A novel small heat shock protein of *Haliotis discus hannai*: characterization, structure modeling, and expression profiles under environmental stresses

Bo-guang Sun^{1,2} · Yong-hua Hu^{1,2}

Received: 12 December 2015 / Revised: 16 February 2016 / Accepted: 28 February 2016 / Published online: 29 March 2016
© Cell Stress Society International 2016

Abstract Small heat shock proteins (sHsps) are a class of chaperones with low molecular weight, feathered by a C-terminal α -crystallin domain (ACD). They participate in reestablishing the stability of partially denatured proteins and therefore contribute to cellular homeostasis. In this work, we identified a sHsp homolog (designated as sHsp19) from *Haliotis discus hannai*, an economically important farmed mollusk in East Asia. sHsp19 possesses a sHsp hallmark domain, which exhibits the typical fold of ACD as revealed by a three-dimensional model constructed through an iterative threading assembly refinement method. The amino acid sequence sHsp19 shares low identities with any other known sHsps, with percentages below 35 %. Besides, sHsp19 shows relatively distant phylogenetic relationships with sHsps of various mollusks, including two other identified sHsps of abalone subspecies. qRT-PCR analysis indicated that the expression of *sHsp19* occurred in multiple tissues. Upon exposure to thermal, oxidative, and multiple toxic metal stresses, the level of *sHsp19* mRNA was rapidly elevated in a persistent fashion, with the maximum increase up to 170.58-, 405.84-, and 361.96-fold, respectively. These results indicate sHsp is a novel sHsp that possesses the distinguishing structural feature of sHsps but has remote homologies with known sHsps. It is

likely to be important in stress adaptation of abalone and may be applied as a bioindicator for monitoring pollution or detrimental changes of environment in abalone culture.

Keywords Mollusk · Abalone · sHsp · Environmental stress · Bioindicator

Introduction

Heat shock proteins (Hsps) are a large group of chaperones distributed in a great variety of organisms including animals, plants, and bacteria. Their main function is to promote protein folding and thereby prevent cellular accumulation of non-native proteins (Hartl and Hayer-Hartl 2002; Mymrikov et al. 2011). Hsps are divided into several families, including Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (sHsps) (Kampinga et al. 2009).

sHsps are the smallest members of the Hsp superfamily with molecular masses ranging mainly from 12 to 43 kDa. Compared to other Hsps, sHsps are highly diverse in their primary sequences except for a conserved C-terminal domain, which is known as the α -crystallin domain (ACD) and consists of 80–100 amino acid residues (Bakthisaran et al. 2015). sHsps can bind to denatured proteins as chaperones in an ATP-independent pattern, protecting them from aggregation to maintain cellular homeostasis. They form a pivotal cellular network to serve as a buffer system for stabilizing the partially unfolded proteins induced by diverse stress factors, such as temperature, heavy metals, organic solvents, reactive oxygen radicals, and so on (Mymrikov et al. 2011; Haslbeck and Vierling 2015). sHsps are known to participate in various physiological processes, such as cell proliferation and differentiation, development, promoting angiogenesis, cytoskeletal organization, apoptosis, and the immune response (Arrigo 2000; Mounier and Arrigo 2002; Kostenko and Moens

✉ Yong-hua Hu
huyonghua@qdio.ac.cn

¹ Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China

² Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

2009; Zhang et al. 2012b). In mollusks, a number of sHsps have been identified and reported to be responsive to diverse environmental stimuli or bacterial infections (Park et al. 2008; Zhang et al. 2010a, b, 2013; Bao et al. 2011; Wan et al. 2012; Li et al. 2013, 2015; You et al. 2014).

Pacific abalone *Haliotis discus hannai* is a commercially valuable mollusk species cultured mainly in China, Korea, and Japan (Li et al. 2012). In recent years, abalone culture has experienced increasing problems with environmental pollution. During the breeding process, the animal has to face multiple abiotic environmental stimuli including temperature variation, heavy metals, and oxidative stress (Nie and Wang 2004). Since the sHsp network plays a vital role in an animal's survival strategy under stress conditions, knowledge regarding the abalone's sHsps is necessary to understand the stress adaptation mechanisms of this mollusk. Animals usually possess multiple sHsps (Haslbeck and Vierling 2015), for example, humans have 10 sHsps and *Caenorhabditis elegans* has 16 sHsps (Bakthisaran et al. 2015). However, so far only two sHsps have been characterized from different abalone subspecies, i.e., the Hsp26 of Pacific abalone *H. discus hannai* (Park et al. 2008), and the Hsp20 of disk abalone *Haliotis discus discus* (Wan et al. 2012). The present work was performed to identify a novel sHsp homolog (sHsp19) from *H. discus hannai*. In addition, to assess the potential of sHsp19 serving as a novel bioindicator, we analyzed its expression profiles in response to multiple environmental stressors including heat shock, oxidative stress, and multiple heavy metal ions.

Material and methods

Animals

Adult *H. discus hannai* (3 years old, averaging 81.2 ± 5.6 mm in shell length) were purchased from an abalone farm (Jiaonan, Qingdao, China). They were kept in aerated seawater (20 ± 1 °C, pH 8.0 ± 0.1 , salinity 30 ± 1 ‰) under a 12-h/12-h light/dark photoperiod and fed with *Laminaria japonica* daily. The animals were acclimatized for 7 days before experimental manipulation. No mortality was observed during the acclimation period and the experiments. Abalones were anesthetized by MS-222 (Sigma, St. Louis, MO, USA) before all experiments, which were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals promulgated by the State Science and Technology Commission of Shandong Province.

