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Alteration of *Phascolosoma esculenta* heat shock protein 90 expression under heavy metal exposure and thermal stress

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ABSTRACT. The full-length complementary DNA (cDNA) of heat shock protein 90 was cloned from *Phascolosoma esculenta* (PeHSP90) using expressed sequence tag and rapid amplification of cDNA end approaches. The cDNA of PeHSP90 was 2521 bp including a 5'-untranslated region of 110 bp, a 3'-untranslated region of 230 bp, and an open reading frame of 2181 bp. All of the characteristic motifs of the HSP90 family were completely conserved in the deduced amino acid of PeHSP90. The expression of PeHSP90 was induced by 3 heavy metals or elevated temperature, under which Zn^{2+} displayed effects were more toxic than those of Cd^{2+} and Cu^{2+}. The polyclonal antibodies generated from the recombinant product of PeHSP90 were specifically identified not only in the recombinant product but also in the native protein from hemocytes. These results strongly suggested that PeHSP90 was involved in heavy metal challenge and thermal stress regulation in *P. esculenta*.

Key words: *Phascolosoma esculenta*; Heat shock protein 90; Heavy metal; Gene expression; Western blot

INTRODUCTION

The exposure of cells to a multitude of stressors in marine environments such as heat, heavy metals, toxins, and anoxia can trigger a set of gene expressions in which phylogenetically conserved proteins known as heat shock protein (HSPs) are predominant. HSPs are molecular chaperones that protect proteomes by recognizing denatured proteins and helping them to enter the repair/folding pathway or undergo proteolysis (de Pomerai, 1996). HSPs can be divided into 4 categories according to molecular mass and function: 1) HSP90 (83-99 kDa), 2) HSP70 (68-80 kDa), 3) HSP60, and 4) a family of small HSPs (15-40 kDa) (Lindquist and Craig, 1998; Mizrahi et al., 2010).

HSP90 is an abundant, highly conserved molecular chaperone compared to its counterparts. It represents 1-2% of cellular proteins in most tissues, even in the absence of stress (Gao et al., 2007). The role of HSP90 in the maintenance of key proteins has been well characterized (Farcy et al., 2007). HSP90 can also be regulated by a range of stressors including heat or cold shock (Palmisano et al., 2000), hyperosmotic stress (Pan et al., 2000), food deprivation (Cara et al., 2005), and heavy metals (Somji et al., 2002; Papaconstantinou et al., 2003; Warchalowska-Sliwa et al., 2005).

Immune and stress responses in aquatic organisms are becoming an important field of research. The effects of heavy metals on immune responses have been reported in many species (Ali et al., 1996; Snyder et al., 2001; Singer et al., 2005; Funes et al., 2006). HSP90 has attracted little attention compared with the focus on its counterparts HSP70 and small HSPs. *Phascolosoma esculenta* is an economically valuable species rich in proteins and a variety of amino acids and trace elements. The effects of environmental conditions such as temperature and water quality on the growth and health of *P. esculenta* must be elucidated to achieve maximal economic benefits. The purposes of this study were 1) to clone the full-length complementary DNA (cDNA) of *P. esculenta* HSP90 from a cDNA library (designated PeHSP90), 2) to investigate the expression of PeHSP90 messenger RNA (mRNA) after heavy metal exposure and thermal stress, and 3) to analyze the native HSP90 in hemocytes using Western blot with a specific antibody for PeHSP90.

MATERIAL AND METHODS

Animals and heavy metal exposure

P. esculenta (3-4 cm long) was collected from Fenghua, Zhejiang Province, China, and maintained in the laboratory for 2 weeks at 20° ± 1°C before processing. The water exchange rate was 100% per day during the entire experimental period. For the heavy metal challenge experiment, 20 *P. esculenta* cultured in seawater were selected as controls. Sixty *P. esculenta* were divided into 3 tanks to which were added 0.5 M stock solutions of $CuCl_2$, $CdCl_2$, and $ZnCl_2$ with final concentrations of 10 mM. After 6, 12, 24, and 48 h of exposure, hemocytes were collected from the control and treated groups for RNA extraction and cDNA synthesis. Three replicates were performed for each time point.

