Comparative Biochemistry and Physiology, Part C 154 (2011) 1–6

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Effects of dietary zinc on gene expression of antioxidant enzymes and heat shock proteins in hepatopancreas of abalone *Haliotis discus hannai*

Chenglong Wu [a,b], Wenbing Zhang [a,*], Kangsen Mai [a], Wei Xu [a], Xiaoli Zhong [a]

[a] The Key Laboratory of Mariculture (Education Ministry of China), Ocean University of China, 5 Yushan Road, Qingdao 266003, P.R. China
[b] The school of life science, Huzhou Teachers College, 1 Xueshi Road, Huzhou 313000, P.R. China

ARTICLE INFO

Article history:
Received 18 January 2011
Received in revised form 7 March 2011
Accepted 7 March 2011
Available online 13 March 2011

Keywords:
Haliotis discus hannai Ino
Antioxidant enzyme
Heat shock protein
Real-time quantitative PCR
Zinc

ABSTRACT

The expression patterns of different genes encoding antioxidant enzymes and heat shock proteins were investigated, in present study, by real-time quantitative PCR in the hepatopancreas of abalone *Haliotis discus hannai* fed with different levels of dietary zinc (6.69, 33.8, 710.6 and 3462.5 mg/kg) for 20 weeks. The antioxidant enzymes include Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD), catalase (CAT), mu-glutathione-s-transferase (mu-GST) and thioredoxin peroxidase (TPx). The results showed that the mRNA expression of these antioxidant enzymes increased and reached the maximum at the dietary zinc level of 33.8 mg/kg, and then dropped progressively. Expression levels of the heat shock proteins (HSP26, HSP70 and HSP90) firstly increased at 33.8 mg/kg dietary Zn level, and reached to the maximum at 710.6 mg/kg, then dropped at 3462.5 mg/kg ($p<0.05$). Excessive dietary Zn (710.6 and 3462.5 mg/kg) significantly increases the Zn content and significantly decreases the total antioxidant capacity (T-AOC) in hepatopancreas ($p<0.05$). These findings showed that dietary Zn (33.8 mg/kg) could highly trigger the expression levels of antioxidant enzymes and heat shock proteins, but excessive dietary Zn (710.6 and 3462.5 mg/kg) induces a high oxidative stress in abalone.

1. Introduction

Zinc (Zn) is one of the essential micro-minerals for normal growth, development, biomineralization and innate immune function in animals, including fish, shrimp and mollusk (Watanabe et al., 1997; Tan and Mai, 2001; Clegg et al., 2005). Depending on the doses and the chemical forms of Zn, it can act as nutrients, antioxidants, or even toxicants (Lemire et al., 2008). It is well known that Zn functions in homeostasis of oxidation/reduction in human and animals, since Zn is cofactors of more than one hundred of metalloenzymes (Evans and Halliwell, 2001). Dietary Zn could induce expression of antioxidant molecules (e.g., superoxide dismutase and metallothionein) and then maintain a better antioxidant status against oxidative stress in human and animals (Fang et al., 2002). However, deficient or excessive zinc has been reported to exert inhibitory effects on immune responses and increase the severity of infections in human and animals (Shankar and Prasad, 1998). Like other heavy metals, deficient or excessive zinc could also induce oxidative stress and cause damage to lipids, protein and DNA (Oteiza et al., 1995; Oteiza et al., 2000). And zinc could increase the production of damaging reactive oxygen species (ROS) through inhibiting activities of lipoamide dehydrogenase in mitochondria (Gazaryan et al., 2002).

In order to protect against oxidative stresses, organisms have evolved many defense mechanisms including antioxidant enzymes and non-enzyme proteins. The former includes superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), thioredoxin peroxidase (TPx), etc. (Yu, 1994). Antioxidant enzymes are known to work at the first line of enzymatic defense mechanism against free radicals in organisms (Roch, 1999; Yu, 1994). The latter include selenium-binding protein (SEBP), heat shock proteins (HSPs), etc. (Limón-Pacheco and Gonsebatt, 2009; Kalmar and Greensmith, 2009; Wu et al., 2010b). HSPs are families of proteins, including HSP26, HSP70 and HSP90 which are involved in the maintenance of protein homeostasis, i.e., protein folding, aggregation, trafficking and anti-oxidative stress (Hightower, 1991; Limón-Pacheco and Gonsebatt, 2009; Kalmar and Greensmith, 2009). Recently, antioxidant enzymes and HSPs have been broadly used as molecular biomarkers to reveal the potential cellular and physiological effects of exogenous factors, such as microorganism, chemicals, deleterious materials in diet, and malnutrition (Labreuche et al., 2006; Verlecar et al., 2007; Woo et al., 2009). However, up to now, there is little published data available on the possible interaction between dietary Zn and antioxidant enzymes and HSPs in mollusk, even in aquatic invertebrates.

Abalone *Haliotis discus hannai* Ino are large algivorous marine gastropods, and the most commercially important species of gastropods in aquaculture for Asia (Mai et al., 1995). However, abalone

* Corresponding author at: The Key Laboratory of Mariculture, Ministry Education of China, Ocean University of China, 5 Yushan Road, Qingdao, Shandong 266003, P.R. China. Tel./fax: +86 532 82032145.
E-mail address: wzhang@ouc.edu.cn (W. Zhang).