Sequence analysis, phylogenetic profiling, and structure modeling

A complementary DNA (cDNA) library of *H. discus hannai* was constructed in a previous work (Qiu et al. 2013a) and subjected to DNA sequencing. One clone was found to comprise the

coding sequence of a sHsp homolog and the 5'- and 3'-untranslated regions (UTR). This gene was designated as *sHsp19* and the sequence has been deposited in the GenBank database under the accession number KT966742. Sequence and phylogenetic analysis was conducted as previously reported (Qiu et al. 2013a). Protein structure prediction was performed with an iterative threading assembly refinement method (Roy et al. 2010).

sHsp19 expression in different tissues under normal physiological conditions

Adductor muscle, digestive gland, foot muscle, gill, hemocyte, and mantle were dissected aseptically from four abalones and immediately frozen in liquid nitrogen and stored at -80 °C. The total RNA was extracted from the tissues with the HP Total RNA kit (Omega Bio-tek, USA). To eliminate any possible DNA contamination, an additional on-membrane DNase I digestion step was included in the extraction, using the OBI DNase I digestion buffer (Omega Bio-tek, USA) according to the manufacturer's instructions. The quality of the RNA was examined by the NanoDrop 2000 (Thermo scientific, USA) and by gel electrophoresis. The purified RNA was adjusted to 0.3 µg/µl with nuclease-free water. One microliter of total RNA was used for cDNA synthesis with the RevertAid™ Reverse Transcriptase (MBI Fermentas, Canada) according to manufacturer's instructions. Quantitative real-time reverse transcriptase-PCR (qRT-PCR) was performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). The reaction was performed in triplicate in a total volume of 20 µl containing 10 µl SYBR Premix buffer, 1 µl cDNA, 0.2 µl each of the primers, and 8.6 µl PCR-grade water. The PCR program was 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s, 59 °C for 15 s, and 72 °C for 30 s. Negative control without cDNA was included in each assay. Melting curve analysis of amplification products was performed at the end of each PCR to confirm the specificity of the amplification. The PCR products were subjected to electrophoresis in 2% agarose gels to verify the sizes of the amplicons. Elongation factor-1- α (EF1A) was used as an internal control for relative quantification (Qiu et al. 2013b). The experiment was repeated three times. The PCR primers for *sHsp19* are Hd-sHsp19F (5'-GACACTTCCCGAGTATTTTCATC-3') and Hd-sHsp19R (5'-TCGTCAGGACACCATCTTTAG-3'). The primers for elongation factor-1- α are EF1AF (5'-TGCTGTCTGATCGTTGCCT-3') and EF1AR (5'-GCTGTCCATCTTGTGATTCCA-3'). The C_t values were analyzed with the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The data were presented as the relative expression levels of *sHsp19* in different tissues compared to the lowest and expressed as means plus or minus standard errors of the means (SE).

***sHsp19* expression in gill under environmental stresses**

The messenger RNA (mRNA) levels *sHsp19* were evaluated when abalones were exposed to three different challenges of abiotic environmental stresses. For the heat shock challenge, abalones were maintained at 28 °C for 1, 2, 4, 8, and 12 h. For the heavy metal challenge, abalones were subjected to 24-h exposure to CuSO₄, HgCl₂, and Pb(NO₃)₂ with final metal ion concentrations of 5, 25, and 125 µg/l in seawater. For the oxidative stress challenge, abalones were exposed to H₂O₂ with a final concentration of 1 mM in seawater for 3, 6, 12, 24, 48, and 72 h. After challenge, four abalones in each

treatment were used for gill collection. Total RNA was extracted from the tissue and used for qRT-PCR was performed as described above with α -tubulin as an internal control (Qiu et al. 2013b). All experiments were repeated three times.

Statistical analysis

All statistical analyses were performed with the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to assess the main effects of indicated treatments. Then the statistically significant differences between controls and treatments were calculated by *t* test (***P*<0.01).

