Full Length Research Paper

cDNA cloning and mRNA expression of heat shock protein 70 gene in blood clam *Tegillarca granosa* against heavy metals challenge

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In this study, the full-length heat shock protein 70 of *Tegillarca granosa* was cloned from cDNA library by rapid amplification of cDNA end (RACE). The open reading frame (ORF) of heat shock protein 70 was 1968 bp, and it encoded a protein of 655 amino acids with a predicted molecular weight of 71.48 kDa and an isoelectric point of 5.25. Basic local alignment search tool (BLAST) analysis showed that the heat shock protein 70 of *T. granosa* shared high similarity with other species, supporting that it is a new member of heat shock protein family. Western blot analysis revealed that the generated polyclonal antibodies could specially detect native protein from whole cell lysate of *T. granosa*. The spatial distribution confirmed that the heat shock protein 70 was abundant in visceral mass, gill and haemocytes, and weakly in foot, mantle and adductor. Heavy metal pollutes such as lead (Pb^{2+}), cadmium (Cd^{2+}) and copper (Cu^{2+}) could induce the gene expression in similar manners by quantitative real-time polymerase chain reaction (PCR). The present results indicate that heat shock protein 70 of *T. granosa* may be involved in environmental pollution challenges and should be considered as one of *T. granosa* promising molecular marker candidates.

Key words: *Tegillarca granosa*, heat shock protein 70, heavy metals, quantitative real-time polymerase chain reaction (PCR).

INTRODUCTION

Heavy metal pollution is one of the environmental stresses that influence living organisms. When the intracellular level of metal ions rises above certain thresholds, the expression of a series of intracellular proteins and the vital processes of these organisms are affected, which can eventually lead to cell death (Eide, 2006). Heat shock proteins (HSPs) are present in both prokaryotic and eukaryotic cells. They are a family of highly conserved stress response proteins that are ubiquitously expressed at low levels under normal physiological conditions (Kiang and Tsokos, 1998; Golli-Bennour and Bacha, 2011). HSPs are designated according to their molecular weight. Several major families are identified, including small heat shock proteins, HSP60s, HSP70s, HSP90s and HSP100s (Franzellitti and Fabbri, 2005). HSP70s are abundant and well-conserved stress inducible proteins that can promote the survival of various organisms under multiple environmental stresses (Padmini and Rani, 2008). They act as activators of innate immunity and play essential roles in *de novo* protein folding, membrane translocation, degradation of misfolded proteins and other regulatory processes (Molina et al., 2000). Moreover, since HSP70 responds to minor environmental perturbations, it has been proposed as a molecular indicator of adverse biological effects (Yoshimia et al., 2009).

The blood clam *Tegillarca granosa* (Linnaeus, 1758) is

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Abbreviations: ORF, Open reading frame; BLAST, basic local alignment search tool; IPTG, isopropyl β-D-1-thiogalactopyranoside; HSP, Heat shock proteins.

widely distributed along the West Pacific, Indian and Atlantic coasts. It is an economical shellfish in Shandong, Zhejiang, Fujian and Guangdong provinces of China. With the expansion of intensive culture of *T. granosa*, pathogens and environmental issues have emerged and resulted in a dramatic decrease in its production (Jin et al., 2011). Environmental stressors such as heavy metals cause the decrease of mitochondrial membrane potential, denaturation of enzymes, generation of reactive oxygen species (ROS) and death (Pourahmad and O'Brien, 2000; Droge, 2002). So, the detoxification of heavy metals for the aquatic organisms is crucial, and HSP70 may act as a key role in the detoxification of heavy metals (Li et al., 2008).

In mollusc, several cDNAs encoding HSP70 have been reported, such as *hsp70s* from bay scallop, *Argopecten irradians* (GenBank accession number AY485261) (Song et al., 2006); oyster, *Crassostrea gigas* (GenBank accession number AB122063) (Boutet et al., 2003); Pacific abalone *Haliotis discus hannai* (GenBank accession number DQ324856) (Cheng et al., 2007) and hard clam, *Meretrix meretrix* (GenBank accession number HQ256748) (Yue et al., 2011). However, the molecular feature and function of HSP70 from *T. granosa* have not been intensively investigated. Hence, this study was aimed at confirming the expression of *hsp70* in response to environmental stressors. The transcription levels of *T. granosa hsp70* (*Tghsp70*) in response to plumbum, cadmium, copper and thermal challenges were analyzed. We also cloned and characterized the HSP70 gene from *T. granosa*. Meanwhile, a polyclonal antibody of *Tghsp70* was generated in mice for future examination. This work also implies that *hsp70* may be selected as a biomarker for the stress level testing.

MATERIALS AND METHODS

Animals and challenge experiments

T. granosa were collected from Ningbo, Zhejiang province, China, and reared for a week at 20 ± 2°C before processing. For heavy metal challenge experiment, 50 blood clams cultured in seawater were used as the control group. Three groups of 50 blood clams were each placed in a tank and treated with 10 μmol/L $CuCl_2$, 10 μmol/L $CdCl_2$ and 10 μmol/L $PbCl_2$, respectively. After 6, 12, 24 and 48 h of exposure, the haemocytes and visceral mass were collected immediately from the control and the treated groups and frozen in liquid nitrogen for RNA extraction and cDNA synthesis. There were three replicates (30 samples mixed as one replicate) for each time point.

For temperature challenge experiment, another three groups were exposed for 12 h at 4, 37°C and room temperature (20 ± 2°C), respectively. Haemocytes and visceral mass were collected and cDNA synthesis was performed following the same procedure.

Cloning of the full-length cDNA of *Tghsp70*

According to the consensus sequence of HSP70, degenerate primers P1, P2, P3 and P4 were designed and used to amplify partial *Tghsp70* cDNA from a SMART cDNA library, which was constructed using a nested polymerase chain reaction (PCR) strategy. Specific

primers P5 and P6 were designed based on the fragment earlier obtained. The 3' end of *Tghsp70* was cloned using sense primer P5 and reverse primer M13-47, while the 5' end of *Tghsp70* was cloned using sense primer M13Rv-M and reverse primer P6. The PCR products were cloned into pMD18-T simple vector (TaKaRa) and sequenced bi-directionally with primers M13-47 and RV-M. The sequencing results were verified and subjected to cluster analysis.

Sequence analysis of *Tghsp70*

The *Tghsp70* gene and deduced amino acid sequences were both analyzed using the BLAST algorithm at NCBI website (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was further analyzed with the Expert Protein Analysis System. The signal sequence of *Tghsp70* was predicted using SignalP 3.0. Multiple alignment of *Tghsp70* was performed with MegAilgn in DNAStar.

Phylogenetic analysis

The deduced amino acid sequences of TgHSP70 were used for phylogenic analysis. A neighbor-joining tree was constructed with Mega 3.1 software package (http://www.Megaso ftware.net/) (Kumar et al., 2004) and ClustalX (1.81). To derive the confidence value for phylogeny analysis, bootstrap trials were replicated 1,000 times.

