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Molecular cloning and expression analysis of heat shock protein 20 (HSP20) from the pearl oyster *Pinctada martensii*

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ABSTRACT. Small heat shock proteins (HSPs) are molecular chaperones with ATP-independent properties. They are involved in a variety of physiological and stress processes. In this study, the full-length HSP 20 (HSP20) from *Pinctada martensii*, designated as *PmHSP20*, was obtained from hemocytes using rapid amplification of cDNA ends technology. The *PmHSP20* cDNA was 952 bp in length, containing an open reading frame of 534 bp that encoded 177-amino acid residues, with an isoelectric point of 5.86 and molecular weight of 20.24 kDa. The sequence of this deduced polypeptide contained typical structure and function domains conserved in the HSP20 family, providing evidence that *PmHSP20* belongs to the *HSP20* family. The *PmHSP20* mRNA expression levels were detected in various tissues of *P. martensii* and in hemocytes after challenges with the bacteria *Vibrio harveyi* and lipopolysaccharide (LPS) using quantitative real-time polymerase chain reaction amplification. The results indicated that *PmHSP20* is constitutively expressed in all tissues tested and might be involved in the immune response. The upregulation of *PmHSP20* after *V. harveyi* and LPS challenge suggests that *PmHSP20* plays an important role in anti-bacterial immunity. Studies on *PmHSP20* are a

valuable resource to further explore the immune system in pearl oysters and might enhance our knowledge of molluscan innate immunity.

Key words: Heat shock protein 20; *Pinctada martensii*; Cloning; Expression; Bacterial challenge

INTRODUCTION

Heat shock proteins (HSPs) are ubiquitously expressed and phylogenetically conserved stress proteins existing in all organisms (Caspers et al., 1995). They play an important role during potentially deleterious stress conditions (Hartl, 1996; Jolly and Morimoto, 1999; Feder and Hofmann, 1999; Kregel, 2002). Many stresses, such as elevated temperatures, altered pH, and oxygen deficit impair the establishment of correct protein structures and may result in the disassembly of certain already structured proteins. HSPs are molecular chaperones, which bind to partially denatured proteins to impede their degradation, contributing to the refolding of these proteins depending on ATP after removal of the stress trigger and facilitating their elimination when they become irreversibly damaged (Thomas et al., 2005; Sun and MacRae, 2005). Incremental HSP expression is regulated at various levels such as RNA synthesis, mRNA stability, and translation efficiency (Mahmood et al., 2010). In recent years, proteins with immunological functions have received increasing amounts of attention; thus, focus has been placed on HSPs, which are potent activators of the innate immune system in all animals (Bezemer et al., 2012).

Based on their molecular mass, HSPs are divided into five families: HSP60, HSP70, HSP90, HSP100, and the small heat shock proteins (sHSPs) (Schlesinger, 1990). sHSPs are a group of proteins that consist of a conserved α-crystalline domain and variable N- and C-terminal extensions. Their molecular weights range from 12 to 43 kDa (Mahmood et al., 2010). Notably, increasing numbers of sHSP functions have been generated along with their evolution. sHSPs have been verified to participate in various physiological processes such as actin and intermediate filament dynamics (Wieske et al., 2001; Quinlan, 2002), cellular stress resistance (Landry et al., 1989), life span (Wood et al., 2010), membrane fluidity (Tsvetkova et al., 2002), and disease prevention (Mackay et al., 2003; Selcen and Engel, 2003; Evgrafov et al., 2004). sHSPs comprise multiple specific protein families; in this study, the HSP20 family, which, as the name implies, have an apparent molecular mass of 20 kDa, are of particular interest. It has been reported that HSP20 has a basic function in the cellular physiology of the stages of erythrocyte infection. Specifically, it has been implicated in the immediate response to pathological events, the extracellular regulation of platelet functions (Gusev et al., 2005), and in cardioprotection (Islamovic et al., 2007). Consistent with this, the ability of HSP20 to strengthen the endurance of fishery animals to disease is becoming clear (Wan et al., 2012).

