Fish and Shellfish Immunology 95 (2019) 635–643

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A 28.6-kD small heat shock protein (MnHSP28.6) protects *Macrobrachium nipponense* against heavy metal toxicity and oxidative stress by virtue of its anti-aggregation activity

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ARTICLE INFO

Keywords:
Macrobrachium nipponense
Small heat shock protein (sHSP)
Heavy metal
Oxidative stress
Chaperone

ABSTRACT

Small heat shock proteins (sHSPs) are ATP-independent chaperones and involved into various physiological and stress processes. In the present study, a 28.6-kD sHSP coding gene, MnHSP28.6, was cloned and characterized from the oriental river prawn *Macrobrachium nipponense*. Tissue distribution analysis via qPCR and western blot revealed that MnHSP28.6 predominantly expressed in muscle. The temporal transcription of MnHSP28.6 in muscle after bacterial challenge, heavy metal exposure and doxorubicin (DOX) injection was investigated by qPCR. The results showed that the expression of MnHSP28.6 were strongly enhanced by both Cd^{2+} and Cu^{2+} exposure, as well as DOX injection, but not by bacterial infection. Aggregation assays showed that recombinant MnHSP28.6 could effectively prevent temperature-induced aggregation of citrate synthase, and reduction-induced aggregation of insulin *in vitro*. MnHSP28.6 also could protect muscle extracts from heat-induced protein denaturation and superoxide dismutase (SOD) inactivation. Expressing MnHSP28.6 in *E. coli* conferred host cell impressive protection against H_2O_2 compared to control. These results suggest a protective role of MnHSP28.6 in maintaining protein homeostasis, preventing aggregation, promoting resistance to heavy metal and keeping redox balance.

1. Introduction

The oriental river prawn *Macrobrachium nipponense* (Decapoda; Palaemonidae) is a typical freshwater crustacean species widely distributing in brackish and fresh waters throughout China and other Asian countries [1]. *M. nipponense* is also one of the most extensively cultured and productive fresh water crustacean species in China with a cultured production of about 272,592 tons in 2016. *M. nipponense* live in diverse environments and consequently have to deal with different stress challenge. The success of *M. nipponense* in their adaptation to changing environments relies on their capacity to survive often stressful conditions. In natural water, *M. nipponense* have to face challenges from often rapid changes in temperature, salinity, pH, availability of oxygen, environment pollution by heavy metal, chemicals, diseases resulted from various pathogens and other environmental stresses. For organisms, stress response is characterized by the induced expression of a series of proteins, particularly the heat shock proteins (HSPs), whose prominent function is to act as molecular chaperones that prevent the aggregation and unfolding of proteins upon stress conditions [2,3].

HSPs were originally described in fruit fly *Drosophila melanogaster* which was exposed to a severely heat-shocked environment and were subsequently demonstrated to be ubiquitously and evolutionarily conserved molecules that are present in all living organisms [4]. HSPs can be classified according to molecular mass as HSP100, HSP90, HSP70, HSP60, HSP40 families and small HSP (sHSP) family [5,6]. The sHSP family consists of heterogeneous proteins characterized by a conserved α-crystalline domain (ACD) of ~90 amino acids which flanked by variable N and C-terminal extensions [7–9]. The ACD is a conserved domain with an ancient origin and widely distributes in all kingdoms [10]. In contrast to ATP-dependent chaperones such as HSP90, HSP70, and HSP60, sHSPs lack refolding activity but function as chaperones that prevent the unspecific aggregation of miscellaneous proteins [11]. In addition, sHSPs can assist HSP90 and HSP70 in facilitating the refolding of the bound substrate protein [12–14].

A total of 10 different sHSP-coding genes (HSPB1-B10) have been identified from the human genome [15]. *D. melanogaster* has four major sHSPs (HSP22, HSP23, HSP26 and HSP27) that display distinct intracellular localization and specific developmental patterns of

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https://doi.org/10.1016/j.fsi.2019.10.053
Received 25 August 2019; Received in revised form 22 October 2019; Accepted 27 October 2019
Available online 31 October 2019
1050-4648/

expression in the absence of stress [16,17]. Functional investigations indicate that sHSPs of animals are involved into cell protection, cell differentiation, apoptosis, interaction with the cytoskeleton, lifespan, diapause, oxidative stress and innate immunity [18–20], and their dysfunction has been related to many diseases such as cancer development, cardiovascular diseases, cataracts, myopathy, and neuron diseases [9,21,22]. Recently, multiple members of sHSP family have been identified in numerous insect species. For example, 10 sHSP genes are known in *Apis mellifera*, 7 in *Anopheles gambiae*, 11 in *D. melanogaster*, 10 in *Tribolium castaneum*, 16 in *Bombyx mori*, 14 in *Plutella xylostella*, 15 in *Choristoneura fumiferana*, and 5 in *Bemisia tabaci* [23]. These homologs of sHSP play pivotal roles in insect development and in tolerance to diverse biotic and abiotic stress factors. While the sHSP genes are still rarely reported in crustaceans with a few exceptions, such as HSP21 in *Penaeus monodon* [24], HSP21 in *Fenneropenaeus chinensis* [24], HSP37 in *Macrobrachium rosenbergii* [25] and p26 in *Artemia franciscana* [26]. A lot of work is still needed to further reveal their functions in crustacean.

The need for healthy breeding has led us to pay attention to the mechanisms of immune defense and stress resistance in the economical aquatic crustaceans. A better knowledge of the structural characteristics, evolutionary relationship and biological function of crustacean sHSPs would provide relevant insights regarding the biology of these organisms and their strategies for defensing adverse environments. In the present study, our main goal is to identify a novel member of sHSP family in *M. nipponense*, characterize its expression profiles under a variety of stresses, and investigate its anti-aggregation activity *in vitro*.

