Aquaculture and Fisheries 2 (2017) 119–123

Contents lists available at ScienceDirect

Aquaculture and Fisheries

journal homepage: www.keaipublishing.com/en/journals/aquaculture-and-fisheries/

Original research article

Hic74, a novel alanine and glycine rich matrix protein related to nacreous layer formation in the mollusc *Hyriopsis cumingii*

Xiaojun Liu [a, 1], Can Jin [a, 1], Leiming Wu [a], Shaojian Dong [a], Shimei Zeng [a], Jiale Li [a, b, *]

[a] Key Laboratory of Genetic Resources for Freshwater Aquaculture and Fisheries, Shanghai Ocean University, Ministry of Agriculture, Shanghai 201306, China
[b] E-Institute of Shanghai Universities, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form
27 February 2017
Accepted 10 March 2017
Available online 12 April 2017

Keywords:
Hyriopsis cumingii
Freshwater mussel
Matrix protein
Pearl
Biomineralization

ABSTRACT

Organic matrix proteins play an important role in the biomineralization of mollusc shells. We have identified and characterized a novel matrix protein, namely hic74, isolated from the mantle of the freshwater mussel *Hyriopsis cumingii*. The hic74 gene encodes a 850 amino acid protein that is rich in alanine (ala, 30.8%), glycine (gly, 25.8%) and serine (ser, 10.6%), with ala mainly existing in poly-ala forms within the ala/gly-rich regions. Quantitative PCR expression analysis demonstrated that *hic74* was specifically amplified from the mantle and *in situ* hybridization showed a strong signal in the epithelial cells in the pallial region of the mantle. Lower levels of expression were detected during the early stages of pearl sac formation but increased and remained constant during nacreous layer formation suggesting that hic74 might be involved in this process.

1. Introduction

Biomineralization is an incredible process in which organisms use raw locally-produced materials obtained from the external environment to form functional minerals (Simkiss & Wibur, 1989). The molluscan shell is a marvellous product that results from calcium carbonate ($CaCO_3$) biomineralization. It is generally composed an inner nacreous layer and an outer prismatic layer and the $CaCO_3$ polymorphs are of calcite and aragonite and only occasionally the less stable polymorphs such as vaterite and amorphous calcium carbonate occur (Marin, Luquet, & Marie, 2008).

In the calcified layer, the organic matrix accounts for 5% (*w*/*w*) of the shell and is incorporated between the $CaCO_3$ crystals indicating their dominant role in the shell formation (Alivisatos, 2000; Falini, Albeck, & Weiner, 1996). During nacreous layer formation, the organic matrix is secreted by the mantle cells to the extrapallial fluid, where the hydrophobic proteins and chitins build an organic framework, and the acidic matrix proteins cover the organic network structure for the assembly of nucleation (Addadi, Joester, & Nudelman, 2006). The essence of framework formation is a complex and tightly regulated process and creates the necessary microenvironments for production of the crystals and supply a surface for specific molecular reorganization. Several framework proteins have been isolated from the nacreous layer. For example, pearlin that is rich in gly and tyr was demonstrated to be associated with the formation of the organic network (Miyashita, Takagi, & Okushima, 2000). MSI7 is a multifunctional matrix protein, its C-terminus is involved in the formation of the framework, N-terminus manipulated to bind Ca^{2+} and the gly-rich region in the middle is responsible for the flexibility of the framework (Feng, Fang, & Yan, 2009; Zhang, Xie, & Meng, 2003a). MSI60 is rich in ala and gly mainly exists in poly-ala/gly forms, which enable the protein to self-assemble into fiber-like structures possessing good extensibility and offering structural support for crystal nucleation and growth, and the asp-rich regions are proposed to be the Ca^{2+}-binding sites (Sudo, Fujikawa, & Nagakura, 1997).

Hyriopsis cumingii is a unique Chinese freshwater mollusc that accounts for 70% of the freshwater pearls and is widely cultured in the Jiangsu, Zhejiang, Jiangxi, Hunan and Anhui provinces (Li & Li, 2009; Wang, Yuan, & Li, 2007). In recent years, *H. cumingii* has become a popular experimental model to study shell biomineralization, and numerous genes associated with this process and shell-matrix proteins were isolated. Alkaline phosphatase is distributed across the mantle and has a vital role in the absorption of calcium

* Corresponding author. 999 Huchenghuan Road, Shanghai 201306, China.
E-mail address: jlli2009@126.com (J. Li).
[1] These two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.aaf.2017.03.005

ions (Tang & Shi, 2000), and two glycoproteins isolated from pearls and shells contribute to crystal morphology (Natoli, Wiens, & Schroeder, 2010). Perlucin is highly expressed in the mantle, adductor muscle gill, and hemocytes and *in situ* hybridization indicated that it was specificity expressed in the pallial region of the dorsal mantle, thereby implying its involvement in nacreous layer formation (Lin, Ma, & Bai, 2013). Nacrein was isolated by fast protein liquid chromatography (FPLC) and *in vitro* crystallization experiments revealed it role in crystal morphology (Han, Li, & Shi, 2010). In addition, to regulating crystal morphology, a few framework proteins have been isolated and their role in mechanisms of organic framework formation explored. Silkmapin is a silk-like protein involved in framework construction and contains a C-terminal asp-X-asp motifs that promote Ca^{2+} binding (Liu, Dong, & Jin, 2015a). Furthermore, a novel prismatic layer protein, hic31, which is structurally similar to collagen I alpha 1 and alpha 2, has recently been cloned and shown to be involved in shell and pearl framework formation (Liu, Zeng, & Dong, 2015b).