Tissue-specific expression of *Tghsp70* mRNA

Quantitative real-time PCR (qRT-PCR) was performed to measure the *Tghsp70* mRNA expression in different tissues with the Rotor-Gene TM 6000 real-time PCR detection system (QIAGEN). Two specific primers P7 and P8 were designed to amplify a fragment of *Tghsp70*. The β-actin gene of *T. granosa* was amplified as an internal control using P9 and P10. Total RNA was isolated from foot, mantle, haemocytes, visceral mass, adductor and gill. The first-strand cDNA was synthesized using the M-MLV reverse transcription system (Promega). A 1:50 dilution of the cDNA mix was used as the template. The amplification volume was 20 μL, containing 10 μL 2× SYBR Premix Ex Taq II (Takara), 5 μL template, 1 μL primers each (10 mmol/L) and 3 μL PCR-grade water. The qRT-PCR parameter was denatured at 95°C for 1 min, followed by 40 cycles of 95°C for 5 s, 53°C for 20 s, and 72°C for 20 s. Melting analysis of the amplified products was used to confirm the specificity of PCR amplification. The ΔΔCt method was used to calculate the expression level of *Tghsp70*. There were three replicates for each tissue.

In vivo exposures to heavy metals and temperature challenges

Haemocytes and visceral mass were selected to investigate the temporal expression of *Tghsp70* transcripts after heavy metal exposure and temperature treatment using qRT-PCR. The amplification and calculation methods were the same as the measurement of tissue-specific expression of *Tghsp70*.

Expression of TgHSP70 recombinant protein

Specific primers P11 and P12 were designed to amplify the open reading frame (ORF) of *Tghsp70* with *Bam*H I and *Sal* I sites at the two ends. The PCR product was cloned into the *Bam*H I/*Sal* I-digested pET-28a (+) vector (Novagen) and named pET-HSP70. The correct insertion direction was confirmed by enzyme digestion and sequencing analysis. For recombinant protein expression, plasmid

Table 1. Primers used in this study.

Name	Length	Sequence (5' - 3')
P1	20	AGYTATGTNGCNTTCACAGA
P2	20	YTGTCRTTDGTRATKGTRAT
P3	20	ACAGTHCCWGCHTAYTTYAA
P4	20	TTRTCRGARTADGTKGTRAA
P5	19	CACAACTGTCCCAACCAAA
P6	18	CCGCCATCATCAATAACT
P7	26	CGGGATCCATGAATCCAACAAACACC
P8	28	ACGCGTCGACTTAATCTACTTCTTCGAT
P9	21	ATTTGAACTGACTGGTATCCC
P10	21	TCATCTTCTGCCTTGTATTTT
P11	18	CCGCTTCTTCATCCTCAT
P12	19	GTGTTGGCGTACAAGTCCT

pET-HSP70 was transformed into *E. coli* BL21 (DE3) (Novagen) and cultured at 37°C in Luria broth (LB) agar containing 50 mg/L kanamycin. The culture was induced with 1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG) at an OD_{600} of 0.6 to 0.8, and incubated for an additional 1, 2, 3, 4 and 5 h under the same conditions. Protein samples were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot (Su et al., 2009)

Preparation of polyclonal antibody

The purified recombinant TgHSP70 (rTgHSP70) was mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich) and injected into five Balb/c mice (Zhejiang Academy of Medical Sciences). The mice were immunized with additional three injections of purified rTgHSP70 with an equal volume of Freund's incomplete adjuvant at 1-week intervals after the first injection. Blood was collected three days after the last injection and centrifuged at 10,000 g for 10 min before incubation at 4°C overnight. The supernatant antibodies were stored at -20°C. At the same time, the negative control group was immunized with phosphate buffered saline (PBS) as antigen using the same method.

Western blot analysis

The total protein extract from *E. coli* BL21 were boiled for 10 min with loading dye and separated by SDS-PAGE. The proteins were then blotted onto a sheet of nitrocellulose membrane by electrophoresis at 20 V for 14 h. After blocking with 5% skim milk in TBST (10 mmol/L Tris-HCl pH 7.5, 100 mmol/L NaCl, 0.05% (w/v) Tween 20) for 4 h at room temperature; the membrane was incubated with the anti-TgHSP70 polyclonal antibody at an 1:5,000 dilution in 5% skim milk for 2 h. The membrane was washed three times with TBS (10 mmol/L Tris-HCl pH 7.5, 100 mmol/L NaCl) and one time with TBST extensively. Secondary antibody (1:10,000) was then added and the membrane was incubated for 1 h at 37°C, followed by three washes with TBST. Signal was developed with 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium chloride (BCIP/NBT) for 30 s and stopped by rinsing the membrane with distilled water.

Statistical analysis

The data were then subjected to analysis by one-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$.

RESULTS

Cloning and analysis of the full-length cDNA of *Tghsp70*

A 1399-bp fragment of *Tghsp70* was amplified using nested PCR from the cDNA library of *T. granosa*. Based on this expression sequence tag (EST), 609- and 929-bp fragments were amplified using primer pairs P5/M13-47 and M13Rv-M/P6, respectively. A 2492-bp full-length cDNA sequence (Figure 1) of *Tghsp70* was assembled by overlapping the 609-bp fragment, the 929-bp fragment and the 1,399-bp EST, and deposited into GenBank under the accession number JN936877.

The ORF of *Tghsp70* was 1968 bp in length, and encoded a 655-amino acid protein with a predicted molecular mass of 71.48 kDa and a pI of 5.25. Amino acid sequence analysis (Figure 1) revealed HSP70 signature motifs (IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK), ATP/GTP-binding site (AEAYLGKT), bipartite nuclear targeting sequence (KRKFKKDITDNKRAVRR), cytosolic *hsp70*- specific motif (EEVD), and degenerate repeats of tetrapeptide GGMP.

Phylogenetic analysis

Multiple sequence alignment (Figure 2) showed that the deduced amino acid sequence of *Tghsp70* was highly similar to those of other HSP70s from *C. gigas*, *H. discus hannai*, *Phascolosoma esculenta*, *Rattus norvegicus*, *Homo sapiens*, *Danio rerio*, *M. meretrix*, *Penaeus monodon*, *Apostichopus japonicus* and *Caenorhabditis elegans*. The evolutionary tree of *hsp70* was constructed based on the protein sequences from different species (Figure 3). The relationships among different HSP70s displayed in the phylogenic tree were consistent with the traditional taxonomy of these species.

