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Characterization and expression of *Sp*Hsp60 in hemocytes after challenge to bacterial, osmotic and thermal stress from the mud crab *Scylla paramamosain*

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ABSTRACT

Hsp60 play a crucial role in the process of pathogenic and protective immune responses and is implicated in autoimmune disease. In order to understand the environmental response and immune response of this gene, we cloned a Hsp60 (*Sp*Hsp60) gene from the mud crab *Scylla paramamosain*, localized *Sp*Hsp60 in hemocytes by in situ hybridization, and detected the expression of *Sp*Hsp60 after stresses in relation to three housekeeping genes (β-actin, 18S rRNA and GAPDH). The full-length of the *Sp*Hsp60 cDNA was found to be 2424 bp. The predicted ORF encoded a protein of 576 amino acids with a predicted molecular mass of 61.19 kDa and a theoretical isoelectric point (pI) of 5.46. It shared high scoring identity 95% with the swimming crab *Portunus trituberculatus*. In situ hybridization assay showed that a higher expression occurred in the granular and semigranular cells when compared to the hyaline hemocytes. It suggested that *Sp*Hsp60 was mainly contributed from the granular and semigranular cells in hemolymph. The expression level of *Sp*Hsp60 in hemocytes showed a clear time-dependent pattern during the 96 h after stimulated by *Vibrio alginolyticus*. During this experiment the gene was induced and the highest expression level was observed at 3 h. The significantly up-regulated expression and rapid response of *Sp*Hsp60 indicated that the crabs were sensitive to bacterial challenge. After osmotic stress, the expression of *Sp*Hsp60 in hemocytes showed that this gene was induced by the high salinity (30‰) and the crabs had its adaptive responses to high salinity, when compared to the normal salinity (15‰). *Sp*Hsp60 mRNA expression in hemocytes was analyzed after thermal stress at 6 h, the highest and the lowest expression levels of *Sp*Hsp60 were observed at 36 and 32 °C, respectively. This study demonstrated that *Sp*Hsp60 was easily induced at the higher temperatures. Based on our research, *Sp*Hsp60 participate in innate immune and environmental response of *S. paramamosain*. It could be used as a biomarker to test the stress caused by the local aquaculture environment.

1. Introduction

Heat Shock Proteins (HSPs) are a family of proteins that exist in all organisms ranging from prokaryotes to mammals and function mostly as molecular chaperons. HSPs are constitutively expressed under the normal physiological conditions of the organisms and take part in many essential life activities, such as metabolism, growth, differentiation, programmed cell death, fertilization and immune diseases [1–4]. A wide variety of "stressful" stimuli, such as anoxia ischemia, toxins, protein degradation, hypoxia, acidosis, microbial damage, high salinity and hyperthermia will lead to their up-regulation [5–7]. In eukaryotes, HSPs are categorized into several families and named according to their function, sequence homology and molecular mass in kilo-Daltons (kDa). The families primarily include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and several smaller Hsp groups [8].

The Hsp60 or chaperonin family is a group of proteins with distinct ring shaped, or toroid quaternary structures [9]. A vital activity of Hsp60 is mediation of the native folding of proteins in an ATP-dependent manner [10]. In invertebrates, some researches

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revealed that the Hsp60 appear to play important roles in responses to bacterial challenge [11], thermal stress [12,13] and salinity stress [13,14] in addition to normal cell functions. As an endogenous stress signal molecules, the Hsp60 plays a crucial role in the immune defense against pathogens [15], and is implicated in autoimmune disease [16,17] in vertebrates. The capacity of Hsp60 as a self-antigen, a carrier of other functional molecules and a ligand for innate toll-like receptor (TLR) signaling is considered [18]. Study on the white shrimp *Litopenaeus vannamei* and the sea anemone *Anemonia viridis* indicated that Hsp60 may play a critical role in mediating the immune responses to bacterial challenge [11,12].

The mud crab, *Scylla* spp. is a group of four commercially important portunid species that are found in intertidal and subtidal sheltered soft-sediment habitats, particularly mangroves, throughout the Indo-Pacific region [19,20]. In much of Southeast Asia, mud crabs are a valuable source of income for coastal communities [21–23]. Considering its high commercial interest, study focused on the bacterium *Vibrio alginolyticus* which caused many diseases, such as exoskeleton ulcer disease and black gill diseases, has received increasing attention [24]. In addition, osmotic and thermal stress can affect the antibacterial activity of *S. paramamosain* [25,26].

Up to the present, most studies of Hsp60 are focused on mammals and typical model organisms. However, very little is known about the response of Hsp60 against stress conditions in crustaceans, except *L. vannamei* [11,13] and the swimming crab *Portunus trituberculatus* [14]. To understand the adaptation mechanisms under a variety of stresses, it is necessary to study the biochemical and molecular nature of Hsp60 in organisms. In this paper, we report the molecular cloning of a full-length cDNA encoding Hsp60 (*Sp*Hsp60) from *S. paramamosain* and analyze the expression level of *Sp*Hsp60 after response to bacterial, osmotic and thermal stress. There are three morphologically different hemocyte types in crustaceans: hyaline, semigranular, and granular cells [27]. The hyaline cells mainly participate in phagocytosis [28,29], while the granular and semigranular cells can be cytotoxic and lyse foreign eukaryotic cells [30], they can store and release the prophenoloxidase (proPO) system [31]. In order to clarify the contribution of *Sp*Hsp60 in different hemocytes, we detected the localization of this gene in hemolymph by in situ hybridization.