Cloning the full-length cDNA of HSP90 from *P. esculenta*

Two sets of degenerate primers (P1: 5'-ATGATYGGGDCARTTYGGYGT-3' and P2:

5'-CYTTCATGATNCKYTCCAT-3'; P3: 5'-GARCARTAYDTSTGGGARTC-3' and P4: 5'-TCRCARTTSTCCATRATRAA-3') were designed based on the conserved sequences of the HSP90 protein family. RNA extraction, cDNA synthesis, polymerase chain reaction (PCR) amplification, and PCR product sequencing were performed according to a procedure described by Wang et al. (2010). Two gene-specific primers - P5: 5'-TGCCTGGTCCTCTTTGATGT-3' and P6: 5'-GAGCACCATTTGACATGTTTG-3' - were synthesized to generate the 5'- and 3'-ends of PeHSP90, respectively.

Sequence analysis of PeHSP90

The PeHSP90 gene sequence was analyzed using the Basic Local Alignment Search Tool algorithm at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast). The conserved motifs and common characteristics of the deduced amino acid sequence of PeHSP90 were analyzed with the online software Expert Protein Analysis System (http://www.expasy.org/). The similarity percentage of the deduced amino acid sequences of PeHSP90 and other HSP90s were calculated using the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html).

Spatial course expression level of PeHSP90 exposure to heavy metals

The mRNA expression of PeHSP90 in hemocytes was measured using quantitative real-time PCR in a Rotor-Gene™ 6000 real-time PCR detection system (Corbett, Cambridge, UK). Two PeHSP90-specific primers - P7: 5'-ACCAAGCCACTATGGACCCG-3' and P8: 5'-AGAAATGCTTGACAGCCAGG-3' - were designed to amplify a product of 112 bp. For normalization of PeHSP90 transcript expression, glyceraldehyde 3-phosphate dehydrogenase was also amplified with primers P9 (5'-CCAGAACATCATCCCAGCA-3') and P10 (5'-ACGAACAGGGACACGGAAG-3') to serve as an internal control. RNA extraction, cDNA synthesis, PCR components, and the amplification conditions matched those described in our previous study (Wang et al., 2010). All data are given in terms of relative mRNA reported as means ± standard deviation.

PeHSP90 expression profiles after temperature elevation

For the temperature challenge experiment, 40 individuals were randomly divided into 2 tanks containing 2 L seawater at 20°C and acclimatized for 1 week. After the acclimation period, the water temperature of one basin was elevated to 37°C and the other stayed at 20°C. Temperature treatment was performed for 6, 12, 24, and 48 h. Hemolymphs were collected individually from the control and treated groups using a syringe and centrifuged at 2000 *g* at 4°C for 10 min to harvest hemocytes. Four replicates were performed for each treatment.

Western blot analysis of recombinant PeHSP90 and native protein in hemocytes

The mature peptide of thePeHSP90 was generated with a pET-28a *in vitro* expression system, and the recombinant product was purified to be homologous using HisTrap Chelating

Columns (Amersham Biosciences). The purified rPeHSP90 was excised from the gel, ground into small pieces, and dissolved in PBS for antibody preparation. The protocols for antibody preparation and Western blot were consistent with those in one of our previous studies (Jin et al., 2011).

RESULTS

Sequence analysis of PeHSP90

The complete sequence of PeHSP90 cDNA contained a 5'-untranslated region of 110 bp and a 3'-untranslated region of 230 bp with a polyA tail. A canonical polyadenylation signal sequence AATAAA was replaced by AATATA in PeHSP90 (italics in Figure 1). An open reading frame of 2181 bp encoded a polypeptide of 726 amino acids residues with a predicted molecular mass of 83.62 kDa. A characterized motif (MEEVD) for cytosolic HSP members was also identified at the C-terminus of PeHSP90 (underlined in Figure 1) (Gupta, 1995). No signal peptide was identified from the deduced amino acid sequence of PeHSP90, which further demonstrated that PeHSP90 is a nonsecreted protein in cells. The characteristic conserved histidine kinase-like ATPases and HSP90 domains were found at 38 to 192 amino acids and 194 to 726 amino acids in the deduced amino acid of PeHSP90 through Simple Modular Architecture Research Tool analysis. The 5 HSP90 protein family signatures-NKEIFLRELISNSSDALDKIR, LGTIAKSGT, IGQFGVGFYSA YLVAD, IKLYVRRVFI, and GVVDSEDLPLNISRE (Gupta, 1995)-were completely conserved in the deduced amino acid sequence of PeHSP90 (shadowed in Figure 1). The sequence was deposited in GenBank accession No. GQ503177.

