The molecular characterization and expression of heat shock protein 90 (Hsp90) and 26 (Hsp26) cDNAs in sea cucumber (*Apostichopus japonicus*)

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Abstract The heat shock proteins (HSPs) are a family of proteins whose expression is enhanced in response to environmental stressors. The *Apostichopus japonicus* *hsp90* and *hsp26* genes were cloned using expressed sequence tag and rapid amplification of cDNA ends techniques. The full-length cDNA of *Aphsp90* and *Aphsp26* contains 3,458 and 1,688 nucleotides encoding 720 and 236 amino acids, respectively. Multiple alignments indicated that the deduced amino acid sequences of ApHsp90 and ApHsp26 shared a high level of identity with Hsp90 and small SHPs (sHSPs) sequences of zebrafish, ant, acorn worms, etc., and shared identical structural features with Hsp90 and sHSPs. The expression profiles of these two genes under heat treatment were investigated by real-time quantitative PCR. It was found that the messenger RNA (mRNA) transcripts of the two *A. japonicus* genes varied among different tissues under normal conditions and heat shock, and that the mRNA expression of the two genes was higher in the intestine compared to other tissues. Heat

shock significantly elevated the expression of *Aphsp90* and *Aphsp26* mRNA in a temperature- and time-dependent manner. The results indicate that *Aphsp90* and *Aphsp26* played important roles in mediating the environmental stress in *A. japonicus*.

Keywords *Apostichopus japonicus* · Hsp90 · Hsp26 · mRNA expression · Heat shock

Introduction

Apostichopus japonicus is a common echinoderm mainly found along the East Asian coasts. It has been consumed since ancient times due to its good flavor and medicinal value (Fu et al. 2005). According to its curative properties, it has been exploited as an important economic resource with good market value. In recent years, as demand increased rapidly, the sea cucumber aquaculture have developed in Japan, Korea, and northern of China (Liao 1997). Production of the sea cucumber in China has increased rapidly during the last several years, the area of sea cucumber farming in China has reached 10,000 ha, and output of dried *A. japonicus* (trepang) reached 5,800 tons in 2000 (Chen 2004). In order to maintain the healthy development of sea cucumber farming, there is a need to understand the interaction of *A. japonicus* with environment factors such as temperature. Living in shallow water, *A. japonicus* undergoes day–night and seasonal temperature fluctuations. Water temperature has a significant impact on growth and energy metabolism in *A. japonicus* (Li et al. 2002; Yang et al. 2006). Some studies focused on the heat response in *A. japonicus* found that high temperature can also significantly influence enzyme activity and antioxidant defense (Dong et al. 2007; Wang et al. 2008). Most of these

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studies concentrated on physiological characterization of *A. japonicus*, there is little information on genes expression.

Exposure of organism to various stressors can elicit a subset of genes expression, in which heat shock proteins (HSPs) are a major group. The HSPs are a highly conserved family of proteins that occur from bacteria to mammals (Craig 1985). They have been identified in a series of metazoans. On the basis of the average molecular mass, they are classified into different families, such as Hsp110, Hsp100, Hsp90, Hsp70, Hsp60 and small heat shock proteins (sHSPs) (Georgopoulos and Welch 1993; Feder and Hofmann 1999). Hsp90, which belongs to a highly conserved molecular chaperone, is an important protein that contributes to folding, maintenance of structural integrity, and regulation of a subset of cytosolic proteins (Picard 2002). Recently, Hsp90 has been shown to function in cell differentiation, development, and signal transduction (Soetaert et al. 2006; Wu and Chu 2008; Li et al. 2009). The sHSPs are a more diverse protein family. Under cellular stress, the sHSPs can aggregate into a high molecular mass of approximately 200–800 kDa and this process is essential for their function as molecular chaperones, involving the assembly and transport of new polypeptides and the removal of misfolding proteins (Arrigo and Landry 1994; MacRae 2000). sHSPs have been shown to be involved in embryogenesis and cell proliferation (Linder et al. 1996; Shirk et al. 1998; Reineke 2005).

Acting as a molecular chaperone, the synthesis of HSPs is changed in response to heat shock, heavy metals, toxicity, or any changes inducing protein damage in the cellular environment (Welch 1992; Sanders 1993). The role of HSPs in cell protection and as stress markers has been well recognized in many organisms (Wahid et al. 2007), and considerable variations of HSPs in response to stressors have also been observed in some marine organisms. The expression level of HSPs messenger RNA (mRNA) significantly increased under osmotic and heat stress in the lobster (Chang 2005), and bacteria and water temperature had an impact on the level of HSPs mRNA expression in shrimp *Penaeus monodon* and *Artemia franciscana* (Crack et al. 2002; Huang et al. 2008; Wanilada et al. 2010). HSPs were upregulated after exposure to thermal stress in Cnidaria, and the variability in the HSPs stress response was also observed in different species (Choresh et al. 2003; Chow et al. 2009; Rosic et al. 2011). Hsp70 expression increased in the coelomocytes of the trauma-stressed common sea star *Asterias rubens* (Pinsino et al. 2007). Thus, the expression variation of HSPs in response to changes in environmental stressors is a critical question in ecological physiology.