Pinctada martensii is an important species of mariculture mollusc in South China owing to its economic value. It is primarily cultured to develop the pearls that are known as "South China Sea Pearls" (Zhao et al., 2012). However, a high mortality in juvenile, mother, and operational cultured pearl oysters has been frequently observed in recent years (Wang et al., 2011), resulting in a severe reduction in seawater pearls (Wang et al., 2009). To date the cause of the mass oyster mortalities has not been elucidated; however, it has been suggested that the causes are connected with multiple factors such as the degradation of water quality, pathogens, and stock degeneration caused by inbreeding (Song et al., 2006).

Monitoring the response pattern of pearl oysters to environmental challenges might help us to further understand the stress resistance mechanisms in pearl shells, provide assistance in determining appropriate biomarkers to assess environmental impact, and establish treatment strategies for disease control (Wang et al., 2009). To date, several *HSP20* gene sequences isolated from mollusc species have been identified, analyzed, and made available in GenBank, including those of *Cyclina sinensis* (AET13647.1) and *Meretrix meretrix* (AFK80359.1) (Li et al., 2013). However, no information is available on the transcriptional level expression of HSP20 in *P. martensii* under bacterial challenge and lipopolysaccharide (LPS) stimulation. Accordingly, the objective of this research was to obtain *HSP20* cDNA from *P. martensii* and to investigate its gene expression patterns in different tissues and its temporal expression after *Vibrio harveyi* challenge and LPS stimulation. The data presented here identify and characterize HSP20 in *P. martensii* and suggest a role for HSP20 in the immune response of this organism.

MATERIAL AND METHODS

Animals and sample collection

Adult *P. martensii* samples obtained from Liushagang, Zhanjiang, and Guangdong in China were approximately two years of age. The pearl oysters were cultured in recirculating seawater of 25° to 27°C for 3 to 5 days prior to the experiment. For the analyses of gene expression in different tissues, the hemocytes, mantle, gill, adductor muscle, foot and hepatopancreas were collected from healthy pearl oysters and then stored immediately in liquid nitrogen. Among these tissues, the hemocytes were used for quantitative polymerase chain reaction (qPCR) expression analyses.

RNA extraction and preparation of cDNA

Total RNA was extracted from all isolated tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) referring to the manufacturer manual. A 1% agarose gel was used to determine the integrity of the RNA. RNA quantity and concentration were measured using a ND1000 spectrophotometer (Thermo, Waltham, MA, USA). cDNA was synthesized following the instruction of the M-MLV reverse transcriptase kit (TaKaRa, Otsu, Japan). The cDNA was used as the template for qPCR.

PCR and cloning of *PmHSP20* cDNA

We applied 5'/3'-rapid amplification of cDNA ends (RACE) to obtain the full-length cDNA of *PmHSP20* using the SMART RACE cDNA amplification kit (Clontech, Kusatsu, Japan). Specific primers were designed based on the nucleotide sequences of the *HSP20* cDNA fragment obtained from the transcriptome of *P. martensii* previously constructed in our laboratory (Zhao et al., 2012). Table 1 shows the primers utilized in the current experiment.

In the outer PCR, the template was the RACE cDNA. The outer amplified product was then used as the template for the inner PCR. PCR cycling conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min; and 72°C for 10 min.

Table 1. Primers utilized in this experiment.