2. Materials and methods

2.1. Prawns and tissue sampling

Wild prawns, *M. nipponense*, with average body weight of approximately 2.0 g ± 0.5 g were obtained from Baiyang Lake, where is the highest dimensional natural habitat of *M. nipponensis* in China. The prawns were cultured in 50-L tanks with aerated freshwater at 21 ± 1 °C and fed with artificial bait twice per day for at least one week prior to experimentation in the laboratory. Hemolymph was drawn using a syringe from healthy prawns in an equal volume of anticoagulant (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH7.0) and haemocyte were immediately isolated by centrifugation at 800*g* for 10 min. Meanwhile, intestine, muscle, gill, and hepatopancreas were dissected out from the prawns. All the haemocytes and tissues were preserved in liquid nitrogen for further detection of gene expression.

2.2. Bacterial challenge, heavy metal exposure and oxidative stress

For immune stimulation experiments, 60 healthy prawns were selected for injecting with a 6.5 μL suspension of *Aeromonas veronii* (5×10^7 CFU/mL) in physiologic saline solution into the last abdominal segment of each individual. The muscle from six surviving individuals was randomly sampled at 0, 6, 12 and 24 h post injection for qPCR analysis.

For heavy metal exposure trials, 100 robust prawns were randomly divided into two equal groups and kept in tank water with 0.1 mg/L $CdCl_2$ and 0.5 mg/L $CuCl_2$ respectively. At 0, 6, 12, 24, and 48 h post heavy metal treatments, 6 prawns per treatment group were randomly selected and their muscle was dissected on ice for qPCR analysis.

Doxorubicin (DOX), a radical-generating agent, was used for oxidative stress treatment, which is believed to disrupt redox balance in cell by interfering with the mitochondrial phospholipid cardiolipin and causing the production of reactive oxygen species (ROS) [27]. Firstly, 60 accommodated prawns were equally divided into 6 groups and injected with about 20 μL DOX solution with doses at 0, 0.5, 1.25, 2.5, 5, and 10 μg/g body weight respectively. 6 individuals from each group were sampled and their muscle was ablated at 24 h post injection for qPCR analysis. Then, 40 healthy prawns were injected with DOX solution as a single dose of 1.25 μg/g body weight. The muscle from six surviving individuals were randomly sampled at 0, 12, 24, and 48 h post injection for qPCR analysis and concentration measurement of ROS and malondialdehyde (MDA).

Table 1
Nucleotide sequences of primers used for amplification in this study.

Primers	Sequences (5′–3′)
AOLP	GGCCACGCGTCGACTAGTACT$_{16}$ (G/A/C)
AP	GGCCACGCGTCGACTAGTAC
HSP-F1	GATGGCTCTTCAATGTCGTCG
HSP-F2	ATGTCATCCTCCAAGTCCCT
HSP-R1	TTACTGCTCAGGCAACTTCA
HSP-qF	GATGGCTCTTCAATGTCGTCG
HSP-qR	ACGACTGCTGGACGGAGGAAC
Act-F	GAGAAATCCTATGAACTTCCCGACG
Act-R	GGATACCGCAAGATTCCATACCCAA
HSP-exF	CGCGGATCCATGTCATCCTCCAAGTCCCT
HSP-exR	CCGCTCGAGTTACTGCTCAGGCAACTTCA

2.3. RNA extraction and reverse-transcription

Total RNA was extracted from haemocytes and different tissues with Trizol reagent (Invitrogen, USA) following the manufacturer's protocol and treated with RQ1 RNase-Free DNase (Promega, USA) to remove contaminated DNA. cDNA was reverse-transcripted from total RNA by M-MLV reverse transcriptase (Promega, USA) following the manufacturer's protocol with a universal primer AOLP (Table 1).

2.4. Cloning and analysis of the MnHSP28.6 gene

Primer HSP-F1 was designed based on the transcript sequence of MnHSP28.6 obtained from *M. nipponense* transcriptome database (SRX142691). 3′RACE was performed with the gene-specific primer HSP-F1 and an anchor primer AP. The PCR products were gel-purified and cloned into pMD19-T vector (TaKaRa, China). After being transformed into the competent cells of *Escherichia coli* DH5α, the positive recombinants were identified through PCR screening and sequenced by Shanghai Sangon Company (China). According to the transcript sequence and the obtained sequence by 3′RACE, a couple of specific primers HSP-F2 and HSP-R1 were designed for verification of the full-length ORF sequence by PCR and sequencing.

The deduced amino acid sequence was obtained using an ORF finder program (http://ncbi.nlm.nih.gov/gorf/gorf.html) and then analyzed with the Expert Protein Analysis System (http://www.expasy.org). The potential signal peptide was predicted through SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The protein domains were searched via web CD-search tools, including SMART for Ensembl database (http://smart.embl-heidelberg.de) and Batch for NCBI database (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). The homology search against the Nr, Swissprot and Landmark database was performed using BLASTp and Smart BLAST algorithms at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were carried out with CLC Main Workbench software. A phylogenetic tree was constructed using the amino acid sequences of MnHSP28.6 and its homologs mainly gained from Swissprot database by Neighbor-joining (NJ) method. One thousand bootstraps were performed to assess nodal support.

2.5. Quantitative real-time PCR (qPCR) analysis

Transcript level of MnHSP28.6 was measured by qPCR on a LightCycler system (Roche, USA) using the SYBR Green kit (TaKaRa, China). The primer sequences used for amplifying target gene (HSP-qF/

qR) and β-actin (Act-F/R) gene as internal control were listed in Table 1. Expression level of the target gene was calculated by comparing the cycle threshold value (Ct) to the reference gene β-actin. The relative mRNA expression was calculated using the ΔΔCt method [28]. All the results were generated with three technical replicates.

