. In addition, we detected acidic GAGs in the secretory cells of the central mantle zone of *P. f. martensii* and neutral GAGs in the mantle of *C. gigas* (unpublished). In this study, the full-length sequence of *CHST11* of *P. f. martensii* was obtained via the rapid amplification of cDNA ends (RACE) technique. We then elucidated the functions of *CHST11* in the formation of chondroitin sulfate.

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2. Materials and methods

2.1. Experimental animals

Adult pearl oysters were obtained from commercial farm (20°250 N, 109°570E) Dajing Xuwen, Zhanjiang, Guangdong Province, China. The animals were cultured at 25–27°C in tanks with recirculating seawater for 2–3 days before the experiment.

2.2. RNA extraction and cDNA synthesis

The animals were sacrificed for tissue sampling. The tissue samples included the marginal zone (ME) and central zone (MC) of the mantle, adductor muscle (A), gill (GI), gonad (GO) and haemocytes (B). The tissues were immediately stored in liquid nitrogen until use.

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA quantity was evaluated by measuring OD260/OD280 with a NanoDrop ND1000 spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA). RNA integrity was determined by fractionation on 1.0% agarose gel.

2.3. Rapid amplification of cDNA ends (RACE)

In this study, the two *CHST11* genes of pearl oyster *P. f. martensii* were designated as *PmCHST11a* and *PmCHST11b*. Primers were designed based on a partial sequence of the *CHST11* gene from the pearl sac transcriptome of *P. f. martensii* [17] and are listed in Table 1. Nested PCR was performed to increase the specificity and sensitivity of the PCR products.

Table 1
Primer list used in this study.

Primer Name	Primer Sequence (From 5′ to 3′)	Application
PmCHST11a-5′ outer	TTGAAGTTCGTTCGCTCCGACTGAATAA	5′ RACE
PmCHST11a-5′ inner	TCGCCAGAATGTAGATCCAATCTTTTCCA	5′ RACE
PmCHST11a-3′ outer	ACCGTCTCAGCATGGACTTTAGAAACAGC	3′ RACE
PmCHST11a-3′ inner	AGGGTGATGTAAGAGACCCACTGATCGC	3′ RACE
PmCHST11b-5′ outer	AAGAAATGTATGACCAAATAAGCCT	5′ RACE
PmCHST11b-5′ inner	GTTTAGCGATTATATCATAAGGTAT	5′ RACE
PmCHST11b-3′ outer	GAGACCGCAAAGGAGATGTTCCCTCTT	3′ RACE
PmCHST11b-3′ inner	TTCAAGGTTACATCCGATCCTCTTTCCAA	3′ RACE
GAPDH-S	GCAGATGGTGCCGAGTATGT	qRT-PCR
GAPDH-A	CGTTGATTATCTTGGCGAGTG	qRT-PCR
PmCHST11a-qPCR-S	TCCAAGGCGAAAAGTCCG	qRT-PCR
PmCHST11a-qPCR-A	TGTTGAAGATTCTAGCACAGCGTA	qRT-PCR
PmCHST11b-qPCR-S	TAGATTTCCATTGGCAACCTTT	qRT-PCR
PmCHST11b-qPCR-A	CGAGTTATTTGTACGGTGTTGATG	qRT-PCR
RNAi-RFP-S	GCGTAATACGACTCACTATAGGG CTGTCCCCCCAGTTCCAGTAC	RNAi
RNAi-RFP-A	GCGTAATACGACTCACTATAGGG CGTTGTGGGAGGTGATGTCCAGCT	RNAi
RNAi-*PmCHST11a*-S	GCGTAATACGACTCACTATAGGGAAAAGTCCG TTTGATATACCACCAG	RNAi
RNAi-*PmCHST11a*-A	GCGTAATACGACTCACTATAGGGGAGCGTTGA AATAGTCATCTACCGT	RNAi
RNAi-*PmCHST11b*-S	GCGTAATACGACTCACTATAGGGCGGGTAGAC TTGTAGATTTCCATTG	RNAi
RNAi-*PmCHST11b*-A	GCGTAATACGACTCACTATAGGGGCGGCAGAA AGATAGTACAAACG	RNAi

Notes: the sequences underlined are the T7 promoter sequence.

To prepare the single-strand cDNA template for RACE reactions, total RNA was extracted from mantle tissue with SMART RACE cDNA Amplification Kit (Takara, Dalian, China) in accordance with the manufacturer's instructions. The synthesized cDNA was used as the template for the first PCR reaction. The product of the first PCR reaction was used as the template for the second PCR reaction. The PCR program was executed as follows: 95°C for 5 min, 35 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 2 min and 72°C for 10 min.

2.4. DNA sequencing and sequence analysis

Purified PCR products were subcloned into a pMD-19T vector (TAKARA, Japan), transformed into DH5α and then sequenced. The full-length cDNA of the *PmCHST11* genes were obtained with DNAMAN software. BLAST program (http://www.ncbi.nlm.nih.gov/) was used to analyze the full-length cDNA of *PmCHST11* genes. The open reading frame (ORF) was identified by using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The amino acid sequence was characterized with DNAMAN. SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi) and the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were used to study the domain of the detected peptide and the transmembrane domain of the *PmCHST11* genes, respectively. Multiple sequence alignments were generated with the sulfotransferase domain amino acid of each sequence by using ClustalX (http://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was constructed using MEGA6.0 program based on the neighbor-joining method with 1000 bootstrap replicates. CHST11 from *Polymorphum gilvum* was used as the out group.

2.5. Quantitative real-time PCR (qRT-PCR) assay

The primer sequences used for the qRT-PCR assay are shown in Table 1. The qRT-PCR assay was performed on Applied Biosystems 7500/7500 Fast Real-Time System (Applied Biosystems, Foster City, CA, USA) with Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific). GAPDH was selected as the reference gene to verify the expression levels of sulfotransferase genes [18,19,20,21]. To detect gene expression patterns of *PmCHST11* genes, the comparative Ct method was used to analyze the expression levels of the genes. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. The Ct values for the *PmCHST11* genes and the GAPDH gene were determined for each sample. The expression of *PmCHST11* genes were calculated by the $2^{-\Delta Ct}$ method with GAPDH as the reference gene. The PCR program was conducted as follows: 5 min at 95°C and 40 cycles (each cycle was for 30 s at 95°C, 15 s at 60°C and 15 s at 72°C).

2.6. RNA interference (RNAi) experiment

Sequence-specific primers for the experiment were designed and are listed in Table 1. An experimental group and two control groups were separately designed to detect the effects of *PmCHST11* expression on nacre formation. Ten animals (5–6 cm in shell length) were used per group.

PCR products were obtained by using specific primers and were purified by using EasyPure Quick Gel Extraction Kit (Thermo Scientific). dsRNA was synthesized from PCR products by using T7 RNA polymerase (Thermo Scientific). Template DNA was digested by RNase-free DNase I (Thermo Scientific). dsRNA integrity and quantity were verified as previously described. dsRNA-*PmCHST11* were diluted to 1000 ng/μL with RNase-free water and injected into the adductor of per oyster. Another injection with the same dose was performed on day 4 after the first injection. The same volume of RNase-free water and 100 μg of dsRNA-RFP in RNase-free water were separately injected [22] into oysters in the two control groups.