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Cloning and expression analysis of a small HSP26 gene of Pacific abalone (*Haliotis discus hannai*)

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Abstract: *Heat shock proteins (HSPs) are evolutionally conserved from micro organism to mammals and play important roles in many biological processes including thermal tolerance. We isolated a homologue of small HSP26 (sHSP26) from a subtracted cDNA library of heat shock-treated abalone (Haliotis discus hannai). The abalone sHSP26 encompossed 793 nt, including a coding region of 501 nt. The deduced amino acid sequence of the abalone sHSP26 contained well conserved α-crystallin domain and showed overall identities of 27-31% with the other species' sHSP proteins. The abalone sHSP26 transcript was induced by heat shock treatment, but not by cold shock treatment.*

Key words: *Pacific abalone, Heat shock protein, Small HSP26, Thermal stress*
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Introduction

Heat shock proteins (HSPs) are well known as stress proteins because various forms of stress enhance their transcriptional activation and biosynthesis in organisms, ranging from bacteria to humans (Lindquist *et al*., 1986). According to their average apparent molecular masses they are designed as HSP100, HSP90, HSP70, HSP60, and small-size HSPs (Parsell and Lindquist , 1993). One of the small HSPs (sHSP), HSP26 represents an early line of defense against stress within cells, binding partially denatured proteins, preventing irreversible denaturation, and, in cooperation with other molecular chaperones, promoting either protein renaturation or protein destruction (Haslbeck *et al*., 2005a; Cashikar *et al*., 2005).

In recent years, cDNAs encoding HSP70 and HSP90 have been described from Pacific abalone (*Haliotis discus hannai*) (HSP70; GeneBank accession no. DQ324856) (Cheng *et al*., 2007) and Tube abalone (*Haliotis tuberculata*) (HSP70; EMBL accession no.; AM283516.1, HSP90; EMBL accession no.; AM283515.1) (Farcy *et al*., 2007). Although the potential importance of sHSPs in stress defense mechanism was raised by many biologists, the sHSPs have not been extensively studied in aquatic invertebrates.

Over the years, changing climate has received much attention in various fields. A warmer climate may affect several biogeochemical processes in all organisms including animals and plants (Estiarte *et al*., 2008). Especially, as temperature is a primary physical factor affecting the life of mollusks (Chen, 1984), it is necessary for studies related to thermal stress. In this study, we have identified a partial fragment of sHSP 26 from a suppression subtracted cDNA library of Pacific abalone (*Haliotis discus hannai*) treated with thermal stress. The expressional changes of sHSP26 were investigated in the heat-shock or cold-shock treated Pacific abalone.

Materials and Methods

Animals: Pacific abalones (*Haliotis discus hannai*) were obtained from Jeju Fisheries Research Institute (Jeju, Republic of Korea) and acclimatized to laboratory conditions for 10 days before starting the experiments. Pacific abalone was exposed to heat-shock or cold-shock from ambient sea water (18°C) to 28°C or 4°C, respectively. The treated abalones (3 individuals per each time point) were sampled at 0, 0.5, 1, 2, 3, 4 and 5 hr in heat-shock treatment, or at 0, 3, 6, 9 and 24 hr in cold-shock treatment. The collected samples were ground immediately under liquid nitrogen for RNA extraction. Total RNA samples were extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions.

Cloning and sequence analysis of sHSP 26: We constructed a suppression subtracted cDNA library of Pacific abalone, heat-shock derived and analyzed expressed sequence tag (EST) of 289 clones (data not shown). The EST clone AHSL2-D11-84, which carries a 793-bp insertion, showed significant sequence homology (31%) to the Artemia (*Artemia sinica*) sHSP26 (Gene Bank accession no. DQ310576.1). The AHSL2-D11-84 contains a 5'-untranslated region (UTR), an open reading frame (ORF) and a 3'-UTR, but not poly A tail.