Primer	Sequence (5'-3')	Application
3'-inner	GGAAACACGAGGAGAAACAGGACGAACA	Inner PCR
3'-outer	TTCCCGACCCCTAGCAGAATTTTCAA	Outer PCR
5'-inner	TTCCCTGAGATGACCACTCTATTGTCCACTACTT	Inner PCR
5'-outer	CAGTTTTGGGGCGGAGACAGATAGCAC	Outer PCR
NUP	AAGCAGTGGTATCAACGCAGAGT	Outer PCR
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Inner PCR
UPM-short	CTAATACGACTCACTATAGGGC	Inner PCR
GAPDH-F	GCAGATGGTGCCGAGTATGT	qRT-PCR
GAPDH-R	CGTTGATTATCTTGGCGAGTG	qRT-PCR
HSP20-F	CTGTTTTGGTGAGGGGAAGG	qRT-PCR
HSP20-R	CTGGGGAGAAATGGGATACG	qRT-PCR
M13-F	CGCCAGGGTTTTCCCAGTCACGAC	Colony PCR
M13-R	AGCGGATAACAATTTCACACAGGA	Colony PCR

DNA sequencing and bioinformatics analysis

The PCR products of the 5'- and 3'-ends were cloned as above, purified using GeneJET PCR purification kit (Thermo), inserted into the pMD-18T vector (TaKaRa), and then sequenced. All the obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The PmHSP20 open reading frame (ORF) was obtained using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The molecular weight and theoretical isoelectric point were analyzed using a ProtParam tool (http://web.expasy.org/cgi-bin/protparam/protparam). The online tools SMART (http://smart.embl-heidelberg.de/) and PROSITE (http://prosite.expasy.org/) were applied to predict the protein domains and functional sites, respectively. MEGA 6.0 software was applied in the cluster analysis of *PmHSP20*.

Stress experiments under two different conditions

For the bacterial challenge experiment, 108 pearl oysters were split at random into two groups. The experimental group was intramuscularly injected with 100 μL *V. harveyi* at 1 x 10^7 cells/mL, whereas the control group was injected with 100 μL phosphate buffered saline. A total of six post injection time points, 0, 2, 4, 6, 8, and 16 h, were selected for expression analysis.

For LPS stimulation, 144 pearl oysters were split at random into two groups. The LPS challenge group was intramuscularly injected with 100 μL (10 μg/mL) LPS diluted with phosphate buffered saline. The treatment method of the control group was same as that in the bacterial challenge experiment. A total of seven time points, 0, 2, 4, 6, 8, 12, 16, and 24 h after injection were selected.

Nine pearl oysters were randomly selected at each time point for analysis.

Real-time qPCR analysis

Real time qPCR was performed using the SYBR® Select Master Mix Kit (Life Technologies, Carlsbad, CA, USA) following manufacturer protocol and was carried out on the Applied Biosystems 7500 Real-Time system (Foster City, CA, USA). The quantity of each

PmHSP20 mRNA was calculated as previously described via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Additionally, the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Statistical analysis

The qPCR data were analyzed using SPSS 19.0 (IBM, Armonk, NY, USA). The Tukey honestly significant difference (HSD) test was used to determine differences among the treatments. P values less than 0.05 were considered to be statistically significant.

RESULTS

Characterization of *PmHSP20* cDNA and protein sequence

Specific primers for *PmHSP20* (Table 1) were designed based on the 534-bp unigene sequence annotated as HSP20 from the *P. martensii* transcriptome database. One pair of nested primers was designed to clone the 5' and 3' nucleotide sequences using 5'- and 3'-RACE technology, respectively. This resulted in the generation of two fragments of 437 and 617 bp, respectively. The integrated *PmHSP20* cDNA reaching a length to 952 bp was obtained by overlapping the fragments. Sequencing and various bioinformatics techniques as described in the Method section allowed further investigation of this polynucleotide. The data suggest that the complete *PmHSP20* cDNA contains a 5'-untranslated region (UTR) of 347 bp, an ORF of 534 bp, and a 3'-UTR of 68 bp, encoding a 177 amino acid polypeptide. The 3'-UTR contained a poly (A) tail of 26 bp and a typical polyadenylation signal (aataaa). The cDNA sequence has been submitted to GenBank and assigned the accession No. AJK26930.1. The deduced amino acids sequence of *PmHSP20* consisted of the characteristic HSP20 family motifs predicted by the SMART program (Figure 1).

