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Molecular cloning, characterization and expression of heat shock protein 90 gene in the haemocytes of bay scallop *Argopecten irradians*

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Abstract Heat shock protein 90 (HSP90) is a highly conserved molecular chaperone that plays key roles in the folding, maintenance of structural integrity and regulation of a subset of cytosolic proteins. In the present study, the cDNA of *Argopecten irradians* HSP90 (designated AiHSP90) was cloned by the combination of homology cloning and rapid amplification of cDNA ends (RACE) approaches. The full-length cDNA of AiHSP90 was of 2669 bp, including an open reading frame (ORF) of 2175 bp encoding a polypeptide of 724 amino acids with predicted molecular weight of 83.08 kDa and theoretical isoelectric point of 4.81. BLAST analysis revealed that AiHSP90 shared high similarity with other known HSP90s, and the five conserved amino acid blocks defined as HSP90 protein family signatures were also identified in AiHSP90, which indicated that AiHSP90 should be a cytosolic member of the HSP90 family. Fluorescent real-time quantitative PCR was employed to examine the expression pattern of AiHSP90 mRNA in haemocytes of scallops challenged by Gram-negative bacteria *Vibrio anguillarum* and Gram-positive bacteria *Micrococcus luteus*. In both bacterial challenged groups, the relative expression level of AiHSP90 transcript was up-regulated and reached maximal level at 9 h after injection, and then dropped progressively to the original level at about 48 h post challenge. The results indicated that AiHSP90 was potentially involved in the immune responses against bacteria challenge in scallop *A. irradian*.

Introduction

Heat shock proteins (HSPs) are ubiquitous and highly conserved stress proteins, occurring in all organisms from bacteria to humans. They play important roles in response to potentially deleterious stress conditions [1–6]. More recently, it has

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also been suggested that HSPs could function as potent activators of the innate immune system [7,8]. According to their apparent molecular mass, HSPs have been classified into several families: HSP90 (85—90 kDa), HSP70 (68—73 kDa), HSP60, HSP47 and low molecular mass HSPs (16—24 kDa).

HSP90 is one of the most abundant cellular proteins even under non-stress conditions, accounting for 1%—2% of cellular proteins in most tissues [9]. It has been demonstrated to play crucial roles in protein folding, protein degradation and signal transduction [10—16]. The most well-known function of HSP90 concerns the maintenance of key proteins, such as steroid receptors and protein kinases, by forming specific complexes [17]. It also participates in the cellular response to environmental stimuli and interacts directly with several signal transducers and their components [18], for example casein kinase (CK) Π [12,13], the Raf and Src components of the mitogen-activated protein (MAP) kinase system [19] and the cell-cycle control serine/threonine kinases Weel [20]. HSP90 is also involved in the immune response especially in lipopolysaccharide (LPS) recognition [21—23], and regulated by a range of stressors such as heat or cold shock [24—27], hyperosmotic stress [28], food-deprivation, reduced oxygen level [29], polychlorinated biphenyl (PCB) [30], arsenates [31], heavy metals [32,33] and diseases [34,35].

Members of the HSP90 gene family have been characterized in numerous phylogenetically diverse organisms. In vertebrates and budding yeast, there exist two types of HSP90 genes, namely HSP90α and HSP90β, which encode two similar cytosolic isoforms [9,17]. HSP90β lacked the glutamine-rich sequence (QTQDQ) at the N-terminus compared with HSP90α [36]. Until now, only one type HSP90 has been reported in invertebrates except *Anopheles albimanus* which contains two HSP90 genes [37]. To our knowledge, most studies of HSP90 are focused on mammals and typical model organisms, while the molecular features and functional studies in mollusk remain deficient and only HSP90s from *Chlamys farreri* and *Haliotis tuberculata* have been cloned and characterized [38,39].