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TACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTGTAAAACGACGGCCAGTAGATCTGTAATACGACTCACTATAGGGCGCTAG        90
CTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGCCGCATAACTTCGTATAGCATACATTATACGAAGTTATC       180
AGTCGACGGTACCGGACATATGCCCGGGAATTCGGCCATTACGGCCGGGGAAGAACAAAGCCGGATTGAATCAAGGGGATAGAAGATCAA       270
AACTGTTCTGTATATTATTTTTACATAAAAACTCAACATGTCCAAGAAATCCAAACAAGCTATCGGAATTGATTTGGGAACAACATACTC       360
                               M  S  K  K  S  K  Q  A  I  G  I  D  L  G  T  T  Y  S
CTGTGTGGGTATATTCCAGCATGGTAAAGTAGAAATAATCGCCAACGATCAGGGCAACAGAACAACCCCTAGTTATGTAGCCTTCACAGA       450
  C  V  G  I  F  Q  H  G  K  V  E  I  I  A  N  D  Q  G  N  R  T  T  P  S  Y  V  A  F  T  D
TACAGAACGTCTGATTGGTGATGCCGCCAAGAACCAAGTGGCTATGAATCCAACAAACACCATTTTTGATGCAAAGCGTTTAATTGGAAG       540
  T  E  R  L  I  G  D  A  A  K  N  Q  V  A  M  N  P  T  N  T  I  F  D  A  K  R  L  I  G  R
AAAATTCACCGATGCATCAGTCCAGTCGGACATGAAACACTGGCCATTTACAGTTATTGATGATGGCGGTAAACCAAAGATCAAAGTTGA       630
  K  F  T  D  A  S  V  Q  S  D  M  K  H  W  P  F  T  V  I  D  D  G  G  K  P  K  I  K  V  E
ATACAAAGGAGAGACAAAAACATTTTATCCAGAAGAAGTGTCATCAATGGTTCTGAACAAAATGAAAGAAACTGCAGAGGCTTACCTTGG       720
  Y  K  G  E  T  K  T  F  Y  P  E  E  V  S  S  M  V  L  N  K  M  K  E  T  A  E  A  Y  L  G
TGTGACAATAACAAATGCAGTAGTCACAGTCCCTGCTTACTTTAATGACTCACAACGTCAAGCAACAAAAGATGCCGGTACTATTTCAGG       810
 V  T  I  T  N  A  V  V  T  V  P  A  Y  F  N  D  S  Q  R  Q  A  T  K  D  A  G  T  I  S  G
AATGAATGTATTGCGTATTATCAACGAACCCACTGCCGCTGCTATTGCATATGGTCTTGACAAAAAGGTTGGTGGTGAAAGGAATGTTCT       900
  M  N  V  L  R  I  I  N  E  P  T  A  A  A  I  A  Y  G  L  D  K  K  V  G  G  E  R  N  V  L
GATCTTTGATCTTGGTGGAGGTACCTTTGATGTCTCTATACTTACAATTGAAGACGGTATATTTGAAGTAAAGTCAACATCTGGTGATAC       990
 I  F  D  L  G  G  G  T  F  D  V  S  I  L  T  I  E  D  G  I  F  E  V  K  S  T  S  G  D  T
TCATTTGGGTGGTGAAGATTTTGACAATCGTATGGTTAATCACTTCATAGATGAATTTAAACGTAAACACAAAAAGGATATTTCAGATAA      1080
  H  L  G  G  E  D  F  D  N  R  M  V  N  H  F  I  D  E  F  K  R  K  H  K  K  D  I  S  D  N
CAAGAGAGCAGTAAGACGTCTTAGAACAGCTTGTGAAAGAGCAAAGAGAACCCTCTCCTCAAGTACACAGGCTAGTGTTGAAATTGATTC      1170
  K  R  A  V  R  R  L  R  T  A  C  E  R  A  K  R  T  L  S  S  S  T  Q  A  S  V  E  I  D  S
TTTGTATGAGGGTATTGACTTTTACACAAGTATCACTAGAGCTCGTTTTGAAGAGTTGAATGCAGATCTTTTCCGTGGTACCTTGGAACC      1260
  L  Y  E  G  I  D  F  Y  T  S  I  T  R  A  R  F  E  E  L  N  A  D  L  F  R  G  T  L  E  P
TGTAGAGAAAGCTTTGAGAGATGCCAAGCAGGACAAGGCCAACATTCATGATATTGTACTTGTTGGTGGTTCCACCAGAATTCCAAAAAT      1350
  V  E  K  A  L  R  D  A  K  Q  D  K  A  N  I  H  D  I  V  L  V  G  G  S  T  R  I  P  K  I
TCAGAAGTTGTTGCAGGATTTCTTCAATGGTAAAGAACTGAACAAATCCATCAACCCAGATGAAGCTGTAGCCTATGGAGCAGCTGTTCA      1440
 Q  K  L  L  Q  D  F  F  N  G  K  E  L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q
AGGAGCCATTTTGTCTGGTGATCAATCTGAAGAGGTGCAGGATCTTCTCTTGTTGGATGTAGCACCATTATCCTTGGGTATTGAGACTGC      1530
  G  A  I  L  S  G  D  Q  S  E  E  V  Q  D  L  L  L  L  D  V  A  P  L  S  L  G  I  E  T  A
CGGTGGTGTAATGACAACACTGATAAAACGTAACACAACTGTCCCAACCAAACAAAACCAGACCTTCACCACATATTCTGACAATCAGCC      1620
  G  G  V  M  T  T  L  I  K  R  N  T  T  V  P  T  K  Q  N  Q  T  F  T  T  Y  S  D  N  Q  P
AGGTGTACTCATCCAGGTCTTTGAGGGAGAACGTGCAATGACCAAAGATAACAACCTCCTTGGTAAATTTGAACTGACTGGTATCCCACC      1710
  G  V  L  I  Q  V  F  E  G  E  R  A  M  T  K  D  N  N  L  L  G  K  F  E  L  T  G  I  P  P
AGCACCTCGTGGTGTTTCACAGATTGAAGTTACCTTTGATATTGATGCTAATGGTATCTTGAATGTGCATGCCATTGACAAGAGTACAGG      1800
  A  P  R  G  V  S  Q  I  E  V  T  F  D  I  D  A  N  G  I  L  N  V  H  A  I  D  K  S  T  G
AAAAGAGAACAAGATCACAATTACAAACGACAAAGGTCGTCTCAGCAAAGATGAAATCGACAGAATGGTTAATGATGCAGAAAAATACAA      1890
  K  E  N  K  I  T  I  T  N  D  K  G  R  L  S  K  D  E  I  D  R  M  V  N  D  A  E  K  Y  K
GGCAGAAGATGACAAACAAAGAGAAAGAATTGGAGCTAAGAATGGACTTGAAAGTTACGCATTCCAAATGAAATCAACTGTGGAAGACGA      1980
  A  E  D  D  K  Q  R  E  R  I  G  A  K  N  G  L  E  S  Y  A  F  Q  M  K  S  T  V  E  D  E
GAAATTGAAGGACAAAATTTCTGAGGATGATAAGAAGTCTATTATTGACAAATGTGATGAAATCATTAAGTGGTTAGATGCCAATAGTTT      2070
  K  L  K  D  K  I  S  E  D  D  K  K  S  I  I  D  K  C  D  E  I  I  K  W  L  D  A  N  S  L
GGCTGAGAAGAAGAGTTTGAACACAAGCAGAAAGAATTGGAAGGTGTCTGTAATCCAATTATCACCAAATTGTACCAGGGTGCTGGAGG      2160
  A  E  K  E  E  F  E  H  K  Q  K  E  L  E  G  V  C  N  P  I  I  T  K  L  Y  Q  G  A  G  G
CGCTGCTGGAGCTGGAGGTATGCCAGGTGGTATGCCTGGAGGATTCCCAGGTGGTGCAGGAGGTCAACAGTCCAGTGGAGGAAGTGGTGG      2250
  A  A  G  A  G  G  M  P  G  G  M  P  G  G  F  P  G  G  A  G  G  Q  Q  S  S  G  G  S  G  G
ACCAACCATCGAAGAAGTAGATTAAATAATGAGAACTTCAATATCTCAAAACAAAAATCATTACAATTAGGTCCATTGCTAAAAATTCTG      2340
  P  T  I  E  E  V  D  .
TTTAGTAATTTGTCTGTTTTCATAGTTACCTGTTCGGAAGTTGGTAATTCTATATTTAAAAATGATTTAATTTCCAAATATTAACAATAG      2430
GAACATCTTAAGATTAAAGTAGTTGTTGAATAACAAGTAAAAAACGGTTAATAAAAGTTAAA                                  2492
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Figure 1. Sequence of *hsp70* of *T. granosa* and its amino acid sequence. ▨ Three classical HSP signature motifs; ▢, ATP/GTP-binding site motif A; ⎯, bipartite nuclear targeting sequence; ▨, GGMP TPR; ⎯, cytosolic HSP70 character sequences; ▢, tailing signal.