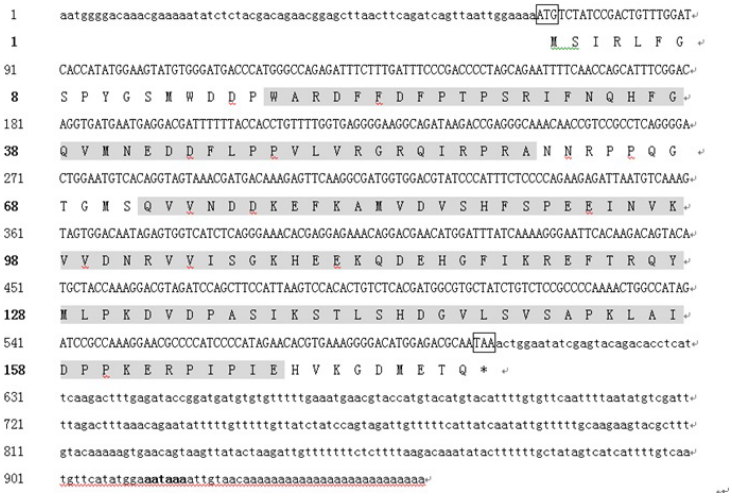

Figure 1. Nucleotide and amino acid sequences of *PmHSP20*. The initiation codon (ATG) and the stop codon (TAA) are boxed. The putative polyadenylation signal (aataaa) is in bold. The amino acid residues shaded gray represent the crystallin motif and the HSP20 domain, respectively.

Homology analysis of *PmHSP20*

The protein sequence of *PmHSP20* demonstrated resemblance with HSP20s of other animals. It shared the highest identity (52%) with HSP20 from the spider *Stegodyphus mimosarum*. Additionally, 47% identity was shown with HSP20 from the nematode *Trichinella spiralis*, 46% with the wasp *Ceratosolen solmsi marchali*, and 45% with the coelacanth *Latimeria chalumnae*. Alignment with these proteins showed that most of the identities were located in the characteristic α-crystallin domain (Figure 2).

