Comparative Biochemistry and Physiology, Part B 203 (2017) 141–148

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Molecular characterization and expression analysis of chitinase from the pearl oyster *Pinctada fucata*

Haimei Li [a,b,1], Deqing Wang [a,b,1], Zhenghua Deng [a,b], Guiju Huang [a], Sigang Fan [a], Daizhi Zhou [a,b], Baosuo Liu [a], Bo Zhang [a], Dahui Yu [a,*]

[a] Key Laboratory of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture and Division of Aquaculture and Biotechnology, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China
[b] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form 19 October 2016
Accepted 31 October 2016
Available online 05 November 2016

Keywords:
Chitinase-1 gene
Expression profile
Shell formation
Biomineralization
In situ hybridization
Larval stage
Pinctada fucata

ABSTRACT

Chitinase is an enzyme that plays an important role in the chitin metabolism of a wide range of organisms. However, the function of chitinase in the pearl oyster *Pinctada fucata* is yet to be determined. In this study, a chitinase gene (named *PfChi1*) was cloned from *P. fucata* and its expression profiles were investigated. The full-length cDNA of *PfChi1* was 2743 bp and consisted of a 2187-bp open reading frame encoding 728 amino acid residues, a 47-bp 5′-untranslated region (UTR), and a 509-bp 3′-UTR. Similar to other known chitinases, the PfChi1 protein is composed of an N-terminal leading signal peptide, a catalytic domain, a linker region, and a C-terminal chitin-binding domain. The results of qRT-PCR showed that *PfChi1* was expressed in a wide range of tissues with relatively high levels in the mantle, muscle, gill, and gonad, and relatively low levels in hemocytes, the intestine, and the digestive gland ($P < 0.05$). In situ hybridization showed that *PfChi1* was mainly expressed in the mantle edge, particularly in the outer epithelial cells of the inner fold, whereas few hybridization signals were detected in the inner epithelial cells of the middle fold. A shell damage experiment indicated that *PfChi1* transcript levels were up-regulated significantly ($P < 0.05$) at 24 h after shell damage and decreased gradually thereafter, followed by shell regeneration, indicating that *PfChi1* is involved in shell formation. In addition, *PfChi1* expression was higher in trochophore larvae than in other developmental stages ($P < 0.05$), indicating a possible association with the formation of prodissoconch shells. To the best of our knowledge, this study is the first to report the potential biomineralization function of a chitinase in *P. fucata*.

1. Introduction

Chitin, a long unbranched insoluble homopolymer composed of 1,4-β-linked *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant biopolymer on earth after cellulose (Renkema et al., 1997; Daimon et al., 2003). Chitin is distributed widely in nature, and is found as a structural constituent of fungal cell walls (Duo-Chuan, 2006); the cuticle and integuments of arthropods (Xia et al., 2016), nematodes (Aranda-Martinez et al., 2016), mollusks (Minamoto et al., 2015), and in some algae (Daimon et al., 2003). In recent years, studies on the functions of chitin in bivalves have concentrated mainly on the Pacific oyster *Crassostrea gigas* (Badariotti et al., 2007; Badariotti et al., 2011), the mussel *Mytilus galloprovincialis* (Weiss et al., 2009), and the pearl oyster *Pinctada fucata* (Suzuki et al., 2007; Suzuki et al., 2009). In both adult and larval developmental stages, chitin is believed to play a fundamental role in the biomineralization of mollusk shells (Weiss et al., 2009). Chitin is synthesized by chitin synthase and degraded or remodeled by chitinases. However, little is known about the functions of chitinase in mollusk shell formation.