Sequence analysis: The nucleotide and deduced amino acid sequences were analyzed using the Genetyx-Win program ver. 4.0 (Genetyx Co., Japan). Multiple alignments of the sHSP were constructed using the ClustalW program (http://www.ebi.ac.uk/Tools/

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  1 ctttggtcttcctcccgaggagaagaaagatttgctccatgggagtcctgataagaagga
 61 agaacctgctgaacagaccacaggtctagacagagaggaacctcgtccggaagacgacaa
121 cacgacggaaacagagtccttgggcgatctaacggatgtcgaggaagagagaagcgagga

181 agagaaaggggaaaaaggagatacctgcctaATGTTGAAATATGACGAGAAGTTGTCTCT
                                    M  L  K  Y  D  E  K  L  S  L 10

241 CACAGATGACGCTGATGACTTCCTGCACGTGGAGAAACAGACGACAGGTGACAGTGCTCC
     T  D  D  A  D  D  F  L  H  V  E  K  Q  T  T  G  D  S  A  P 30

301 AGAGAAGACGACAGTCCGTATTATGCACGAAGACGAAGATGTAGCACGATTGATGTCTGA
     E  K  T  T  V  R  I  M  H  E  D  E  D  V  A  R  L  M  S  D 50

361 TGGAGAGGAAGATGTTGACAGGACTCAGGCCGAAGCCGATGAGAAAGCTCCTGCCCTCTC
     G  E  E  D  V  D  R  T  Q  A  E  A  D  E  K  A  P  A  L  S 70

421 AACATCACAATATTTCCAAATTCGGTTTGATCTGTCCAGCTACAAACCAGAAAACATCCG
     T  S  Q  Y  F  Q  I  R  F  D  L  S  S  Y  K  P  E  N  I  R 90

481 GGTAGTTGTTAGAAGTGGTGACGTCATCGTGGAAGCCGAGCACGAGAGCACGTGTGACAG
     V  V  V  R  S  G  D  V  I  V  E  A  E  H  E  S  T  C  D  S 110

541 TTACAGCGAGACTGAGACACTGCGAAGGCGCATTCGTCTCCCGGACAAAGTGGACCAGTC
     Y  S  E  T  E  T  L  R  R  R  I  R  L  P  D  K  V  D  Q  S 130

601 CATGTTGACGAGTGTCCTGAATGCAGAGGGCGAGATGACCATTCAGGCGCCATTCCTGTC
     M  L  T  S  V  L  N  A  E  G  E  M  T  I  Q  A  P  F  L  S 150

661 TCAGGCCATCAGTGGGAAGGACGAGAAAATTGTCCCGATTATTTGGGAGTGAtttgagat
     Q  A  I  S  G  K  D  E  K  I  V  P  I  I  W  E  *          166

721 cgtgaaggatatatgaatctcagtgttccattagctgaggtggtgttatcacattgctga
781 ctcagtatttatc
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Fig. 1: cDNA and predicted amino acid sequence of the abalone sHSP26. Numbering of nucleotides and amino acids are given at the left and the right margins, respectively. Putative transcription factor-binding sites are underlined. The nucleotide sequence has the accession number EF472916 in the GenBank database

clustalw/). Bootstrap values for phylogenetic tree analysis were built through ClustalW and visualized with MEGA program (ver. 3).

Expression studies using RT-PCR: Total RNA samples were extracted from the tissues using TRIzol reagent (Invitrogen, USA). Subsequently, first-strand cDNA synthesis was carried out using the Advantage RT-for-PCR Kit (Clonetech, USA). The levels of sHSP26 expression were detected by RT-PCR using specific primers (sHSP26-RT-F; 5'-CAGAGTCCTTGGGCGATCTAAC-3' and sHSP26-RT-R; 5'-GCAGGAGCTTTCTCATCGGCTTC-3'), which were based on the nucleotide sequence of the abalone sHSP26 cDNA. For appropriate comparison, PCR of abalone HSP70 was conducted with the same cDNA samples using the abalone HSP70 primers (HSP70-RT-F; 5'-CAGCTGTGGACAAGAGCACC-3' and HSP70-RT-R; 5'-CCTTCTCGGCAAGCTGGTTAG-3').