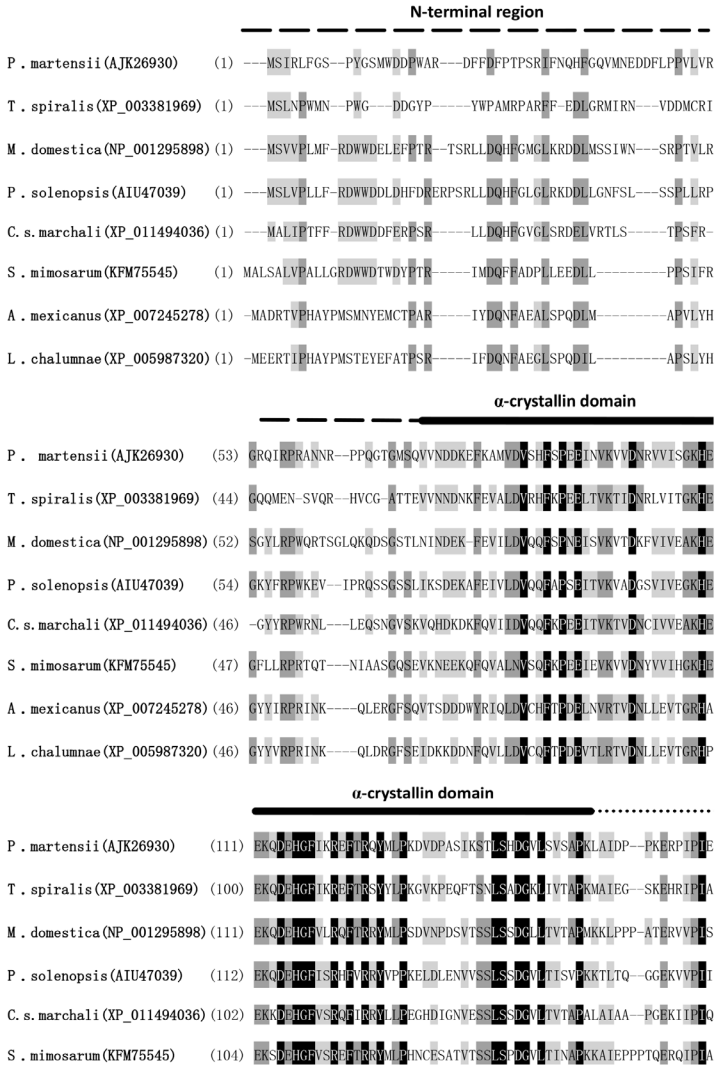

Figure 2. Alignment of HSP20 protein sequences from *Pinctada martensii* and other invertebrate species. The highly conserved α-crystallin domain is marked using a bold black line. The data further show that the N- and C-terminal regions are variable.

A neighbor-joining phylogenetic tree was built in order to analyze the evolutionary relationship of PmHSP20 with other HSP20 (Figure 3). According to phylogenetic taxonomy, the tree was divided into different clades such as fish, mammals, arthropods, and mollusca. PmHSP20 belongs to the mollusca HSP20 subgroup. The relationships presented in the phylogenetic tree conformed to traditional taxonomy.

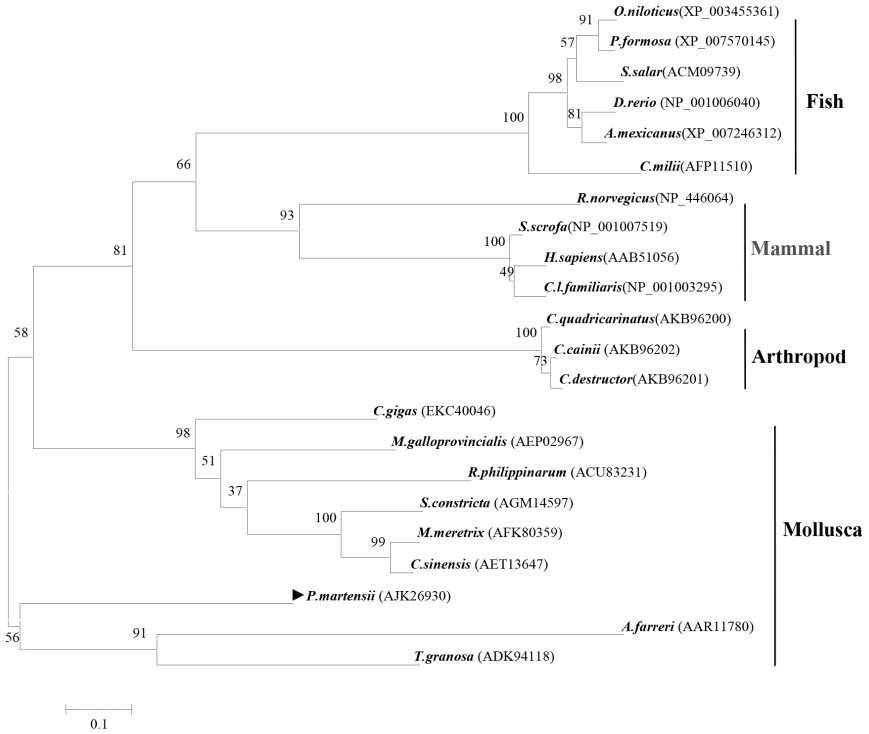

Figure 3. Phylogenetic analysis of PmHSP20. A phylogenetic tree of HSP20 was built using the neighbor-joining method through the program MEGA 6.0 based on the protein sequences of different species. The percentage bootstrap values are indicated by numbers at the branch points.