Expression of *Tghsp70* mRNA in different tissues

To examine the tissue distribution profile of *Tghsp70*, total RNA was isolated from the foot, mantle, haemocytes, visceral mass, adductor and gill of unchallenged *T. granosa*. *Tghsp70* transcripts were detected in all examined tissues (Figure 4). The highest expression level was detected in visceral mass, followed by gill, haemocytes and mantle.

Moreover, the expression levels of *Tghsp70* in foot and adductor were lower than those in others.

Transcriptional responses of *Tghsp70* to various stresses

The expression patterns of *Tghsp70* in haemocytes and

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Majority                  MS-KG--PAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPTNTVFDAKRLIGRK
Tegillarca granosa        ..-.KSKQ.I...........I.......................................................I..........    79
Crassostrea gigas         ..-.PAQQ.I...................................V..............N..I..........               79
Haliotis discus hannai    ..-.---Q.....................................................E..I..........R              76
Phascolosoma esculenta    ..-.TA-Q.....................................S...............S.....E.....                 78
Rattus norvegicus         ..-..--.....................................................................R             77
Homo sapiens              ..-..--.....................................................................R             77
Danio rerio               ..-..--.....................................................................R             77
Meretrix meretrix         ..-.A--.....................................................I..........                  77
Penaeus monodon           .A-.A--.....................................................S..........                  77
Apostichopus japonicus    .PG..TS.I....................T...............................I..........S                 80
Caenorhabditis elegans    ..---TCK.I...........IY.N....L..SE..K.........V.....D.A.R..E..........R                  77


                          FDDAVVQSDMKHWPFTVINDAGKPKIQVEYKGETKTFYPEEISSMVLTKMKETAEAYLGKTVTNAVVTVPAYFNDSQRQA
Tegillarca granosa        .T..S............D..G.....K............V.....N..........V.I................              159
Crassostrea gigas         .N..S.............Q.S..M.K......E...SA..V.....N...........IN..............                159
Haliotis discus hannai    .EE.N.........N.LS.G.......N..D.P............Q....I.D.....                               156
Phascolosoma esculenta    Y..PS..........VSEG.........FA...............IN...I.....                                 158
Rattus norvegicus         ...........M.V....R..V.........S.....V.........I.....                                    157
Homo sapiens              ...........M.V....R..V.........S.....V.........I.....                                    157
Danio rerio               ...G.........N....NSR..V........S.S............I.......S...I.....                        157
Meretrix meretrix         ...PG.........E....QT..LR.......S.F.......N.......F.....                                  157
Penaeus monodon           .E.HT..........I..EST.........DK.........I.........S..KD.....                            157
Apostichopus japonicus    .S.TA......N...K...K....VL.A.HMN.L...N...........S..QK..D.....                           160
Caenorhabditis elegans    ..EET....I......KGKQ...VVE..V...KRE.NA....A...Q........V..HS.RD..I.....                  157


Majority                  TKDAGTISGLNVLRIINEPTAAAIAYGLDKKVG----GERNVLIFDLGGGTFDVSILTIEDG-IFEVKSTAGDTHLGGED
Tegillarca granosa        .........M...............----..................-......S....                              234
Crassostrea gigas         .............................NQSQ..............-......S....                              238
Haliotis discus hannai    .............................----..............-......                                  231
Phascolosoma esculenta    .............................----.A............-......                                  233
Rattus norvegicus         .......A.....................----A..............-......                                  232
Homo sapiens              .......A.....................----A..............-......                                  232
Danio rerio               .............................----A..............-......                                  232
Meretrix meretrix         .............................----..............-......S....                             232
Penaeus monodon           ................G............----..............-......                                  232
Apostichopus japonicus    .....V.A...........L......LK----..QH.................C.D..-M.....                        235
Caenorhabditis elegans    ....A..A...AI........L.....GIT----D.K.I...........S.AE.S....                             233


Majority                  FDNRMVNHFIQEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTSITRARFEELNADLFRG
Tegillarca granosa        ...........D....................................V........................                314
Crassostrea gigas         ...................E...................S........F.....................                   318
Haliotis discus hannai    .............................................F..V.Y................                      311
Phascolosoma esculenta    .........V......F....T...........................                                       313
Rattus norvegicus         .........A.......E...............................                                       312
Homo sapiens              .........A.......E...............................                                       312
Danio rerio               .........T.......................................                                       312
Meretrix meretrix         ..............M..................................Y............S.....                     312
Penaeus monodon           .............Y...P.E...SL................V...F................C.....                     312
Apostichopus japonicus    ...IL.D..SA.........TG.A..L.....S........A..NV.....F..V.........D.CS.Q..K                 315
Caenorhabditis elegans    ..S.VLQ..MT.....TG....S.P..I..................E.TV.V...FD.T...SK.........C.....K          313


Majority                  TLEPVEKALRDAKLDKAQIHDIVLVGGSTRIPKIQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGDKSEEVQDLL
Tegillarca granosa        .................Q...N...............................................G.....Q........     394
Crassostrea gigas         .M.....................................................................                 398
Haliotis discus hannai    ...............A...S................................C........................H.........    391
Phascolosoma esculenta    ..........S....GA......................................................                 393
Rattus norvegicus         ..D...........S.......................................................N.........         392
Homo sapiens              ..D...........S.......................................................N.........         392
Danio rerio               ..D......M.................Y..........................................N.........         392
Meretrix meretrix         ..D....S...MG.DA......................................................H.........         392
Penaeus monodon           .......S...M.........................................................C.....A.....       392
Apostichopus japonicus    C.......II...IS.SA.DT...............M.T......D........................Q....K.I            395
Caenorhabditis elegans    ...........T..GR.DEV................KE.....D..C........F.......V...V.DDTIK.V             393
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Figure 2. Alignment of HSP70 amino acid sequences of *T. granosa*. Amino acid residues that were instead of dot at least six sequences.

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Majority                  LLDVTPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVP
Tegillarca granosa        ....A...........T......V...N.....................F..........................S     474
Crassostrea gigas         ....................N.............................................................     478
Haliotis discus hannai    ...............................................................I..................     471
Phascolosoma esculenta    ....................A.............................F................................     473
Rattus norvegicus         ..................................................................................     472
Homo sapiens              ..................................................................................     472
Danio rerio               ..................................................................................     472
Meretrix meretrix         ....A...........S..........................L......................................     472
Penaeus monodon           ....................A...........................................S.................     472
Apostichopus japonicus    .V..A.....L.......T..E...SR..C.KE...S.F....T..S...F.....L....R..Q...S..L.......     475
Caenorhabditis elegans    .V..V...H..........N..D...R..A.ASK.....A........S............R..HR..T.........     473

Majority                  QIEVTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVNEAEKYKAEDEKQRDRISAKNGLESYAFNMKST
Tegillarca granosa        ...................H.I..............DE.D....D........D...E..G...........Q....     554
Crassostrea gigas         ...................................DE.D........Q......E..A.................     558
Haliotis discus hannai    .......................M.............E........N........K...Q...............     551
Phascolosoma esculenta    ...............................D....K...A...D.DA.....Q...A.....Y.....       553
Rattus norvegicus         .....................................................Q.........KV.S..S.........A.     552
Homo sapiens              .....................................................Q.........KV.S..S.........A.     552
Danio rerio               .................M...................................Q.........DV...KV.........     552
Meretrix meretrix         ...................T.C...............D....D.....Q.....K...T...Q....S......     552
Penaeus monodon           ..................................E......QD......D........S....C......     552
Apostichopus japonicus    K....Y.............K.S....S.T..............E.D...A...Q.....DA..E..T.R.Q..N..YSV..A     555
Caenorhabditis elegans    .VD................A.....RS.R...R.E.....QA..D......KQFER..AA......SR.Q..A...QV.QA     553