2.6. Concentration measurement of ROS and MDA

To verify the oxidative stress resulted from DOX injection, the total proteins from muscle were extracted using a KEYGEN protein extraction kit (Keygen, China) and subjected to ROS and MDA analysis as biomarkers for oxidative stress, according to a previously described method [29]. Specifically, the ROS levels were quantified according to reactive oxygen species assay kit instructions (Nanjing Jiancheng Bioengineering Institute, China). A MDA detection kit was chosen to figure out the MDA content as a marker of lipid peroxidation according to the instructions (Nanjing Jiancheng Bioengineering Institute, China).

2.7. Recombinant expression, purification and western blot analysis

The DNA fragment encoding MnHSP28.6 was obtained by PCR with a pair of primers HSP-exF/R with restriction enzyme sites for *Bam*H I and *Xho* I at the 5′-end. Both the amplified fragment and the expression vector pET-30a were digested with *Bam*H I and *Xho* I, and ligated together with T4 DNA ligase. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for expression. After being induced by 1 mM isopropyl β-D-thiogalactoside (IPTG) at 28 °C for 5 h, the cells were harvested by centrifugation and the recombinant MnHSP28.6 (rMnHSP28.6) protein was purified using Ni-NTA Resin (GenScript, China). The proteins were separated by reducing 12% SDS-PAGE, and visualized with Coomassie bright blue R250. The concentration of purified rMnHSP28.6 was quantified by the Bradford method. The purified rMnHSP28.6 was used as an antigen to produce rabbit polyclonal antibody by the traditional method [30].

Proteins of different tissues from healthy prawns were isolated and subjected to western blot for MnHSP28.6 expression profiles analysis. The total proteins of prawn were extracted with a KEYGEN protein extraction kit (Keygen, China) and analyzed by SDS-PAGE. After electrophoresis, the peptides were transferred to nitrocellulose membranes with a Mini Trans-Blot system (Bio-Rad, USA). The rabbit anti-MnHSP28.6 polyclonal antibody (1:100 dilution) was used as the primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) (1:300) was used as the secondary antibody. GAPDH was used as the reference.

2.8. Anti-aggregation assays toward model substrates

Aggregation assays of the model proteins citrate synthase (CS) and insulin were performed according to previously described methods [15] with slight modification. Thermal aggregation of CS (200 μM) (Sigma, USA) was monitored in HEPES buffer (40 mM HEPES, pH7.5, 1 mM DTT) at 50 °C. Reduction-inducted aggregation assay of insulin (200 μM) was performed at 37 °C in the presence of 20 mM dithiothreitol (DTT) in PBS butter. To analyze the effect on the aggregation of model substrates, purified rMnHSP28.6 were added at different concentrations to a reaction mix and preincubated 5 min at experimental temperature followed by adding the substrate protein. All reactions were continuously monitored at 360 nm for CS or 412 for insulin in a Synergy HTX multifunctional microporous plate detector (BioTek, USA).

2.9. Prevention of protein aggregation in muscle extracts

The muscle of prawn was dissected on ice and transferred to a microfuge tube and snap-freeze by immersing in liquid nitrogen. About 5 mg tissue was homogenized using an electric homogenizer in 1 mL ice-cold PBS containing 1 mM DTT and protease inhibitor PMSF. Tissue debris was removed by centrifugation at 16, 000 × g for 10 min at 4 °C. The total protein concentration was determined by Bradford assay using BSA as a standard. Thermal aggregation of tissue extract was analyzed according to an adapted method [15]. In brief, the muscle lysate was incubated for 90 min at 45 °C in a water bath at a concentration 3 mg/mL without or with the addition of rMnHSP28.6 to the final concentration at 4 μM–64 μM. Then the samples were centrifuged for 10 min at 8, 600 × g to separate insoluble proteins. Pellets were washed twice with ice-cold PBS, dissolved in 1-fold loading buffer, heated in boiling water for 2–5 min, and analyzed by 10% SDS-PAGE. Gels were stained with Coomassie bright blue R250, scanned with CanoScan 5600F (Canon, Japan), and the amount of insoluble proteins in each lane was quantified by densitometry using the one-dimensional gel analysis tool in the Quantity One software (Bio-Rad, USA). The amount of insoluble proteins in each sample was normalized to that in sample without rMnHSP28.6 and the resulting values were plotted against the rMnHSP28.6 concentration. Each assay was repeated at least three times, and the mean ± S.D. was calculated.

2.10. Superoxide dismutase assay

In addition to aggregation assays, the total protein extracts from prawn muscle was also used for determination of superoxide dismutase (SOD) activity by using a SOD assay kit (WST-1 method) (Nanjing Jiancheng Bioengineering Institute, China). SOD activities in the muscle extracts were measured after incubation at 45 °C for 90 min with or without rMnHSP28.6.

2.11. H_2O_2 tolerance assay of MnHSP28.6-expressing E. coli

The antioxidant ability of MnHSP28.6 *in vivo* was further tested via H_2O_2 tolerance assay according to the method described by Tang et al. [31]. Briefly, both *E. coli* expressing MnHSP28.6 and strain containing empty vector pET30a were cultured in LB medium with kanamycin at 37 °C till OD_{600} reaches 0.6. Protein expression was then induced for 1 h with IPTG at final concentration of 1 mM at 28 °C. Then the bacteria were diluted into equal density (OD at 600 nm). 200 μL cells expressing MnHSP28.6 or not were mixed with 200 μL H_2O_2 at concentrations of 0, 10, 20, 25 and 30 mM and incubated with gentle shaking at 28 °C for 1 h. Subsequently, a 20 μL droplet of each trial was plated onto LB medium and incubated overnight at 37 °C. Bacterial growth was observed and recorded. All the experiments were repeated three times.

2.12. Statistical analysis

All data were expressed as mean ± S.D.. Statistical analysis was performed using SPSS software (Ver 18.0) and statistical significance at $p < 0.05$ was analyzed using one-way ANOVA.