Chitinases (EC 3.2.1.14) are *endo*-β-1,4-*N*-acetylglucosaminidases that cleave a bond between the C1 and C4 atoms of two consecutive *N*-acetylglucosamines of chitin (Flach et al., 1992; Okada et al., 2013). Chitinase is an important enzyme in both chitin-containing and chitin-less organisms (Mali et al., 2004) and is essential for the normal function of organisms. In vertebrates, chitinase is generally synthesized for defense against chitin-containing pathogens (Gooday, 1999; Malaguarnera et al., 2003), molting in arthropod (Watanabe and Kono, 1997; Arakane et al., 2003), and digestion of chitinous food (Spindler-Barth et al., 1990; Kramer and Muthukrishnan, 1997). There have, however, been few studies on molluscan chitinases, with just a small number of reports on *C. gigas* (Badariotti et al., 2007; Badariotti

Abbreviations: Chi, chitinase; *PfChi*, chitinase gene from *Pinctada fucata*; ORF, open reading frame; UTR, untranslated region; qRT-PCR, quantitative real-time polymerase chain reaction; RACE, rapid-amplification of cDNA ends; DIG, digoxigenin; GSP, gene-specific primer; CBM14, carbohydrate-binding module family 14.

* Corresponding author at: Key Laboratory of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture, Guangzhou 510300, China.
E-mail address: pearlydh@163.com (D. Yu).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.cbpb.2016.10.007
1096-4959/

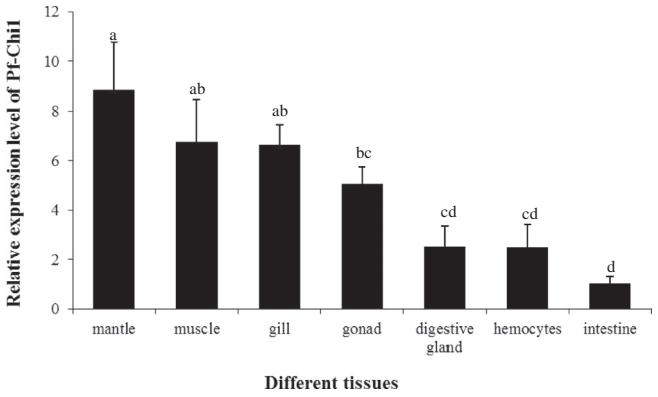

Fig. 1. Relative expression levels of *PfChi1* in various tissues of *Pinctada fucata*. Gonad tissue was derived from females (oocyte stage). Three biological replicates were analyzed for each tissue, and three technical replicates were analyzed in each PCR reaction. Data are expressed as the mean ± S.D. Different letters indicate a significant difference among tissues at $P < 0.05$.

et al., 2011; Okada et al., 2013) and *Hyriopsis cumingii* (Wang et al., 2012), but none on *P. fucata*.

P. fucata is an economically important marine pearl-producing species in China and Japan. The shell of *P. fucata* consists of two mineralized layers, the nacreous and prismatic layers. Mantle tissue in bivalves has a wide array of functions, including biomineralization (Simkiss and Wilbur 1989). Previous studies have shown that chitin is the major component of organic matrices in the nacreous and prismatic layers (Levi-Kalisman et al., 2001; Suzuki et al., 2007). These two layers are formed by the mantle center (MC) and mantle edge (ME), respectively, by epithelial cell secretion (Sudo et al., 1997). The epithelial cells from mantle tissue secrete organic components such as proteins and polysaccharides, which play important roles in the spatial and chemical controls of crystal nucleation, and regulation of crystal growth, morphology, and shell microstructure (Wilt, 2005; Wilt et al., 2003). The mantle edge is further characterized into three terminal folds: the outer fold (OF), middle fold (MF), and inner fold (IF) (Wada, 1999). However, to date, the functions of chitinase in the shell formation and larval development of *P. fucata* have not been investigated.

In this study, the cDNA sequence of the chitinase in *P. fucata* was cloned and characterized and the potential functions were investigated by examining tissue-specific expression and the response to shell damage. The precise regions of gene expression in the mantle were detected via in situ hybridization. Finally, to better understand the potential roles of chitinase in larval development, we investigated its expression levels in different developmental stages. Our results thus provide an insight into the functions of chitinase in the shell formation and larval development of *P. fucata* at the molecular level.