1532-0456/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2011.03.003

culture has suffered serious problems of mortality from disease outbreaks (Wang et al., 1997; Ye et al., 1997), environmental contamination and decreased innate immunity of abalone (Hooper et al., 2007). It is of importance to alleviate stresses and enhance the animal's innate immunity through nutritional methods (Mai et al., 2004). Previous studies of the relationship between nutrition and immunity of abalone mainly focused on the effects of dietary micronutrients (selenium, zinc and copper, etc.) on the anti-oxidation responses at post-translational level. It has been shown that abalone *H. discus hannai* fed diet with 35 mg/kg of Zn could reach the maximum growth (Tan and Mai, 2001). Furthermore, dietary zinc (35 mg/kg) could significantly increase the expression of selenium-dependent glutathione peroxidase and selenium-binding protein in abalone *H. discus hannai* (Wu et al., 2010a, 2010b). To clarify the relationship between dietary Zn and the homeostasis of oxidation/antioxidation, however, the data of the effects of dietary Zn on the gene expression of the other antioxidant enzymes or heat shock proteins in *H. discus hannai* are needed.

As the metabolic organ and constantly metabolizing various materials that contains levels of nutrients and toxins, the hepatopancreas is the main defense organ against oxidative stress caused by excessive ROS (Wu et al., 2010a, 2010b). And the anti-oxidative response at translational level is an important part of innate immune functions, therefore, the aim of the present study was to investigate the transcriptional profiles of antioxidant enzymes (Cu/Zn-SOD, Mn-SOD, CAT, Mu-GST and TPx) and HSPs (HSP26, HSP70 and HSP90) in hepatopancreas of abalone fed with graded levels of dietary Zn.

2. Materials and methods

2.1. Animals, experimental diets and treatments

Abalone *Haliotis discus hannai* Ino were obtained from farmed stock. There were four dietary zinc treatments, and each treatment was conducted in three replicates. Artificial diets with graded levels of supplemented zinc (0, 35, 700 and 3500 mg/kg) were formulated to feed the experimental animals. The compositions of the basal diet are presented in Table 1. Zinc sulfate ($ZnSO_4 \cdot 7H_2O$) was used as the source of dietary Zn. Procedures of diet preparation were modified from previous studies on abalone (Tan and Mai, 2001). Final zinc concentrations in the four experimental diets were 6.69 mg/kg, 33.8 mg/kg, 710.6 mg/kg and 3462.5 mg/kg, respectively, as determined by inductively coupled plasma–atomic emission spectrophotometer (ICP-OES; VISTA-MPX, VARIAN) (n = 3) (Zhang et al., 2003).

Prior to initiation of the feeding trial, the abalone were acclimated to laboratory conditions for 2 weeks. Then the abalone juveniles (initial mass: 0.35 ± 0.03 g) were assigned to a flow-through system using a completely randomized design with four triplicated groups. Each replicate was stocked with 30 abalone in one acrylic tank (100 L). Each diet was fed to satiation to abalone once daily (17:00) for 20 weeks. Every morning, feces and excess diets were removed. During the experimental period, water temperature ranged from 12.5 to 21.0 °C, salinity of 30–34 ppt., pH 7.6–7.9, and dissolved oxygen was not less than 7.0 mg/L.The zinc concentration in the seawater flowing into the rearing system was 4.0 μg/L determined by ICP-OES (n = 3).

2.2. Sample collection

At the termination of the feeding trial, animals were not fed for 3 days. Ten abalone were randomly sampled from each tank. Hepatopancreas were excised from the abalone and pooled in each replicate. The sampled hepatopancreas were immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation and subsequent analyses.

Table 1
Ingredient and proximate composition of the basal diet (on dry weight basis).

Ingredients	Percents in diet (%, dry weight)
Casein[a]	25.00
Gelatin[a]	6.00
Dextrin[b]	33.50
CM-cellulose[b]	5.00
Sodium alginate[b]	20.00
Vitamin mix[c]	2.00
Zn-free mineral mix[d]	4.50
Choline chloride[b]	0.50
SO/MFO[e]	3.50
Proximate analysis (n = 3)	
Crude protein	31.47
Crude lipid	3.38
Ash	7.68

[a] Sigma Chemical, St. Louis, MO, USA.
[b] Shanghai Chemical Co., Shanghai, China.
[c] Vitamin mix, each 1000 g of diet contained: thiamin HCl, 120 mg; riboflavin, 100 mg; folic acid, 30 mg; PABA, 400 mg; pyridoxine HCl, 40 mg; niacin, 800 mg; Ca pantothenate, 200 mg; inositol, 4000 mg; ascorbic acid, 4000 mg; biotin, 12 mg; vitamin E, 450 mg; menadione, 80 mg; B12, 0.18 mg; retinol acetate, 100000 IU; cholecalciferol, 2000 IU; and ethoxyquin, 400 mg.
[d] Zn-free mineral mix, each 1000 g of diet contained: NaCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 6.0 g; $NaH_2PO_4 \cdot 2H_2O$, 10.0 g; KH_2PO_4, 20.0 g; $Ca(H_2PO_4)_2 \cdot H_2O$, 8.0 g; Fe–citrate, 1.0 g; $MnSO_4 \cdot H_2O$, 64.8 mg; $CuSO_4 \cdot 5H_2O$, 12.4 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; KIO_3, 1.2 mg; and Na_2SeO_3, 0.4 mg.
[e] Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

