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# A heat shock protein 90 β isoform involved in immune response to bacteria challenge and heat shock from *Miichthys miiuy*

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ABSTRACT

Heat shock protein 90 (HSP90) is highly conserved molecular chaperone that plays a critical role in cellular stress response. In this study, we reported the identification and functional analysis of a heat shock protein 90 gene from miiuy croaker (designated Mimi-HSP90). Mimi-HSP90 contained five conserved HSP90 protein family signatures and shared 89.6%–99.5% similarity with other known HSP90 β isoform. Homology analysis and structure comparison further indicated that Mimi-HSP90 should be β isoform member of the HSP90 family. The molecular evolutionary analysis showed that HSP90 was under an overall strong purifying select pressure among fish species. Mimi-HSP90 gene was constitutively expressed in ten examined tissues, and the expression level of liver was higher than in other tissues. The expression level of Mimi-HSP90 gene under bacterial infection and heat shock were analyzed by real-time quantitative RT-PCR, resulted in significant changes in liver, spleen, and kidney tissues. The purified recombinant pET-HSP90 protein was used to produce the polyclonal antibody in mice. The specificity of the antibody was determined by Western blot analysis. All results suggested that Mimi-HSP90 was involved in thermal stress and immune response in miiuy croaker.



## 1. Introduction

Heat shock proteins (HSPs) are ubiquitous and highly conserved stress proteins, and exist widely from bacteria to mammals and plants. They play key roles in response to potential stress conditions [1,2]. According to their apparent molecular mass, HSPs can be grouped into several families: HSP110, HSP90, HSP70, HSP60 and low molecular weight HSP [3,4]. Among all HSP family, HSP90 is a highly conserved and the most abundant cellular protein, accounting for 1%–2% of cellular proteins in most tissues under non-stress conditions [5]. HSP90 participates in some cellar process, such as cell signaling, signal transduction, protein folding, and protein degradation under normal metabolism and stressful conditions [6–9]. As a molecular chaperone, HSP90 plays a key role in folding newly synthesized and refolding denatured proteins under stress environment [10], and it is also involved in the immune response. There are two cytoplasmic isoforms of HSP90, namely HSP90 α (inducible type) and HSP90 β (constitutive type) in cell, which are the consequences of gene duplication in 500 million year ago [11–14]. HSP90 β lacks the glutamine-rich sequence (QTQDQ) at the N-terminus and is larger than HSP90 α, while they exhibit different molecular chaperone functions [15]. The studies of HSP90 have demonstrated that expression of HSP90 is inducible and can be regulated by all kind of environmental stresses, such as heat shock [16,17], bacterial challenge [18], and heavy metals [19].

The aquatic environment is the more complex system. Aquatic animals usually face a variety of environmental stress, such as thermal shock, bacteria, virus, and oxygen levels [20]. Therefore, the aquatic animals must develop an effective and helpful system to adapt to bad environments. A mass of evidence have demonstrated that HSP is responsible for protecting animals from harm. HSP90 is a member of the HSP family and has received some attention in research. To our knowledge, several HSP90s have been reported and the expression level of HSP90 gene is shown in scallop and fish, such as bay scallop [21], carp [22], senegalese sole [23], and rainbow trout [24]. However, the report about the response of HSP90 gene at functional level in teleost remains deficient.

Miiuy croaker, *Miichthys miiuy*, is an economically important fish as it is extensively distributed from the western Japan Sea to the East China Sea. After it flourishes for several years, diseases of cultured miiuy croaker have occurred frequently. Meantime high temperature also restrains fish healthy developing. These factors

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limit the profitability and development of miiuy croaker [25], and result in a great loss of miiuy croaker in aquaculture. In order to elucidate the molecular immune mechanisms in miiuy croaker, a series of immune response and evolution mechanism studies of the immune-related genes have been carried out and reported [26–34]. However, the stress response of miiuy croaker HSP90 against bacterial infection and heat shock is largely unknown. It is important to understand the functions and expression characteristics of HSP90 under environmental stress. In this study, for the first time, we report the isolation of the HSP90 cDNA of miiuy croaker (designated Mimi-HSP90). The gene expression analysis is used to clarify the role of HSP90 in response to *Vibrio anguillarum* infection and heat shock. Meanwhile, HSP90 protein expression situations are analyzed from miiuy croaker. To understand possible evolutionary process of the HSP90, an evolutionary study of HSP90 among fish species is discussed.

## 2. Materials and methods

### 2.1. Animals, immune challenge and heat shock stress

Miiuy croakers (mean weight 810 g) were collected from Zhoushan Fisheries Research Institute (Zhejiang, China) and maintained in aerated seawater at 20 °C for two weeks before processing. Ten tissues (kidney, liver, spleen, fin, brain, heart, intestine, gill, muscle and eye) from miiuy croaker were removed and kept at −80 °C until use. Challenge of miiuy croaker with *V. anguillarum* was performed as described by Xu et al. [35]. Miiuy croaker were anaesthetized by immersion in MS222 and injected intraperitoneally with 1 ml bacterial suspension. The infected fish were killed at 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h after injection. For heat shock treatment experiment, ten miiuy croaker fish were put into aerated aquaria at different temperature (25 °C, 28 °C, 31 °C, 34 °C, 37 °C, respectively) for 2 h and then moved back to natural environment for 30 min. At 37 °C, experimental fish would die rapidly. Meantime, non-heat shock fish were kept in the 20 °C as a control group. Finally, tissues (liver, spleen, and kidney) of experiment under bacterial challenge and heat shock stress and control fish were removed and kept at −80 °C until use.