Because of fast growth rate and strong tolerance to diseases, bay scallop *Argopecten irradians* was introduced from America, and has been cultured extensively in the coastal provinces of China. After flourished for several years, the bay scallop culture in China is now suffering from the problem of mortality. Better understanding of the immune mechanisms may allow more efficient control of the disease, as well as the intensive breeding and long-term sustainability of scallop farming. The main objectives of the present study are: (1) to clone the cDNA of *A. irradians* HSP90 (designated AiHSP90); (2) to investigate the mRNA expression of AiHSP90 in haemocytes of bay scallop challenged by *Vibrio anguillarum* or *Micrococcus luteus*, and (3) to understand the response of HSP90 against bacterial infection.

Materials and methods

Animals, immune challenge and haemolymph collection

Bay scallops *A. irradians*, averaging 55 mm in shell length, were purchased from Nanshan Market, Qingdao, China and maintained in aerated seawater at 18 °C for 1 week before processing. For the bacterial challenge experiment, 100 individuals were kept in each aerated tanks. A total of 50 μL of live *Vibrio anguillarum* or *Micrococcus luteus* resuspended in 0.1 mol L^{-1} PBS (pH6.4, $OD_{600} = 0.4$) was injected into the adductor muscle of scallops. The non-injected scallops and scallops received an injection of 50 μL PBS were used as blank group and control group, respectively. The injected scallops were returned to seawater tanks and nine individuals were randomly collected at 3, 6, 9, 12, 24, 48 and 72 h post-injection. Haemolymph from three individuals were pooled as a replicate to minimize individual variability, and three replicates were employed for each time point during the challenge experiment. The haemolymph from the blank, control and stimulated groups were collected using a syringe from the adductor muscle and centrifuged at 800 × *g*, 4 °C for 10 min to harvest the haemocytes. The haemocyte pellets were immediately subjected to RNA extraction using TRIzol reagent (Invitrogen).

cDNA library construction and AiHSP90 cloning

A cDNA library was constructed from the whole body of a bay scallop challenged by *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene) [40].

Two degenerated primers F1 5′-GDGTGTTYATCATGGAC AAYTGTGA-3′ and R1 5′-TTCATGATYCTYTCCATGTTDGC-3′ were designed based on the conserved sequence of known HSP90s to amplify the partial fragment of AiHSP90 gene from bay scallop. PCR reaction was performed in a 20 μL reaction volume containing 2 μL of 10× PCR buffer, 1.2 μL of $MgCl_2$ (25 mmol L^{-1}), 1.6 μL of dNTP (2.5 mmol L^{-1}), 1 μL of each primer (10 μmol L^{-1}), 12 μL of PCR-grade water, 0.2 μL (1 U) of Taq polymerase (Promega) and 1 μL of cDNA template. The PCR temperature profile was 94 °C for 5 min followed by 33 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gel and purified by the PCR fragment purification kit (TaKaRa). The purified PCR product was ligated into the pMD18-T vector (TaKaRa), and transformed into competent *Escherichia coli* cells. The recombinants were identified through blue-white color selection in ampicillin-containing LB plates and screened with M13 forward and reverse primers. Three positive clones were sequenced on an ABI377 Automated Sequencer (Applied Biosystem), and the resulting sequences were verified and subjected to cluster analysis.

The 5′ end of AiHSP90 cDNA was obtained by RACE technique. Two specific reverse primers, R2 5′-TGTTAGCG GACCAACCATACTG-3′ and R3 5′-CTGTGATGTATGGTATCG GAGG-3′ were designed based on the partial sequence amplified by degenerated primers. The PCR amplification was performed using the same reaction system as described before with oligo (dG)-adaptor and R2 by the 5′ RACE system (Invitrogen), and then a nested PCR was carried out using oligo (dG)-adaptor and R3.

The 3′ end of AiHSP90 was amplified by using sense primer F2 5′-AAGATGAGAAGCCAAAGGTCG-3′ and T5 primer 5′-TGTGCTGCAAGGCGATTAAG-3′ with 1 μL of cDNA library mix as template. The full-length of the sequence was verified

by sequencing the fragment amplified by the primers F4 5′-ATCAGGCAGAAACTAACACCG-3′ and R4 5′-AACGAGAAGAAATGCCAAACG-3′ (located at 5′ UTR and 3′UTR of AiHSP90).