2.3. Synthesis of abalone cDNA

Total RNA was extracted using the Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. RNA quality was checked by spectrophotometry and agarose gel electrophoresis to confirm the suitability for cDNA synthesis. Ratios of the absorbance at 260 and 280 nm ranged from 1.6 to 1.8, and ratios of the absorbance at 230 nm and 260 nm ranged from 1.8 to 2.0. First-strand cDNA was synthesized from 3 μg of total RNA using 25 μM of anchored oligo dT20 (Invitrogen, USA) (Table 2), 500 μM dNTPs (Takara, Japan), 200 units of SuperScript™ II RT reverse transcriptase with provided buffer (Invitrogen, USA). Reactions were incubated for 1.5 h at 42 °C followed by 70 °C for 10 min and in a final volume of 20 μL. cDNA mix was diluted to 1:3 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR).

2.4. mRNA quantification by real-time quantitative PCR

Changes in the genes expression of the antioxidant enzymes and heat shock proteins were quantified using fluorescence real-time quantitative PCR. Analyses were performed in triplicate using a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). These analyzed genes are as follows: Cu/Zn-SOD, Mn-SOD, CAT, Mu-GST, TPx, HSP26, HSP70 and HSP90.

Primers used to amplify the target genes are listed in Table 2. Nucleotide sequences were selected as primers using known sequences for the abalone species *H. discus hannai* Ino (GST, TPx, HSP26, HSP70, and HSP90) and *H. discus discus* (Cu/Zn-SOD, Mn-SOD, and CAT) (Table 3). The ribosomal protein S9 gene was used as an internal control. TE buffer and DEPC-water for the replacement of cDNA template was used as the negative control. Meanwhile, the specificity of real-time PCR amplification using primers was conducted by detecting cDNA mixture from the hepatopancreas of abalone, TE buffer and DEPC-water.

Real-time PCR amplification was carried out in triplicate in a total volume of 25 μl, containing 12.5 μl of 2 × SYBR Green Real-time PCR Master Mix (Takara, Japan), 2 μl of 1:3 diluted cDNA and 0.2 mmol

Table 2
Abalone antioxidant enzymes and heat shock proteins' genes analyzed in this study.

Gene	Basic function	Cellular localization	Reference
Cu/Zn-SOD	Detoxification of O_2^-	Cytoplasm	Kim et al. (2007)
Mn-SOD	Detoxification of O_2^-	Mitochondria	Ekanayake et al., (2006)
CAT	Detoxification of H_2O_2	Cytoplasm, nucleus mitochondria	Ekanayake et al., (2008)
Mu-GST	Detoxification of H_2O_2	Cytoplasm, nucleus mitochondria	Wan et al. (2008)
TPx	Detoxification of H_2O_2	Cytoplasm, nucleus mitochondria	Wickramaarachchilage et al., (2008)
HSP26	Chaperone, Antioxidant Biomarker of stress	Cytoplasm, nucleus	Selsby and Dodd, 2005 Brerro-Saby et al., 2010
HSP70	Chaperone, Antioxidant biomarker of stress	Cytoplasm, nucleus	Cheng et al., 2007
HSP90	Chaperone, Antioxidant biomarker of stress	Cytoplasm, nucleus	Zhang et al., 2011

Cu/Zn-SOD: Cu/Zn-superoxide dismutase, Mn-SOD: Mn-superoxide dismutase, CAT: catalase, Mu-GST: Mu-glutathione-s-transferase, TPx: thioredoxin peroxidase, HSP26: heat shock protein 26, HSP70: heat shock protein 70, and HSP90: heat shock protein 90.

each of primers. The real-time PCR temperature profile for genes was 95 °C for 2 min followed by 35 cycles of 5 s at 95 °C, 15 s at 59 °C, and 20 s at 72 °C. Fluorescent data were acquired during each annealing phase. During the detection, each sample was run in quartic, and the PCR-grade water which replaced the template was the negative control. The expression levels of genes were calculated by $2^{-\Delta\Delta CT}$ method, and the value stood for an n-fold difference relative to the calibrator (Livak and Schmittgen, 2001).

2.5. Zn concentration and the total antioxidant capacity (T-AOC) in the hepatopancreas

Final Zn contents in the hepatopancreas were determined by ICP-OES as previously described by Zhang et al. (2003). The total antioxidant capacities in the hepatopancreas were determined using a commercial T-AOC Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as previously described by Wu et al. (2010a). The data of T-AOC are expressed as T-AOC unit/mg tissue protein.

2.6. Statistical analysis

All data were presented as mean ± SD (standard deviation). Data of the fold change of expression were logarithmically transformed before subjected to a one-way analysis of variance (one-way ANOVA) using SPSS 16.0. When overall differences were significant, Tukey's test was conducted to compare the means between individual treatments. For statistically significant differences, $p<0.05$ was required.

Table 3
Real-time quantitative PCR primers for antioxidant enzymes and heat shock proteins' genes and Ribosomal protein S9 gene of abalone *Haliotis discus hannai* and *H. discus discus*.