In order to explore initial molecular change following exposure to heat stress in *A. japonicus*, in this study, we investigated the expression patterns of *hsp90* and *hsp26*,

which are assumed to be involved in prevention of cellular damage during heat stress. The results of our study will provide insight for investigators of the stress-related cellular response in Echinodermata, and may also be useful for identifying the potential biomarkers of environmental stressors in *A. japonicus*.

Materials and methods

Heat stimulation and sample preparation

A. japonicus (60.8±5.7 g) were collected in China from Jiaozhou Bay in the Yellow Sea in June 2010 (average temperature, 18.5°C) and cultured in the laboratory for 1 week in seawater (30 ppt salinity) at 20°C, with continuous aeration. Half of the water was exchanged for fresh seawater daily, and the samples were fed twice a day with a laboratory-made formula (36.36±0.39% (w/w) water, 63.64±0.26% (w/w) dry matter, which included 5.04±0.19% (w/w) crude protein, 0.26±0.05% (w/w) fat, and 72.20±0.19% (w/w) ash).

Specimens of sea cucumbers were used in rapid temperature change treatments. Four temperatures, 20°C, 22°C, 24°C and 26°C, were chosen to determine the levels of mRNA expression in various tissues at these temperatures, which had been used in earlier studies of heat stress in *A. japonicus* (Liu 2008). After heat exposure for 2 h, six individuals in each temperature group were randomly selected and various tissues, including body wall, intestine, and respiratory tree were frozen in liquid nitrogen. To determine the expression levels after exposure for different lengths of time to heat shock treatment, six individuals were chosen at random at 0, 1, 2, 4, 6, 8, 10, 12, 24, 48, h at 26°C heat shock treatment. The intestine was removed from each sample at each time point then frozen in liquid nitrogen and stored at −80°C.

RNA extraction

Total RNA from various tissues was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. mRNA was isolated with Oligotex mRNA kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and concentration of RNA were determined by electrophoresis in 1% (w/v) agarose gel under denaturing conditions.

Cloning of *Aphsp90* and *Aphsp26* full-length cDNA

A 737 base pair (bp) expressed sequence tag (EST) sequence for *hsp90* and a 951 bp EST sequence for *hsp26* were obtained from a subtractive hybridization cDNA

library of *A. japonicus*. The cDNA from body wall tissue of the heat-treated group was used as the tester and the cDNA from the control group was used as the driver during the subtractive hybridization procedure with a PCR-Select cDNA Subtraction kit (Clontech, Mountain View, CA, USA). The two EST sequences showed a high level of similarity to the *hsp90* and *sHSPs* genes of mouse, carpenter ant, acorn worms, etc. The sequences are deposited in the EST database with accession number HO054976 for *Aphsp90* and HS400471 for *Aphsp26*.

The 5' and 3' rapid amplification of the cDNA ends (RACE) PCR technique was used to obtain the full-length *A. japonicus hsp90* (*Aphsp90*) and *A. japonicus hsp26* (*Aphsp26*) cDNAs. Ten gene-specific primers (F1–F5 and R1–R5; Table 1) were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). First-strand cDNA was synthesized using a Smart RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA). For 5'-RACE, total mRNA (1 µl) was reverse transcribed at 42°C for 1.5 h in a 10 µl volume containing 1 µl of SMART II oligo (Table 1), 2 µl of 5× first-strand buffer, 1 µl of DTT (20 mM), 1 µl of dNTP mix (10 mM), 1 µl of 5'-CDS primer A (Table 1), 0.25 µl of RNase inhibitor (10 U), 1 µl of SMARTscribe reverse transcriptase (100 U) and 1.75 µl of sterile water. For 3'-RACE, 1 µl of total mRNA was reverse transcribed under the same conditions with 2 µl of 5× first-strand buffer, 1 µl of DTT (20 mM), 1 µl of dNTP mix (10 mM), 1 µl of 3'-CDS primer A (Table 1), 0.25 µl of RNase inhibitor (10U), 1 µl of SMARTscribe reverse transcriptase (100 U), and 2.75 µl of sterile water.

The 5' and 3' ends of the *Aphsp90* and *Aphsp26* sequences were cloned by PCR using cDNA as the template. The 3' end of *Aphsp90* sequence was amplified with primer F1, F3, F5, and the 3' end of the *Aphsp26* sequence was amplified with primers F2 and F4. The 5' end of the *Aphsp90* and *Aphsp26* sequences was amplified with primer R1, R3, R5 and primer R2, R4, respectively. PCR was done with 5 µl of 10× Universal Primer A Mix (UPM), 1 µl of gene-specific primer (Table 1), 5 µl of 10× advantage 2 PCR buffer, 1 µl of dNTP mix, 1 µl of advantage 2 polymerase (Clontech, Mountain View, CA, USA), 2.5 µl of cDNA template, and 34.5 µl of PCR-grade water. The amplification cycle was 94°C for 1 min, then 35 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min, and a final extension at 72°C for 3 min on a programmable thermal control cycler (MyCycler, Bio-Rad, Hercules, CA, USA).