3. Results

3.1. Sequence analysis of MnHSP28.6

Based on the *M. nipponense* transcriptome database search, a transcript homologous to sHSP gene was identified. Its full-length cDNA was cloned via RACE method and verified by PCR and sequencing. Sequence analysis showed that this gene encoded a protein of 263 amino acid residues with a theoretical size of 28.6 kD and a predicted isoelectric point of 5.05. So we named this protein as MnHSP28.6. No putative signal peptide was present at the N terminal of the protein. Protein domain analysis indicated that MnHSP28.6 protein contained a typical α-crystalline domain (ACD) (from Q77 to K160) (Fig. 1A), which consists of 84 amino acid residues. Smart BLAST analysis against the Landmark database indicated that the ACD sequence of MnHSP28.6 shared 41.2%, 35.8%, 32.7%, and 32.2% identities with those of zebra

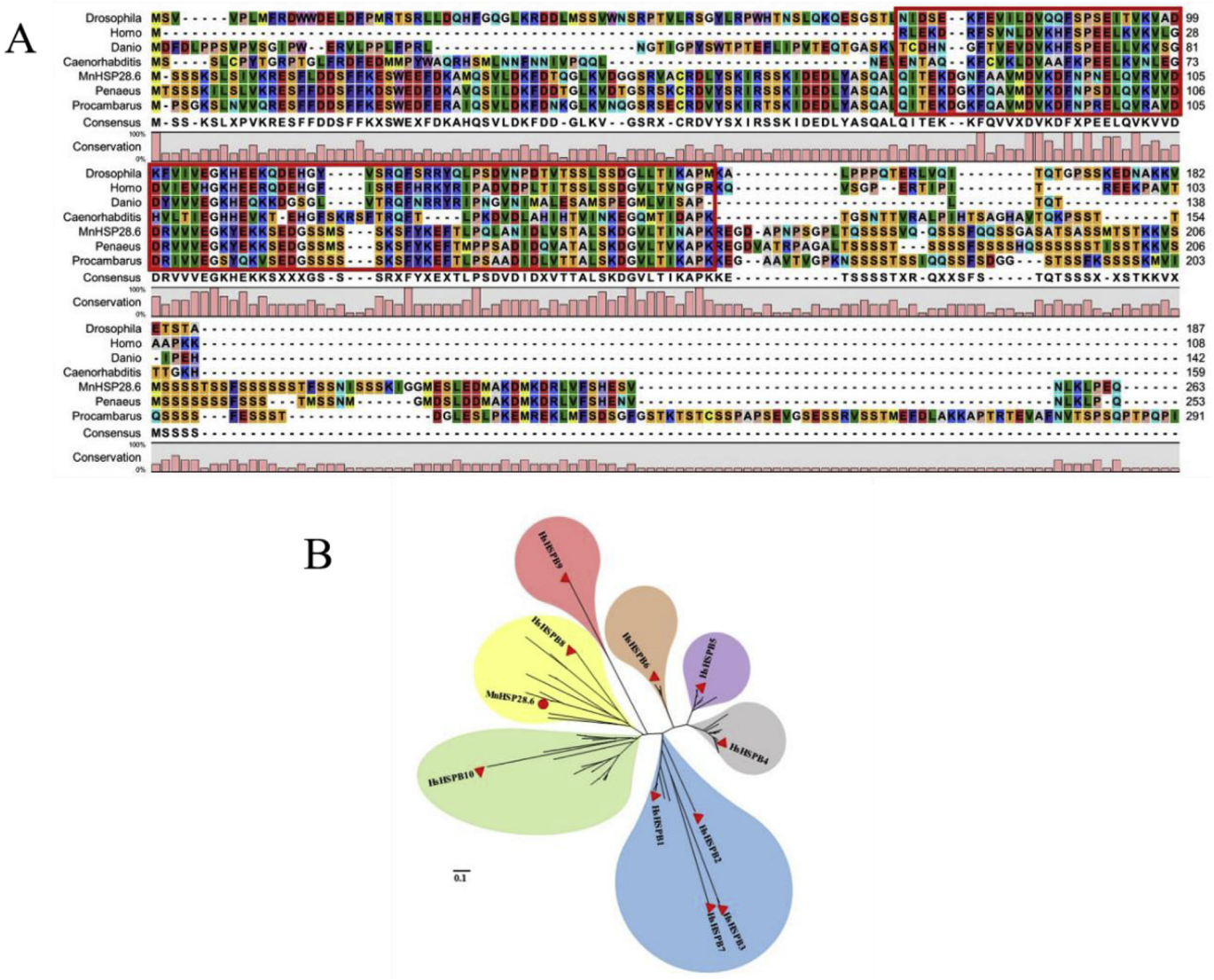


Fig. 1. Molecular characterization of MnHSP28.6. (A) Multiple sequence alignments of MnHSP28.6. The amino acid sequences of homologs were from *Danio rerio* (NP_001094428), *Homo sapiens* (NP_001317308), *Drosophila melanogaster* (NP_523827), *Caenorhabditis elegans* (NP_499316), *Penaeus* (*Litopenaeus*) *vannamei* (XP_027239519) and *Procambarus clarkii* (AGZ84434). The deduced α-crystalline domain (ACD) is boxed. (B) Phylogenetic analysis of sHSPs and their homologs proteins gained from the Swissprot database using the neighbor-joining method with a bootstrap value of 1000.