Gene		Nucleotide sequence (5′-3′)	Size (bp)	Reference
Cu/Zn-SOD	F	CCCACTTCAACCCTTTCG	199	*Haliotis discus discus* (DQ530214)
	R	TTGCCCAGGTCATCCACT		
Mn-SOD	F	CGCCTACGTCACAAACCT	151	*Haliotis discus discus* (DQ530210)
	R	GCTAAGCACCTCCCAGAA		
CAT	F	CAGCACAGAGGATGAAGACAAC	138	*Haliotis discus discus* (DQ530211)
	R	GCACGCTTCTGGATGAACTC		
Mu-GST	F	CGGCAGCAAGGTGACAGT	106	*Haliotis discus hannai* (EU734743)
	R	GAAGGCTAACAGCGTGGG		
TPx	F	TCAGACTACAGAGGGAAATA	134	*Haliotis discus hannai* (DQ118780)
	R	CATCCAAGGACCTCACAG		
HSP26	F	TTCGGTTTGATCTGTCC	199	*Haliotis discus discus* (EF472916)
	R	GTCATCTCGCCCTCTG		
HSP70	F	ATGCCAATGGTATCCTC	134	*Haliotis discus hannai* (DQ324856)
	R	GTAATTCTCAGCCTCGTT		
HSP90	F	CACTGTGGACCAGAAATGC	141	*Haliotis discus hannai* (GU014545)
	R	ACAGCAAAGCACGGAACT		
RP S9	F	CAGAATCCGAAAGTCAGCC	125	*Haliotis discus hannai* (EU247757)
	R	TCATCTTGCCCTCGTCCA		

F: Forward, and R: Reverse.

3. Results

3.1. Antioxidant enzymes gene expression in response to graded levels of dietary zinc

Real-time quantitative PCR was used to investigate the mRNA levels of antioxidant enzymes genes (Cu/Zn-SOD, Mn-SOD, CAT, Mu-GST and TPx) in the hepatopancreas of abalone fed graded levels of dietary Zn for 20 weeks. Transcriptions of Cu/Zn-SOD were significantly 0.88-fold, 0.98-fold and 0.48-fold higher in the abalone fed with 33.8, 710.6 and 3462.5 mg/kg dietary Zn, respectively, than those in the dietary Zn-deficient treatment (6.69 mg/kg) ($p<0.01$) (Fig. 1A). Similar expression patterns were found in CAT (Fig. 1C). Compared with those in the Zn-deficient group, CAT mRNA levels were approximate increases of 1.41-fold, 1.53-fold and 1.07-fold in treatments with 33.8, 710.6, and 3462.5 mg/kg of dietary Zn, respectively. In addition, Mn-SOD (Fig. 1B), mu-GST (Fig. 1D) and TPx (Fig. 1E) were significantly higher in treatment with dietary Zn (33.8 mg/kg) than those in the other treatments ($p<0.05$). The minimum mRNA levels of Mn-SOD, mu-GST and TPx were respectively found in the excessive dietary Zn (3462.5 mg/kg) group. Mn-SOD, mu-GST and TPx mRNA levels in the treatment with dietary Zn (33.8 mg/kg) were approximate 1.14-fold, 0.9-fold and 1.36-fold higher than those in the Zn-deficient (6.69 mg/kg) group, respectively.

3.2. Heat shock proteins' gene expression in response to graded levels of dietary zinc

The effects of graded dietary Zn on the mRNA levels of HSP26, HSP70 and HSP90 were shown in Fig. 1F, G and H, respectively. There were similar expression profiles for HSP26, HSP70 and HSP90. Compared with that in the Zn-deficient group (6.69 mg/kg), HSP26 mRNA levels were increased by approximately 0.43-fold, 1.02-fold and 0.65-fold in treatments with 33.8, 710.6 and 3462.5 mg/kg of dietary Zn, respectively ($p<0.05$) (Fig. 1F). Both HSP26 and HSP90 mRNA levels significantly increased to the maximum levels in the group with 710.6 mg/kg of dietary Zn, and then significantly dropped when dietary Zn increased to 3462.5 mg/kg. The HSP70 and HSP90 mRNA levels in the treatment with 33.8 mg/kg of dietary Zn showed approximately 1.85- and 1.35-fold higher than those with deficient dietary Zn (6.69 mg/kg), respectively (Fig. 1G, H). Moreover, these