Majority                  VEDEKLKDKISEEDKKTILDKCNEIISWLDSNQLAEKEEFEHKQKELEKVCNPIITKLYQAAGGAPG---GGMPGGMP--
Tegillarca granosa        ..............D...S.I...D...K...A.S................G...........G....A.A--........GG     632
Crassostrea gigas         .D.........G.........E...K.M.Q....D............G...........S.....---........N-     634
Haliotis discus hannai    ...........D....T....DV...........D...........G.............G.M-P.......GG     630
Phascolosoma esculenta    ...........D....M....T...........H...........T...............G.M-P.......GG     632
Rattus norvegicus         .....QG..ND...QK...........K..T.......Q.................S...M..----....F.--     626
Homo sapiens              .....QG..ND...QK...........N...K..T.......Q.................S...M..----....F.--     626
Danio rerio               ..........G...D...QK......V.G...K..T..R.....Q.................S...M..GMPE.....F.--     630
Meretrix meretrix         ...........D....A.I.....V...........Q.................-......G-A.......N-     629
Penaeus monodon           .....F.E.....RNK...ET...T.K...M...G....Y......I.Q...........M.A......P---.....F.GG     629
Apostichopus japonicus    .Q.-APEG.L.Q..RE.VMKLV..TTT......L....D.CTY.FE..Q.TVA..M..IH.NG.S.SD----.EA.PSS--     628
Caenorhabditis elegans    L.EHG--SLL.A..A.RAK.AVEDTLR.MER.T..D.D.I.A.D...KSI.QD.L..MH.QEAQSGSG--C.N..-----     624

Majority                  --G--GGGAPSG-GGSSGGPTIEEVD-
Tegillarca granosa        FP.GA..QQS..-.S--..........     656
Crassostrea gigas         FG.GAP..GAP.-...G..........     660
Haliotis discus hannai    -A.GLP...DGQT..............     656
Phascolosoma esculenta    MP.GMP..GAA.T.TGGS.........     659
Rattus norvegicus         --.--..AP...-.A...-........     647
Homo sapiens              --.--..AP...-.A...-........     647
Danio rerio               --.--A.A..-.-.....-........     650
Meretrix meretrix         FG.--A......-.............     653
Penaeus monodon           AP.--A...AP.A.....-........     653
Apostichopus japonicus    -----C.SQSNQGPSQ.H...V....     650
Caenorhabditis elegans    -----S..FH.S--NYPQ...V.....     644
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Figure 2. Contd.

visceral mass were measured at 0, 6, 12, 24 and 48 h after heavy metal exposures. In haemocytes (Figure 5), the expression level of *Tghsp70* was significantly ($P<0.01$) up-regulated at 12 h after Pb^{2+} treatment to 3.23-fold higher than that in the control group. Similar expression profile was found after Cd^{2+} treatment; the *Tghsp70* expression level increased to a maximum of 4.64-fold at 12 h than that in the control group. After Cu^{2+} treatment, the peak expression level was detected at 12 h with a 3.32-fold increase in comparison with that in the control group. The effect of heavy metal stress on the expression of *Tghsp70* in visceral mass is shown in Figure 6. *Tghsp70* expression after Pb^{2+}, Cd^{2+} and Cu^{2+} exposure was 2.45-fold higher at 24 h, 5.98-fold higher at 48 h and 8.77-fold higher at 24 h than that in the control group,

respectively.

Furthermore, thermal challenge at 37°C and cooling at 4°C for 48 h induced a 4.87-fold and an 1.87-fold increase in *Tghsp70* expression in haemocytes than that in the control group, respectively (Figure 7). In visceral mass, *Tghsp70* expression increased to a level of 2.31 fold higher after cold stress and 8.22 fold higher after heat treatment than that in the control group, respectively.

Expression of recombinant *Tghsp70* protein and Western blotting analysis

The recombinant plasmid pET-HSP70 was transformed into *Escherichia coli* BL21. After IPTG induction, protein was purified and subject to SDS-PAGE electrophoresis.
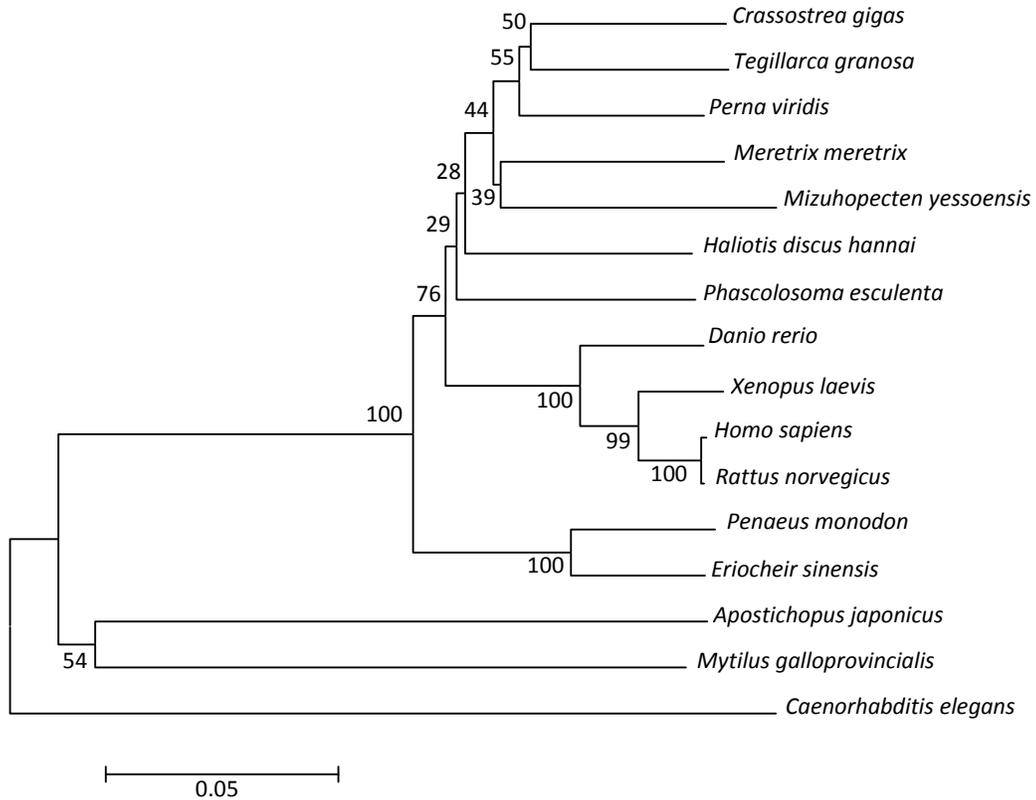
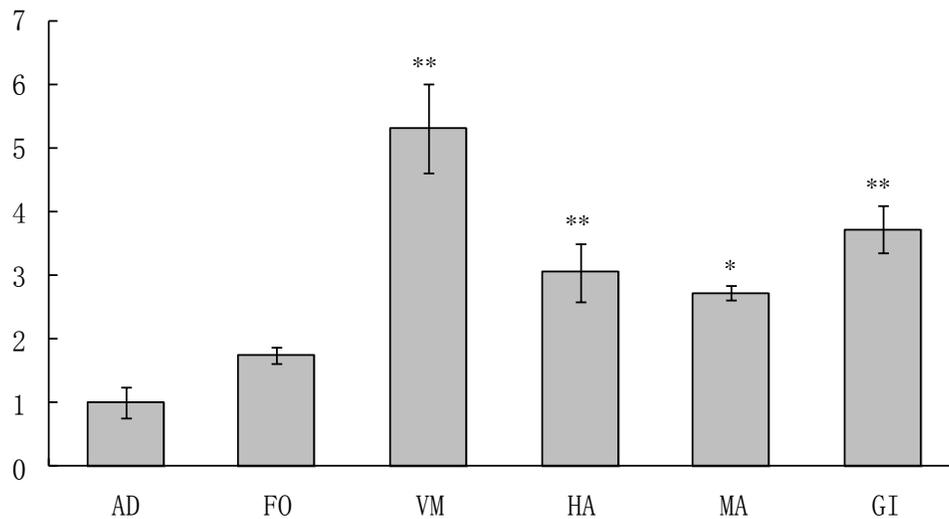**Figure 3.** The phylogenetic tree based on the HSP70 sequences from different species.