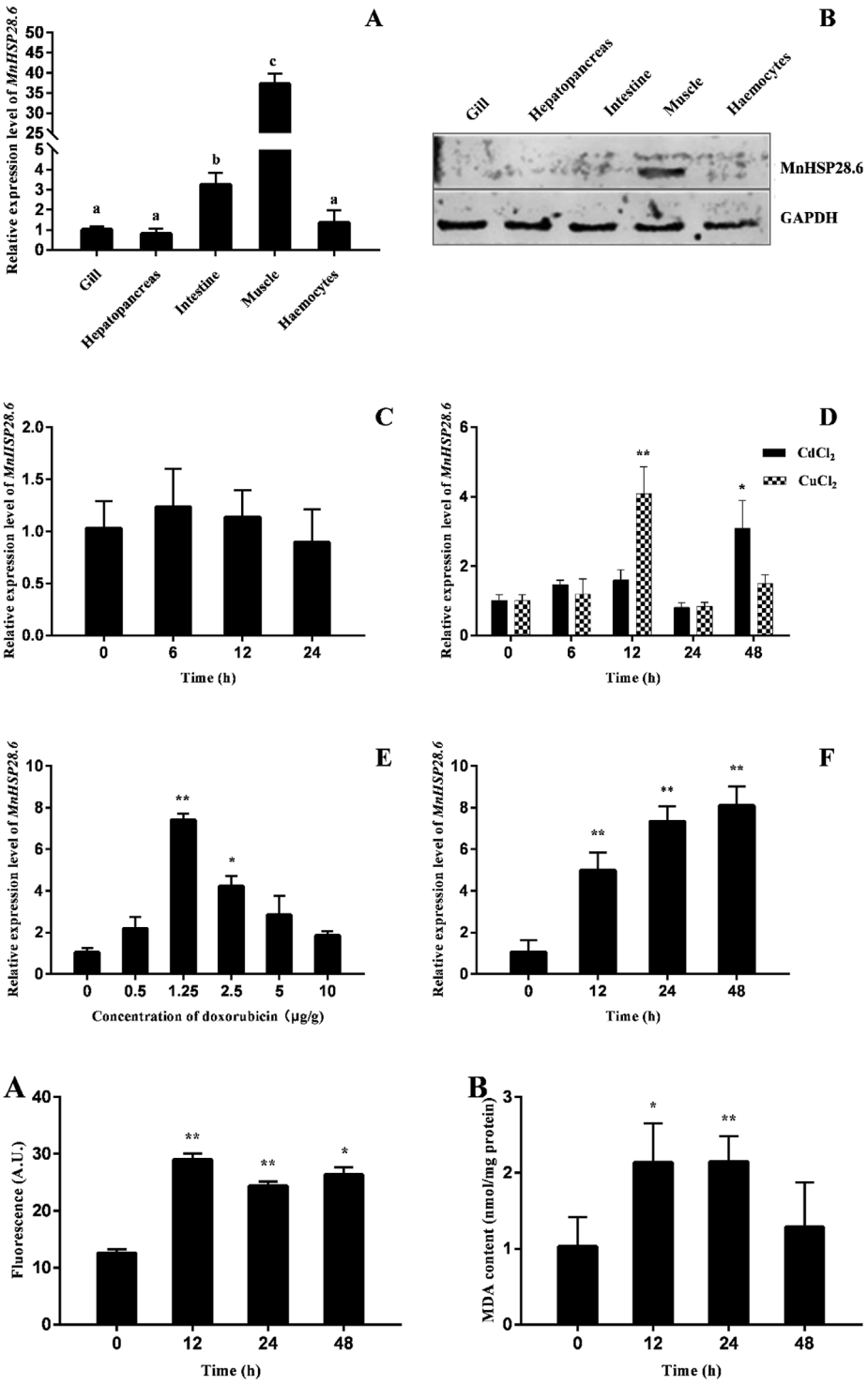
fish heat shock protein β-6 (NP_001094428), human α-crystallin B chain isoform 2 (NP_001317308), fruit fly lethal (2) essential for life, isoform A (NP_523827) and nematode stress-induced protein 1 (NP_499316). BLASTp analysis against Nr database displayed that MnHSP28.6 were highly homologous with protein lethal (2) essential for life from *Penaeus vannamei* (XP_027239519; identity = 70.2%) and heat shock protein 27 from *Procambarus clarkii* (AGZ84434; identity = 79.4%). A multiple alignment among these sequences highlighted the conservatism of the ACD (Fig. 1A). Homologous protein sequences (S1), including 10 human sHSPs (HSPB1-10), screened mainly from Swissprot library were used for building a phylogenetic tree (Figs. 1B and S2). When the 10 human sHSPs were highlighted in the tree, we found that six main groups could be recognize: the mammalian HSPB1, 2, 3 and 7 were brought together to form an outer group of the entire system tree, other five discernible groups contained HSB10, 8, 6, 5 and 4 respectively. Lonely HSPB9 existed independently and constituted the root of groups HSPB10 and 8. MnHSP28.6 was first aggregated with three other crustacean homologs and then combined with fish and amphibian homologs to form a branch of group HSPB8. That is to say, MnHSP28.6 has the highest homology with mammalian HSPB8, which also known as HSP22, H11 protein kinase or product of E2IG1 gene.

3.2. Expression levels of MnHSP28.6 in different tissues

The distribution of MnHSP28.6 in different tissues was assessed by qPCR and western blot assay. The result of qPCR showed that the transcription of MnHSP28.6 gene occurred predominantly in muscle tissue with weak mRNA signals detected in other tissues including intestine, gill, hepatopancreas and haemocytes (Fig. 2A). While MnHSP28.6 protein was only detected in muscle, not in other tissues by western blot analysis (Fig. 2B).

3.3. Expression profiles of MnHSP28.6 in response to various stresses

The relative expression levels of MnHSP28.6 mRNA in muscle were analyzed using qPCR following immune challenge, heavy metal exposure and oxidative stress resulted from *A. veronii* infection, Cd^{2+} and Cu^{2+} administration, and DOX injection. During bacterial infection, there was no significant alteration in MnHSP28.6 expression, only a



Figur 2. Spatial and temporal expression analysis of MnHSP28.6. Tissue distribution of MnHSP28.6 in prawn was analyzed by qPCR. β-actin was used as the internal control (A). Tissue distribution of MnHSP28.6 in prawn by western blot with GAPDH as the internal reference (B). Expression profiles of MnHSP28.6 mRNA relative to β-actin was analyzed by qPCR in the prawn muscle during bacteria *A. veronii* infection (C), heavy metal Cd^{2+}, Cu^{2+} exposure (D), and oxidative stress induced by doxorubicin (DOX) (E, F). The value was shown as mean ± S.D. and bars with different letters were significantly different ($p < 0.05$). * denotes significant difference ($p < 0.05$); ** denotes highly significant difference ($p < 0.01$). Six biological replicates were carried for each sample. Each experiment was performed at least 3 times.