Fig. 1 Sequence analysis of *sHsp19*. In the cDNA sequence, the translation start and stop codons are shown in **bold** and the polyadenylation signal is **boxed**. In the amino acid sequence, the α -crystallin domain is underlined. The predicted residues on the putative dimer interface are marked by *triangles*

```

GGGGACGA
-240 GAGGAGAATATTATCGTTAGCGCAGTTTTCGATAAGAAAGCGCCAGTGTGTCTTCATTT
-180 AGTACTGGGAGAAGATATCTAAGGCAAGTTGAAGACGTGTGGAACGGATCCATTACACGG
-120 GCATTTCACATTGTGTCAAGTTTGTAAAAATAGCTTGGATTGTTCTGGGCTAGACCTT
-60 GGCCTTGCTGTACTTCCGCATTGGAGCGTCAGCCAACGAGACATCCATCAAGCCGCGTC
-248
1 ATGTGCGTCGAACGGGTTACATTCTCTGCTCTTCTCGACTTCCACTACTTCGATGACATC
1 M S L E R V H I P A L L D F H Y F D D I
61 TTCAAGATGCTCACCAGAAATTTGCAAAGATGCACCAAGACATGCGATCAGCGACGCTT
21 F E D A H R E F A K M H Q D M R S A T L
121 CGTCTTACACCGCCGGACCAGCTGATTGAAGCAATAATTCACACACACAGAGGCTGGAG
41 R L T P P D Q L I E G N N S H T Q R L E
181 GCATTATGAAACAGTTGTCTACTAACGAGGACGGCAGTCGGGAATTTCTGCTCAAACCTG
61 A F M K P V V T N E D G S R E F L L K L
241 GATGTCAGTACATTTAAACCAGAAGAAATTCAGTGAAAACGGACGGAAATGAACCTCTCA
81 D V S T F K P E E I Q V K T D G N E L S
301 ATCGAAGCCAAACATGAGGATAAGAATGACACTTCCCGAGTATTTTCATCAATTCTCCCGC
101 I E A K H E D K N D T S R V F H Q F S R
361 CACTATACCTGCGCTGAGGGCATAGTGACCGAAGCACTGGCCTCGACTTTATCTAAAGAT
121 H Y T L P E G I V T E A L A S T L S K D
421 GGTGTCCTGACGATCAAAGCCCCCTTTATTGCCGACAACGCAATTGAGGGACCACCGTCC
141 G V L T I K A P F I A D N A I E G P P S
481 AAGAAACCAGCCCTTGAGAACCAGTGAACGGCGAGAGAATAAACATTGAACGATTATTAA
161 K K P A L E N Q
541 ATTTTATGGAACGGCGATTTTGATTTTTTTCGGCCACATATATTGCCGACAATACAGTTA
601 GCTACTTTAGGTATGTTATCCATGATACGGTACAATTTAATGGAAGTGAAGTTATCTTTC
661 AGTCATTTTCGCTTCTTTTAGTCTTGAGCTATAGAGGCACGCACTAAGTATGTATACCTTG
721 CTTTTTGCCCTTAGATGGTTGGTATATTTAATTTTTTCCAGTTTAAAGAAGATGCAAGT
781 TTAAACTTGTTTTACGGTGCACGTGATCTGTGATTATTGTCACCAATATTACCTGTA
841 TGTTTGTAATAAAATTTGTTGTTGACGTACATGGGCATAGTTTCATGACTAGATTACGCTT
901 TTTTCATATTCTAGTTAACATTACTATGTTTACTTTGAACCTGCGTGGGGATCCATGCTGG
961 TGCTTGTTTATTTAAATCTGAATTGAAAAAACAGGGTTTTTTTCATTTCCTGAGTAC
1021 TTTAGCGACATTAAGTTGAAAACAGTTTTCAAAAGGAATAAATGCAGTTAATATACAAA
1081 ATATATAAATATTCAAACAACCAATTAATTAGTACTAATTGGGCCTTGGCACAATAACA
1141 GCGTATCAAAGGTTTTTATGTCTTTTTACACGTAAGAGTGTGTAATAATATGAACAAAAC
1201 AGACTATTGTGAACAGAGCATAACCAGGTGTTAGTAGATCAAGGTGAATGTCCATGACC
1261 TATGACACCTTGACGACAGGTGAAAACCGGTGCTTGAATCCCAATGATGTGATTCGTC
1321 AGTGGTTAGTGATTGTGAGTGGCCTTGTGGGATAATGAATCATTGTCAAATATCCGAA
1381 AGTAAAGTAACATTTTTTCCCTTTTCAAACAACAAATTTTCATGTCCCTTTTTTTTGTACA
1441 TAAACATAGTGTTTTTTTTTTTGTGTTTGTGATAAATTTTTTCCCGGGTAATTTGCAAA
1501 AAGCCTTTAGCCTGTTTTTGCCCGTCAATTTGGGATTGTTTTTTTTTTGAAAGGTGAA
1561 AGGGCCTTCAAATTTCCCTCCCGTAAAGTACCTTTTGTTTTGGGCCCTGGGGTAAATTTG
1621 CAAATCCCGGATAATACCTTTCCCGGTGTTTGTGTTTTTTTGTGTTTACATTTAAGTAATG
1681 GTTTGGGTTGGTTTTTAAATCCCGCAAAACCGGGTTTTTTCCCTTTCCCTGGGTACTTTA
1741 AGAATTTTGGGGTTGGGGTGGGGCATAATTTTCCTTTGTTCCTGAATAAAAGTTTTTGGC
1801 CCAGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
1861 AAA

```


sHsp19	MSLERVHIPALLDFHYFDIIFEDAHREFAKMHQDMRSATLR	41
<i>Venerupis philippinarum</i> isoform 1	MSWNLVPLLRDFGFFDRORDLESDWVKEFDEDEWKSMDFESSR	43
<i>Sinonovacula constricta</i>	MSWSIVPLAFRDFGFFDRORDLESTWMKEFDDDFKSMDFDTSF	43
<i>Cyclina sinensis</i>	MSWKIVPLFKRDFGFFDRORDLESDWVKEFDEDEWKSMDFESSR	43
<i>Meretrix meretrix</i>	MSWKIVPLFKRDFGFFDRORDLESDWVKEFDEDEWKSMDFESSR	43
<i>Crassostrea gigas</i>	MSLVPVDFDFWGYWDKORSLEPSFEREFARSFEFEFDREL	40
<i>Homo sapiens</i>	MEIPVPVQPSWLRASAPLPGLSAPGRLFQDQ	32
<i>Mytilus galloprovincialis</i> Hsp22	MIGIFFDKQMDMPSFKNDFDKDFPKDAKHSS	32
<i>Venerupis philippinarum</i> isoform 2	MSWVLVPVGMKDFGYEDRORDMPSDWLHLFDDSWRGLGMEDSV	43
<i>Tegillarca granosa</i>	MSGIIPRLCRQVQAIHCPRRMSYRRNLVVFQWGRRRRPFDDLEFPSNALFDH	52
<i>Haliotis discus discus</i>	MRPEFRSSIEDHFRDMN	17
<i>Haliotis discus hannai</i> Hsp26	MLKYDEKLSITDADDLHVEKQTTGDSAPEKTTVRI	37
<i>Chlamys farreri</i>	YRARSVVPRLCRHVQAVARLPLRYRSNIPKQFFFRPKTCRDPWESNFSKGRSFDKHFRSI	61
<i>Argopecten irradians</i>	YRANSVVPRLCRQLRAAARQLRCQSNYPNPFWRMLENRKPWSLHENRSHEFDKHFRSM	61
<i>Mytilus galloprovincialis</i> Hsp24.1	DKKWTSYVKIGDFDAQHVKKVDNGNVIIHAKYTDGNDENGGDTVERKRTVKVPENVDAAEK	120