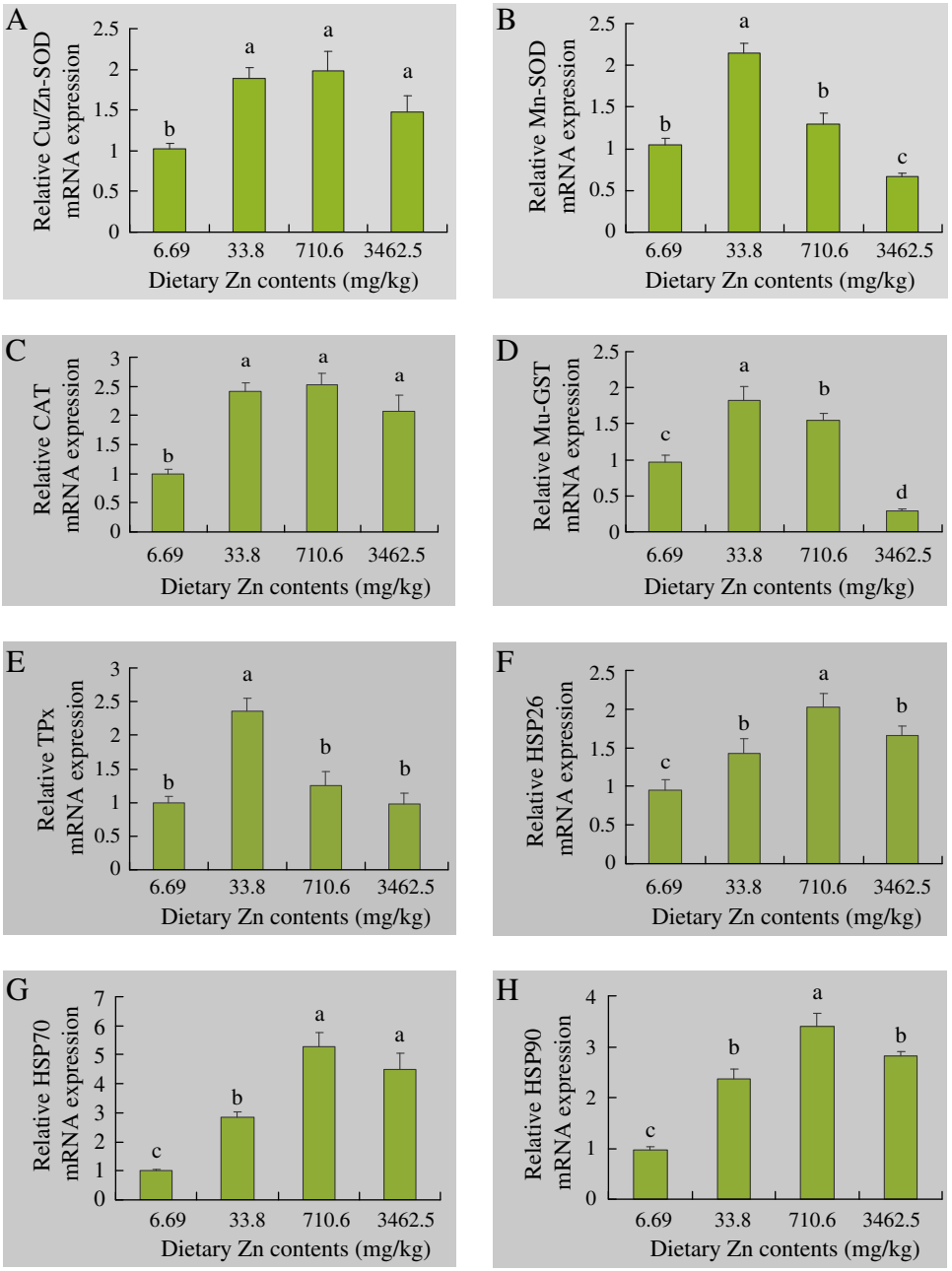

Fig. 1. Relative mRNA expression levels of antioxidant enzyme and heat shock proteins' genes in the hepatopancreas of abalone *Haliotis discus hannai* fed with graded levels of dietary Zn for 20 weeks, respectively. All values represent the mean ± SD (n = 3 replicates, and 3 abalone per replicate). Bars bearing different letters are significantly different ($p<0.05$; Tukey's test).

levels in treatments with 710.6 mg/kg of dietary Zn showed approximately 4.3-fold and 2.4-fold higher than those in treatment with deficient dietary Zn (Fig. 1G, H).

3.3. Zn concentration and T-AOC levels in hepatopancreas

Dietary zinc significantly increased the Zn concentration in hepatopancreas ($p<0.05$) (Table 4). The highest value of the Zn concentration in hepatopancreas was found to be 1.01 ± 0.02 mg/kg in the treatment with 3462.5 mg/kg of dietary Zn.

There was no significant difference in relative T-AOC levels between groups fed with 6.69 and 33.8 mg/kg of dietary Zn. When the dietary Zn levels were higher than 33.8 mg/kg, the relative T-AOC levels significantly decreased in abalone fed with 710.6 mg/kg of

Table 4
Effects of dietary Zn on the Zn contents and the total antioxidant capacity (T-AOC) in hepatopancreas of abalone *Haliotis discus hannai* (mean ± SD, n = 3).

Dietary Zn content (mg/kg)*	Hepatopancreas Zn content (mg/kg)*	Hepatopancreas T-AOC (U/mg)
6.69	0.51 ± 0.01^{c}	0.63 ± 0.02^{a}
33.8	0.64 ± 0.01^{b}	0.63 ± 0.02^{a}
710.6	0.71 ± 0.02^{b}	0.58 ± 0.01^{b}
3462.5	1.01 ± 0.02^{a}	0.42 ± 0.02^{c}
ANOVA		
F Value	77.378	34.183
P Value	0.000	0.000

*Values of the tissue samples are expressed on a dry-weight basis. Means in the same column sharing a common superscript letter were not significantly different ($P>0.05$) as determined by Tukey's test.

dietary Zn, and fall to the minimum with excessive dietary Zn (3462.5 mg/kg) ($p<0.01$).