Figure 4. Tissues distribution of *Tghsp70* transcripts measured by quantitative real-time. AD, Adductor; FO, foot; VM, visceral mass; HA, haemocytes; MA, mantle; GI, gill. Each symbol and vertical bar represent the mean ± SD (n=3). Significant differences between other tissues and adductor were indicated by an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.
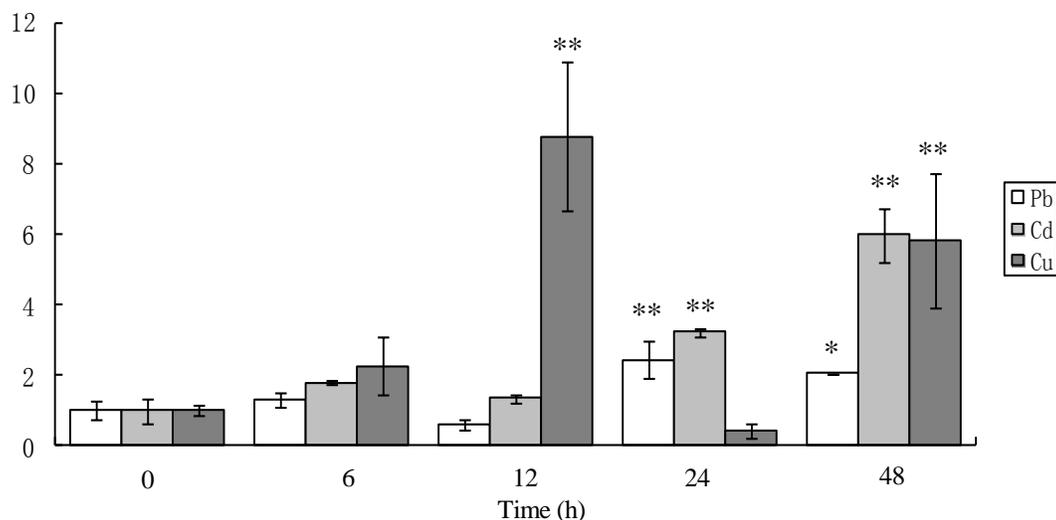
Coomassie brilliant blue staining revealed a distinct band with a molecular weight of about 70 kDa (Figure 8). The peak expression level of recombinant protein was observed at 4 h after IPTG induction. This band was excised with a clean blade and soaked in PBS for Coomassie brilliant blue staining revealed a distinct band
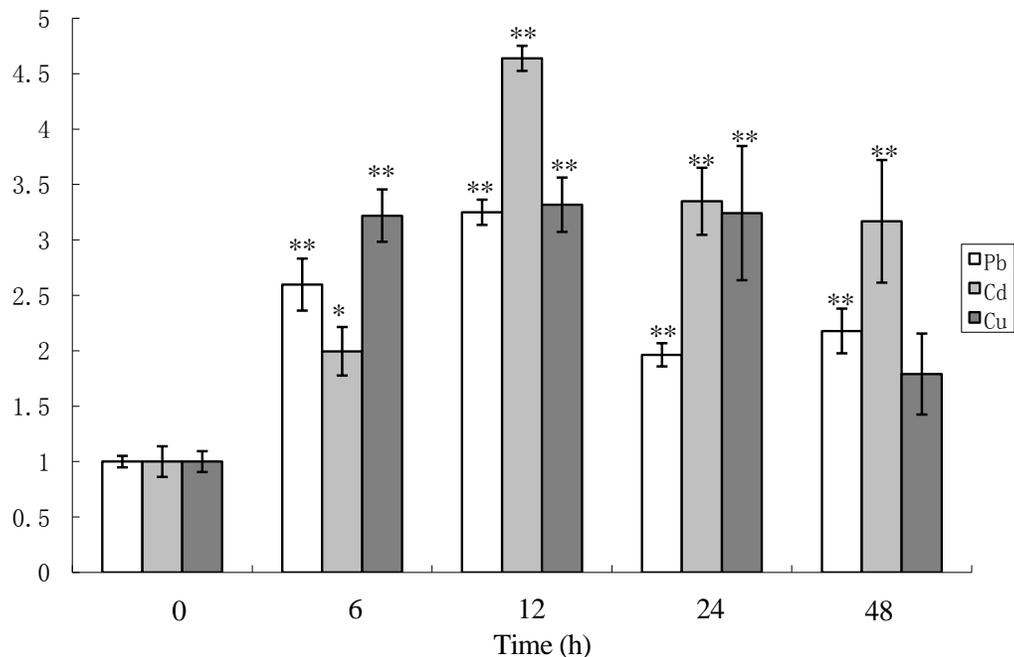
Figure 5. Real-time PCR of *Tghsp70* gene in haemocytes exposed to heavy metals. Each symbol and vertical bar represent the mean ± SD (n = 3). Significant differences between other time points and 0 h were indicated by an asterisk at P < 0.05 and two asterisks at P < 0.01.

Figure 6. Real-time PCR of *Tghsp70* gene in visceral mass exposed to heavy metals. Each symbol and vertical bar represented the mean ± SD (n = 3). Significant differences between other time points and 0 h were indicated by an asterisk at P < 0.05 and two asterisks at P < 0.01.

