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The cooperative expression of Heat Shock Protein 70 KD and 90 KD gene in juvenile *Larimichthys crocea* under *Vibrio alginolyticus* stress

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ABSTRACT

Heat shock proteins (HSPs) play significant roles in the immune response of fish in defending against diverse environmental threats or stresses. In this study, two complete *HSP70* and *HSP90* genes of *Larimichthys crocea* (designated as *LycHSP70* and *LycHSP90*) were identified and characterized (GenBank accession no. KT456551 and KT456552). The complete open reading frame (ORF) fragments of *LycHSP70* and *LycHSP90* were 1917 bp and 2151 bp, encoding 638 and 716 amino acids residues respectively. Many significant functional domains and motifs were found, such as Hsp70 family signatures, Hsp90 family signatures, ATP-GTP binding site and EEVD motif regions, and they were associated with relative functions. Phylogenetic relationship and BLASTp analysis interpreted that they were unambiguously assigned to *HSP70* and *HSP90* family. The total length DNA of *LycHSP70* was 7889bp, *LycHSP90* was 5618 bp, and the gene location mapping were analyzed based on the whole-genomic DNA sequence of *L. crocea*. *LycHSP70* and *LycHSP90* were constantly expressed in eight tested tissues, with their expression peaks appearing in liver. Spleen, brain and head kidney also witnessed higher expression level. *LycHSP70* and *LycHSP90* were significantly induced by pathogenic bacteria *V. alginolyticus*, and they were both up-regulated in liver and spleen from 0 to 72 h post-injection. All the findings would contribute to better understanding the biologic function of HSPs in defending against pathogenic bacteria challenge and further exploring the innate immune response in fish.

1. Introduction

Marine fish are significant components in maintaining the stability of marine ecosystems [1] and provide high quality proteins for human beings [2]. Many of them have become valuable economical species and have been used in artificial culture of the marine system. For instance, large yellow croaker (*Larimichthys crocea*), which belongs to the *Larimichthys* genus and Sciaenidae family, is an abundant commercial marine-cultured fish in China from the 1960s due to its high nutritional quality, fine palatability and high production. Due to overfishing in the 1970s its population collapsed and it almost become endangered species [3]. With the advance of artificial culture, *L. crocea* has been gradually cultured in the southeast region of China, such as Fujian, Zhejiang, Guangdong and Guangxi province. However, the expansion of artificial breeding scale and density from the 2000s further leads to the decline of its immunity and cause fish to face more threats with fewer resistance to disease from survival and aquatic environment. Many biological and non-biological factors, such as pathogenic bacteria, heavy metals, parasites, virus and prokaryotes, could easily kill them and contribute to tremendous economic losses when the serious diseases break out in the fish farming industry.

Vibrio genus is one of the most common bacterial species in marine farming circumstance. Several members of them [4] (*Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio vulnificus* and *Vibrio harveyi*) are the pathogenic and potential killers of marine animals, such as marine fish and shellfish [5]. Some literature has indicated Vibriosis is a serious prevalent disease leading to huge economic loss in aquatic production, even threatening human health [6,7]. *V. alginolyticus* is a halophilic motile gram-negative bacterium, which could infect marine vertebrate and invertebrate animals, even human beings as an opportunistic pathogen [2,8], and its infection damages the healthy lung and liver and changes the level of IL-1β, IL-6 and other liver or hematological indicators [9]. In fact, the immunity of fish is generally poorer than mammals in defending

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against pathogenic threats. Wu et al. [10] had indicated that the adaptive immunity of *L. crocea* was poorer than innate immunity because some important genes linking with adaptive immunity (such as CD4 and CD8) were partial and incomplete. Therefore, the study of immunity in *L. crocea* and new mechanisms of fish against pathogenis is necessary for protecting the marine health and declining economic loss.

Heat shock proteins (HSPs) have been identified in almost all animals and play significant roles in many cellular processes, such as cell growth, individual development, protein folding, cell immunity, signal transduction, etc [11]. HSPs range in size from 27 to 110 kDa and are divided into six subfamilies, including HSP100 (~100 kDa), HSP90 (85–90 kDa), HSP70 (68–73 kDa), HSP60, HSP47 and low molecular mass HSPs (16–24 kDa) [12,13]. Some previous studies have interpreted HSPs were conserved proteins participating in innate and adaptive immune processes under pathogenic stress or against serious diseases [14]. HSP90 and HSP70 are two crucial multigenic families involved in immune regulation and signal transduction. HSP70 consists of an N-terminal ATP-dependent chaperones/ATPase domain (~44 kDa), a substrate binding domain (SBD, ~18 kDa) and a variable C-terminal domain (CTD, ~10 kDa) [11,15]. Likewise, HSP90 is also a dimeric protein including four highly conserved structural domains: an N-terminal domain (NTD, ~25 kDa) that is involved in ATP-binding, a middle domain (a “charged linker” region, ~35 kDa), a protein-binding middle domain (MD) and a C-terminal dimerization domain (CTD, ~12 kDa) containing a highly conversed MEEVD motif for binding and interacting with some key co-chaperones [16,17]. Now HSP90 and HSP70 have been identified and characterized in many marine fish, shellfish and mammals [18], such as rainbow trout [19], *Sciaenops ocellatus* [20], *Haliotis discus hannai* [21], flatfish [22], *Sepiella maindroni* [12] human [23], etc. Additionally, heat shock response is generally essential for protecting individuals from a variety of threats within life cycle. However, there are no adequate investigation of HSP90 and HSP70 in *L. crocea*. Hence, the main objectives of this paper are: (1) to clone the open reading frame (ORF) of HSP70 and HSP90 from *L. crocea* (designated as *LycHSP70* and *LycHSP90* respectively), (2) to analyze their molecular features, gene structures, line construction, domain or motif regions and genetic locus, (3) to investigate their distribution patterns in different tissues and inhibit the cooperative and regulative expression profiles after live pathogenic bacteria *V. alginolyticus* infection.