sHsp19	L.TPPDCLIEGNN.....SHTQRLEAFMKPFVVTNEDSREFLLKLDVSTKKEETQVK	93
<i>Venerupis philippinarum</i> isoform 1	S.RFDRELERIRK..DMFKLDTGSSMLSIEKFFVTDVPVGNKLSIRFDCSQKKEEISVK	100
<i>Sinonovacula constricta</i>	K.KFDEELEQIRR..SMFKLDSGSSMLKVDREFFVTDPTGKKLALRFDCSQKKEEISVK	100
<i>Cyclina sinensis</i>	L.KFDRELEKIKR..DLFKLDTGSSMLKVDREFFVTDPTGKKLALRFDCSQKKEEISVK	100
<i>Meretrix meretrix</i>	M.KFDRELEKIKR..DLFKLDTGSSMLQVERFFVTDPAKNKLAIRFDCSQKKEEISVK	100
<i>Crassostrea gigas</i>	..RMKDEMHKLAP....KELKKDNFLNISNPYVEDASGNKKLHLKFDSCDFKKEEINVK	93
<i>Homo sapiens</i>	FGEGILLEAELALC.PTTLAPYYLRAPSVLPVAVQVPTDPGHFSLVDVKHFSPEETAVK	91
<i>Mytilus galloprovincialis</i> Hsp22	...MDEEIAARMKR..EMFSLTTPESLSKVQDFVQDFTGDKMAIRFDCSQKKEEISVK	87
<i>Venerupis philippinarum</i> isoform 2	R.RFDSELDVRD..EMHRMDVQVELDVRHFFIVDPEGNHKSIRFDCSQKKEEIEVK	100
<i>Tegillarca granosa</i>	MKDFVDCQFKMER..LMDPFARARNMGEILSGEAEVKYDLELFQKLDVROKKEEINVK	110
<i>Haliotis discus discus</i>	K.ARDEMMRRMASNRPFMHNRSTPGQTTITRTEAEVKYDKNREFFVQVQKEVHLKVS	76
<i>Haliotis discus hannai</i> Hsp26	MHEDEDVARILMSD....GEEDVDRTOAEADEKAPALSTSQYFQIRFELSLSKKENIRV	92
<i>Chlamys farreri</i>	F.DEIGQMTRLAFGETASEMPRGGRHVLARSEAAELNVKKAFLSKMNMREDQENVKTK	120
<i>Argopecten irradians</i>	L.DEMGQMTKLVFGDAMEEMPRGRHVLARSEAAELKFDKKAFLSKMNVKDEQEQHVKK	120
<i>Mytilus galloprovincialis</i> Hsp24.1	MHSFMRSDBGTMVLEAPYLHPEERQIQVVPHEGGALVSSLSHSNLMKENVENREFEVKVS	180

sHsp19	TDGNEISIEAKHEDKNDTS.RVFFHQSRRHYTIPGIVTEALASTSKDGVITIKAEFIAD	152
<i>Venerupis philippinarum</i> isoform 1	TMDKRCVHAKHTEESFGR.KVYREFTKEYTLPNAIDPLRTSILSKDGVICQIEAFAAP..	157
<i>Sinonovacula constricta</i>	TMDKRCVHAKHTEESFGR.SVYREFTREYTLQSVDPRLRTSTLSKDGVICQIEAFAAP..	157
<i>Cyclina sinensis</i>	TMDNRCVNAKHTEESFGR.KVYREFTKEYTLPKSVDPRLRTSTLSKDGVIMVEAFAAP..	157
<i>Meretrix meretrix</i>	TMDNRCVHAKHTEESFGR.KVYREFTKEYTLPKNVDPRLRTSTLSKDGVIMVEAFAAP..	157
<i>Crassostrea gigas</i>	TDDRNVVHAKHEEGSKGQ.KVYKEETQTLYLPEGVDPQKQVSTLTNDGVICVEAFAAP..	150
<i>Homo sapiens</i>	VVGEHVEVHARHEERPDEHGFVAREFHRRYRPPGVDPAAVTSALSPFGVLSIQAAPAS.	150
<i>Mytilus galloprovincialis</i> Hsp22	TMDKQTVHAKHEEVSPGR.KVYREFTKSYTLQDQVPLSKSSLTNDGFCQVEAFAAP..	144
<i>Venerupis philippinarum</i> isoform 2	TLDKKQVHAKHVQDEGR.KTQEQFTREFTLPENVDPNKLKSHLSESGVICQIEAFAVPPP	159
<i>Tegillarca granosa</i>	LTDDRVIITGKHEQKADEHGYVSRRESREFVLQNLMDTFSSTLSEDCVITIIQARAKG.	169
<i>Haliotis discus discus</i>	QLENKIVISGKHEARADDHGFVSREFTREELLPENVDTESMTSRTEDCVILIEAKMKG.	135
<i>Haliotis discus hannai</i> Hsp26	VRSGDIVAEHESTCDSY.SETETRRRIRFPDKVDQSMITSVNAEGENTIQAEFLS.	150
<i>Chlamys farreri</i>	IDKDRVITAKQNAESSG.RVSREITREELLPENIDKETINAAVTDEGLVVRIRIKG.	178
<i>Argopecten irradians</i>	INNRRVISAHNPNPDGNG.CVSRREITREELLPENIDVESMKAVTEEGLVNVGRVKG.	178
<i>Mytilus galloprovincialis</i> Hsp24.1	CKDGLITVQAEKQSEDCG.HRESVWRQMTVPRSVLGKNIECLKDEEGNTIRAFVVED	239