As an internal control, β-actin was amplified using the appropriate primers (β-actin-RT-F; 5'-GCCGCTTGACTCTT GTGTGC-3 and β-actin-RT-R; 5'- CTCCTCTGGTGCAACGCGG-3'). The PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final step of 72°C for 7 min. Ten microliters of PCR products were analyzed on a 1.5% (w/v) agarose gel (Amresco, USA) containing ethidium bromide (100 ng/ml) using a Mini-Gel Electrophoresis System (Cosmo bio, Japan).

Results and Discussion

We employed a PCR-based technique, namely suppression-subtractive hybridization (SSH), to identify genes differentially expressed in abalone treated heat shock, in an attempt to understand the molecular processes involving the response to environmental stressors in mollusks.

Among the 289 of EST clones, the AHSL2-D11-84 was revealed to be homologous to known sHSP26 genes of other species using GeneMaster 3.0 software (Ensoltek, Korea). The nucleotide and deduced amino acid sequences of sHSP26 are shown in Fig. 1. The abalone sHSP26 cDNA (GenBank accession no. EF472916) spans 793 nt and comprises the 221 nt 5'-UTR, 61 nt 32 -UTR, and the 501 nt ORF that encodes a polypeptide of 166 amino acids (Fig. 1). The deduced polypeptide has a molecular weight of 18.82 kDa and an expected isoelectric point of 4.46. We also performed PCR of genomic DNA to determine the abalone sHSP26 gene structure using the specific primers (sHSP26-RT-F and sHSP26-RT-R). Similarly, the result was that the abalone sHSP26 gene did not include intron. The intronless-gene strucuture is found in other sHSP genes (Franck *et*