Fig. 3. Analysis of oxidative stress induced by DOX injection. Intracellular levels of ROS (A) and MDA (B) in prawn muscle after injection of DOX at 1.25 μg/g body weight. Data shown above were the mean values ± S.D. of the three separate experiments. * denotes significant difference ($p < 0.05$); ** denotes highly significant difference ($p < 0.01$).

slight but not significant transient up-regulation appeared in muscle at 6 h post bacterial injection (Fig. 2C). However, dramatic up-regulations of MnHSP28.6 transcript were recorded post stimulations by other stress factors, including Cd^{2+}, Cu^{2+} and DOX with different temporal patterns. A similar about 5-fold increase of MnHSP28.6 mRNA compared to untreated control group was observed at 12 h during either Cd^{2+} or Cu^{2+} exposure (Fig. 2D). In the oxidative stress experiment, a significant up-regulation of MnHSP28.6 transcript was detected in muscle responding to DOX injection in a dose- and time-dependent manner (Fig. 2E and F). The highest expression level presented at 48 h post injection of DOX at 1.25 μg/g body weight. Meanwhile, significantly increased contents of ROS and MDA in muscle verified the occurrence of oxidative stress resulted from DOX injection (Fig. 3).

3.4. Recombinant expression of MnHSP28.6

For biological function analysis, MnHSP28.6 was subjected to recombinant expression in *E. coli* BL21 (DE3). After 8 h induction with 0.1 mM IPTG, the target protein rMnHSP28.6 with a His tag was verified by SDS-PAGE and western blot analysis using a rabbit His-tag antibody (data not shown). The soluble recombinant protein (rMnHSP28.6) was purified using the immobilized nickel-affinity

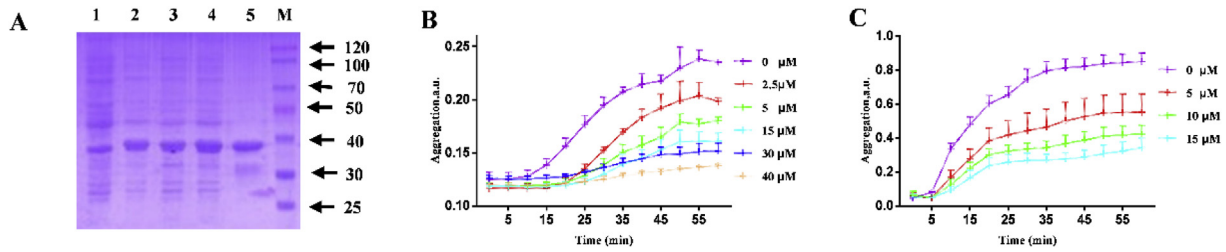


Fig. 4. Recombinant expression of MnHSP28.6 and its anti-aggregation activity against model substrates. (A) SDS-PAGE analysis of rMnHSP28.6 protein. Lane 1: negative control for rMnHSP28.6 (without induction); Lane 2–4: IPTG induced rMnHSP28.6; Lane 5: purified rMnHSP28.6; Lane M: protein molecular standard. (B) The effect of rMnHSP28.6 on the thermal aggregation of citrate synthase (CS) at 50 °C. (C) The effect of rMnHSP28.6 on the chemical aggregation of insulin by dithiothreitol (DTT). The model substrates CS and insulin were incubated alone or with different concentration rMnHSP28.6 under denatured conditions. The aggregation kinetics were followed by monitoring the apparent opticaldensity at 360 nm for CS or 412 nm for insulin. Data shown above were the mean values ± S.D. of the three separate experiments.

chromatography. The molecular size and expression quantity of the recombinant protein were confirmed via SDS-PAGE (Fig. 4A). The antiserum against MnHSP28.6 was prepared using the recombinant protein.

3.5. Chaperone activity of MnHSP28.6 toward model substrates

A standard chaperone assay was employed to test the anti-aggregation activity of rMnHSP28.6 using citrate synthase (CS) and insulin as model substrate proteins. The temperature-induced aggregation of CS, and reduction-induced aggregation of insulin were measured by detecting increased light scattering. Aggregation processes were monitored for 60 min (until reaching the plateau) following the increase in light scattering by recording the absorbance at 360 or 412 nm. The effects on the aggregation kinetics of the model substrates (Fig. 4B and C) demonstrated rMnHSP28.6 could suppress aggregation of both CS and inulin effectively.

3.6. Chaperone activity of MnHSP28.6 toward tissue proteins

To understand the chaperone activity of MnHSP28.6 in a biologically complex environment, we used muscle extracts of prawn as substrates in the chaperone assay. Incubation at 45 °C resulted in evident protein aggregation of tissue extracts as indicated by the presence of insoluble proteins. As expected, pre-addition of rMnHSP28.6 into the tissue extracts effectively reduced the thermal aggregation of proteins (Fig. 5A). The maximum aggregation inhibition rate was about 58% (Fig. 5B).

3.7. Protection of SOD activity in prawn muscle by MnHSP28.6

In order to further clarify the role of MnHSP28.6 in antioxidants, the activity of SOD as a representative in prawn muscle was detected following thermal treatment at 45 °C. The result showed that addition of rMnHSP28.6 remarkably blocked the SOD inactivation insulted from heat treatment (Fig. 5C).