The expected DNA fragment were eluted from the agarose gel, ligated to the pMD18-T vector (Takara, Shiga, Japan) and transformed into DH-5α competent cells (Tiangen, Beijing, China). Transformed cells were grown overnight on LB agar plates containing 100 µg/ml

ampicillin. White clones were selected and 1 µl of culture was added to a 25 µl reaction volume with 12.5 µl of Go Taq Hot Start Master Mix (Promega, Madison, WI, USA), 1 µl each of universal primers M13-47 and M13-48 (Table 1) and 9.5 µl of PCR-grade water. The amplification profile included denaturation at 94°C for 3 min, then 30 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 90 s, followed by a final extension at 72°C for 5 min. The positive recombinant clones were sequenced by Beijing Mao Jian United Stars Technology Co., Ltd. (Beijing, China). The resulting sequences were verified and subjected to cluster analysis. The sequences obtained after 5'- and 3'-RACE were assembled by DNASTar Lasergene 7.1 software (DNASTar Inc., Madison, WI, USA) to obtain the full-length cDNA.

Sequence analysis, multiple sequence alignment, and phylogenetic analysis

The full-length cDNA sequences of *Aphsp90* and *Aphsp26* were analyzed for similarity with the BLAST programs (Altschul et al. 1997) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). The deduced amino acid sequences were obtained using Translate tool software at the ExPASy server of the Swiss Institute of Bioinformatics (<http://www.expasy.org/tools/dna.htm>). The SMART program (<http://smart.embl-heidelberg.de/>) was used to predict the functional sites or domains in the amino acid sequence. The molecular mass and theoretical isoelectric point was predicted using the compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Three-dimensional domain structures were predicted by the SWISS-MODEL server (<http://www.expasy.org/swissmod/SWISS-MODEL.html>). Multiple sequence alignment was done using the program Clustal W (<http://www.ebi.ac.uk/clustalW>) and phylogenetic analysis was done with Clustal X and Mega4.0 software using the neighbor-joining algorithm, and the tree topology was evaluated by 1,000 replications bootstraps.

Quantitative analysis of *Aphsp90* and *Aphsp26* mRNA expression

The mRNA expression profiles of *Aphsp90* and *Aphsp26* in three tissues (body wall, intestine, and respiratory tree) at different temperatures were examined using real-time quantitative PCR (qPCR). The mRNA expression of *Aphsp90* and *Aphsp26* at different exposure times under heat stress (26°C) was examined with the same method.

Total RNA in each sample was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and reverse transcribed using

Hamburg, Germany). The 25- μ l amplification volume contained 2 μ l of cDNA (100 ng), 0.2 μ mol of each gene-specific primer and 12.5 μ l of SYBR® Green PCR Master Mix (Takara, Shiga, Japan). Each reaction was run in triplicate. The profile for real-time qPCR was 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 59°C for 20 s, 72°C for 30 s. Each product generated a single discrete peak in the melting curve analysis used to demonstrate the specificity of the PCR products. The average C_t value of each triplicate reaction was calculated using Realplex software version 2.2 (Eppendorf, Hamburg, Germany) with the β -actin gene as the reference gene.

Fig. 2 Multiple alignment analysis of ApHsp90 with other Hsp90s. The deduced protein sequences were used in the analysis using Clustal W sequences alignment program. The signature motifs are underlined. The *asterisk* indicates identical amino acids. The GenBank accession number for these proteins are as following: *D. rerio* (Zebrafish, *Danio rerio*,

[illegible]

Statistical analysis

The $2^{-\Delta\Delta CT}$ method was used to determine the expression level of *Aphsp90* and *Aphsp26* mRNA from *A. japonicus*. The variation among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using the SPSS statistical package 13 (SPSS Inc., Chicago, IL, USA). All data are reported as mean±SD and the level of statistical significance were set at $p<0.05$.

Results

Identification of *Aphsp90* full-length cDNA

The 3,458 nucleotides of the full-length cDNA of *Aphsp90* included a 5' untranslated region (UTR) of 93 bp, a 3'UTR of 1,205 bp with a canonical polyadenylation signal sequence AATAAA, and an open-reading frame (ORF) of 2,160 bp (positions 94–2,253) encoding a protein of 720 amino acids with a predicted molecular mass of 82.94 kDa and a theoretical isoelectric point of 5.45 (Fig. 1). The cDNA sequence of the *Aphsp90* gene is deposited in the GenBank database with accession number HQ689677. All five conserved amino acid motifs defining the Hsp90 protein family signature (NKEIFLRELISN[S/A] SDALDKIR, LGTIA [K/R] SGT, IGQFGVGFYSA[Y/F] LVA [E/D],

IKLYVRRVFI and GVVDS [E/D] DLPLN [I/V] SRE) and the consensus sequence (MEEVD) at the C-terminus were present in the ApHsp90 sequence. An adenosine triphosphate (ATP)-binding domain and functional domains typically found in Hsp90 (Gupta 1995; Caplan 1999) were detected at positions 39–194. The amino acid comparison indicated that the ApHsp90 shared a high level of similarity with the Hsp90 sequences reported for acorn worms, zebrafish, mouse, shrimp, etc. (Fig. 2).