4. Discussion

In the present study, the mRNA expression levels of antioxidant enzymes (Cu/Zn-SOD, Mn-SOD, CAT, mu-GST and TPx) were higher in the dietary Zn (33.8 mg/kg) treatment than those in the deficient dietary Zn group, respectively (Fig. 1A-E). In previous studies, it has been demonstrated that Zn could increase the production of ROS ($O_2^{\bullet-}$ and H_2O_2) in cell (Gazaryan et al., 2002; Dineley et al., 2005), then induce the expression levels of antioxidant enzymes (SOD, CAT, GST and GPx) (Woo et al., 2009). And it is a basic function of zinc to activate defense mechanisms through metal responsive elements (MRE) motifs in the 5′ regulatory regions of these antioxidant genes (Chung et al., 2005). SOD could catalyze the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and oxygen. In turn, H_2O_2 is converted by CAT and GPx into H_2O and molecular oxygen in animal cells (Nordberg and Arner, 2001). In addition, several studies have shown that higher expression or activities of antioxidant enzymes correlated with the better life span and healthy status of aquatic animals (Fang et al., 2002; Wu et al., 2010b). Moreover, the homeostasis states between ROS and antioxidants (e.g., Zn) are suitable for the health and survival of hosts (Fang et al., 2002). In our previous studies, it has been demonstrated that abalone have better growth or anti-oxidation status when fed with 35 mg/kg of dietary Zn (Tan and Mai, 2001; Wu et al., 2010a, 2010b). Combined with these findings, it is implied that dietary Zn (33.8 mg/kg) could highly trigger the expression levels of antioxidant enzymes, and keep the homeostasis of oxidation/reduction in abalone.

However, in the present study, it was also found that Cu/Zn-SOD, Mn-SOD, CAT, mu-GST and TPx mRNA levels decreased at excessive dietary Zn (3462.5 mg/kg) compared with dietary Zn (33.8 mg/kg) ($p<0.05$). Moreover, the mRNA levels of Mn-SOD and mu-GST were significantly lower in excessive the dietary Zn (3462.5 mg/kg) group than those in the Zn-deficient (6.69 mg/kg) group (Fig. 1B, D). It has been demonstrated that a high content of Zn induced excessive accumulation of ROS and caused oxidative stress (Kindermann et al., 2005; Cabreiro et al., 2009). Zn^{2+} could trigger mitochondrial ROS production through Ca^{2+}-permeable AMPA/kainate channels (Sensi et al., 1999) and depress activities of lipoamide dehydrogenase (Gazaryan et al., 2002), subsequently, induce a drastic increase in oxidized lipids and proteins (Lemire et al., 2008) and cause the dysfunction of mitochondria (Dineley et al., 2005). As for the up-regulation of Cu/Zn-SOD and CAT at excessive dietary Zn (710.6 mg/kg), it indicated that they were enhanced to remove excessive ROS under weak oxidative stress (Jo et al., 2008). Although Mn-SOD could convert excessive $O_2^{\bullet-}$ to H_2O_2, it is difficult to completely remove $O_2^{\bullet-}$ and H_2O_2 due to the decreasing expression of Mn-SOD, mu-GST and TPx in mitochondria. In addition, the mRNA expression levels of GPx and SEBP in the hepatopancreas of abalone were also decreased by a high content of dietary Zn (710.6 mg/kg) (Wu et al., 2010a; 2010b). In the present study, the Zn content in hepatopancreas increased with dietary Zn levels (Table 4). On the contrary, the T-AOC levels in the hepatopancreas significantly decreased at the excessive dietary zinc levels (710.6 and 3462.5 mg/kg) (Table 4). These results indicated that strong oxidative stresses were induced by the excessive dietary Zn (3462.5 mg/kg). Animals could lose appropriate metabolic functions under strong oxidative stress when heavy metals accumulated beyond a certain threshold concentration in a living body (Jo et al., 2008; Wu et al., 2010a, 2010b). This could be used to explain why the excessive dietary Zn decreased the mRNA levels of antioxidant enzymes in the present study.

In addition, similar transcriptional patterns were also observed in the HSPs genes (HSP26, HSP70 and HSP90) in abalone fed with graded levels of dietary Zn (Fig. 1F, G and H). The expression levels of HSPs firstly increased at 33.8 mg/kg of dietary Zn, and rearched to the maximum at 710.6 mg/kg, then decreased at excessive dietary Zn (3462.5 mg/kg) (Fig. 1F, G and H). It was shown that HSPs could be up-regulated by dietary Zn because HSPs influence the folding of newly synthesized polypeptides by providing a chaperoned environment coupled to translation (Frydman, 2001; Cousins et al., 2003; Moore et al., 2003). Meanwhile, up-regulation of HSPs were induced by lots of denatured proteins caused by accumulation of toxic ROS, and then used to refold and reassemble these proteins (Dean et al., 1991; Fukuda et al., 1996; Choi et al., 2008; Zhang et al., 2009). In addition, HSPs could modulate the redox status of the cytosol in cells due to their reactive cysteine groups toward cytochrome c (Nardai et al., 2000; Kalmar and Greensmith, 2009). Furthermore, defense mechanisms could be activated under weak oxidative stress (Zhang et al., 2004). Therefore, the up-regulation of HSPs could be ascribed to a long term protective mechanism (Gao et al., 2007; Zhang et al., 2011). However, the mRNA level of HSPs transcripts decreased in abalone fed with 3462.5 mg/kg of dietary Zn compared with those in the 710.6 mg/kg of dietary Zn (Fig. 1F, G and H). It could be due to the negative regulation mechanism (Morimoto et al., 1993). When the HSPs reach a high abundance, the heat shock transcription factors (HSFs) will bind to the HSPs and lose the DNA binding activity to the heat shock elements (HSEs) (Ali et al., 1998; Zou et al., 1998). And then the expression levels of HSPs decreased (Morimoto et al., 1993).