Homology analysis of PeHSP90

The deduced amino acid sequence of PeHSP90 was highly similar to that of HSP90s in invertebrates and vertebrates (more than 77% similarity in all matches). This similarity was also observed in the HSP90 of *Danio rerio* (77%), *Eriocheir sinensis* (81%), *Haliotis asinina* (83%), *Mytilus galloprovincialis* (84%), *Portunus trituberculatus* (78%), and *Sesamia nonagrioides* (79%).

mRNA expression of PeHSP90 after heavy metal exposure

The temporal expression profiles of PeHSP90 after heavy metal exposure are shown in Figure 2. In the Zn^{2+} treatment groups, the expression level of PeHSP90 increased during the 1st 12 h and reached peak expression with an approximately 20.0-fold increase compared to that in the control group ($P < 0.01$). As time progressed, the expression of PeHSP90 decreased significantly and returned almost to the original level at 48 h. A similar expression pattern was detected in the Cd^{2+} challenge groups. The maximal expression level was also detected at 12 h with an 8.0-fold increase ($P < 0.05$). In the Cu^{2+} groups, HSP90 mRNA expression was upregulated with a 2.2-fold increase in the 1st 6 h. Then the expression of PeHSP90 was sharply downregulated to its minimum at 48 h with only a 0.22-fold increase compared to that in the control group ($P < 0.01$).