Sequence analysis of AiHSP90

The searches for nucleotide and amino acid sequence similarities were conducted with BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Multiple alignment of AiHSP90 was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A phylogenic tree was constructed using the programs of CLUSTAL X1.83 [41] and MEGA3.1 [42] based on the sequences of AiHSP90 and other known HSP90 sequences. Bootstrap analysis was used with 1000 replicates to test the relative support for the branches produced by the neighbor-joining analysis.

Quantitative analysis of AiHSP90 mRNA expression

The expression of AiHSP90 transcript in haemocytes after bacterial challenge was recorded by fluorescent real-time RT-PCR. Total RNA was isolated from the haemocyte pellets as described above. The cDNA first-strand synthesis was carried out based on Promega M-MLV RT Usage information (Promega). cDNA mix was diluted to 1:5 and stored at −80 °C for subsequent fluorescent real-time PCR. Two AiHSP90 gene-specific primers AiRTF 5′-TCAGTATGGTTGGTCCGCTAA-3′ and AiRTR 5′-CGGTTGCCTTTTCCTTCAGA-3′ were used to amplify a product of 149 bp. A constitutive expression gene, β-actin gene, was used as internal control to verify the quantitative real-time PCR reaction. Two scallop β-actin primers AF 5′-CCCTCTATGCCTCTGGTCGT-3′ and AR 5′-TTCTCTCTCGGCTGTGGTTG-3′ were used to amplify a 200 bp fragment of bay scallop β-actin gene. PCR grade water for the replacement of cDNA template was used as negative control.

The fluorescent real-time PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). The amplifications were performed in triplicates in a 25 μL reaction volume containing 12.5 μL of 2× SYBR Green Master Mix (Applied Biosystems), 1 μL (each) forward primer and reverse primer (10 μmol L^{-1}), 1 μL of 1:5 diluted cDNA, and 9.5 μL of PCR grade water. The thermal profile for real-time PCR was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 1 min. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, fluorescent real-time PCR data from three replicate samples were analyzed with 7300 System SDS Software v1.3.0 (Applied Biosystems, USA). To maintain consistency, the baseline was set automatically by the software. The comparative C_T method was used to analyze the expression level of AiHSP90 [43]. All analyses were based on the C_T values of the PCR products. The C_T was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. The C_T for the target amplification of AiHSP90 and the C_T for the internal control β-actin were determined for each sample. Differences in the C_T for the target and the internal control, called ΔC_T, were calculated to normalize the differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT-PCR. The blank group was used as the reference sample, called the calibrator. The ΔC_T for each sample was subtracted from the ΔC_T of the calibrator; the difference was called $\Delta\Delta C_T$ value. The expression level of AiHSP90 could be calculated by $2^{-\Delta\Delta CT}$, and the value stood for an *n*-fold difference relative to the calibrator. The data obtained from real-time PCR analysis were subjected to Student's *t*-test to determine differences in the mean values between blank and treated groups. Significance was concluded at $P < 0.05$.

Results

cDNA cloning and sequencing of the scallop AiHSP90 gene

The PCR product amplified by the degenerated primers was of 745 bp, and its nucleotide sequence was homogeneous to other known HSP90s. AiHSP90-specific primers R2, R3 and F2 were designed based on the above sequence, and used for the full-length cDNA cloning. By RACE and nested PCR approaches, two fragments corresponding to the 5′ and 3′ end of the AiHSP90 cDNA were amplified. A 2669 bp nucleotide sequence representing the complete cDNA sequence of AiHSP90 was obtained by cluster analysis of the above fragments.