These results will be useful for identifying the potential function of *Sp*Hsp60, to mediate the immune and environmental responses, meanwhile, to be a biomarker in mud crab aquaculture.

2. Materials and methods

2.1. Animal and sample treatment

Crabs (*S. paramamosain*), averaging 5.8 ± 0.6 cm in carapace length, 9.0 ± 0.8 cm in carapace width and 120 ± 8 g in body weight, were collected from a commercial farm in Zhangzhou, Fujian province, China. Briefly, all the samples were acclimated to normal culture conditions (15‰, 23 ± 2 °C) for two days. All of the animals were vigorous no injury, with both claws and appendages intact.

For the bacterial stress, 120 crabs were divided into two groups with 60 in each pool. The size of pools is 4 m (length) × 8 m (width) × 1 m (height) with a running water system at ambient temperature. The temperature and salinity were monitored by thermometer and salimeter three times a day (6. 12. 18 o'clock). In this study, *V. alginolyticus* was cultured on 2216E solid medium at 25 °C, overnight. Then, the bacteria were diluted in saline (0.85% (w/v) pH 7.0) to an OD_{600} of 0.2, which is approximately equivalent to 10^7 CFU/ml. The trial group was injected with 20 μl live *V. alginolyticus* into the arthrodial membrane of last walking leg. In the control group, each crab was injected with 20 μl sterile saline. To determine the expression levels after *V. alginolyticus* challenge, four crabs of each group were randomly sampled at 0, 3, 6, 12, 24, 36, 72 and 96 h post-injection, respectively.

For the osmotic stress, a total of 100 crabs (the original culture salinity is 15‰) were employed. Compared to 15‰ (normal salinity), 30‰ (high salinity) was selected as the stress salinity to test. Half of them were put into normal salinity water, and the rest were treated at high salinity water. The normal culture condition was as same as the bacterial stress trial (see above). At selected times (0, 12, 24, 36, 72 and 96 h) after the osmotic stress, four specimens in each treatment were randomly sampled to analyze the expression levels of *Sp*Hsp60.

For the thermal stress, a total of 50 crabs were employed, 10 crabs were used at each of the selected temperatures (10, 15, 25, 32 and 36 °C). They were equally cultured in 5 plastic tanks, the 10 and 15 °C groups were placed in low temperature incubator equipment, the 25 °C group was cultured in ambient temperature, the temperature of 32 and 36 °C groups were maintained by heating rod. The size of tanks is 0.8 m (length) × 0.6 m (width) × 0.4 m (height). The normal temperature for the culture of crabs is 25 °C, but water temperature and salinity will change with seasons at about 11–36 °C and 5–33‰ respectively. After 6 h (at the normal salinity 15‰), four individuals in each treatment were randomly sampled to analyze the expression levels of *Sp*Hsp60.

2.2. RNA extraction and cDNA synthesis

All tissues were removed immediately and flash frozen in liquid nitrogen. Total RNA was extracted from the mud crab tissues using Trizol RNA isolation reagent (Invitrogen) according to the manufacturer's instructions and the quality was monitored by agarose gel electrophoresis. The total RNA concentration, quality and integrity were determined using a NanoDrop 1000 spectrophotometer. Genomic DNA was removed by DNase I (TaKaRa) digestion and the RNA concentrations were determined using UV spectrophotometry. First-strand cDNA was synthesized from 2 μg of total RNA using the Revert Aid™. First-strand cDNA Synthesis Kit (Fermentas) using the oligo $(dT)_{18}$ primer.

2.3. Cloning of the full-length cDNA of SpHsp60

Degenerate primers 6H1F and 6H1R (Table 1) were designed, based on the conserved amino acid sequences of Hsp60s in the National Centre for Biotechnology Information (NCBI) database, to clone the middle fragment of the *Sp*Hsp60 cDNA by PCR. The PCR

Table 1
Primers used for polymerase chain reaction (PCR) amplification (D = A/T/G, R = A/G, K = G/T).

Name	Sequence (5′-3′)	PCR objective
6H1F	CAAGGCGGTGGAGCTGAAGGACAA	cDNA homolog cloning
6H1R	TTACATCATGCCDCCCATRCCKCCC	cDNA homolog cloning
6H2	CGAGAAGAAGGACCGTGTGAAT	3′ RACE
6H3	GGTGAAGGTCTGGGTTTGT	5′ RACE
6H4F	ATGCCTTGGTTCTCCTGTCTG	Real-time PCR and in situ hybridization
6H4R	ATGGTGTTCTTGCGGTTGTCG	Real-time PCR and in situ hybridization
GF	AATGCCATCACAATAGAAAAATC	Internal control
GR	GGAACAATCAACACTACCACACC	Internal control
β-actin F	GAGCGAGAAATCGTTCGTGAC	Internal control
β-actin R	GGAAGGAAGGCTGGAAGAGAG	Internal control
18SF	CAGACAAATCGCTCCACCAAC	Internal control
18SR	GACTCAACACGGGGAACCTCA	Internal control