The three-dimensional structure analysis of ApHsp90 showed that the transcript contained two separate domains attached to each other by a highly charged loop. The N-terminal domain that is the ATP-binding site contains an eight-stranded β -sheet and six α -helices. The similarity of domains with other animals indicated the same function of ApHsp90. Ala117, Gly118, Ala119, Phe127, Gly128, and Val129 were located in the loop chain, which was the binding site for ATP/ADP, antibiotic, substrate polypeptides, and substrate target proteins etc. The middle domain consisted of two $\alpha\beta\alpha$ domain at the N- and C-terminus of the construct. Nests such as Thr304, Asn305, Asp306, Glu363, Leu364, and Zle365 were located in the loop (Li et al. 2009; Fig. 3).

Identification of *Aphsp26* full-length cDNA

The 1,688 bp of the *Aphsp26* full-length cDNA included a 5' UTR of 180 bp, a 3'UTR of 800 bp and an ORF of 708 bp

Fig. 3 Structure of ApHsp90 in *A. japonicus*. α Helices are showed in blue, β -sheets are showed in purple and loop in pink. **a** Depiction of ApHsp90 N-terminal domain structure. Red arrow shows the position of bound ADP/ATP; **b** the ATP-binding site in the N-terminal domain is shown in red; **c** depiction of ApHsp90 middle domain structure. The red arrow shows the position of bound substrate polypeptides; **d** the position of bound substrate polypeptides in the middle domain of ApHsp90 is shown in red

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1  ACATGGGGATTCAATCACAGCTTCAGAGGGCAGCTAGTAAAGAAAACATGATTTGTGG
61  TTCTAATAGGACGTTTCACAGCCAGACAAATTTGCTCAATCTGCTTATACGATATTTTATTG
121  ACTACTCACTAAACAACTCCATCTTTGAAGACAGTCAAGAAGCAAACTACTTGGAAA
181  ATGGCATCGGTATTGTATTCCATCATTGGCCTTCAGCGTACCATATCCTTGCTGC
1  M A S V F V I P S F G L Q R T T Y P C C
241  GGTATCACGGTTCCTGAAGACCGCATATCAATACCGTCGTCAGCGGAAAATCCATCT
21  G Y H G S V R P R Y Q Y R R Q A E N P S
301  TCTTTGAAACATCTCGCAGTTGCTGCAATCGCTTGGCGATGACGAGTTTGATAATAAA
41  S F E T I S Q L L Q S L A D D E F D N K
361  GACATTACTTTTCCATCCTGCTCTCAAATTAAGACAGCGAGTTAAACAAGAGGAGACT
61  D I T F P S L L L K L R Q R V N K K E T
421  CAGTCAGCTGAAGGAGTAGCGAAATCGGTGTAGTCTTCACTCAACGAACAAACACAGAT
81  Q S A E G V A K S V E S S L N E Q T P D
481  AAATTCAAAGTCTCAGTAGATCTGGCGAGGCATTAAACCTGACGAAATATCAGTCAA
101  K F K V S V D L G E A F K P D E I S V K
541  ATCGTTGACAAAACCTCTGAAATAGAAGGAAAACAGAAAGGAGAGAGATGGTGAT
121  I V D K T L K I E G K H E E K G E D G D
601  TTCACTCTCGGCAATTTACCGGCTTATCGTCTTCCAGATGACATTAACCTTGAACAA
141  F T S R Q F T R S Y R L P D D I N L E Q
661  CTGACGTCACTATTATCATTGTAGGGGCTGCTTAACGTGAGGCCCAAGACTGGTGAAG
161  L T S S L S F E G V L N V E A P R L V K
721  GAGGAACCTAAACAGCTGAGAGGAATATCCGATCGAACAAGGACAGACAGTTGACGC
181  E E L K P A E R N I P I E Q G Q T S S A
781  ATCAAGACCAAGCAAGAACTAGAGGGGGAGAGGATAAAGTAGAGGAGCGAAATCTGAC
201  I K T N E E L E G G E D K V E G A K S D
841  GCAGAAGCGATGGAAGAAGAACTGCTGTAAGTTGAGAACTAACTGAGAAAAAACAG
221  A E A M E E E S G K V R E T N *
901  GCGTCAATTAACGATTGAAGAAGTCTGATATCTGCTGTCAACAGTGGCTCCTTCATGT
961  GATTCATGCAATATACAGTTACTAATATGGACACTACAGCTGCTTTTGCAAACTTTTC
1021  TATTTTAACTGTTTAAACAGAAAATCAAGTTCGAGTTGTTTGTATTATTATTGTAATT
1081  ATGTTTAACTATTTTTACTTACTCACTATACGATTAAATTCATTATTTCATAAACCA
1141  TTAATATTCGCTAGCACTTATACCTTAAAGATCATGGCTGAACCTAATCTCTATTAA
1201  TTGCACTTTGAGTTGAAAATGAAGTGTACTCACAATATTATGTTTATGTATAAATGCT
1261  TATTATATGTACGACGTTTTCGAGTTTACAGTTTGAACCACTGCAGTCCAATCTTTGAT
1321  TTTTCTTAAAGCTTTCTATCAATTTGTGAATTGTTTAAATCAAGAATAGGTGACCT
1381  GGTCTAGTGTATCTGTAGTGTACTATAGTTGTAAGTATCATCTTGAATTTCTTGT
1441  TGTAGTTTGTTCATTCTGTAAGAGATACATTTTGTATGTTGTTTCTCAGCAGCA
1501  TTATTATGACAGACATTAAATGATACAACATGACCAAGATTATCAAAATATGTTCTA
1561  TTTATCTTATATTATAGATATTATAATTAATATGTATGCTGCTGTAACCTATATATT
1621  CATTCTCTGAACTAAATACAGATTTTACAAGTAACTAAAAAAAAAAAAAAAAAAAAA
1681  AAAAAAA