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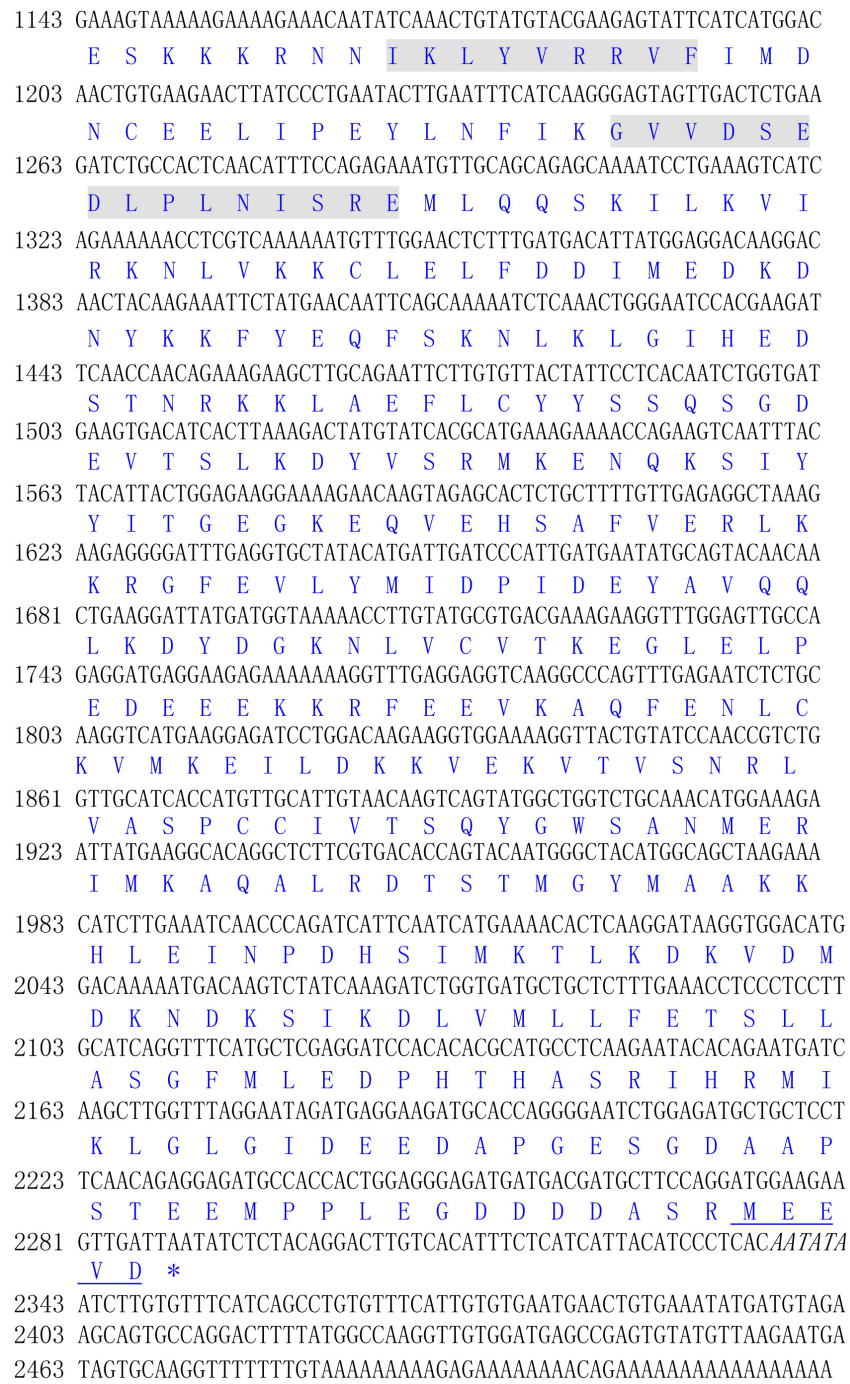
1   AAGGCAGCGTTTGTTGACCGCGCCAAGAACATTCGGCGTGTAACCTGCGACACTCGTGGAAC
63  CATTGCACTTGACTGAAAAAAGAAAGATGCTGAAATGCTGCAAAAAAAATGCCACCAATG
                                                    M  P  P  M
123 CCAGATTTGGAACAAATGGAGGACTCGGAGGTGGAAACCTTTGCCTTCCAGGCAGAAATT
      P  D  L  E  Q  M  E  D  S  E  V  E  T  F  A  F  Q  A  E  I
183 GCCCAGCTGATGAGTTTGATTATCAACACTTTCTACTCAAATAAAGAAATTTTCTTGAGA
      A  Q  L  M  S  L  I  I  N  T  F  Y  S  N  K  E  I  F  L  R
243 GAGCTGATCTCCAACTCATCTGATGCATTGGACAAGATCAGGTATGAGTCCCTGACCGAT
      E  L  I  S  N  S  S  D  A  L  D  K  I  R  Y  E  S  L  T  D
303 CCATCAAAGTTGGACAGTGGGAAGGATTTGCAGATCCGAATCATTCCTGACAAAAGCAGC
      P  S  K  L  D  S  G  K  D  L  Q  I  R  I  I  P  D  K  S  S
363 AACACCCTGACCATTATTGATACTGGTATTGGTATGACCAAAGCAGACCTGGTAAACAAC
      N  T  L  T  I  I  D  T  G  I  G  M  T  K  A  D  L  V  N  N
423 CTTGGAACCATTGCCAAATCGGGAACCAAAGCTTTCATGGAGGCACTCCAGGCCGGTGCT
      L  G  T  I  A  K  S  G  T  K  A  F  M  E  A  L  Q  A  G  A
483 GATATCTCTATGATTGGACAATTTGGTGTCGGCTTCTACTCTGCCTACCTGGTCGCAGAC
      D  I  S  M  I  G  Q  F  G  V  G  F  Y  S  A  Y  L  V  A  D
543 AAGGTTACAGTAACTTCCAAACACAATGATGATGAGCAATACACATGGGAATCCTCTGCG
      K  V  T  V  T  S  K  H  N  D  D  E  Q  Y  T  W  E  S  S  A
603 GGTGGTTCTTTTACTGTGAGACCTGATACTGGAGAGTCCCTTGGTCGTGGTACCAGAATT
      G  G  S  F  T  V  R  P  D  T  G  E  S  L  G  R  G  T  R  I
663 GTGCTCTACATCAAAGAGGACCAGGCAGAATACCTTGAGGAAAGGCGCATCAAAGAGGTG
    V  L  Y  I  K  E  D  Q  A  E  Y  L  E  E  R  R  I  K  E  V
723 GTAAAAAAACACTCACAGTTCATTGGCTACCCAATCAAGCTGCTAGTAGAGAAGGAACGT
      V  K  K  H  S  Q  F  I  G  Y  P  I  K  L  L  V  E  K  E  R
783 GATAAGGAGATTGAGGATGATGAAGAAGAAGAAGAAAAGAAAGAAGAGGGGGAAAAAGAC
      D  K  E  I  E  D  D  E  E  E  E  E  K  K  E  E  G  E  K  D
843 AAGGAAGACAAGCCAAAGATTGAGGATCTTGATGAAGATGATGAAGATGAGGACAAGGAC
      K  E  D  K  P  K  I  E  D  L  D  E  D  D  E  D  E  D  K  D
903 AAAGGCAAGAAAAAGAAAAAGATCAAGGAAAAATACACAGAAGACGAAGAGTTAAACAAA
      K  G  K  K  K  K  I  K  E  K  Y  T  E  D  E  E  L  N  K
963 ACCAAGCCACTATGGACCCGCAATCCAGATGACATCTCGACAGAGGAATATGGAGAATTC
      T  K  P  L  W  T  R  N  P  D  D  I  S  T  E  E  Y  G  E  F
1023 TATAAGTCACTTACAAATGATTGGGAGGACCACCTGGCTGTCAAGCATTTCTCAGTTGAA
       Y  K  S  L  T  N  D  W  E  D  H  L  A  V  K  H  F  S  V  E
1083 GGACAGCTTGAGTTCCGCGCCCTGCTGTTTGTTCCAAAAAGAGCACCATTTGACATGTTT
       G  Q  L  E  F  R  A  L  L  F  V  P  K  R  A  P  F  D  M  F
```