Quantitative analysis of *PmHSP20* gene expression in the sampled tissues

Real time qPCR was conducted to determine the expression of *PmHSP20* mRNA in all sampled tissues. Samples were normalized to the expression of an internal reference, *GAPDH*. The highest expression was detected in the hepatopancreas, which is involved in the defense system of mollusks, whereas a relatively lower expression level was determined in the adductor muscle, hemocytes, gill, and foot (Figure 4).

Temporal expression pattern of *PmHSP20* after *V. harveyi* challenge and LPS stimulation

To further investigate the function of *PmHSP20* in the immune response, we analyzed the temporal expression of the *PmHSP20* mRNA in hemocytes after *V. harveyi* injection using

real time qPCR. *PmHSP20* expression levels were upregulated and reached a maximum level at 2 h post-infection (1.74-fold, $P < 0.05$), then fell to their lowest level at 6 h. From 6 to 16 h, the expression of *PmHSP20* elevated gradually but significantly at each time tested and peaked at 16 h (1.44-fold, $P < 0.05$). Thus, *PmHSP20* expression in hemocytes is significantly upregulated after infection by *V. harveyi* (Figure 5).

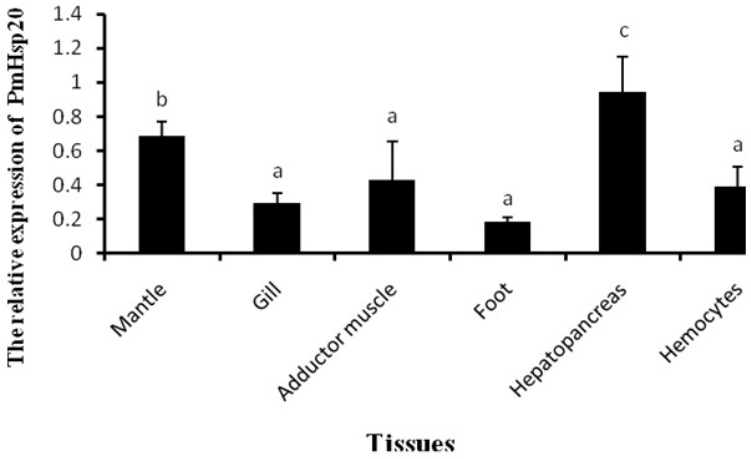

Figure 4. Expression patterns of *PmHSP20* mRNA in all sampled tissues. The *Pinctada martensii GAPDH* gene was used as a reference control. Significant difference is indicated by different letters ($P < 0.05$).

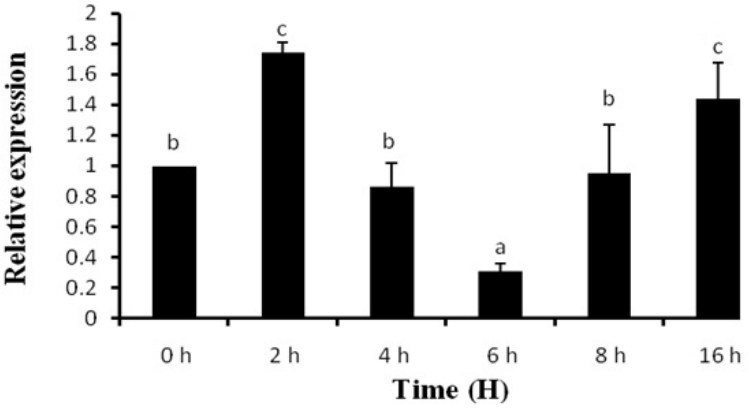

Figure 5. Expression patterns of *PmHSP20* mRNA after *Vibrio harveyi* challenge. Real time qPCR was performed using cDNA samples generated from hemocytes 0, 2, 4, 6, 8, and 16 h post-infection. *Pinctada martensii GAPDH* was used as an internal reference. Significant difference is indicated by different letters ($P < 0.05$).

