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Molecular cloning and expression analysis of five heat shock protein 70 (HSP70) family members in *Lateolabrax maculatus* with *Vibrio harveyi* infection

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ABSTRACT

Heat shock proteins 70 (HSP70s) are molecular chaperones that aid in protection against environmental stress. In this study, we cloned and characterized five members of the HSP70 family (designated as HSPa1a, HSC70-1, HSC70-2, HSPa4 and HSPa14) from *Lateolabrax maculatus* using rapid amplification cDNA ends (RACE). Multiple sequence alignment and structural analysis revealed that all members of the HSP70 family had a conserved domain architecture, with some distinguishing features unique to each HSP70. Quantitative real-time (qPCR) analysis revealed that all members of the HSP70 family were ubiquitously and differentially expressed in all major types of tissues, including testicular tissue. This indicated that HSP70s have vital and conserved biological functions, and may also function in the development of germinal cells. The expression of mRNA of the five HSP70 family members mRNA expression was significantly increased in the head kidney, intestine and gill after *Vibrio harveyi* challenge, suggesting that HSP70s play an important role in the immune response.

1. Introduction

Heat shock proteins (HSPs) are a family of conserved cellular proteins that are found in all organisms from bacteria to mammals [1,2]. HSPs play important biological roles both under normal and stress conditions. Under normal conditions, HSPs act as molecular chaperones and are involved in protein folding and transport, cell cycle regulation, apoptosis, and spermatogenesis [3,4]. When organisms are subjected to stressors, such as changes in temperature, dissolved oxygen levels and osmotic pressure, and the presence of heavy metals and microbial infections, HSPs expression increase significantly, enabling resistance to these stressors, and maintaining homeostasis [5–8].

According to their molecular weight, HSPs can be classified into HSP110 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), small HSPs (HSPB) [9,10]. Among HSPs, the heat shock protein 70 (HSP70) family is one of the most conserved, and has been studied extensively. HSP70a has been reported to activate innate immunity and play crucial roles in the appropriate response to stress and survival during stress-induced damage [2,5]. The HSP70 family contains two different genes, an inducible type HSP70 and a constitutive type HSC70 (heat shock cognate 70) [11,12]. HSC70 is actively expressed by non-stressed cells and remains unchanged or only mildly induced with stressful stimuli, whereas HSP70 is highly induced by stress [5,12]. Fewer studies have been conducted on HSC70 than HSP70 for their ability to protect against environmental stress. Recent studies indicated that HSC70 also plays important role in organism development [13] and in response to environmental stressors, such as heat shock, heavy metal exposure and bacterial infection [5,12]. Recent studies have identified multiple HSP70 family members in fish, such as heat shock protein 70a1 (HSPa1), heat shock 70 kDa protein 2-like (HSPa2), heat shock 70 kDa protein 4-like (HSPa4), heat shock 70 kDa protein, heat shock cognate 70 (HSPa8), and heat shock 70 kDa protein 14-like (HSPa14) [14–16]. Members of the HSP70 family are involved in embryonic and gonadal development, as well as spermatogenesis, and protection against environmental stress. HSPa2 plays important roles during meiosis and post-meiosis [17–20]. In addition, HSPa8 and HSPa4 are involved in embryogenesis [21].

Abbreviations: HSP, heat shock protein; HSC, heat shock cognate; HSPa4, heat shock protein 70 kDa 4-like; HSPa14, heat shock protein 70 kDa 14-like; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; qPCR, quantitative real-time polymerase chain reaction; UTRs, untranslated regions; ORF, open reading frame; NBD, nucleotide-binding domain.

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In aquaculture, fish often encounter stressors such as changes in temperature, crowding, and viral and bacterial invasion, which sometimes result in serious losses [22]. These factors are closely related with the expression levels of heat shock proteins in fish [13]. It is necessary to investigate the functions and expression characteristics of HSP70s under various stresses because of the important roles of HSP70s in disease and stress resistance. Recently, HSP70s have been used as biomarkers to monitor environmental changes. *Lateolabrax maculatus* is one of the most economically important species of marine fish in China. However, these fishes are commonly infected by various viruses and bacteria, which have resulted in a drastic decline of their natural resources. Therefore, there is an urgent need to identify novel immune-related genes involved in specific signaling pathways in this species. In this study, five members of the HSP70 family were cloned using RACE. The sequences obtained were analyzed and compared with their homologues in fish, and a phylogenetic tree was constructed. In addition, we used qPCR to confirm the mRNA expression of these HSP70s in different tissues and under bacterial infection. The effects of bacterial infection on HSP70 mRNA levels we re investigated to determine anti-stress mechanisms of *L. maculatus*.

2. Materials and methods

2.1. Experimental animals

We purchased mature male *L. maculatus* (100 ± 20 g) from Ningbo Aquatic Products Market (Ningbo, China). The water temperature of the tanks was 20 °C. The liver, testes, gills, spleen, head kidney, muscle, heart and intestines were dissected from *L. maculatus*, quickly dropped into liquid nitrogen, and subsequently stored at −80 °C. The Institutional Animal Care and Use Committee at the Zhejiang Laboratory Animal Research Center and Ningbo University approved this study.