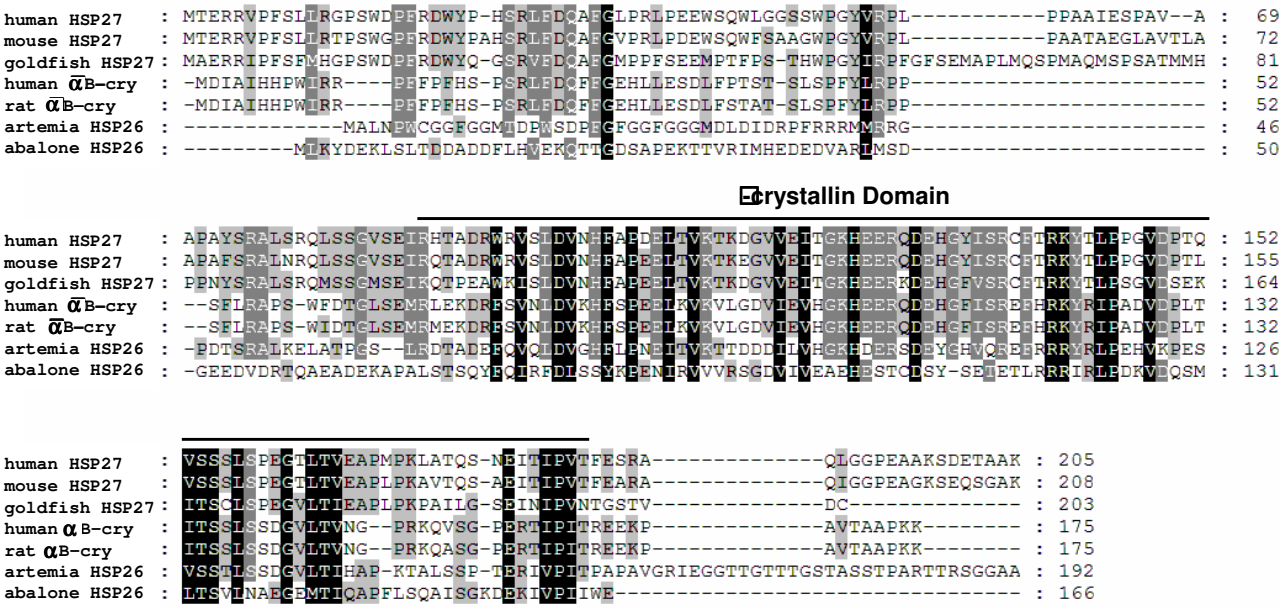

Fig. 2: Amino acid alignment of the abalone sHSP26 with known sHSP sequences. Location of the α-crystallin domain is indicated above the sequence. The origin and accession number (DDBJ, EMBL and GenBank) for each of the retrieved sHSP sequences in this study are as follows:
Human HSP27, NM_001540.2; mouse HSP27, NM_013560.1; human αB-crystallin, NM_001885.1; rat αB-crystallin, NM_012935.2; goldfish HSP27, AB239443.1; artemia HSP26, DQ310577.1; abalone HSP26, EF472916

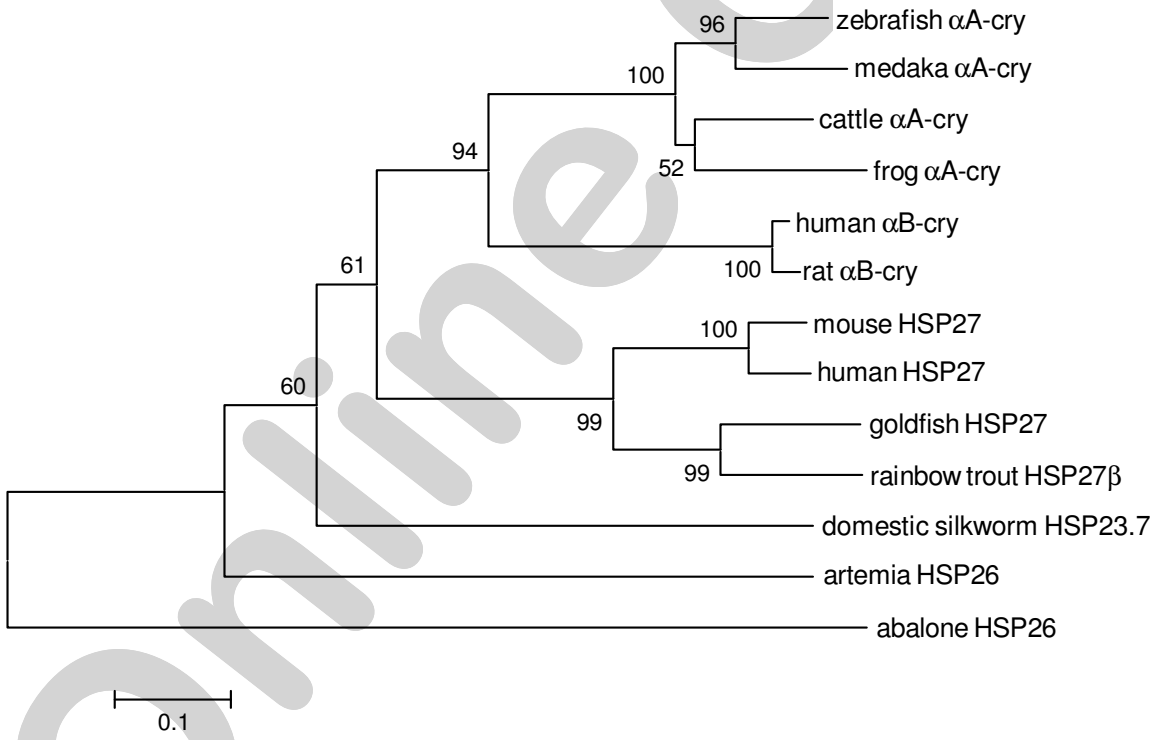

Fig. 3: Phylogenetic relationship based on the deduced abalone sHSP26 amino acid sequences of various animals. A phylogenetic tree of the aligned sequences was constructed using the Neighbor-Joining algorithm within MEGA (version 3.0). The degree of confidence for each branch point was determined by bootstrap analysis (1000 repetitions). The origins and accession numbers of the sHSP amino acid sequences are:
Human HSP27, NM_001540.2; mouse HSP27, NM_013560.1; human αB-crystallin, NM_001885.1; rat αB-crystallin, NM_012935.2; frog αA-cry D88185.1; domestic silkworm HSP23.7, NM_001043477.1; zebrafish αA-crystallin, NM_152950.2; cattle αA-crystallin, NM_174289.2; rainbow trout HSP27β, EF067864.1; medaka αA-crystallin, AJ000940.1; goldfish HSP27, AB239443.1; artemia HSP26, DQ310577.1; abalone HSP26, EF47291