Characterization of AiHSP90

The cDNA sequence of AiHSP90 was deposited in GenBank under accession no. EF532406. The full-length cDNA of AiHSP90 was of 2669 bp, including a 5′-terminal untranslated region (UTR) of 90 bp, a 3′-terminal UTR of 404 bp with a canonical polyadenylation signal sequence AATAAA and a poly (A) tail, and an open reading frame (ORF) of 2175 bp encoding a polypeptide of 724 amino acids with predicted molecular mass of 83.08 kDa and theoretical isoelectric point of 4.81. The five amino acid blocks defining HSP90 protein family, (NKEIFLRELISN[S/A]SDALDKIR, LGTIA[K/R]SGT, IGQFGVGFYSA[Y/F]LVA[E/D], IKLYVRRVFI, GVVDS[E/D]DLPLN[I/V]SRE) and the consensus sequence MEEVD at the C-terminus were highly conserved in AiHSP90 sequence. SMART program analysis revealed that the typical histidine kinase-like ATPases domain, which was ubiquitous in all HSP90 family members, located from position 35—188.

Homology analysis of AiHSP90

The deduced amino acid sequence of AiHSP90 was close matched to other HSP90s in invertebrates and vertebrates. It displayed high similarity to HSP90s of Zhikong scallop (96.8%), abalone (89.7%), human (86% to α isoform and 86.7% to β isoform), zebrafish (84.6% to α isoform and 85% to β isoform), fruitfly (83.4%) and so on. The common names, species names and the GenBank accession numbers were

listed in Table 1. Multiple sequence alignment of AiHSP90 with other known HSP90 proteins revealed that they were highly conserved, especially in the regions of HSP90 family signatures.

Based on the sequences of HSP90s, a phylogenetic tree was constructed using the programs of CLUSTAL X1.83 and MEGA3.1 (Fig. 1). Plant and animal HSP90 were separated and formed two distinct branches in the tree. In the branch of animal, all the vertebrates were clustered together and formed two branches (HSP90α and HSP90β isoform groups). All the vertebrates and arthropods were clustered together and formed a sister group to the branch of mollusk consisting of bay scallop, Zhikong scallop and abalone. The relationships displayed in the phylogenic tree were in good agreement with traditional taxonomy.

Quantitative analysis of AiHSP90 gene expression

Fluorescent real-time quantitative PCR was employed to measure the temporal expression of AiHSP90 transcript in haemolymph of scallops challenged by *V. anguillarum* or *M. luteus*. After *V. anguillarum* challenge, AiHSP90 mRNA level increased and reached the maximum (6.8-fold higher than blank group) at 9 h post injection (Fig. 2). As time progressed, the expression level of AiHSP90 mRNA dropped back to the original level at 48 h post injection. Significant differences in the expression level of AiHSP90 were observed at 3 h, 9 h, 12 h, 24 h and 72 h after injection compared with the blank group ($P < 0.05$). Same expression pattern of AiHSP90 transcript was also observed in the *M. luteus* challenged group. AiHSP90 mRNA level increased and reached maximal level at 9 h after injection and was 3-fold higher than that observed in the blank group. Then the expression level of AiHSP90 transcript dropped gradually to nearly the original level at 48 h post injection. Significant differences of the expression level of AiHSP90 were observed at 9 h, 12 h, 24 h and 72 h after injection compared with the blank group ($P < 0.05$).