2.2. Bacterial challenge and sample preparation

Twenty L. *maculatus* were selected for the bacterial (*Vibrio harveyi*) challenge. According to the methods described by Shi et al. [23], *V. harveyi* activated twice was diluted with sterile phosphate buffer solution (PBS) to a final concentration of 3.8×10^6 CFU/mL. Each fish was injected with 100 μL of suspended bacteria intraperitoneally. Another twenty fish each received the same amount of PBS, and they were used as the control group. Head kidney, gill, and intestine samples of five fish were randomly collected at 3, 12, 24 h, respectively, and stored at −80 °C.

2.3. Cloning HSP70 family genes

Total RNA was extracted with Trizol reagent from the testis. Reverse transcriptions were executed using an oligo (dT) primer according to the BioRT cDNA First Strand Synthesis Kit (Bioer) manual. A pair of degenerate primers (Table 1) was used for cloning HSP70 cDNA. PCR was conducted as follows: 5 min at 94 °C, 35 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s) and 10 min at 72 °C for the final extension. Ultimately, the products were detached by agarose electrophoresis, DNA gel green was used for showing the bands, from which the expected products were subsequently obtained and purified using an AxyPrep DNA Gel Extraction Kit (Axygen) and AxyPrep PCR Cleanup Kit (Axygen). The purified fragments were then cloned into pMD18-T vectors (Takara), propagated in *Escherichia coli* DH5α (Takara) and sequenced by Biosune Company, Shanghai, China.

To obtain the full sequence of HSP70 cDNA, RACE was conducted to determine the 5′ and 3′ cDNA ends, using the Smart RACE cDNA Amplification Kit (CloneTech) and 3′ Full RACE Amplification Kit (Takara). The primers used for RACE contained several gene specific primers (Table 1) designed according to the obtained HSP70 cDNA fragment and several primers provided in the RACE Kit. The PCR program was properly modified, based on the instructions in the kit. The PCR program of 5′ and 3′ RACE were run as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 2 min; then 10 min at 72 °C for the final extension. Extraction, purification, and sequencing of the expected fragments were performed as mentioned above.

2.4. Sequence analysis

Sequence similarity analyses of five genes were performed using the Blast program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequences of five genes were analyzed with the expert protein analysis system (http://www.expasy.org/). The open reading frames (ORFs) of five genes were determined using the Primer Premier 5 and translated into amino acid sequence. The molecular mass (MM) and theoretical Isoelectric point (pI) of the proteins were calculated based upon their deduced amino acids by the ProtParam tool (http://www.expasy.ch/tools/protparam.html). The multiple sequence alignments of *L. maculatus* HSPa1a, HSC70-1 and HSC70-2 were produced by Vector NTI 10 software. The three dimensional protein structures were predicted by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) Phylogenetic relationships were inferred by the Neighborjoining (NJ) method implemented from pairwise amino acid distances in MEGA5.1.

2.5. Quantification of mRNA expression

Real-time quantitative PCR was used to detect the expression levels of five HSP70 family member genes in different tissue and the temporal expressions in head kidney, intestine and gill after *V. harveyi* challenge. Total RNA from testis, liver, gill, spleen, head kidney, muscle, heart and intestine were extracted with Trizol reagent (Tiangen). Reverse transcription was performed with BioRT cDNA First Strand Synthesis Kit (Takara). Five pairs of primers (Table 1) were used for analyzing mRNA expression respectively. qPCR was run as follows: 95 °C for 30 s; 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s for 40 cycles. The melting curves of the PCR products were obtained by gradual heating of the samples from 55 to 95 °C, furthermore, reaction specificity was determined when there was only one peak in the melting curve. The qPCR reaction was performed in triplicate for each sample, a mean value was used to calculate mRNA levels. Three biological replicates were measured for each group. The expression ratios were determined by method $2^{-\Delta\Delta Ct}$. The excel software was employed to statistical analysis, and one-way ANOVA was performed to determine the significant difference using SPSS 17.0 (SPSS Inc. Chicago, USA). Significant differences were considered as $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Cloning and sequence analysis of L. maculatus HSP70 genes

Five full-length cDNA of HSP70 family genes were obtained by the RACE method. All sequences were submitted to GenBank and denoted as heat shock protein 70a (HSPa1a; KT989368), heat shock cognate 70-1 (HSC70-1; KT279199), heat shock cognate 70-2 (HSC70-2; KT279200), heat shock 70 kDa protein 4-like (HSPa4; KX926427) and heat shock 70 kDa protein 14 (HSPa14; KT438510). The full-length cDNAs ranged from 1690 to 3523 bp, including open reading frames of 1521–2517 bp encoding HSP70 family proteins of