			Identities (%)
sHsp19	NAIEGEPSSKKPALENQ.....	168	--
<i>Venerupis philippinarum</i> isoform 1	ASVDAFREFLPIEKL.....	173	34.8
<i>Sinonovacula constricta</i>	DSVEAFREHLPIEKL.....	173	34.5
<i>Cyclina sinensis</i>	ASVDAFREFLPIEKL.....	173	33.9
<i>Meretrix meretrix</i>	ANVDAFREFLPIEKL.....	173	33.3
<i>Crassostrea gigas</i>	KAVEAEKERVPIEYMKKK.....	169	32.6
<i>Homo sapiens</i>	..AQAEPPAAAK.....	160	28.7
<i>Mytilus galloprovincialis</i> Hsp22	KTCVARKEIIEPIEKLMLK.....	162	28.5
<i>Venerupis philippinarum</i> isoform 2	PEPKTKEIMPIEIHLESKTKPKKEAKDESKEGKEEPKGATEEPKEGTSEKK	210	27.4
<i>Tegillarca granosa</i>	..AEIGKERTIKIEKQKQKQKAELEEGKK.....	197	26.0
<i>Haliotis discus discus</i>	..AEDSTERVIEIQKEGTNKEGKTD.....	160	24.7
<i>Haliotis discus hannai</i> Hsp26	QAISGKDEKIVPIIWE.....	166	23.3
<i>Chlamys farreri</i>	..FTEEDMKVIEIER.....	191	19.4
<i>Argopecten irradians</i>	..VTEELEKVIEIQREPLA.....	195	18.3
<i>Mytilus galloprovincialis</i> Hsp24.1	VEMEQKK.....	247	14.9

Fig. 2 Alignment of amino acid sequences of sHsp19 and representative sHsp homologs of human and mollusks. The Genbank accession numbers of the aligned sequences are as follows: *Venerupis philippinarum* isoform 1 (ACU83231), *Sinonovacula constricta* (AGM14597), *Cyclina sinensis* (AET13647), *Meretrix meretrix* (AFK80359), *Crassostrea gigas* (XP_011448781), *Homo sapiens* (NP_653218), *Mytilus galloprovincialis* Hsp22 (AEP02967), *Venerupis philippinarum* isoform 2 (ACU83232), *Tegillarca granosa* (HM125895), *Haliotis discus discus* (Wan et al. 2012), *Haliotis discus hannai* Hsp26 (EF472916), *Chlamys farreri* (AY362760), *Argopecten irradians* (EU277735), and *Mytilus galloprovincialis* Hsp24.1 (AEP02968)

Results and discussion

Sequence analysis, phylogenetic profiling, and structural modeling of sHsp19

The cDNA of *sHsp19* contains a 5'-UTR of 248 bp, an ORF of 507 bp, and a 3'-UTR of 1356 bp with a putative polyadenylation signal (AATAAA) localized at 15 bp upstream of the poly-A tail. The deduced amino acid sequence of sHsp19 is composed of 168 residues and has a theoretical molecular mass of 19.1 kDa and a pI of 5.22. Domain prediction revealed that sHsp19 possesses an α -crystallin domain, located at the C-terminal of the sequence (residues 68–158) (Fig. 1).

Amino acid sequence alignment indicated that sHsp19 shares low overall identities with known sHsps of human and mollusks, with identities ranging from 34.8 (Hsp22 isoform-1 of *Venerupis philippinarum*) to 14.9 % (Hsp24.1 of *Mytilus galloprovincialis*). Interestingly, sHsp19 also shares low identities with the two

identified abalone sHsps, i.e., 24.7 % with HdHSP20 of disk abalone *H. discus discus* and 23.3 % with Hsp26 of *H. discus hannai* (Fig. 2). Phylogenetic analysis indicated sHsp19 is distantly divergent from other sHsps of mollusks, such as *Argopecten irradians*, *Chlamys farreri*, *Crassostrea gigas* (Zhang et al. 2012a), *Meretrix meretrix*, *Tegillarca granosa*, and *V. philippinarum* (Fig. 3). Notably, the phylogenetic tree also revealed unexpected high evolutionary divergence among sHsp19 and two other identified sHsps of abalones (Fig. 3).

The three-dimensional structure predicted by threading modeling indicated that the N-terminal of sHsp19 is mainly composed of α -helices, and the C-terminal region forms a β -sandwich constituted by two anti-parallel sheets of three and four β -strands (Fig. 4). This β -sandwich fold is highly consistent with the structural feature of ACD domain of typical sHsps (Haslbeck and Vierling 2015).