In conclusion, dietary Zn could modulate the mRNA expression of antioxidant enzymes and heat shock proteins in abalone. And dietary Zn (33.8 mg/kg) could better maintain the oxidation/antioxidation homeostasis in the hepatopancreas. These findings will enable a better understanding of the genetic response of abalone in response to dietary Zn. However, longitudinal intervention studies directed at exploring oxidative stress and antioxidant status are needed to be investigated, such as microRNA intervention studies.

Acknowledgments

This research was financially supported by grants from the National Natural Science Foundation of China (no. 30972262).

References

Ali, A., Bharadwaj, S., O'Carroll, R., Ovsenek, N., 1998. HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. Mol. Cell. Biol. 18, 4949–4960.
Brerro-Saby, C., Delliaux, S., Steinberg, J.G., Boussuges, A., Gole, Y., Jammes, Y., 2010. Combination of two oxidant stressors suppresses the oxidative stress and enhances the heat shock protein 27 response in healthy humans. Metabolism 59, 879–886.
Cabreiro, F., Picot, C.R., Perichon, M., Friguet, B., Petropoulos, I., 2009. Overexpression of Methionine Sulfoxide Reductases A and B2 Protects MOLT-4 Cells Against Zinc-Induced Oxidative Stress. Antioxid. Redox. Sign. 11 (2), 215–226.
Cheng, P.Z., Liu, X., Zhang, G.F., He, J.G., 2007. Cloning and expression analysis of a HSP70 gene from Pacific abalone (*Haliotis discus hannai*). Fish Shellfish Immunol. 22, 77–87.
Choi, Y.K., Jo, P.G., Choi, C.Y., 2008. Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*. Comp. Biochem. Physiol. C 147, 286–292.
Chung, M.J., Walker, P.A., Brown, R.W., Hogstrand, C., 2005. Zinc-mediated gene expression offers protection against H_2O_2-induced cytotoxicity. Toxicol. Appl. Pharmacol. 205, 225–236.
Clegg, M.S., Hanna, L.A., Niles, B.J., Momma, T.Y., Keen, C.L., 2005. Zinc deficiency-induced cell death. IUBMB Life 57, 661–669.
Cousins, R.J., Blanchard, R.K., Moore, J.B., Cui, L., Green, C.L., Liuzzi, J.P., Cao, J., Bobo, J.A., 2003. Regulation of zinc metabolism and genomic outcomes. J. Nutr. 133 (5 Suppl 1), 1521S–1526S.
Dean, R.T., Hunt, J.V., Grant, A.J., Yamamoto, Y., Niki, E., 1991. Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants, and target proteins. Free Radic. Biol. Med. 11, 161–168.
Dineley, K.E., Richards, L.L., Votyakova, T.V., Reynolds, I.J., 2005. Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria. Mitochondrion 5, 55–65.
Ekanayake, P.M., De Zoysa, M., Kang, H.S., Qiang, W., Jee, Y., Lee, Y.H., Kim, S.J., Lee, J., 2008. Cloning, characterization and tissue expression of disk abalone (*Haliotis discus discus*) Catalase. Fish Shellfish Immunol. 24, 267–278.
Ekanayake, P.M., Kang, H.S., De Zoysa, M., Jee, Y., Lee, Y.H., Lee, J., 2006. Molecular cloning and characterization of Mn-superoxide dismutase from disk abalone (*Haliotis discus discus*). Comp. Biochem. Physiol. B 145, 318–324.