Figure 1. Complete cDNA sequence of HSP90 from *Phascolosoma esculenta* and its deduced amino acid sequence. Nucleotides were numbered from the first base at the 5'-end. Asterisk indicates the stop codon. The potential polyadenylation signal is in italic. Shadowed regions indicate five HSP90 family signature sequence. The consensus sequence MEEVD is underlined.

Continued on next page

Figure 1. Continued.



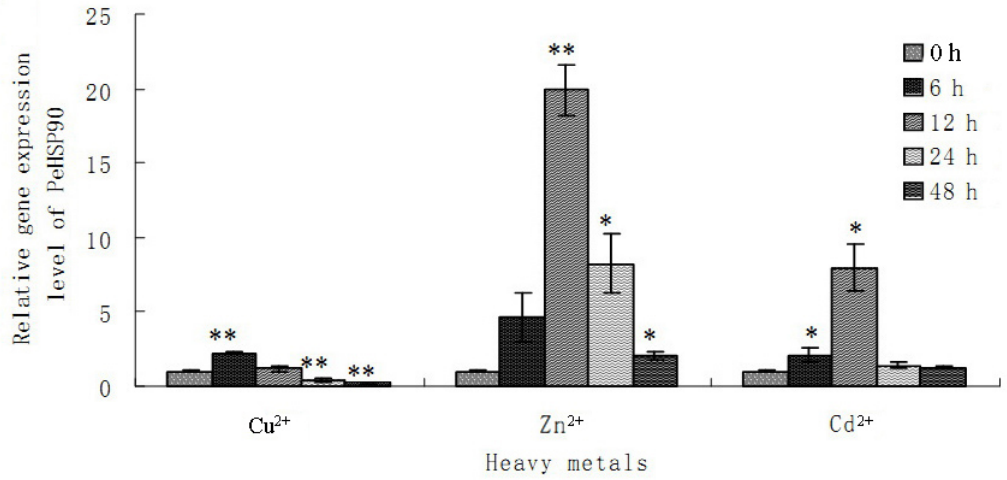


Figure 2. Time-course expression level of PeHSP90 transcripts in hemocytes after thermal stress measured by quantitative real-time PCR at 0, 6, 12, 24, and 48 h. Each symbol and vertical bar represent means ± SD (N = 3). *P < 0.05 and **P < 0.01 for significant differences compared with the control group.

mRNA expression of PeHSP90 after thermal stress

The effects of elevated temperature on the mRNA expression of PeHSP90 are shown in Figure 3. The expression level of PeHSP90 gradually increased during the 1st 12 h, and the maximal expression point was detected at 12 h with a 7.0-fold increase compared to that in the control group (P < 0.01). After that period, a sharply decreased expression profile was identified at 24 h, and expression dropped nearly to its original level at 48 h.

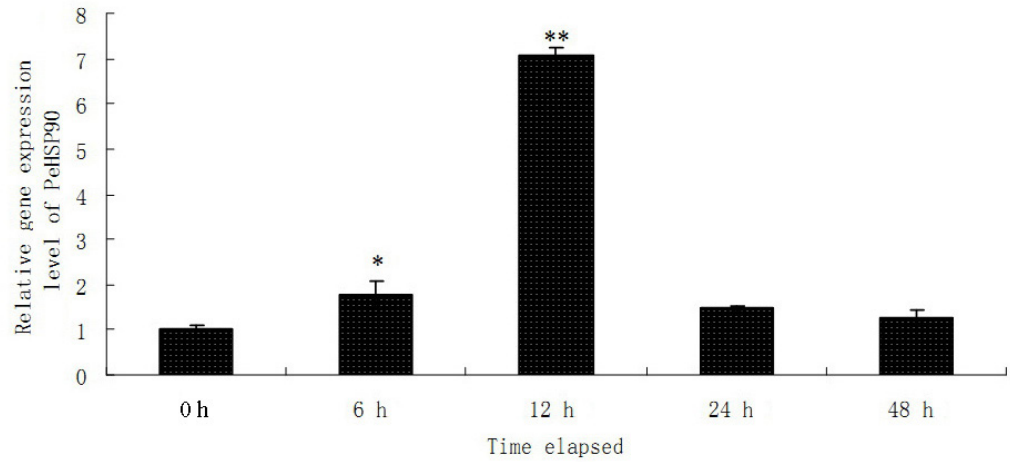


Figure 3. Time-course expression level of PeHSP90 transcript in hemocytes after exposure to three heavy metals measured by quantitative real-time PCR at 0, 6, 12, 24, and 48 h. Each symbol and vertical bar represent means ± SD (N = 3). *P < 0.05 and **P < 0.01 for significant differences compared with the control group.

Western blot

A unique band with an apparent molecular mass of 90 kDa was detected in the positive transformant. This mass was consistent with the predicted molecular weight for recombinant proteins of PeHSP90. Using a polyclonal antibody generated from purified recombinant PeHSP90-immunized mice, we analyzed the native PeHSP90 from hemocytes and the recombinant PeHSP90 by Western blot (Figure 4). The results showed that a specific band (around 90 kDa) appeared in the samples of recombinant PeHSP90 and whole hemocytes lysate of *P. esculenta*. However, this band was not detected in non-induced *Escherichia coil* BL21 or induced *E. coil* BL21 with an empty vector (see Figure 4).

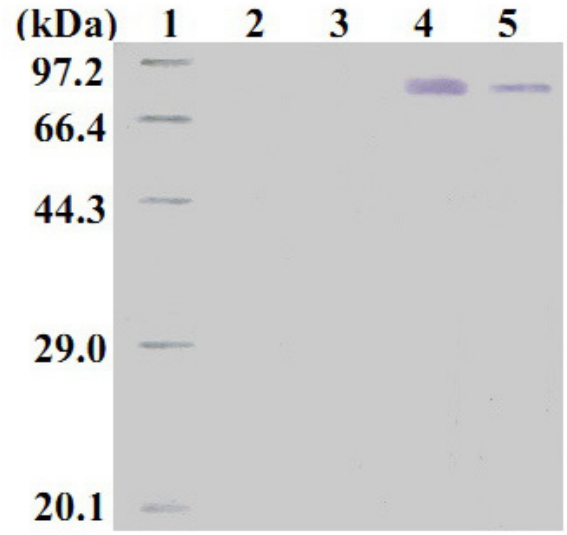


Figure 4. Specificity of rPeHSP90 polyclonal antibody determined by Western blot. *Lane 1* = Protein molecular standard; *lanes 2*, *3* = negative control for rPeHSP90; *lane 4* = rPeHSP90; *lane 5* = total protein extraction from hemocytes of *Phascolosoma esculenta*.