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Fig. 4 The full cDNA and deduced amino acid sequence of *A. japonicus hsp26*. The nucleotides and amino acid residues are numbered on the left. The start and stop codons are included in a box, and the classical polyadenylation signal in the 3'UTR is double underlined. α -Crystallin domain of small heat shock protein is underlined. The IXI/V motif (I-P-I) and conserved site Arg148 are highlighted as yellowed regions

(positions 181–888) with the canonical polyadenylation signal sequence ATTTA (Fig. 4). It encoded 236 amino acids with a predicted molecular mass of 26.37 kDa and a theoretical isoelectric point of 4.87. The IXI/V motif (I-P-I) relating to stabilization of assemblies was present in the sequence and the most conserved site in sHSPs, Arg148, was also found. α -

Crystallin domain typically found in small heat shock proteins was detected at positions 97–193 (Fig. 5). The cDNA sequence of the *Aphsp26* gene is deposited in the GenBank database with accession number HQ689678.

The three-dimensional structure of ApHsp26 was analyzed. The immunoglobulin-like α -crystallin domain (positions 97–193) flanked by N- and C-terminal extension, which is the most conserved element in sHSPs was present in the sequence. The α -crystallin domain consists of a β -sandwich comprising two antiparallel β -sheets, followed by a short C-terminal extension and a long loop extending from the β -sandwich (van Montfort et al. 2001; Fig. 6).

Phylogenetic analysis of the ApHsp90 and ApHsp26 proteins

A phylogenetic tree was constructed by analyzing the amino acid sequences of *A. japonicus* HSPs and similar HSPs of other invertebrate and vertebrate species. The result indicated that ApHsp90 and ApHsp26 belong to the HSPs family. As shown in Fig. 7, *A. japonicus* Hsp90 shares greater identity with the acorn worm *Saccoglossus kowalevskii* than it does with insects, whereas *A. japonicus* Hsp26 shares greater identity with insects.

Quantification of *Aphsp90* mRNA expression

The expression of β -actin mRNA under heat stress was found to be almost stable in our earlier experiments (Liu 2008), so we chose β -actin as the reference gene. Each of the amplified products in this experiment gave a single peak in the dissociation curve, which was verified as the target product by electrophoresis and sequencing. The *Aphsp90* mRNA level in different tissues was determined and the result showed that at normal temperature (20°C) the mRNA level in the intestine of *A. japonicus hsp90* was higher than it was in other tissues (Fig. 8a), which is consistent with the suggestion that the intestine is the main tissue to respond to environmental stressors (Liu 2008).

The mRNA expression of *Aphsp90* showed a temperature-dependent response under heat stress (Fig. 8a). The expression level of *hsp90* increased significantly with increased temperature in all *A. japonicus* tissues; the expression of *Aphsp90* at 26°C was significantly higher ($p < 0.05$) than that at the other temperatures tested.

To determine whether *Aphsp90* expression was time-dependent under heat shock, six sea cucumbers were sampled at each time point. It was found that the expression of *Aphsp90* in *A. japonicus* under heat shock had a curvilinear trend with time. The mRNA level reached a peak at 4 h and remained high at 6 h under heat stress, then decreased gradually to the pretreatment level at 48 h (Fig. 9a).

A. mellifera -----MSLIPLLFSD-WWEDLDRPHRLLDQNFGLGLYPEQLLNSN-----ILDQY--IL 46
D. buzzatii -----MSLSQLLS---LVNELQEPRAPLYD-YGLGIHPIQLRPKTTTRQVLLNPWNCVH 49
C. quinquefasciatus -----MSIIPILFRDSFMDDFMRP-SIMEKSLFDDEFPANLLVAN-----I 40
D. rerio MAERRIPFSFMRSPSWDPFRDWYQGS-RLFDQSFGMPALSEMLTFPSTHWPGYMRPFGH 59
X. laevis MSERRIPFTFLRTPSWDPFRDWYQGSSRLFDQSFGMPRIPEDWHQWPSTSWPGYVRLFPS 60
A. japonicus -MASVFVIPSFGLQRTTYPCCGYHGSVRPRYQYRRAENPSSFETISQLLQSLADDEFDN 59