3.8. H_2O_2 tolerance assay of E. coli expressing MnHSP28.6

As shown in Fig. 6, *E. coli* expressing MnHSP28.6 and strain containing empty pET30a plasmid showed almost the same viability in normal LB medium. When pretreated with 10–30 mM H_2O_2 for 1 h at 28 °C, the clone number of overnight cultured *E. coli* expressing MnHSP28.6 was significantly more than that of the empty vector control. The result suggested that MnHSP28.6 endowed *E. coli* vigorous resistance against oxidative stress induced by H_2O_2.

4. Discussion

Small heat shock proteins (sHSPs) constitute a diverse and widespread family of molecular chaperones, which are ubiquitously present in nearly all living organisms, including virus, bacteria, fungi, plants and animals. The human genome encodes 10 major sHSPs, which possess distinctive developmental expression patterns, tissue distributions, intracellular localizations, and substrate specificities [15]. Here, we identified a sHSP gene, named MnHSP28.6 according to the molecular weight of the protein it encodes, and investigated its expression features as well as the structure and chaperone function of the encoded protein.

MnHSP28.6 protein contains a typical α-crystallin domain (ACD), which is flanked by a 76-AA (amino acid) N-terminal region (NTR) and a 103-AA C-terminal extension (CTE). The ACD represents the conserved signature motif of sHSPs. Multiple alignment analysis of sHSP homologs from mammal, fish, insect, crustacean and nematode shows

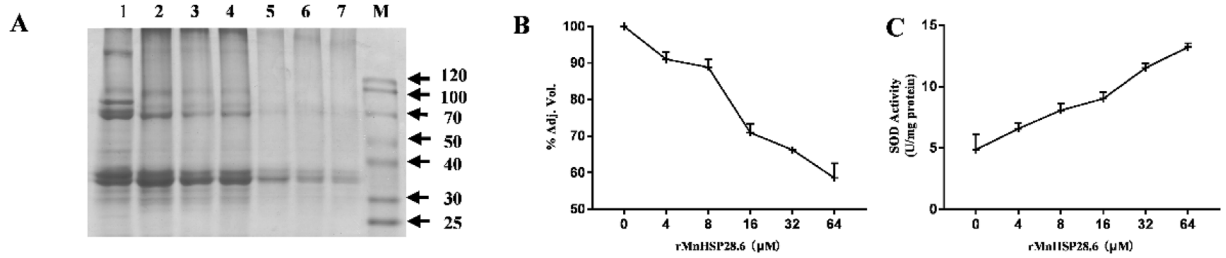


Fig. 5. The protective effects of rMnHSP28.6 against the temperature-induced protein aggregation and SOD inactivation of prawn muscle extracts. (A) SDS-PAGE analysis of precipitated proteins. 3 mg/ml soluble muscle extract was incubated at 45 °C for 90 min in PBS. After incubation, precipitated protein was collected by centrifugation and analyzed by 10% SDS-PAGE. Lane 1: the total soluble proteins of the muscle extract. Lane 2–7, the precipitated protein after thermal treatment in the presence of rMnHSP28.6 at 0, 4, 8, 16, 32 and 64 μM. (B) The relative amount of insoluble proteins in each sample was quantified by densitometry using the one-dimensional gel analysis tool in the Quantity One software (Bio-Rad, USA) and normalized to the total amount of insoluble proteins in samples without rMnHSP28.6. (C) The SOD activity in muscle extract after heat treatment at 45 °C for 90 min in the presence of different concentrations of rMnHSP28.6. Each assay was repeated at least three times, and the mean ± S.D. was calculated.

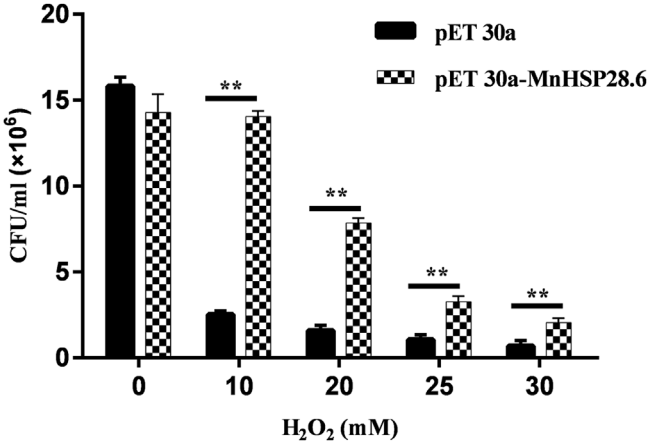


Fig. 6. H_2O_2 resistance analysis of MnHSP28.6 *in vivo*. *E. coli* expressing MnHSP28.6 or containing empty plasmid as the control was cultured on solid LB medium following pretreatment by H_2O_2. Numbers of plate colony were plotted against each concentration of H_2O_2. Bars represent the mean ± S.D. (n = 3).

that their ACD sequences are conservative. Structurally, ACD is composed of β-strands forming a compact antiparallel β-sandwich highly similar in three-dimensional folding structure [32]. Besides the central ACD structure, the multiple alignment highlights a conserved NTR and an extended Serine-rich CTE in the crustacean sHSPs. The multiple functional roles of the variable and unstructured N- and C-terminal regions in mammalian sHSPs have been proposed, such as regulating interactions with target proteins during chaperone action, protecting the ACD from deleterious amyloid fibril formation, and providing solubility for sHSPs under chaperone and nonchaperone conditions [33]. The characteristic flanking regions of the crustacean sHSPs may imply some distinct roles for adaption to unique selection pressures.

sHSPs tend to have specific tissue distribution, such as human HSPB2, 3 and 7 are mainly expressed in cardiac and skeletal muscle, HSPB6 mainly in smooth and cardiac muscle, HSPB5 mainly in eye lens and muscles, HSPB4 in eye lens, HSPB8 mainly in brain and muscles, HSPB9 and 10 specifically in testis, while HSPB1 has a ubiquitous distribution in various tissues [15]. Here, MnHSP28.6 is predominantly expressed in prawn muscle. So, muscle samples will be taken for the following work.