### 2.2. RNA isolation, cDNA synthesis

Total RNA was extracted from various tissues of miiuy croaker using RNAiso Reagent (TaKaRa) according to the manufacturer's instructions. Then it was resuspended in DEPC-treated water. Quality of the RNA was assessed by electrophoresis on 1.0% agarose gel. Finally, the cDNA was synthesized using Quantscript RT kit (TIANGEN) according to the manufacturer's instructions. Then the cDNA was used for PCR reactions in gene expression and cloning.

### 2.3. Cloning the full-length of the Mimi-HSP90

One EST (GW669537), similar to other fish species HSP90 gene, was acquired from the spleen cDNA library of miiuy croaker by EST analysis in our laboratory [35]. To obtain the complete cDNA sequence of miiuy croaker, Two specific sense primers, HSP90-outer-F 5′-TCTGACCAATGACTGGGAGG-3′, HSP90-inner-F 5′-GTGCCCTGCTCTTCATTC-3′ (Table 1), were designed based on the sequence of EST to clone the 3′ end of Mimi-HSP90 cDNA sequence. RACE-PCR was performed using a Smart RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. The reaction condition of PCR were follows: predenaturalization at 95 °C for 5 min; 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 2 min at 72 °C, and a final step of 10 min at 72 °C. The obtained PCR products were purified by a PCR purification kit (TIANGEN). The purified fragments were ligated into PMD-19T vectors (Takara) and cloned to TOP10 cell according to the standard protocol. Positive clones were screened via PCR with M13± primers. At least three clones were sequenced from both strands on an ABI3730XL Automated Sequencer with M13 primer.

**Table 1**
Primers sequences used in this study and their application.

| Name | Sequence (5′–3′) | PCR objective |
|---|---|---|
| Mimi-HSP90-outer-F | TCTGACCAATGACTGGGAGG | 3′RACE |
| Mimi-HSP90-inner-F | GTGCCCTGCTCTTCATTC | |
| Mimi-HSP90-RT-F | ATGACCAAAGCCGACCTG | Real time RT PCR |
| Mimi-HSP90-RT-R | GTGAAAGAACCTCCAGCA | |
| Mimi-HSP90-Actin-F | GTGATGAAGCCCAGAGCA | Real time RT PCR |
| Mimi-HSP90-Actin-R | CGACCAGAGGCATACAGG | |
| Mimi-HSP90-F | TAGGATCCATGCCTGAAGAAATGCACC | Recombinant |
| Mimi-HSP90-R | GTCGAATTCAATCAACTTCTTCCATGCGT | expression |

### 2.4. Sequence analysis of Mimi-HSP90

The searches for nucleotide and amino acid sequence similarities were conducted with BLAST programs at the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment of Mimi-HSP90 was performed with the Clustal W Multiple Alignment program (http://www.ebi.ac.uk./clustalw/). The sequence identities between Mimi-HSP90 and vertebrates were calculated by the software MegAlign (DNAStar, USA). The SignalP3.0 was used for predicting Mimi-HSP90 signal peptide (http://www.cbs.dtu.dk/services/Signalp/). Additional the domain structure of Mimi-HSP90 was predicted by SMART (http://smart.embl-heidelberg.de/).

### 2.5. Expression of Mimi-HSP90 protein and purification of the recombinant proteins

The open reading frame (ORF) of Mimi-HSP90 was amplified with specific primers (Table 1) and cloned into the pMD-18T vector. The recombination plasmid DNA containing the Mimi-HSP90 ORF was digested with *BamH* I and *EcoR* I enzymes, and then ligated into the pET-28a vector (digested with the same enzymes). The forming recombinant plasmid pET-HSP90 was transformed into *Escherichia coli* BL21 cells, and correct insertion was determined by enzyme digestion and sequencing. Positive clone with correct insertion was cultured overnight in 5 mL LB medium containing kanamycin at 37 °C. Overnight culture was diluted at (1:20) and incubated at 37 °C with shaking at 180 rpm until the cell density ($OD_{600}$) achieved 0.6–0.7. In order to obtain over-expressed recombinant protein, a different time point's induction with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and an IPTG induction using different concentrations (0.05 mM, 0.1 mM, 0.3 mM, 0.5 mM and 0.7 mM) at 37 °C were both performed. After the IPTG induction, protein samples were analyzed by 12% SDS-PAGE. The recombinant protein was purified according to the instruction manual supplied with His-Bind purification kit.

### 2.6. Production of polyclonal antibodies against the recombinant HSP90

Five male BALB/c mice (4-week-old) were used for immunizing with recombinant protein HSP90 mixed with equal volume Freund's complete adjuvant. Subsequently, Mice were booster immunized with the mixture of antigen and Freund's incomplete adjuvant. The mice anti-HSP90 serum was collected after the fourth immunization. It was clarified by overnight incubation at 4 °C, and centrifuged at 1600 rpm for 10 min. Finally the supernatant was