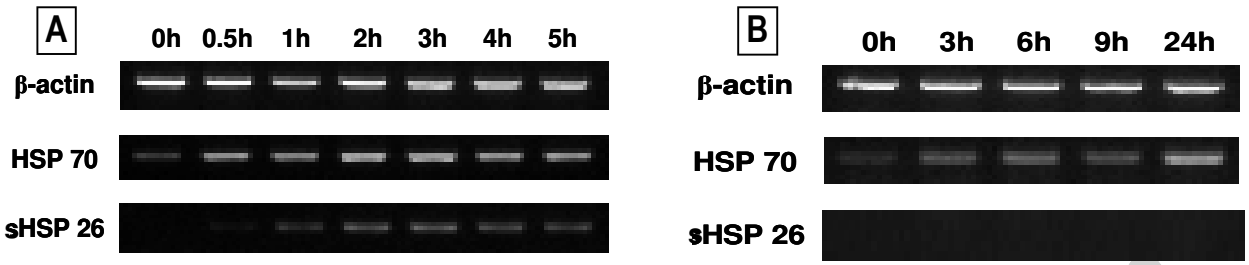

Fig. 4: Expression analysis of the abalone sHSP26 mRNA after exposed to heat-shock (28°C) or cold shock (4°C). A 1μg of total RNA from heat shock treated abalone for different time was used for cDNA synthesis. The β-actin was used as a control to ensure that equal quantities of total RNA were used in the RT-PCR

al., 2004) and HSP 70 gene of lower vertebrate (Molina *et al*., 1993). Intron splicing is normally required for translocation of most mRNAs from the nucleus to the cytoplasm, however, RNA-splicing was inhibited by stress was (Yost and Lindquist, 1986, 1991; Bond, 1988). It was suggested that the absence of introns in stress-induced genes allows to compensate for an inhibition of RNA-splicing thereby enabling preferential expression of these proteins during cellular stress, the nuclear export signal being probably provided by the mRNA secondary or tertiary structure (Huang *et al*., 1999).

Multiple alignment with other species' sHSP reveals to be conserved highly α-crystallin domain (Fig. 2). The α-crystallin domain mediates formation of dimmers, fundamental units of oligomerization for many sHSPs (Haslbeck *et al*., 2005b; Sun and MacRae, 2005). The sHSP26 showed overall identity of 27-31% with the other sHSP in amino acid level. The phylogenetic tree shows that the abalone sHSP 26 forms a single cluster, which is part of a larger group that contains sHSP genes from various vertebrate and invertebrate species (Fig. 3).

RT-PCR analyses were performed to determine the expression pattern of sHSP26 during heat shock or cold shock. We also compared the expression of sHSP26 with that of HSP70 (GeneBank accession no. DQ324856) in heat shock or cold shock treated abalone. The sHSP26 mRNA expression showed an increase after 1 hr of exposure to heat shock, then a decrease was observed from 4 hr. The HSP70 expression was increased significantly after exposure to heat shock (Fig. 4A). Interestingly, the expression sHSP26 was not induced by cold shock even that of HSP70 was strongly induced (Fig. 4B). These results indicate that the abalone sHSP26 exhibits strongly heat-shock specific expression.

In conclusion, this present study has identified and characterized the abalone sHSP26 gene. The deduced amino acid sequence of abalone sHSP26 contains the α-crystallin domain which is conserved in all sHSP of other species. It is considered that the intronless gene structure of abalone sHSP26 is necessary for more effective expression by heat-stress. The results of thermal stress experiments suggest that the abalone sHSP26 specifically response to only heat-stress not cold-stress.

Acknowledgments

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