DISCUSSION

The biology of marine organisms is constantly influenced by changing environmental conditions. HSPs buffer this environmental variation by minimizing the biochemical, physiological, and histological alteration of the host and are therefore important factors in the maintenance of homeostasis across environmental regimes (Sørensen et al., 2003). In recent years, the expression of HSPs under a range of contaminants, including heavy metals (Li et al., 2010), polycyclic aromatic hydrocarbons (Song et al., 2006), and pesticides (Singer et al., 2005) has been intensively investigated. Among the involved HSPs, much attention has been paid to HSP70, but HSP90 has not been extensively studied (Gao et al., 2007).

In this study, the complete cDNA sequence of PeHSP90 was determined. The full-length cDNA of PeHSP90 is 2521 bp, including an open reading frame of 2181 bp that encodes a polypeptide of 726 amino acids without a signal peptide that shares functional char-

acteristics similar to those described in other species. Conserved sequences and characteristic motifs, such as HSP90 family signatures, ATP and geldanamycin binding domains, and the major structural and functional domains typically found in HSP90, were all present in the deduced amino acid sequences of PeHSP90. The MEEVD sequence at the end of PeHSP90 was strictly conserved and shared with the other members of the HSP90 family. Searching for sequence similarities revealed that the deduced amino acid sequence of PeHSP90 shared high identity and similarity with other known HSP90s (more than 77% similarity in all matches). The conserved motifs and higher sequence similarity to other known HSP90 sequences together suggest that PeHSP90 is a member of the HSP90 family.

Growing evidence suggests that environmental contaminants such as Cd^{2+}, Zn^{2+}, and Cu^{2+} induce several HSPs at the transcriptional level (Efremova et al., 2002; Andrew et al., 2003). In scallop, HSP90 responds to various heavy metal stresses (Cd^{2+}, Cu^{2+}, Pb^{2+}) with dose- and exposure time-dependent expression patterns (Gao et al., 2007). An increased expression profile of HSP90 has also been observed in Pacific abalone after feeding with dietary selenium (Zhang et al., 2011). In our study, although all 3 tested metal ions upregulated the expression of PeHSP90 to some extent, distinct effect patterns occurred with respect to each metal. At the same concentration of the 3 metals, the