2. Material and methods

2.1. Larimichthys crocea

Healthy juvenile *L. crocea* with average body length of 25 ± 5 cm and body weight of 375 ± 25 g were collected from the Dongji aquacultural farm in Zhoushan, Zhejiang province, P. R. China, and transferred to the laboratory immediately. These animals were maintained with aerated seawater (salinity, 32 ppt) at 25 °C in static tanks with 100 L for one week before processing. Two groups of 30 individuals each were taken as experimental animals for challenge. One group was injected intramuscularly with 100 μL of live *V. alginolyticus* (1×10^8 CFU/mL, which was resuspended in PBS with pH = 7.4), the other, was accordingly injected with 100 μL PBS as control.

2.2. Cloning of the complete cDNA ORF of LycHSP70 and LycHSP90 in L. crocea

The complete cDNA ORF sequences of *LycHSP70* and *LycHSP90* were amplified with special primers (Table 1, designed by software primer 5.0) based on the *L. crocea* whole-genome data [10]. Total RNA was isolated from liver with the Trizol Total RNA Kit and cDNA synthesis and the cDNA synthesis was performed by M-MLV RTase cDNA Synthesis Kit (TaKaRa, China). The reaction system was performed in 20 μL volume, including 10 × PCR Buffer 2.5 μL, Mg^{2+} 2.5 μL, dNTPs 2 μL, primer-F 0.8 μL, primer-R 0.8 μL, template cDNA (100 ng μL/) 1 μL, Taq DNA polymerase 0.4 μL and ddH$_2$O 10 μL. PCR amplification was conducted on the Thermal Cycler (Bio-Rad, USA) and amplification conditions were: 4 min at 94 °C, followed by 40 cycles of 60 s at 94 °C, 30 s at 58–65 °C, and 2 min at 72 °C, with a final extension of 10 min at 72 °C. PCR products were gel-purified by NucleoTrap GelExtraction Kit (TIANGEN, China) after 1.2% agarose gel electrophoresis and sequenced at Shanghai Invitrogen Biological Technology Company (P.R.China).

Table 1
PCR primer sequences for all HSPs members from *L. crocea*.

Primer	Sequences
For the complete cDNA ORF	
LycHSP70-F	5′-ATGTCAGCTAAAGGAATATCTATTG-3′
LycHSP70-R	5′-TTAGTCAACTTCCTCAATAGTGG-3′
LycHSP90-F	5′-ATGCCTGAAGAAATGCACCAAGAG-3′
LycHSP90-R	5′-TTAATCGACTTCTTCCATGCGTG-3′
For qRT-PCR	
qLycHSP70-F	5′-CGCAGTCATCACAGTTCCG-3′
qLycHSP70-R	5′-TGCCGTCTTCAATGGTCAG-3′
qLycHSP90-F	5′-CCGCTACGAAAGCCTGACTGA-3′
qLycHSP90-R	5′-ACGACCACCTTCTCGGCAAC-3′
β-Lyc actin-F	5′-TCGTCGGTCGTCCCAGGCATCAG-3′
β-Lyc actin-R	5′-ATGGCGTGGGGCAGAGCGTAACC-3′

2.3. Sequence analysis

The amino acid sequences of *LycHSP70* and *LycHSP90* were deduced by the Expert Protein Analysis System (http://www.expasy.org/). The nucleotide homology search was conducted with BLASTn program (http://www.ncbi.nlm.gov/BLAST/). The theoretical MW and *pI* were determined by the Expasy-ProtParam online tool (http://www.expasy.org/tools/protparam.html). Multiple sequence alignments were performed with Clustal W v1.8 (http://pbil.ibcp.fr/htm/index.php). Protein structure was predicted by SMART online tool (http://smart.embl-heidelberg.de/). A phylogenetic tree was constructed by MEGA v6.0 using the Maximun Parsimony method. The functional sites in proteins were predicted with the Eukaryotic Linear Motif resource online tool (http://elm.eu.org/). The genome data of the other organism were obtained from UCSC online tool (http://genome.ucsc.edu/).

2.4. The mRNA distribution of LycHSP70 and LycHSP9 in different tissues

Total RNA was extracted from heart, gills, liver, intestine, muscle, spleen, brain and head kidney, and every tissue was collected from at least 5 healthy animals. Two micrograms of total RNA were reverse transcribed in a final 40 μL with PrimeScript™ RT reagent kit (Tli RNaseH Plus, TaKaRa, China) according to the manufacturer's instructions. For each sample, the test and control reactions were run in triplicate. The qRT-PCR was performed in a reaction mixture of 20 μL, containing primer-F 0.8 μL, primer-R 0.8 μL, 2 × SYBR® Premix Ex TaqTM II 10 μL, cDNA sample (100 ng/μL) 0.8 μL, ROX II 0.4 μL, ddH$_2$O 7.2 μL. The standard cycling conditions were: 95 °C for 1 min (initial polymerase activation), followed by 40 cycles of 10 s at 95 °C, 45 s at 61 °C. PCR specificity was checked with dissociation curve analysis from 55 to 95 °C, and β-actin of *L. Crocea* was used as the internal standard. All qRT-PCR primers were