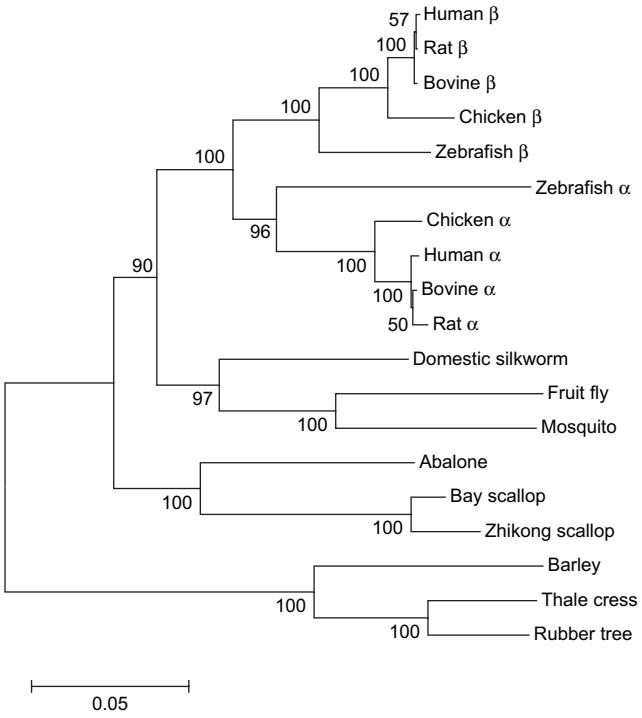

Figure 1 A phylogenetic tree of HSP90 family members constructed with the neighbour-joining method. The common names, species names and the GenBank accession numbers are the same as those in Table 1. Numbers at each branch indicate the percentage of times a node is supported in 1000 boostraps pseudoreplication by neighbour joining.

Discussion

In this paper, the complete cDNA sequence of HSP90 gene from *A. irradians* was reported. Conserved sequences and characteristic motifs, such as HSP90 family signatures,

Table 1 Sequences used in the homology analysis and phylogenetic tree

GenBank no.	Name	Common name	Isoform
EF532406	*Argopecten irradians*	Bay scallop	—
AAR11781	*Chlamys farreri*	Zhikong scallop	—
AM283515	*Haliotis tuberculata*	Abalone	—
CAA27435	*Drosophila melanogaster*	Fruit fly	—
AAB05639	*Anopheles albimanus*	Mosquito	—
BAB41209	*Bombyx mori*	Domestic silkworm	—
NP_571403	*Danio rerio*	Zebrafish	HSP90α
O57521	*Danio rerio*	Zebrafish	HSP90β
P11501	*Gallus gallus*	Chicken	HSP90α
CAA49704	*Gallus gallus*	Chicken	HSP90β
NP_005339	*Homo sapiens*	Human	HSP90α
NP_031381	*Homo sapiens*	Human	HSP90β
NP_786937	*Rattus norvegicus*	Rat	HSP90α
P34058	*Rattus norvegicus*	Rat	HSP90β
NP_001012688	*Bos taurus*	Bovine	HSP90α
BAC82488	*Bos taurus*	Bovine	HSP90β
AAP87284	*Hordeum vulgare*	Barley	—
AAQ08597	*Hevea brasiliensis*	Rubber tree	—
BAA00615	*Arabidopsis thaliana*	Thale cress	—

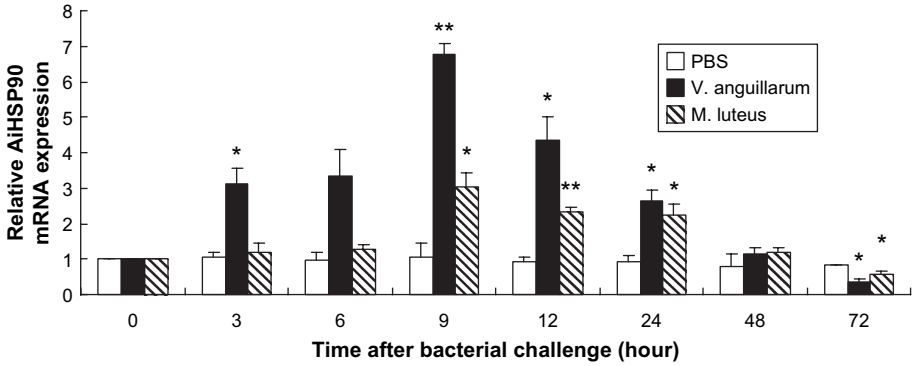

Figure 2 Temporal expression of AiHSP90 mRNA relative to β-actin analyzed by real-time PCR in scallop haemolymph after *V. anguillarum* and *M. luteus* challenge. The values are shown as means ± S.E., $n = 3$. Significant differences between challenged group and blank group are indicated by an asterisk ($P < 0.05$) and two asterisks ($P < 0.01$), respectively.