PeHSP90 gene expression in response to Zn^{2+} was approximately 2.5 times higher than that to Cd^{2+} and 17 times than that to Cu^{2+}. In another words, the same induction intensity of gene expression corresponded to higher concentrations of Cd^{2+} and Cu^{2+} or longer exposure times than those of Zn^{2+}, which suggested that Zn^{2+} could be more toxic than Cd^{2+} and Cu^{2+} for *P. esculenta*.

Temperature change is a permanent event in the marine environment. The common molecular response to temperature elevation includes a dramatic change gene in expression patterns and elevated synthesis of HSPs to eliminate denatured proteins and assist in establishing proper protein conformations. In the present study, mRNA levels of PeHSP90 increased greatly and reached a maximal level during the 1st 12 h, with a 7.1-fold increase compared to that in the control group ($P < 0.01$). After the peak, a decreased expression profile was detected, and levels dropped to control levels by the end of the experimental period.

These results suggest that the expression profile of the PeHSP90 transcript in circulating hemocytes was probably positively correlated to the change in PeHSP90-producing hemocytes. In shrimp, heavy metal exposure decreases hemocyte count during the 1st 8 h, and the greatest decrease in hemocyte numbers (hemocytopenia) is induced by Pb, followed in descending order by Zn, Hg, Cr, Cu, and Cd (Lorenzon et al., 2001). Future research should be conducted to determine hemocyte count at various time points under heavy metal or thermal challenge. These rapid and reversible inductions of PeHSP90 in response to heat shock-like abalone HSP90 also demonstrate its innate advantage under thermal stress (Wang et al., 2011).

Although sequence and expression profile analyses indicated that PeHSP90 belonged to the HSP90 family, the identification and validation of the existence of native HSP90 in *P. esculenta* are also important. Therefore, the recombinant PeHSP90 was produced *in vitro* to generate specific polyclonal antibodies for Western blot analysis. The antibodies were specifically detected in recombinant PeHSP90 and native HSP90 from *P. esculenta* hemocytes (see Figure 4), further informing us that PeHSP90 was a novel HSP90 member in *P. esculenta*. The polyclonal antibodies generated from the study will be of considerable help in the investigation of the biological functions of PeHSP90 at the protein level in our future studies.

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