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Molecular cloning and expression analysis of a cytosolic heat shock protein 70 gene from mud crab *Scylla serrata*

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ABSTRACT

Heat shock protein 70s (Hsp70s) play important roles in resisting environmental stresses and stimulating innate immune system. To understand the immune defense mechanisms of *Scylla serrata*, a full-length cytosolic Hsp70 cDNA of *S. serrata* (designated as SSHsp70) was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) coupled with rapid amplification of cDNA ends (RACE). The full-length of SSHsp70 cDNA was 2235 bp, with a 5′ untranslated region of 105 bp, a 3′ untranslated region of 174 bp, and an open reading frame of 1956 bp encoding a polypeptide of 651 amino acids with an estimated molecular mass of 71.3 kDa and an estimated isoelectric point of 5.55. The cloned SSHsp70 belonged to a cytosolic Hsp70 family. Three typical Hsp70 signature motifs were detected in SSHsp70 by InterPro analysis. Quantitative PCR (qPCR) was used to detect tissue distribution and mRNA expression levels of SSHsp70 under different stress conditions. The obviously high levels of SSHsp70 transcript were in hemocyte, heart, hepatopancreas and gill, whereas low levels were detected in muscle, eyestalk, stomach, and gut. In different temperature treatments, the expression levels of SSHsp70 in low or high temperatures were higher than those in temperate temperature. In pathogen challenge treatments, the mRNA expression level of SSHsp70 reached a maximum level after 18 h and then dropped progressively. In different salt concentration treatments, the mRNA expression level of SSHsp70 had a minimum level at 25‰ salt concentration and high expression levels at high or low salt concentration. In different nitrite concentration treatments, the mRNA expression level of SSHsp70 increased progressively with the increase of nitrite concentration. The results confirmed Hsp70 could be used as a tool for evolution and phylogenetic analysis, a kind of potential biomarker, and a disease resistance factor used in application.

1. Introduction

Under inherently stressful environment, organisms have to face different stress factors. In the process of adaptation, organisms must make appropriately physiological and biochemical changes in order to deal with environmental stresses, at the same time, organisms upregulate expression of stress genes and down-regulate expression of protein synthesis-related genes to resist stresses. Organisms respond to stress at the cellular level with the rapid synthesis of a number of so-called stress proteins (heat shock proteins, Hsps).

Hsps are highly conserved throughout evolution and play an essential role in protecting cells, folding and translocating nascent proteins, refolding denatured proteins, disassembling already formed protein aggregates under both stress and non-stress conditions. Numerous stressful factors including physical, chemical, and biological stressful factors can induce the overexpression of Hsps [1]. Hsp70s, one of important Hsp family members, have a variety of physiological functions in organisms and potential applications in environmental monitoring, disease treatment, genetic breeding [2,3].

Several members of the Hsp70 family have been identified from various Crustacea [4–14]. Well-recognized members of the Hsp70 multigenic family are two closed cytosolic forms which coexist inside Eukaryota: cognate Hsc70 and inducible Hsp70 [15]. They share common structural features including a 44-kDa N-terminal ATPase domain and a 30-kDa C-terminal region [16]. Compared to Hsc70 constitutively expressed regardless of stress, Hsp70 is inducible and involved not only in protein folding and cytoprotection, but also in specific immune responses against infectious agents and in innate immune responses [17]. Hsps played as

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regulators in the immune response and activators in the innate immune system [18,19]. The expression of Hsp70 was transiently induced by pathogen stimulation [20,21].

Scylla serrata, also known as mud crab have a high economic value in the international market [22,23]. This species has been cultured throughout the Indo-Pacific regions for more than 50 years. With the enlargement of cultivation scale and worsening self-pollution, the kinds of *S. serrata* disease and mortality rate become more and more serious, which results in lots of economic losses. In this study, a cytosolic Hsp70 cDNA of *S. serrata* was obtained by RT-PCR and RACE. The different tissue expression levels of SSHsp70 was analyzed. For better understanding the mechanism of *S. serrata* response to the environmental stresses such as pathogenic infect, temperature stress, nitrite stress, mRNA expression levels of SSHsp70 were investigated using real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR).

2. Materials and methods

2.1. Sample collection and treatment

Adult specimens of *S. serrata* (100 ± 10 g) were collected from a commercial farm in Taizhou, China, cultured at 25 ± 1 °C in an aerated plastic tank and fed on clam meat once daily at night for one week before processing. A crab was kept in 35 °C for 1 h for extracting total RNA of gill and cloning full-length cDNA of SSHsp70 gene.

For the different tissue expression analyses, total RNA was isolated from various tissues including hemocytes (about 1.0 ml of haemolymph per crab), heart, hepatopancreas, gill, muscle, eyestalk, stomach, and gut of five unchallenged crabs. The hemocytes were harvested from the last walking leg using a syringe, quickly added to an equal volume of anticoagulant modified Alsever solution (30 mM sodium citrate, 300 mM NaCl, 140 mM glucose, 10 mM EDTA, pH 7.0) [24], and immediately centrifuged at 1000 × *g*, 4 °C for 10 min to collect the hemocytes. All collected tissues were stored at −80 °C for subsequent RNA extraction.