PmHSP20 gene expression after LPS challenge was also monitored. The expression of *PmHSP20* elevated significantly and reached a peak value at 2 h post-injection (2.68-fold, $P < 0.05$), then fell to its lowest level at 6 h. From 6 to 24 h, the amount of *PmHSP20* transcripts heightened gradually at each time tested and peaked at 12 h (1.65-fold, $P < 0.05$). Subsequently, the expression of *PmHSP20* decreased gradually and returned to the original level (Figure 6).

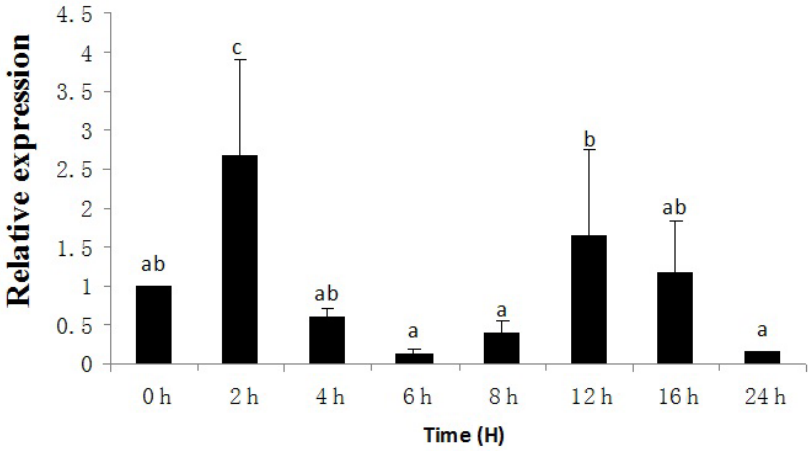

Figure 6. Expression patterns of *PmHSP20* mRNA after LPS stimulation. Real time qPCR was performed using the cDNA samples generated from hemocytes 0, 2, 4, 6, 8, 12, 16, and 24 h post-infection. *Pinctada martensii GAPDH* was used as an internal reference. Significant difference is indicated by different letters ($P < 0.05$).

DISCUSSION

Molluscs are chronically exposed to variable environmental conditions. HSPs act as a buffer by minimizing the biochemical, physiological, and histological stress of the host; therefore, they can function as significant factors to maintain homeostasis across environmental conditions (Li et al., 2010). The present study successfully cloned and characterized the gene encoding HSP20 of *P. martensii* as well as identifying it as a member of the α-crystallin family. The protein sequence of PmHSP20 contained two known structure and function domains; namely, a crystallin motif and an HSP20 motif. The result of multiple-alignment of protein sequences of PmHSP20 with other HSP20 orthologs showed that it contained a conserved α-crystallin domain, which revealed that PmHSP20 belongs to the sHSP family.

To explore the biological function of *PmHSP20* in *P. martensii*, tissue expression patterns were investigated using real time qPCR. *PmHSP20* was detected in different healthy *P. martensii* organisms with highest expression in the hepatopancreas and lowest in the adductor muscle, hemocyte, gill, and foot (Figure 4). The expression pattern of *PmHSP20* in tissues was somewhat similar to previously reported HSP20 expression patterns in the disk abalone (Wan et al., 2012), but different to those previously reported in shrimp (Huang et al., 2008), bloody clam, and razor clam (Bao et al., 2011; Zhang et al., 2013). It has been speculated that different species-or-origin or various tissue functions result in the different expression of sHSPs in tissues. To date, six types of HSP20 in cutworm have been verified to participate in various physiological processes such as thermal stress, development, and 20-hydroxyecdysone induction (Shen et al., 2011).