Positive expression regulation of sHSPs during infection by viral and bacterial pathogens has been reported in crustaceans and molluscs. For example, *Macrobrachium rosenbergii* sHSP (MrHSP37) is potentially involved in the immune responses against the infectious hypodermal and hematopoietic necrosis virus (IHHNV) [25]. A 23.2-kD sHSP (Tg-sHSP) of bloody clam (*Tegillarca granosa*) involved in the immune response against *Vibrio parahaemolyticus* and lipopolysaccharide [34]. In contrast, HSP21 gene of *Penaeus monodon* is down-regulation after the white spot syndrome virus (WSSV) infection [24]. In *Fenneropenaeus chinensis*, the expression of FcHSP21 was inhibited by muscle-injecting WSSV while induced by feeding WSSV [35]. In the present study, the expression of muscle-specific MnHSP28.6 appears no change in response to bacterial infection within 48 h post bacterial injection.

However, the expression of MnHSP28.6 is strongly enhanced by both Cd^{2+} and Cu^{2+} treatments. Heavy metals are one of the most common abiotic stress factors leading to hazardous effects in organisms, which have been well-documented to increase the expression of heat shock protein genes. In particular, HSP70 has been proposed as a potential biomarker of heavy metal pollution because of its up-regulation under cadmium exposure in invertebrates, such as *Musca domestica* [36], *Marsupenaeus japonicus* [37], *Litopenaeus vannamei* [38], *Daphnia magna* [39], the freshwater snail *Physa acuta* [40]. In recent years, some small heat shock genes have been described as biomarkers for heavy metal exposures in a few invertebrates, such as *M. domestica* [41], *Oxya chinensis* [42], *Sinonovacula constricta* [43,44], *Chironomus riparius* [45], *Caenorhabiditis elegans* [46]. Although the potential roles of sHSPs in protecting from metal intoxication have been widely declared, the underlying mechanism remains to be fully understood. An important toxic effect of heavy metal ions is inhibition of the chaperone-assisted refolding of chemically denatured and heat-denatured proteins [47]. HSP70 can properly fold nascent proteins and re-fold soluble proteins, whereas sHSPs can disassemble aggregate proteins; both work to correct non-native protein interactions [48]. So we propose that the elevated expression of MnHSP28.6 function as an inhibitor for prevention of heavy metal-induced aggregation. The further anti-aggregate assays of recombinant MnHSP28.6 against both model proteins as well as total proteins from tissue extracts strongly support this view. The results clarify the chaperone role of MnHSP28.6 in inhibition of both single protein and protein mixture denature.

Another toxicity of heavy metals is related to induction of oxidative stress, an imbalance between the production and elimination of reactive oxygen species (ROS). Abundant evidence supports the view that ROS play a dual role in biological systems, since they can be either deleterious or beneficial to living systems [49,50]. The beneficial effects of ROS are manifested in the involvement of immune responses to infection and many cellular signaling systems. On the other hand, excessive accumulation of ROS in cells that experience oxidative stress can cause radicals related damage of DNA, proteins and lipids, and play a key role in the development of a variety of diseases, such as cancer, atherosclerosis, arthritis and neurodegenerative diseases [51]. The harmful effects of ROS can be balanced by the antioxidant action of enzymatic and non-enzymatic antioxidants [52]. In addition to these classical antioxidants, small heat shock proteins have recently been implicated in the repair of, and protection against oxidative damage [53–56]. Recombinant heat shock protein 27 (HSP27/HSPB1) can protect against cadmium-induced oxidative stress and toxicity in human cervical cancer cells [48]. A small heat-shock protein, p26, from the crustacean *Artemia* confers mammalian cells (Cos-1) enhanced resistance to oxidative damage [53].

To investigate the antioxidant roles of MnHSP28.6, oxidative stress is induced in *M. nipponense* by injection with DOX, which has been successfully applied in the establishment of oxidative stress model of housefly and prawn [29,57]. Increased levels of ROS and MDA following DOX administration also demonstrate the effectiveness of the experiment. During this process, a dose-dependent up-regulation of MnHSP28.6 is recorded with the most effective induction concentration of DOX at 1.25 μg/g body weight, suggesting its potential role in oxidation resistance. It is further confirmed through an *in vitro* experiment, in which the resistance of rMnHSP28.6 expressing *E. coli* to H_2O_2 is detected. The result shows that rMnHSP28.6 confers host bacteria impressive protection against H_2O_2. Our study displays a positive role of MnHSP28.6 in resistance to oxidative stress. However, it is a topic for future study whether this protection is from chaperoning, ROS scavenging or signal regulating by MnHSP28.6. Like other protective HSPs, the antioxidant mechanism of MnHSP28.6 is unfathomed, and it may be act as a cofactor of other cellular antioxidants and/or HSPs [48,58]. In the present study, SOD activity protection experiment answers this question to some extent, as indicated by the result that rMnHSP28.6 fairly block the heat inducted inactivation of SOD. Given the nonspecific molecular chaperone function, it is not difficult to explain that the heterogeneously expressed recombinant sHSP in bacteria also exhibits appreciable antioxidant ability.

5. Conclusion

We identified and characterized a new sHSP gene (named MnHSP28.6) from *M. nipponense*, which encodes a 28.6-kD protein containing a typical α-crystalline domain (ACD). MnHSP28.6 predominantly expresses in muscle tissue and whose transcription can be

strongly enhanced by both heavy metal exposure and DOX-induced oxidative stress, but not by bacterial infection. Recombinant MnHSP28.6 displays potent chaperone activity *in vitro* and protects against H_2O_2-induced oxidative stress and toxicity in host *E. coli* cell. Our results highlight the protective roles of MnHSP28.6 from *M. nipponense* against protein aggregation, heavy metal toxicity and oxidative stress.

Acknowledge

This work was supported by the National Natural Science Foundation of China (No. 31572327); and the Natural Science Foundation of Hebei Province, China (No. C2019201194).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.053.

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