In pathogen challenge treatments, crabs were injected with 200 μl of live *Vibrio alginolyticus* resuspended in 0.1 mol/L PBS (pH7.0, 10^6 CFU/ml) into the arthrodial membrane of the last walking leg. Untreated crabs and crabs injected 200 μl PBS were served as the blank and control respectively. The crabs were returned to the seawater tanks and five individuals were randomly sampled 0, 3, 6, 12, 18, 24, 36 and 48 h post-injection. Total RNA was isolated from the hemocytes of all the untreated and treated crabs at each sampling time point to investigate the effects of pathogen challenges on expression levels of SSHsp70 mRNA.

In different temperature treatments, crabs were kept in 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, respectively for 2 h to investigate the effects of temperatures on expression levels of SSHsp70 mRNA.

In salt concentration challenge treatments, crabs were kept at 25 °C in different salt concentrations (0‰, 5‰, 10%, 15‰, 20‰, 25‰, 30‰, 35‰, 40‰, and 45‰, respectively) for 2 h to investigate the effects of salt concentration challenges on expression levels of SSHsp70 mRNA.

In nitrite concentration challenge treatments, crabs were kept at 25 °C in different nitrite concentrations (0.01, 0.05, 0.1, 0.2, 0.3, and 0.4 mg/L, respectively) for 2 h to investigate the effects of salt concentration challenges on expression levels of SSHsp70 mRNA.

2.2. RNA extraction and purification

All crabs' tissues were homogenized in Trizol reagent (Invitrogen, CA, USA) and the total RNA was extracted according to the manufacturer's instructions. A 10 μl reaction volume containing 1 μl DNase reaction buffer, 2 μl RNA sample and 1 μl RQ1 DNase I (Promega, WI, USA) was incubated at 37 °C for 30 min. After inactivating the DNase at 65 °C for 10 min, 10 μl DNase digested RNA was collected according to the manufacturer's protocol.

2.3. SSHsp70 cDNA cloning

First-strand cDNA was synthesized from 1 μg of total RNA using the Superscript™/First-strand cDNA Synthesis Kit (Invitrogen, CA, USA). PCR was carried out in a programmable mastercycler personal thermocycler (Eppendorf, Hamburg, Germany) in a 10 μl reaction volume containing 1.0 μl of 10× RT buffer, 2.0 μl of $MgCl_2$ (25 mM), 1.0 μl of each dNTP (10 mM), 3.75 μl of RNase-free dH_2O, 0.25 μl of RNase inhibitor, 0.5 μl of AMV reverse transcriptase, 0.5 μl of oligo dT-adapter primer (2.5 μM), and 1.0 μl of total RNA (about 1.0 μg). Reaction was incubated at 30 °C for 10 min, then incubated at 42 °C for 30 min, terminated by heating at 99 °C for 5 min, and subsequently stored at −80 °C or used for PCR. Two degenerate primers dHspF and dHspR (Table 1) were designed based on the conserved sequence of the known decapoda Hsp70 genes (*Scylla paramamosain* EU754021, *Portunus trituberculatus* FJ527835, *Eriocheir sinensis* EU857483, and *Marsupenaeus japonicus* EF091692) for amplifying a SSHsp70 cDNA fragment of about 1094 bp. PCR was performed in a 50 μl reaction volume containing 3 μl of cDNA as a template, 10 μl of 5× PCR buffer, 33.75 μl of dH_2O, 0.25 μl of TaKaRa Ex Taq HS and 1.5 μl of each primer (10 mM). PCR was carried out at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The interest fragment of PCR product was excised, purified by agarose gel DNA fragment recovery kit (TaKaRa, ToKyo, Japan), subcloned into PMD-18T vector (TaKaRa, Tokyo, Japan) and sequenced on an ABI3730 Automated Sequencer (Applied Biosystems, CA, USA). The obtained sequences were verified and analyzed for similarity with other known Hsp70 sequences using BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast).

2.4. Rapid amplification of cDNA ends

Sequence obtained by RT-PCR was used to design specific primers, GSP-3 and GSP-5 (Table 1) to perform 3′ and 5′ rapid amplification of cDNA ends (RACE), respectively. Both 3′ RACE and 5′ RACE were carried out using a Smart RACE cDNA amplification Kit (Clontech, CA, USA) according to the manufacturer's instructions. Amplifications were performed on a mastercycler personal thermocycler (Eppendorf, Hamburg, Germany). Touchdown PCR was used for RACE amplification. The PCR programmes were carried out 5 cycles of 94 °C for 30 s, 72 °C for 2 min, followed by 5 cycles of 94 °C for 1 min, 70 °C for 30 s, 72 °C for 2 min, then 28 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 2 min; a final

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

Name	Sequence (5′–3′)	PCR objective
dHspF	GGTGTCAATGTGCTGCRTATCAT	cDNA cloning
dHspR	TCATCCTCKGCCTTGTACTTCTC	cDNA cloning
GSP-3	GGTGTATGAAGGAGAGCGAGCCATGAC	3′RACE
GSP-5	GGCTCGCTTGCTCTCAGATGGATCCTTC	5′RACE
Hsp70-F	AACCTACTCCTGCGTGGGTGTCTT	Real-time RT-PCR
Hsp70-R	GAACTTCCTGCCAATCAGCCTCTT	Real-time RT-PCR
Actin-F	CCACCACGGCTGAGCGAGAAAT	Real-time RT-PCR
Actin-R	GGAAGGAAGGCTGGAAGAGGGA	Real-time RT-PCR