The expression of HSPs as stress proteins fluctuates when the organism faces variable abiotic stressors and biological stressors such as pathogens and LPS (Li et al., 2010). Generally, the quantity of HSPs increases significantly after bacterial challenge such as has been observed for HSP90 in oyster (*Crassostrea hongkongensis*) (Fu et al., 2011) and for HSP70 in both bay scallop (*Argopecten irradians*) (Song et al., 2006) and pearl oyster

(*Pinctada fucata*) (Wang et al., 2009). LPS is believed to be an endotoxin because it constitutes the outer leaflet of the outer membrane of most gram-negative bacteria (Wang and Quinn, 2010). LPS has bacterial virulence, inducing a strong response in normal animal immune systems. The response mechanisms after bacterial, viral, and LPS challenge have not been fully revealed, whereas bacterial phagocytosis is generally observed concomitant with the generation of reactive oxygen species (ROS) or oxygen free radicals (Zhang et al., 2010). ROS is an effective defensive factor against bacteria but is simultaneously harmful to the host cell, which could result in protein denaturation or proteotoxicity. It has been suggested that ROS production and the accumulation of denatured proteins in the host cell after bacterial stimulation might motivate HSP expression. The subsequent upregulation of HSPs potentially represents a defense mechanism because HSPs associate with damaged or misfolded proteins in order to restore their original structure (Gao et al., 2008).

To further explore the biological function of *PmHSP20* in stress response, its mRNA expression was monitored at different time points after bacterial infection and LPS stimulation. Since hemocytes have been shown to be the primary defense mechanism against pathogens in bivalves (Roberts et al., 2009), the present study utilized them to explore the function of *PmHSP20* in the innate immune response. The temporal expression patterns reported here indicate that *PmHSP20* expression in hemocytes was significantly elevated after challenge with *V. harveyi* and LPS stimulation. Furthermore, a second, more dramatic upregulation of *PmHSP20* expression appeared at 16 h post infection. Although the exact mechanisms of transcriptional upregulation of *PmHSP20* in hemocytes following *V. harveyi* challenge and LPS stimulation are still unknown, the data presented in this manuscript show a correlation between infection and *PmHSP20* gene induction. Generally, HSPs increase dramatically in quantity after bacterial or LPS infection. For example, in scallop, HSP22 transcription was found to be upregulated and reached a maximal level at 12 h after *Vibrio* challenge, then declined progressively to the original level at 48 h (Zhang et al., 2009). In the pearl oyster *P. martensii*, HSP90 expression levels increased significantly and reached a peak at 3 h in response to LPS challenge, then decreased gradually and returned to the original level at the end of experiment (48 h) (Liang et al., 2015). In the present study, the expression level of *PmHSP20* mRNA showed a wave-like pattern after challenge with the biological stressors, which might have resulted from two processes: an acute response related to the immune system and/or a chronic response related to body reparation. This wave-like pattern of HSP expression is similar to previous findings in other species such as HSP20 in clam (*Venerupis philippinarum*) (Li et al., 2010) and HSP90 in oyster (*C. hongkongensis*) (Fu et al., 2011). Although the exact transcriptional mechanisms of upregulation of *PmHSP20* expression in hemocytes are still unknown, the sequential expression pattern of *PmHSP20* might enhance its protective ability against environmental pathogens in *P. martensii.* The different expression profiles between HSPs further elucidated that they belonged to different subtypes of the HSP family and originated from different molluscs. The data presented here begin to elucidate the innate immune system of *P. martensii* by focusing on the role of a conserved protein known to be involved in various stress responses. A better understanding these response mechanisms might alleviate some of the strain on developing aquaculture because diseases caused by bacteria or viruses are still considered as a major constraint to the sustainable development of aquaculture worldwide (Baruah et al., 2010).

In conclusion, *PmHSP20* was cloned from the pearl oyster *P. martensii.* Studies indicate that *PmHSP20* mRNA is constitutively expressed in all tissues tested. In addition,

the expression pattern of *PmHSP20* under bacterial and LPS challenge indicates that there is a correlation between bacterial exposure and *PmHSP20* mRNA levels, therefore suggesting that *PmHSP20* might play a significant role in immune defense. Extrapolation of these results might lead to further understanding of the defensive mechanisms marshaled against the wide range of environmental stressors in *P. martensii*.

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