: . . .
A. mellifera PNRNQRLRNPLIYYRPWGELLRKNEGGGT---STVKADKDKFQVILDV-QQFKPDEINV 101
D. buzzatii PECPTSPVGQILAGRNRDLAKQTSAGADGWNWPLSQVGKDGQVCMQDV-TQFTPSSELSV 108
C. quinquefasciatus PTRRGPK-----RRRCTCVTENQQN-N---AVQKKSRESFEVSIDV-QNFKPEEISV 87
D. rerio PEFASLMQGGPPVMPMMTPSYGRALSRLSSGMSEVKQTGDSWKISLDV-NHFSPEELNV 118
X. laevis QSMDEVPPPTTAPAGAPAPDFSRALSRLSSGISEIRQTSQWKISLDV-NHFAPEELVV 119
A. japonicus KDITFPSLLKLRLQRVNKKETQSAEGVAKSVSESSLNEQTPDKFKVSVDLGEAFKPDEISV 119

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A. mellifera KIVDKCVVVEGKHEEKQDEHGWISRQFTIRRYMIEQCDDIDQVTSSLSSDGVLNITAPRKE 161
D. buzzatii KVVDCNCVIVEGKHEEREDDHGYISRHFVRRYALPGYDGDQVSSLSDDGVLTVSVPKPQ 168
C. quinquefasciatus KMVDNYITVEGKHEEKQDEEGFVSRHFVRKYRLPEGHDLKVVASSLSDDGVLTIAPRLA 147
D. rerio KTKDGVLEITGKHEERKDEHGFISRCFTRKYTLPPGVDSSEKISSCLSPGVLTVVEAP-LP 177
X. laevis KTKDGIVEITGKHEEKQDEHGFISRCFTRKYTLPPGVAINAVASSLSPDGILTVEAP-LP 178
A. japonicus KIVDKTLKIEGKHEEKGEDGDFTSRQFTIRSYRLPDDINLEQLTSSLSFEGVLNVEAPRLV 179

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A. mellifera QPK-IQNERNITIEQTG-KPALKENTEEKKEEKKEEN----- 196
D. buzzatii PVEDKSKERVVQIQQVGPAHLNIRKNEDESKEDKEKEKEKEKKKTATNGNASK--- 221
C. quinquefasciatus LPEVPAKERTIPIVRTE-----QIAEDEPKSQKTAD----- 178
D. rerio KPAIQAPEVNIPVN-----KTTVSTTK----- 199
X. laevis KPAIQSAEISIPITFQS-----RAEIGTSEAKKGEEAAKK----- 213
A. japonicus KEELKPAERNIPIEQGQTSSAIKTNEELEGGEDKVEGAKSDAEAMEEESGKVRETN 235

* : :

Fig. 5 Multiple alignment analysis of ApHsp26 with sHSPs. The deduced protein sequences were used in the analysis using Clustal W sequences alignment program. The IXI/V motif is *underlined*. The conversed site in sHSPs is in *box*. The *asterisk* indicates identical amino acids. The GenBank accession number for these proteins are as following: *A. mellifera* (honey bee, *Apis mellifera*,

Quantification of *Aphsp26* mRNA expression

The expression of *Aphsp26* mRNA in different tissues was assessed by real-time PCR. The results showed that under normal condition (20°C) *Aphsp26*, like *Aphsp90*, were

XP_001120194), *D. buzzatii* (fruit fly, *Drosophila buzzatii*, ABX80642), *C. quinquefasciatus* (southern house mosquito, *Culex quinquefasciatus*, XP_001847194), *D. rerio* (zebrafish, *Danio rerio*, CAM12245), and *X. laevis* (African clawed frog, *Xenopus laevis*, NP_001087285)

highly expressed in the intestine compared to the body wall and the respiratory tree (Fig. 8b).

To determine whether *Aphsp26* was inducible by heat shock, six holothurians were selected at each different temperature. It was found that the mRNA expression of

Fig. 6 Structure of ApHsp26 in *A. japonicus*. α Helices are showed in blue, β -sheets are showed in purple and loop in pink. **a** Depiction of ApHsp26 α -crystallin domain structure; **b** the IXI/V motif (I-P-I) relating to stabilization of assemblies and the most conserved site in sHSPs Arg148 are shown in red