The sHsp protein family is composed of a number of highly diverse members, all of which possess the characteristic ACD domain (Edwards et al. 2011). In our work, both the domain architecture analysis and homology modeling indicated sHsp19 contains an ACD, the hallmark of sHsp family. Nevertheless, the primary sequence of sHsp19 exhibits low overall identities with other sHsps, including the other known homolog of *H. discus hannai*. In addition, phylogenetic profiling implied that sHsp19 has remote evolutionary relationships with other sHsps of various molluscan species, such as clams, mussels, oyster, scallops, and even abalone subspecies. Taken together, these observations suggest sHsp19 is a novel member of sHsp family.

Fig. 3 Evolutionary relationships of sHsp homologs. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The evolutionary distances were computed using the JTT

matrix-based method. Evolutionary analysis was conducted with MEGA6. A total of 18 sHsp sequences were subjected to analysis. Fifteen of them are the same as presented in Fig. 2. The other three sequences are as follows: *Mus musculus* (NP_034094), *Danio rerio* (NP_001002670), and *Xenopus laevis* (NP_001086479). The sequences of abalones are labeled by triangles

Fig. 4 Structure model of sHsp19. The model was generated by the I-TASSER server and presented as a solid ribbon using the discovery studio visualizer software

sHsp19 expression under normal physiological conditions

qRT-PCR analysis indicated *sHsp19* expression occurs in all six examined tissues under normal physiological conditions. The lowest expression of *sHsp19* occurs in hemocyte. In comparison, *sHsp19* expression was found increased in gill, mantle, adductor muscle, foot muscle, and digestive gland by 1.66-, 1.89-, 4.73-, 5.54-, and 23.44-fold, respectively (Fig. 5). Human sHsps have been categorized as class I and class II. Class I sHsps, i.e., HspB1 (Hsp27), HspB4 (α A-

Fig. 5 *sHsp19* expression in abalone tissues under normal physiological conditions. *sHsp19* expression in adductor muscle, digestive gland, foot muscle, gill, hemocyte, and mantle was determined by qRT-PCR. The data were analyzed with the mRNA level compared to that of elongation factor-1- α (the internal control) in each sample and presented relative to the lowest (means $\pm$ SE, $N=3$)

crystallin), HspB5 (α B-crystallin), HspB6 (Hsp20), and HspB8 (Hsp22), are distributed in various tissues, while class II *sHsp* expression is tissue-specific (Bakthisaran et al. 2015). In mollusks, most identified *sHsps* were detected in multiple tissues (Zhang et al. 2010a, b; Bao et al. 2011; Wan et al. 2012; Li et al. 2013). Our results indicated that *sHsp19* is ubiquitously expressed in abalone tissues. In addition, *sHsp19* mRNA was most abundant in digestive gland; this observation is consistent with the tissue distribution analysis of a hard clam (*M. meretrix*) sHsp (Li et al. 2013).

sHsp19 expression in response to multiple environmental stimuli

Given that gill is the primary site exposed to external environments, this tissue was used for evaluating the expression variations of *sHsp19* when abalones were challenged by different environmental stressors, i.e., high temperature, oxidative stress, and heavy metals, and the findings are presented below. The exposure of abalone to high temperature resulted in a rapid induction of *sHsp19* expression, as a 1-h exposure significantly upregulated the expression. The maximum induction of *sHsp19* occurred at a 4-h postexposure (170.58-fold). After 12 h of heat shock, the mRNA levels of *sHsp19* remained significantly higher than the control group (Fig. 6). In mammals, the ubiquitously expressed class I sHsps (Hsp27, α B-crystallins, Hsp20, and Hsp22) are mostly heat-inducible and are pivotal chaperones participating in animals' survival mechanism in detrimental environments (Bakthisaran et al. 2015). Some sHsp have been shown to be able to prevent thermal aggregation of various target proteins (Raman et al. 1995; Rajaraman et al. 1996, 2001). Moreover, multiple sHsp members have been reported to display enhanced chaperone activity at elevated temperatures (Bakthisaran et al. 2015), which benefit from increased exposure of hydrophobic residues caused by heat-induced conformational alteration (Das and Surewicz 1995; Raman and Rao 1997; Chowdary et al. 2004; Lelj-Garolla and Mauk 2006). In mollusks, the expression of a number of sHsps including Hsp26 of *H. discus hannai* and

Fig. 6 *sHsp19* expression in response to high temperature. Pacific abalones were subjected to heat shock at 28 °C. *sHsp19* expression in gill was determined by qRT-PCR at various time points. The mRNA level of *sHsp19* was normalized to that of α -tubulin. Data are shown as means $\pm$ SE ($N=3$). ** $P<0.01$

Fig. 7 *sHsp19* expression in response to oxidative stress. Pacific abalones were subjected to H_2O_2 treatment. *sHsp19* expression in gill was determined by qRT-PCR at various time points. The mRNA level of *sHsp19* was normalized to that of α -tubulin. Data are shown as means $\pm$ SE ($N=3$). $**P<0.01$

HdHSP20 of *H. discus discus* was experimentally reported to be heat-induced (Park et al. 2008; Wan et al. 2012). In addition, *Escherichia coli* producing HdHSP20 exhibited increased thermotolerance (Wan et al. 2012). In the present work, a rapid and intensive induction of *sHsp19* expression was observed upon a relatively mild temperature elevation (28 °C). Although the actual role of *sHsp19* in abalone's survival strategy under unfavorable thermal conditions needs further study, it seems reasonable to speculate that *sHsp* is probably involved in coping with the heat-induced protein denaturation.