ATP and geldanamycin binding domain (from 35 to 188 amino acid residues) [44], as well as the major structural and functional domains typically in HSP90 [45,46], were found in the deduced AiHSP90 amino acid sequence. The EEVD sequence at the N-terminus of AiHSP90 was strictly conserved and shared with the other members of HSP90 family. It has been reported that this peptide is recognized by TPR domains of HOP (HSP70 and HSP90 organizing protein), an adapter protein which mediates the association of HSP70 and HSP90 into a multichaperone complex [47]. Furthermore, the presence of sequence MEEVD on the C-terminus is a character shared by all of the cytosolic HSP90 proteins. Searching for sequence similarities revealed that the deduced amino acid sequence of AiHSP90 shared high similarity with other known HSP90s (more than 77% similarity in all the matches), especially with those from Mollusca, *C. farreri* and *H. tuberculata*. Based on the sequence alignment, structure comparison and phylogenetic analysis, AiHSP90 was concluded to be a cytosolic member of HSP90 family.

In vertebrates, there exists two different cytosolic isoforms of HSP90 gene (HSP90α and HSP90β) which are different in the structure of glutamine-rich sequence (QTQDQ) at the N-terminus, a site of phosphorylation by a dsDNA-dependent kinase [36,48]. In contrast, most invertebrates possess only one HSP90 gene. Homology analysis demonstrated that AiHSP90 shared higher similarity with HSP90β. Moreover, like all vertebrate β-isoforms, there was no QTQDQ sequence at AiHSP90 N-terminus. This observation suggested that AiHSP90 was more closely related to the vertebrate β-isoforms.

The mechanisms underlying HSPs induction and the role of HSPs during bacterial infection have not been fully elucidated. Phagocytosis of bacteria or bacterial products is usually coupled with the generation of oxygen free radicals or reactive oxygen species (ROS) [49]. Generation of ROS is an effective defense against bacteria, while it is also detrimental for the host cell, which could result in protein denaturation or proteotoxicity. As a result of bacterial infection and subsequent ROS production, accumulation of denatured proteins in the host cell might trigger HSPs expression. Up-regulation of HSPs is perhaps a protective mechanism since HSPs could bind to damaged or misfolded proteins to restore their original structure [34,50].

The recent data indicated that stress proteins possessed the ability to modulate the cellular immune responses and played key roles in protecting organisms from pathogenic stress [51,52]. In mammals, there were several studies showing that bacteria or bacterial products induced expression of stress proteins in host cells [53—55]. The increased HSP90 expression and tissue-specific HSP90 response in the western painted turtle *Chrysemys picta bellii* during bacterial infection also suggested a role of HSP90 in immunopathological events in reptiles [34]. The study on protozoa *Acanthamoeba castellanii* suggested that HSP90 was involved in phagocytosis or bactericidal activity against bacteria in host cells [56].

In the present study, the mRNA transcripts in haemocytes were measured after scallops were challenged by *V. anguillarum* or *M. luteus*. In both bacterial challenged groups, the expression of the AiHSP90 mRNA increased and reached the maximum at 9 h, and then dropped progressively. The increase of AiHSP90 transcript was higher in *V. anguillarum* challenged groups than that in *M. luteus* challenged groups, which suggested that *V. anguillarum* was probably a stronger inducer for AiHSP90. It was consistent with the fact that *V. anguillarum* is one of the disease-causing bacteria in bay scallop [57]. This is the first time such a response of HSP90 has been observed in a molluscan species, indicating that AiHSP90 is potentially involved in scallop immune responses to bacterial infection. These data would be helpful to understand the significance of HSP90 to scallop immune defense.

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