Fig. 7 Phylogenetic analysis of ApHsp90, ApHsp26 relative to heat shock proteins of other invertebrates and vertebrates. The arrows denote *A. japonicus* Hsp90 and Hsp26. The tree topology was evaluated by 1,000 replications bootstraps, and numbers on each branch of the tree represent bootstrap support value. The species names, common name, and the GenBank accession numbers encoding sHSPs are as follows: *Drosophila buzzatii* (fruit fly, ABX80642), *Culex quinquefasciatus* (southern house mosquito, XP_001847194), *Danio rerio* (zebrafish, CAM12245), *Carassius auratus* (goldfish, BAE93468), *Tribolium castaneum* (red flour beetle, XP_974390), *Poeciliopsis lucida* (Desert topminnow, AAB46593), *Locusta migratoria* (migratory locust, ABC84494), *Bemisia tabaci* (sweet potato whitefly, ACH85196), *Liriomyza sativae* (vegetable

leafminer, ABE57139), *Oncorhynchus mykiss* (rainbow trout, NP_001118137), *Branchiostoma lanceolatum* (common lancelet, CAE83570), *Nasonia vitripennis* (Parasitic wasp, XP_001604562), and *Bombyx mori* (Silk moth, NP_001036984). The species names, common name and the GenBank accession numbers encoding HSP90 are as follows: *S. kowalevskii* (acorn worm, NP_001164703), *Camponotus floridanus* (ant, EFN72016), *Harpegnathos saltator* (jumping ant, EFN88374), *Apis mellifera* (honey bee, XP_395168), *Dicentrarchus labrax* (European seabass, AAQ95586), *Metapenaeus ensis* (Greasyback shrimp, ABR66910), *Eriocheir sinensis* (Chinese mitten crab, ACJ01642), and *P. monodon* (Giant tiger prawn, ABM54577)

Fig. 8 Relative expression levels of *A. japonicus* Hsp90 and Hsp26 cDNAs in different tissues under various temperatures. **a** *Aphsp90*, **b** *Aphsp26*. The *Aphsp90* and *Aphsp26* mRNA expression levels relative

to β -actin mRNA levels were analyzed using real-time PCR. Different lowercase letters indicate significant differences ($P < 0.05$). All data as mean $\pm$ SD. $N = 6$ sea cucumbers treatment⁻¹

Aphsp26 was temperature-dependent in all *A. japonicus* tissues. The mRNA expression level peaked at 26°C in all tissues ($p < 0.05$), but the intestine showed a more sensitive response to heat stress; the level of expression increased rapidly with increased temperature (Fig. 8b). This result was consistent with that found for the expression of *Aphsp90*.

To determine the relationship between expression level and duration of heat shock, six sea cucumbers were sampled at each time point. As shown in Fig. 9b, the level of *Aphsp26* mRNA expression was time-dependent. The transcripts of *Aphsp26* increased significantly in the first few hours, reached maximum expression after heat shock for 6 h then decreased gradually and reached the pretreatment level at 48 h.

Discussion

During the life cycle of most organisms, they are confronted with numerous environmental stressors, such as high or low

temperature, that have significant impact on their survival. Many genes including HSPs are involved in the protection of cells from environmental stress. *A. japonicus* is epibenthic and temperature fluctuation can affect its growth. In an attempt to better understand the response to heat stress in this holothurian, the *Aphsp90* and *Aphsp26* genes were cloned and the expression patterns induced by heat stress were analyzed.

In this study, the full-length cDNAs of the *Aphsp90* and *Aphsp26* genes were identified and characterized for the first time. The sequence of *Aphsp90* contains 3,458 bp and encodes 720 amino acids. Alignment of multiple sequences revealed that ApHsp90 is highly homologous to other species, including vertebrates and invertebrates. ApHsp90 contains three major domains: an N-domain with an ATP-binding motif, which is the main typical structural feature of Hsp90 members; a central domain with a major site for client protein interactions forming a catalytic site that interacts with ATP bound in the N-domain; and a C-domain that plays an essential role in Hsp90 dimerization. The well-conserved C-terminal motif MEEVD was identi-

Fig. 9 Relative expression levels of *A. japonicus* Hsp90 and Hsp26 cDNAs after exposure for different lengths of time under the same heat shock (26°C). **a** *Aphsp90*, **b** *Aphsp26*. The *Aphsp90* and *Aphsp26* mRNA expression levels relative to β -actin mRNA levels were analyzed using real-time PCR. Different lowercase letters indicate significant differences ($P < 0.05$). All data as mean $\pm$ SD. $N = 6$ sea cucumbers treatment⁻¹

fied in this sequence, which is suggested to bind to many co-chaperones with tetratricopeptide repeat domains, such as immunophilins Cyp40, Hop/Sti1 and FKBP (Pearl and Prodromou 2006). Five conserved amino acid motifs previously regarded as the Hsp90 protein family signature were also identified in the ApHsp90 sequence. Thus, it was confirmed that the function of ApHsp90 is similar to that of Hsp90 in other organisms.

The full-length *A. japonicus* *Aphsp26* cDNA contains 1,688 bp with an ORF of 708 bp encoding a putative peptide of 236 amino acids, and the ApHsp26 sequence shared homology with the sHSPs sequences reported in red flour beetle (47%), migratory locust (50%), southern house mosquito (39%), and goldfish (30%) by multiple sequence alignments. The sequence contains the conserved IXI/V motif (I-P-I) relating to stabilization of assemblies and the α -crystallin domain, which is a mainly functional domain in the sHSPs family, and the main structural features of ApHsp26 include a β -sandwich comprising two antiparallel β -sheets, followed by a short C-terminal extension and a long loop extending from the β -sandwich (van Montfort et al. 2001). This structural feature is essential for aggregation to form a high molecular mass structure. Thus, it was identified that the function of ApHsp26 is similar to that of sHSPs in other invertebrates.