with a molecular weight of about 70 kDa (Figure 8). The peak expression level of recombinant protein was observed at 4 h after IPTG induction. This band was excised with a clean blade and soaked in PBS for polyclonal antibody preparation. The polyclonal antibody generated from the immunized mice was used as the primary antibody for Western blot analysis. The result shows that the mouse antisera specifically identified not only the recombinant protein, but also the native protein from the whole cell lysate of *T. granosa*. The specific bands were not detected in the un-induced BL-21 harboring pET-HSP70 or induced BL-21 with pET-28a (Figure 9). The serum of negative control group did not react with the protein and no band was detected.
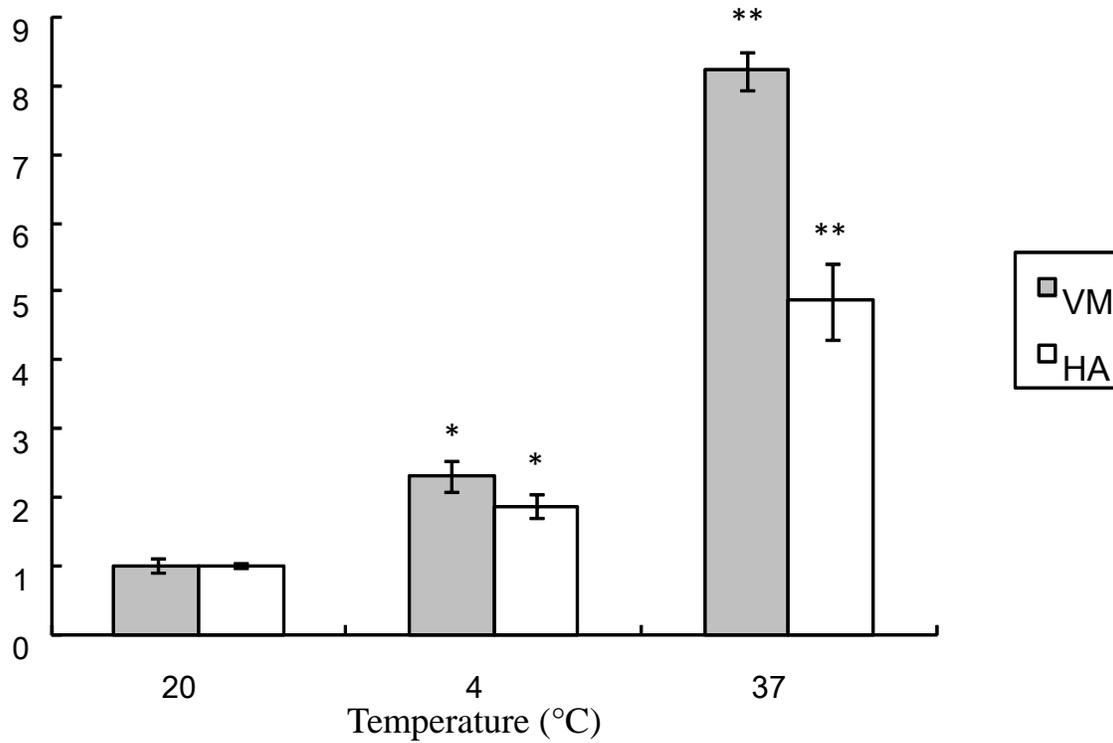
Figure 7. Real-time PCR of *Tghsp70* gene exposed to 4 and 37°C sea water (VM, visceral mass; HA, haemocytes). Each symbol and vertical bar represent the mean ± SD (n = 3). Significant differences between other temperatures and 20°C were indicated by an asterisk at P < 0.05 and two asterisks at P < 0.01.
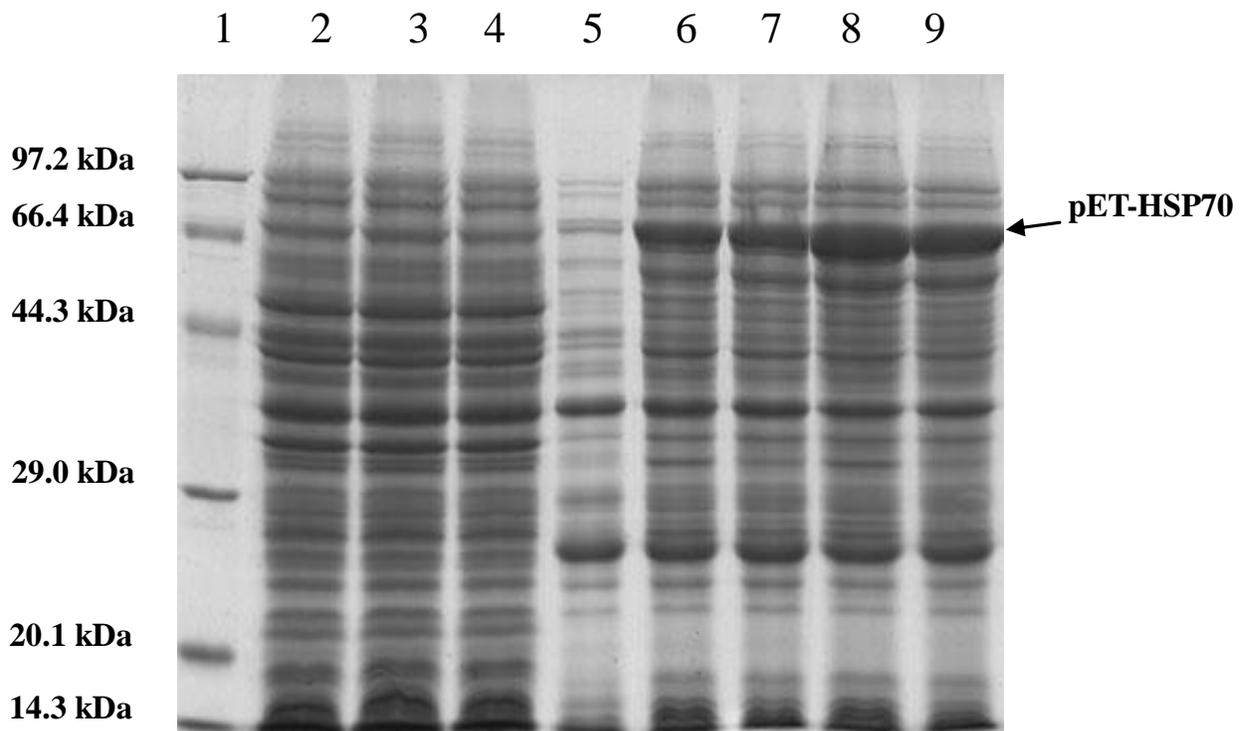
Figure 8. Expression and purification of HSP70 recombinant protein. Lane 1, Low molecular marker; lane 2, total protein of non-induced pET-28a; lane 3, total protein of pET-28a induced for 5 h; lane 4, total protein of non-induced pET-HSP70; lanes 5 to 9, total protein of pET-HSP70 induced for 1, 2, 3, 4 and 5 h.
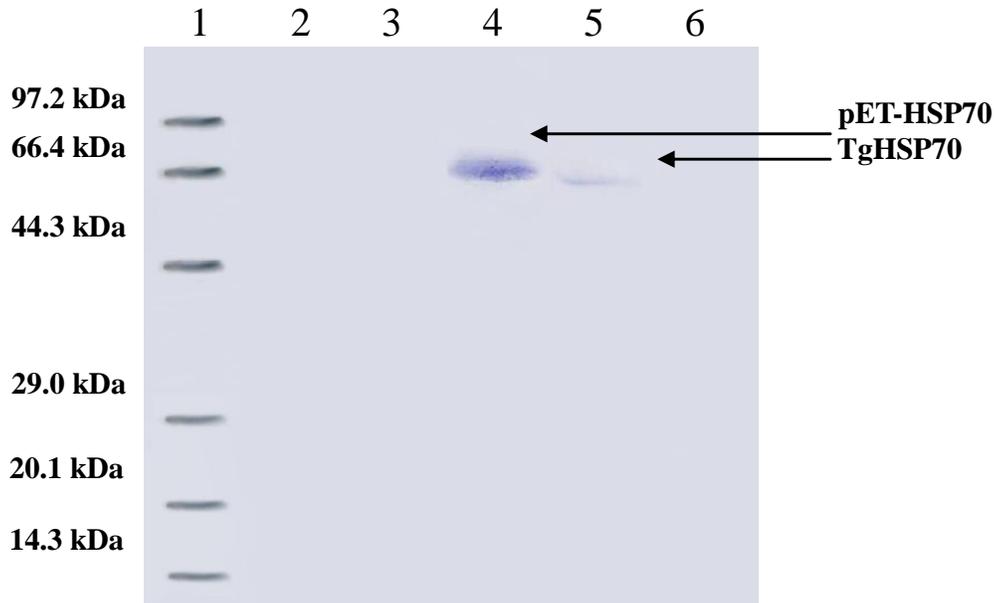
Figure 9. Specificity of HSP70 polyclonal antibody was determined by Western blot. Lane 1, Low molecular marker; lane 2, IPTG induced *E. coil* BL21 with pET-28a for 4 h; lane 3, un-induced *E. coil* BL21 with pET-HSP70; lane 4, IPTG-induced *E. coil* BL21 with pETHSP70 for 4 h; lane 5, cell lysate of *T. granosa*; lane 6, PBS-immunized mice was used in replacement of the primary antibody.