The oxidative stress induced by H_2O_2 promoted significant upregulation of *sHsp19* expression, with the maximum induction (405.84-fold) occurring 3 h after H_2O_2 treatment, then the expression levels declined in a time-dependent manner (Fig. 7). A number of lines of evidence have pointed out mammalian *sHsps* could facilitate cell viability under conditions of oxidative stress (Mymrikov et al. 2011). For instance, *Hsp27* could provide cellular protection against oxidative stress via multiple mechanisms, including decrease of ROS level via increasing reduced glutathione (Arrigo 2007); enhanced proteasome-dependent degradation of oxidized proteins (Arrigo et al. 2005); protecting the cytoskeleton components from oxidative damage (Dalle-Donne et al.

2001). However, the functionality of *sHsps* in molluscan defense mechanism against oxidative stress is currently unknown. It was mentioned in a proteomic study that the protein level of a *sHsp* homolog in *Perna viridis* was induced by H_2O_2 treatment (Leung et al. 2011), which seems to be the only report to date regarding molluscan *sHsp* under oxidative pressure. In the present work, we observed quick and persistent elevation of *sHsp19* mRNA upon H_2O_2 stimulation, suggesting a possible significant role of this protein in abalone's survival under oxidative environment.

Exposure of the abalone to heavy metals (Cu, Hg, and Pb ions, respectively) enhanced the expression *sHsp19* in a dose-dependent manner. Of the three heavy metal stressors, *sHsp19* was more sensitive to Hg^{2+} stimulation (maximum 361.96-fold upregulation); the maximum upregulation induced by Cu^{2+} and Pb^{2+} was 93.75- and 77.89-fold, respectively (Fig. 8). In mammals, *sHsp* have been implicated in association with toxic heavy metal ion-induced pathological processes (Bakthisaran et al. 2015). For instance, Cu^{2+} -binding α A- and α B-crystallin were reported to exhibit redox silencing properties and confer cytoprotectivity (Ahmad et al. 2008). In mollusks, transcriptional enhancement of *sHsps* upon

Fig. 8 *sHsp19* expression in response to toxic metal ions. Pacific abalones were treated with Cu^{2+} , Hg^{2+} , and Pb^{2+} , respectively. *sHsp19* expression in gill was determined by qRT-PCR at various time points. The mRNA level of *sHsp19* was normalized to that of α -tubulin. Data are shown as means $\pm$ SE ($N=3$). $**P<0.01$

heavy metal stimulation was documented in a number of species, such as *A. irradians* (Zhang et al. 2010a), *H. discus discus* (Wan et al. 2012), *M. meretrix* (Li et al. 2013), *M. galloprovincialis* (You et al. 2014), *Sinonovacula constricta* (Zhang et al. 2013), and *V. philippinarum* (Li et al. 2010). In our work, an intensive transcriptional induction of *sHsp19* was observed upon administration of Cu^{2+} , Hg^{2+} , and Pb^{2+} , respectively, pointing toward a likely involvement of *sHsp19* in the abalone's detoxification process against heavy metal pollutants.

Conclusions

In summary, in this report we identified a *sHsp* homolog (*sHsp19*) from *H. discus hannai*. *sHsp19* was identified as a novel *sHsp* by the analyses of its domain architecture, sequence homology, phylogenetic origin, and structural feature, in comparison with other known *sHsps*. The expression of *sHsp19* was ubiquitous in different tissues and was found to increase rapidly to multiple environmental stimuli including thermal, oxidative, and heavy metal stressors. Therefore, *sHsp19* is likely involved in abalone's defense mechanisms against environmental stress induced damage and may serve as a useful bioindicator for environmental monitoring in abalone culture and in marine ecotoxicological studies.

Acknowledgments This work was supported by the grant of Taishan Scholar Program of Shandong Province.