Research involving matrix proteins continues to accumulate experimental data in order to formulate a theoretical model for shell biomineralization. In this study, we have identified and characterized a novel matrix protein, hic74, from the nacreous layer of *H. cumingii*. Our results indicated that hic74 is specifically expressed in dorsal epithelial cells of the pallial mantle and may play an important role in pearl and shell formation.

2. Materials and methods

2.1. Samples

Two-year-old *H. cumingii* of approximately 7.5–8.0 cm in length were purchased from the Wuyi pearl farm (Jinhua, Zhejiang Province, China). To avoid stress, the mussels were maintained in the river way for 5 days before being used in the experiment.

2.2. Rapid amplification of cDNA ends (RACE)

The mantle stored in TRIzol was broken by QuickPrep Adapter (MP Biomedicals, Santa Ana, CA, USA) and total RNA extraction was performed according to the instructions of TRIzol Reagent Kit. 5′ and 3′ RACE were conducted using the SMART RACE cDNA Amplification Kit (Clontech) and the Advantage 2 cDNA Polymerase Mix (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. 5′ RACE degenerate primer was designed based on amino acid repeats AAAAAAA of MSI60, a framework protein in nacreous layer (Table 1). According to 5′ RACE sequencing results, we designed the 3′ RACE gene-specific primer (Table 1). The isolated transcript was sequenced and its identity was confirmed by searching with the putative encoded amino acid sequence against GenBank, Swiss-Prot and other databases using the BLAST program (http://www.ncbi.nlm.nih.gov/).

2.3. In silico analysis

Nucleotide and amino acid sequence similarity searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/). The open reading frame of hic74 was deduced using the ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), the signal-peptide sequence was deduced using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), and the protein properties determined by ProtParam (http://web.expasy.org/protparam/). The secondary structure analysis was performed by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.4. Pearl-nucleus-inserting experiment

In September 2015, 200 mussels (shell length: 7.5–8.0 cm) were selected for pearl-nucleus-inserting experiments. The outer epithelial cells from the mantle were implanted into the mantle connective tissue of breeding pearl mussels. On days 3, 5, 8, 11, 15, 18, 22, 25, 30, 37 and 50 after grafting, six mussels were collected at each time point. The pearl sacs in the mantle tissue were carefully peeled off and immediately placed in RNAzol (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Gene expression analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Numerous tissues, including gills, foot, adductor muscle, intestine, liver, kidneys and mantle were sampled and frozen in RNAzol (Thermo Fisher Scientific) at −80 °C. Total RNA of the pearl sacs in the pearl-nucleus-inserting experiment was extracted using a TRIzol Reagent Kit (Thermo Fisher Scientific) according to manufacturer instructions. The first strand of cDNA was synthesized using a reverse transcriptase kit (Promega, Madison, WI, USA) and the EF-1α was the housekeeping gene (Bai, Lin, & Ma, 2014). The primers used for *EF-1α* and *hic74* are listed in Table 1. The reaction contained 10 μL SYBR Premix Ex Taq II (Takara Bio. Inc., Japan), 0.8 μL of 10 μM primer, 80 ng cDNA, and 6.4 μL of RNase-free water. Reactions were performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the program used was 95 °C for 3 min, followed by 40 cycles at a melting temperature of 95 °C for 10 s, an annealing temperature of 58 °C for 30 s, then 5 s from 65 °C to 95 °C for dissociation curve analysis. Relative expression levels were calculated according to the equation $2^{-\Delta\Delta C_T}$.

2.6. In situ hybridization

To assess hic74 expression in the mantle, the digoxigenin (DIG) RNA Labelling Kit (Roche) was used to produce DIG-labelled RNA probes. Sense and antisense probes were synthesized using the SP6 and T7 RNA polymerases (TaKaRa). Fresh mantle tissue was soaked in 4% paraformaldehyde for 6 h and incubated in 25% sucrose buffer

Table 1
List of the primers used for the amplification reactions.

Gene	Sense (5′–3′)	Antisense (5′–3′)	Application
hic74		NGCNGCNGCNGCNGCNGC (N = A/T/G/C)	5′-RACE
	CCGCTTCATCGTCATCAAATG		3′-RACE
	TGCCGAAGGACAGGCTAATG	ATGCGGACGCAGAGGAAGAG	ISH
	TGGTCTGGCTTCGGTCCTCG	CTGCTGCTGTCGTCATCATACTCG	qRT-PCR
EF-1α	GGAACTTCCCAGGCAGACTGTGC	TCAAAACGGGCCGCAGAGAAT	qRT-PCR