DISCUSSION

In this study, a full-length *Tghsp70* was cloned from a cDNA library of *T. granosa* constructed previously. Sequence analysis of the *Tghsp70* gene (Figure 1) showed several specific motifs, and three of them (IDLGTTYS, IFDLGGGTFDVSIL and IVLVGGSTRIPKIQK) were highly conserved. Generally, specific sequences at the C-terminal portion of HSP70 suggest different localization of the protein in a cell. EEVD is a characteristic of cytosolic HSP70; HDEL is a characteristic of endoplasmic reticulum HSP70, and PEAEYEEAKK is the characteristics of mitochondrial HSP70 (Vayssier et al., 1999; Su et al., 2009). An EEVD motif at the C-terminus of TgHSP70 indicates that the protein primarily localizes in the cytoplasm. The sequence IDLGTTYSCV is also conserved according to the signal of DNAK subfamily (motif nDLGTTnSnV). The tetrapeptide repeat (TPR) and the two repeats of GGMP at the end of the protein upstream of EEVD mediate the association of HSP70 with HSP90 into a multi-chaperone complex (Scheufler et al., 2000). The sequences of the ATPase domains of HSP70s from various species are highly conserved, while the C-terminal domains are less conserved than the ATPase domains (Figure 2) (Emilie et al., 2007). Amino acid alignment analysis revealed significant homology of TgHSP70 with HSP70s from other mollusc. For example, TgHSP70 is 91% similar to HSP70 from *C. gigas* (AF144646.1), 90% similar to HSP70 from *M. meretrix* (ADT78476.1), 89% similar to HSP70 from *Pteria penguin* (ABJ97377.1) and 88% similar to HSP70 from *H. discus*

hannai (ABC54952.1). Phylogenetic analysis indicates that TgHSP70 is similar to those from other clams and organisms.

SDS-PAGE and Coomassie blue staining revealed that the size of TgHSP70 was approximately 70 kDa and the amount of the induced protein expression increased sharply over time. Polyclonal antibodies of TgHSP70 were generated with the purified recombinant protein to detect TgHSP70, and our Western blot results demonstrated the specificity of the polyclonal antibodies. *Tghsp70* transcripts were detectable in all the tissues examined. The ubiquitous expression of *Tghsp70* might be attributed to its multiple functions in response to diverse environmental stressors in various tissues. The maximum *Tghsp70* expression level was found in visceral mass. The expression level of *Tghsp70* in gill was higher than those in any other tissue except for that in visceral mass, while the expression of *Tghsp70* was lower in the adductor and foot. In pacific abalone (*H. discus hannai*), the highest level of *hsp70* expression is detected in mantle, followed by gill, sex gland, muscle and digest gland (Cheng et al., 2007).

Oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and the system's ability to readily detoxify the reactive intermediates and/or repair the resulting damage (Li et al., 2011). Bivalve mollusks produce ROS upon exposure to heavy metal and temperature (Abele et al., 2002). HSP70 over-expression provides a protective effect against endogenously or exogenously generated ROS (Chong et al., 1998). The increased synthesis of HSP70 in response

to physical or chemical stressors is preceded by a transcriptional activation of the HSP70 gene, which leads to elevated levels of *hsp70* mRNA (Su et al., 2010). In order to investigate the response of *Tghsp70* to heavy metal and thermal stressors, *T. granosa* was challenged by 10 µmol/L $CuCl_2$, 10 µmol/L $CdCl_2$, 10 µmol/L $PbCl_2$, 37 and 4°C seawater, respectively. Temporal expression of *Tghsp70* was analyzed using real-time PCR. The *Tghsp70* gene expression in haemocytes was up-regulated after 6 h of exposure to Pb^{2+}, and the peak expression level was detected at 12 h post exposure. The expression level of *Tghsp70* was down-regulated smoothly in the next 36 h.

In visceral mass, the transcriptional level of *Tghsp70* was 0.62 fold after 12 h, 2.44 fold after 24 h, and 2.04 fold after 48 h more than those in the control group. Similar expression pattern was detected in haemocytes treated with Cd^{2+}. The highest level of *Tghsp70* expression was found at 12 h (4.64 fold). The expression patterns of *Tghsp70* in haemocytes after Cu^{2+} treatment was similar with the patterns after Cd^{2+} and Pb^{2+} treatments. The transcriptional level of *Tghsp70* in visceral mass reached the peak at 12 h and down-regulated in the next 36 h. The mRNA expression patterns of *Tghsp70* in visceral mass exposed to the three heavy metals were different. Compared with that in the control group, the *Tghsp70* gene was slightly up-regulated 12 h after Cu^{2+} treatment and the expression level of *Tghsp70* decreased only 0.43 fold at 24 h, and then recovered. Unlike Cu^{2+} treatment, Pb^{2+} treatment led to significant decrease in *Tghsp70* mRNA level after 12 h, and then recovered and reached the peak at 24 h. The expression of *Tghsp70* increased slightly after Cd^{2+} treatment, and the peak expression level was detected at 48 h, which was 5.98 fold higher than that in the control group. These results indicated that TgHSP 70 was a keen response factor to marine heavy metal stress. It may be a candidate biomarker for marine heavy metal pollutant. Cold stressor also resulted in increases in *Tghsp70* mRNA transcripts in haemocytes and visceral mass. The level of cold-induced *hsp70* was also increased in *Leptinotarsa decemlineata* from three different latitudes (Lyytinen et al., 2012). Cold stress increases liver *hsp70* mRNA, thereby affecting the physiological function and non-specific immune ability of GIFT tilapia (Liu et al., 2011).

Conclusion

In this study, the HSP70 gene of *T. granosa* was cloned and the transcription levels of *Tghsp70* in response to plumbum, cadmium, copper and thermal challenges were analyzed. On the whole, a polyclonal antibody of *Tghsp70* was generated in mice for future examination. HSP70 is one of the crucial factors that protect cells from damages.

It is necessary to standardize the classification of the HSP70s found in the bivalve to facilitate the comparison of the functions of similar HSP70s among different bivalve species and to understand the function of different HSP70 members (Yue et al., 2011). Due to the responses of HSP70s to pollutions and toxins in the habitat, they may be used as potential biomarkers of the environment. The identification and expression analysis of TgHSP70, and the production of anti-TgHSP70 polyclonal antibody are useful for further research of native HSP70 detection, and lay the foundation of studies in the molecular mechanisms of further detailed expression.

ACKNOWLEDGEMENTS

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