References

- Ahmad MF, Singh D, Taiyab A, Ramakrishna T, Raman B, Rao CM (2008) Selective Cu^{2+} binding, redox silencing, and cytoprotective effects of the small heat shock proteins αA - and αB -crystallin. *J Mol Biol* 382:812–824
- Arrigo AP (2000) *sHsp* as novel regulators of programmed cell death and tumorigenicity. *Pathol Biol* 48:280–288
- Arrigo AP (2007) The cellular “networking” of mammalian *Hsp27* and its functions in the control of protein folding, redox state and apoptosis. *Adv Exp Med Biol* 594:14–26
- Arrigo AP, Virot S, Chaufour S, Firdaus W, Kretz-Remy C, Diaz-Latoud C (2005) *Hsp27* consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. *Antioxid Redox Signal* 7:414–424
- Bakthisaran R, Tangirala R, Rao CM (2015) Small heat shock proteins: role in cellular functions and pathology. *BBA Proteins Proteomics* 1854:291–319
- Bao YB, Wang Q, Liu HM, Lin ZH (2011) A small HSP gene of bloody clam (*Tegillarca granosa*) involved in the immune response against *Vibrio parahaemolyticus* and lipopolysaccharide. *Fish Shellfish Immunol* 30:729–733
- Chowdary TK, Raman B, Ramakrishna T, Rao CM (2004) Mammalian *Hsp22* is a heat-inducible small heat-shock protein with chaperone-like activity. *Biochem J* 381:379–387
- Dalle-Donne I, Rossi R, Milzani A, Di Simplicio P, Colombo R (2001) The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic Biol Med* 31:1624–1632
- Das KP, Surewicz WK (1995) Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α -crystallin. *FEBS Lett* 369:321–325
- Edwards HV, Cameron RT, Baillie GS (2011) The emerging role of *HSP20* as a multifunctional protective agent. *Cell Signal* 23:1447–1454
- Hartl FU, Hayer-Hartl M (2002) Protein folding—molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295:1852–1858
- Haslbeck M, Vierling E (2015) A first line of stress defense: small heat shock proteins and their function in protein homeostasis. *J Mol Biol* 427:1537–1548
- Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 14:105–111
- Kostenko S, Moens U (2009) Heat shock protein 27 phosphorylation: kinases, phosphatases, functions and pathology. *Cell Mol Life Sci* 66:3289–3307
- Lelj-Garolla B, Mauk AG (2006) Self-association and chaperone activity of *Hsp27* are thermally activated. *J Biol Chem* 281:8169–8174
- Leung PT, Wang Y, Mak SS, Ng WC, Leung KM (2011) Differential proteomic responses in hepatopancreas and adductor muscles of the green-lipped mussel *Perna viridis* to stresses induced by cadmium and hydrogen peroxide. *Aquat Toxicol* 105:49–61
- Li C, Wang L, Ning X, Chen A, Zhang L, Qin S, Wu H, Zhao J (2010) Identification of two small heat shock proteins with different response profile to cadmium and pathogen stresses in *Venerupis philippinarum*. *Cell Stress Chaperones* 15:897–904
- Li JQ, He QG, Sun H, Liu X (2012) Acclimation-dependent expression of heat shock protein 70 in Pacific abalone (*Haliotis discus hannai* Ino) and its acute response to thermal exposure. *Chin J Oceanol Limnol* 30:146–151
- Li HJ, Liu SX, He CB, Gao XG, Yuan XT (2013) Identification of a small HSP gene from hard clam *Meretrix meretrix* and its potential as an environmental stress biomarker. *Aquat Biol* 18:243–252
- Li JK, Wu XW, Tan J, Zhao RX, Deng LW, Liu XD (2015) Molecular cloning of the heat shock protein 20 gene from *Paphia textile* and its expression in response to heat shock. *Chin J Oceanol Limnol* 33:919–927
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25(4):402–408. doi:10.1006/meth.2001.1262
- Mounier N, Arrigo AP (2002) Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell Stress Chaperones* 7:167–176
- Mymrikov EV, Seit-Nebi AS, Gusev NB (2011) Large potentials of small heat shock proteins. *Physiol Rev* 91:1123–1159
- Nie ZQ, Wang SP (2004) The status of abalone culture in China. *J Shellfish Res* 23:941–945
- Park EM, Kim YO, Nam BH, Kong HJ, Kim WJ, Lee SJ, Jee YJ, Kong IS, Choi TJ (2008) Cloning and expression analysis of a small *HSP26* gene of Pacific abalone (*Haliotis discus hannai*). *J Environ Biol* 29:577–580
- Qiu R, Liu X, Hu YH, Sun BG (2013a) Expression characterization and activity analysis of a cathepsin B from Pacific abalone *Haliotis discus hannai*. *Fish Shellfish Immunol* 34:1376–1382
- Qiu R, Sun BG, Fang SS, Sun L, Liu X (2013b) Identification of normalization factors for quantitative real-time RT-PCR analysis of gene expression in Pacific abalone *Haliotis discus hannai*. *Chin J Oceanol Limnol* 31:421–430

- Rajaraman K, Raman B, Rao CM (1996) Molten-globule state of carbonic anhydrase binds to the chaperone-like alpha-crystallin. *J Biol Chem* 271:27595–27600
- Rajaraman K, Raman B, Ramakrishna T, Rao CM (2001) Interaction of human recombinant alphaA- and alphaB-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation. *FEBS Lett* 497:118–123
- Raman B, Rao CM (1997) Chaperone-like activity and temperature-induced structural changes of alpha-crystallin. *J Biol Chem* 272:23559–23564
- Raman B, Ramakrishna T, Rao CM (1995) Temperature-dependent chaperone-like activity of alpha-crystallin. *FEBS Lett* 365:133–136
- Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 5:725–738
- Wan Q, Whang I, Lee J (2012) Molecular and functional characterization of HdHSP20: a biomarker of environmental stresses in disk abalone *Haliotis discus discus*. *Fish Shellfish Immunol* 33:48–59
- You LP, Ning XX, Chen LL, Zhang LB, Zhao JM, Liu XL, Wu HF (2014) Expression profiles of two small heat shock proteins and antioxidant enzyme activity in *Mytilus galloprovincialis* exposed to cadmium at environmentally relevant concentrations. *Chin J Oceanol Limnol* 32:334–343
- Zhang L, Wang LL, Song LS, Zhao JM, Qiu LM, Dong CH, Li FM, Zhang H, Yang GP (2010a) The involvement of HSP22 from bay scallop *Argopecten irradians* in response to heavy metal stress. *Mol Biol Rep* 37:1763–1771
- Zhang L, Wang LL, Zhao JM, Qiu LM, Song LS, Dong CH, Li FM (2010b) The responsive expression of heat shock protein 22 gene in zhikong scallop *Chlamys farreri* against a bacterial challenge. *Aquac Res* 41:257–266
- Zhang G, Fang X, Guo X, Li L, Luo R, Xu F et al (2012a) The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* 490:49–54
- Zhang XW, Wang XH, Zhu HY, Kranias EG, Tang YL, Peng TQ, Chang J, Fan GC (2012b) Hsp20 functions as a novel cardiokine in promoting angiogenesis via activation of VEGFR2. *PLoS ONE* 7(3), e32765. doi:10.1371/journal.pone.0032765
- Zhang A, Lu Y, Li C, Zhang P, Su X, Li Y, Wang C, Li T (2013) A small heat shock protein (sHSP) from *Sinonovacula constricta* against heavy metals stresses. *Fish Shellfish Immunol* 34:1605–1610