The *hsp* genes have highly conserved sequences and are widely used for evolutionary and phylogenetic analysis (Zhang and Denlinger 2010). The neighbor-joining method was used to conduct a phylogenetic analysis including Hsp90, Hsp70, and sHSPs from vertebrate and invertebrate species. The results indicated that ApHsp90 and ApHsp26 belong to the Hsp90 and sHSPs clusters, respectively, confirming that they are similar to Hsp90 and sHSPs, respectively.

The distribution pattern of *Aphsp90* and *Aphsp26* were determined in different *A. japonicus* tissues. Under normal condition, the two genes are both expressed in all tissues of *A. japonicus*, suggesting that these gene products are required to maintain cell homeostasis. Although these two genes are expressed in different tissues, the expression level is much higher in some tissues than in others. Real-time PCR showed that the expression levels of *Aphsp90* and *Aphsp26* in the intestine were higher compared to the levels in the body wall and respiratory tree at all temperatures tested. Differential levels of expression in different organs have been observed in other species. The expression level of Hsp90 and sHSPs are higher in the gut compared to other tissues in the black tiger prawn *P. monodon* and the orange-spotted grouper *Epinephelus coioides* (Huang et al. 2008; Chen et al. 2010). The intestine is a major organ in the sea cucumbers that plays important roles in digestion, absorption, and secretion of nutrients (Liao 1997). Owing to its function in metabolism, the intestine can be sensitive to environmental changes, which was proved by observation of

the atrophied stage of the digestive tract during aestivation. Under heat shock, reactive oxygen species can accumulate, which is harmful for the activity and structure of enzymes (Abele et al. 2002; Heise et al. 2003). In order to maintain the normal level of metabolic activity in *A. japonicus*, expression of the *Aphsp90* and *Aphsp26* genes is induced in response to the increase of misfolding proteins. Our results suggest that the *A. japonicus* intestine is a heat-sensitive tissue and we used it as the target in a time-dependent study.

Determination of temperature-course expression pattern of *Aphsp90* and *Aphsp26* was done with real-time PCR and the results indicated that the expression levels of the two genes are temperature-dependent. Heat stress impacts on the folding and transformation of proteins and the HSPs play a role in maintaining biological processes in the organism. Hsp90 contributes to the maintenance of structural integrity, and the sHSPs aggregate and form multimeric complexes that attract proteins denatured by stress. The damage to an organism is increased with elevated temperatures and in order to maintain structural integrity at high temperature this holothurian needs more molecular chaperones, so the transcripts of these two genes were increased.

The time-course expression pattern of *Aphsp90* and *Aphsp26* was determined in the *A. japonicus* intestine by real-time PCR. It was found that the transcripts of the two genes were elevated with exposure time, which indicated a possible response linked to protein misfolding. At 26°C, the expression level of *Aphsp90* reached peak at 4 h and remained high at 6 h, whereas the mRNA expression of *Aphsp26* reached a maximum at 6 h. The difference expression profiles of the two genes indicated their different functions under heat shock. The elevated levels of the two genes could enhance the thermal tolerance of *A. japonicus* and strengthen the correction of misfolding protein, which has been reported in earlier studies of other species (Parsell and Lindquist 1993; Feder and Hofmann 1999; Tomanek 2002). However, the continued synthesis of HSPs requires a great deal of energy and has an impact on the synthesis of other proteins and on the growth of the organism (Krebs and Feder 1997; Viant et al. 2003). Thus, for the maintenance of biological processes, the transcripts of the two *A. japonicus* genes decreased gradually after 6 h, and returned to the untreated level at 48 h.

However, it was indicated in higher vertebrate models that the transcription of message and the translation of HSPs are in apparent disagreement and it has been proved that the transcriptional activation of HSPs might not be paralleled by protein synthesis (Hensold et al. 1990; Bruce et al. 1993). More work on the characterization of the response to thermal stress in *A. japonicus* Hsp90 and Hsp26 is needed at the protein level.

HSPs are chaperones that have important roles under environmental stress. This is the first study in which *A.*

japonicus *hsp90* cDNA and *hsp26* cDNA have been cloned, and it was found that the deduced amino acid sequences of ApHsp90 and ApHsp26 shared a high level of homology with the amino acid sequences of zebrafish, acorn worms, mouse, etc. The data showed also that *Aphsp90* and *Aphsp26* mRNAs were expressed more highly in the intestine compared to other tissues, which is consistent with the atrophied stage of the digestive tract during aestivation. Furthermore, the expression levels of the two genes were altered with the time and temperature, a response possibly linked to the repair of misfolding proteins and to maintaining homeostasis of the cellular metabolism in *A. japonicus* under heat stress. Therefore, the results presented here provide useful insight for investigating stress-related cellular response and for identifying potential biomarkers of environmental stressors in *A. japonicus*, which is an important economical species in China (Liao 1997).

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