2. Materials and methods

2.1. Sample preparation

All experimental larvae and healthy adults of *P. fucata* were obtained from the pearl oyster culture base of the South China Sea Fisheries Research Institute at Xincun Port, Hainan Province, China. Tissues (adductor muscle, mantle, digestive gland, gill, hemocytes, intestine, and gonad) and larvae (trochophore, D-veliger, umbo veliger, pediveliger, and metamorphosis larvae) were collected and preserved with sample protector (TaKaRa, Dalian, China). For the shell damage experiment, 48 healthy oysters were used, of which 24 individuals were damaged on the shell edge in accordance with the method described by Mount et al. (2004), and the other 24 individuals were used as controls. Damage entailed cutting a V-shaped notch in the shell margin close to the adductor muscle of the oyster. The damaged oysters and controls were randomly divided into six groups, respectively, and cultured in a tank containing separate cages for each group of four individuals. A piece of cross section of the mantle tissue near the cut was collected from each individual in the six groups at 0 h, 6 h, 12 h, 24 h, 36 h, and 48 h post notching, respectively. Additionally, the mantle tissues of four individuals without shell notching were collected at each time point as controls. All tissues were frozen in liquid nitrogen before storing at −80 °C.

2.2. RNA isolation and first-strand cDNA synthesis

Total RNA was extracted from each tissue using a TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was determined by separating on 1.2% formaldehyde denaturing agarose gels. The quantity of RNA was determined by measuring absorbance at an OD of 260 nm using a NanoDrop ND-1000 UV–Visible Spectrophotometer. Subsequently, 1 μg of total RNA from each tissue was used as a template for a reverse transcription reaction using a SMART™ RACE cDNA amplification kit (Clontech, USA). First-strand cDNA was synthesized and used as a template for further PCR analysis.

2.3. Cloning of the full-length cDNA of PfChi1

On the basis of a 1631-bp fragment of the chitinase gene obtained from the transcriptome library of *P. fucata*, the full-length cDNA was cloned using the rapid amplification of cDNA ends (RACE) method with the SMART™ RACE cDNA amplification kit according to the manufacturer's protocols. RACE PCR was performed using primer sets designed from the adapter sequences of the kit and two pairs of gene-specific nested primers (3′GSP1/3′GSP2 and 5′GSP1/5′GSP2) designed from the initial 1631-bp fragment sequence (Supplement Table).

3′-RACE reactions were run in a 50-μL reaction volume containing 15.5 μL PCR-grade water, 25 μL 2× SeqAmp Buffer, 1 μL SeqAmp DNA Polymerase, 2.5 μL 3′-RACE-Ready cDNA, 5 μL 10× Universal Primer A Mix (10 μM), and 1 μL 10 μM gene-specific primer (3′GSP1) under the following conditions: 94 °C for 3 min; 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; and a final extension at 72 °C for 10 min. Five microliters of the primary PCR product was diluted into 245 μL of Tricine-EDTA buffer. The second PCR amplification was performed as described above using 5 μL of the diluted primary PCR product as a template, 1 μL of the Universal Primer

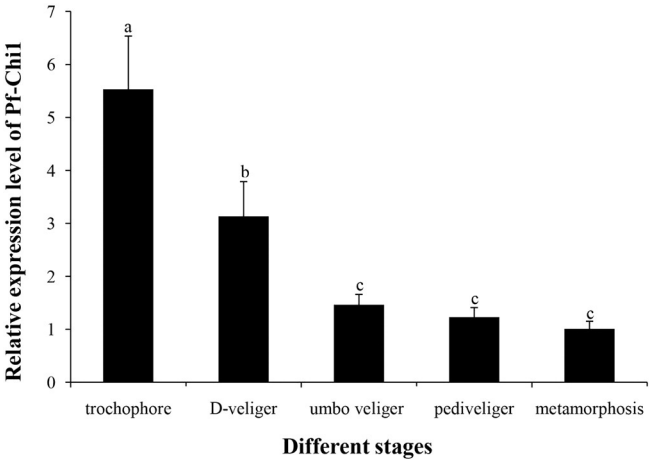

Fig. 2. Relative expression levels of *PfChi1* at different larval stages. Three biological replicates were analyzed for each developmental stage, and three technical replicates were analyzed in each PCR reaction. Data are expressed as the mean ± S.D. Different letters indicate a significant difference among larval development stages at $P < 0.05$.