Evans, P., Halliwell, B., 2001. Micronutrients: oxidant/antioxidant status. Br. J. Nutr. 85, 67S–74S (Suppl).
Fang, Y.Z., Yang, S., Wu, G.Y., 2002. Free radicals, antioxidants, and nutrition. Nutrition 18, 872–879.
Frydman, J., 2001. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu. Rev. Biochem. 70, 603–647.
Fukuda, A., Osawa, T., Oda, H., Tanaka, T., Toyokuni, S., Uchida, K., 1996. Oxidative stress response in iron-induced acute nephrotoxicity: enhanced expression of heat shock protein 90. Biochem. Biophys. Res. Commun. 219, 76–81.
Gao, Q., Song, L., Ni, D., Wu, L., Zhang, H., Chang, Y., 2007. cDNA cloning and mRNA expression of heat shock protein 90 gene in the haemocytes of Zhikong scallop *Chlamys farreri*. Comp. Biochem. Physiol. B 147, 704–715.
Gazaryan, I.G., Krasnikov, B.F., Ashby, G.A., Thorneley, R.N., Kristal, B.S., Brown, A.M., 2002. Zinc is a potent inhibitor of thiol oxidoreductase activity and stimulates reactive oxygen species production by lipoamide dehydrogenase. J. Biol. Chem. 277, 10064–10072.
Hightower, L.E., 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 6, 191–197.
Jo, P.G., Choi, Y.K., Choi, C.Y., 2008. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. Comp. Biochem. Physiol. C 147, 460–469.
Hooper, C., Day, R., Slocombe, R., Handlinger, J., Benkendorff, K., 2007. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. Fish Shellfish Immunol. 22, 363–379.
Kalmar, B., Greensmith, L., 2009. Induction of heat shock proteins for protection against oxidative stress. Adv. Drug Deliv. Rev. 61, 310–318.
Kim, K.Y., Lee, S.Y., Cho, Y.S., Bang, I.C., Kim, K.H., Kim, D.S., Nam, Y.K., 2007. Molecular characterization and mRNA expression during metal exposure and thermal stress of copper/zinc- and manganese superoxide dismutases in disk abalone, *Haliotis discus discus*. Fish Shellfish Immunol. 23, 1043–1059.
Kindermann, B., Döring, F., Fuchs, D., Pfaffl, M.W., Daniel, H., 2005. Effects of increased cellular zinc levels on gene and protein expression in HT-29 cells. BioMetals 18, 243–253.
Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., Nicolas, J.L., 2006. Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. Microbes Infect. 8, 2715–2724.
Lemire, J., Mailloux, R., Appanna, V.D., 2008. Zinc toxicity alters mitochondrial metabolism and leads to decreased ATP production in hepatocytes. J. Appl. Toxicol. 28, 175–182.
Limón-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res. 674, 137–147.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.
Mai, K.S., Ai, Q.H., Xu, W., Liufu, Z.G., 2004. Stress in aquaculture and its prevention with emphasis on nutritional methods. Period. Ocean. Univ. China. 34 (5), 767–774 (in Chinese with English abstract).
Mai, K.S., Mercer, J.P., Donlon, J., 1995. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. III. Response of abalone to various levels of dietary lipids. Aquaculture 134, 65–80.
Moore, J.B., Blanchard, R.K., Cousins, R.J., 2003. Dietary zinc modulates gene expression in murine thymus results from a comprehensive differential display screening. Proc. Natl Acad. Sci. U.S.A. 100, 3883–3888.
Morimoto, R.I., Tissieres, A., Cesrgopoulos, C., 1993. Cell in stress: transcriptional activation of heat shock genes. Science 259, 1409–1410.
Nardai, G., Sass, B., Eber, J., Orosz, G., Csermely, P., 2000. Reactive cysteines of the 90 kDa heat shock protein, Hsp90. Arch. Biochem. Biophys. 384, 59–67.
Nordberg, J., Arner, E.S.J., 2001. Reactive oxygen species, antioxidants and mammalian thioredoxin system. Free Radic. Biol. Med. 31, 1287–1312.
Oteiza, P.I., Clegg, M.S., Zago, M.P., Keen, C.L., 2000. Zinc deficiency induces oxidative stress and AP-1 activation in 3 T3 cells. Free Radic. Biol. Med. 28, 1091–1099.
Oteiza, P.I., Olin, K.L., Fraga, C.G., Keen, C.L., 1995. Zinc deficiency causes oxidative damage to proteins, lipids and DNA in rat testes. J. Nutr. 125, 823–829.
Roch, P., 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. Aquaculture 172, 125–145.
Sensi, S.L., Yin, H.Z., Carriedo, S.G., Rao, S.S., Weiss, J.H., 1999. Preferential Zn^{2+} influx through Ca^{2+}-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proc. Natl Acad. Sci. U.S.A. 96, 2414–2419.
Shankar, A.H., Prasad, A.S., 1998. Zinc and immune function: the biological basis of altered resistance to infection. Am. J. Clin. Nutr. 68, 447S–463S (Suppl).
Selsby, J.T., Dodd, S.L., 2005. Heat treatment reduces oxidative stress and protect muscle mass during immobilization. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R134–R139.
Tan, B.P., Mai, K.S., 2001. Zinc methionine and zinc sulfate as sources of dietary zinc for juvenile abalone, *Haliotis discus hannai* Ino. Aquaculture 192, 67–84.
Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2007. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chem. Biol. Interact. 167, 219–226.
Wan, Q., Whang, I., Lee, J., 2008. Molecular characterization of mu class glutathione-S-transferase from disk abalone (*Haliotis discus discus*), a potential biomarker of endocrine-disrupting chemicals. Comp. Biochem. Physiol. B 150, 187–199.
Wang, B., Li, X., Gou, C., 1997. Infection of spherical viruses from *Haliotis discus hannai* Ino. Virol. Sin. 12, 360–363.
Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. Aquaculture 151, 185–207.
Wickramaarachchilage, A.P., De Zoysa, M., Kang, H.S., Oh, C., Whang, I., Kim, S.J., Lee, J., 2008. Comparative study of two thioredoxin peroxidases from disk abalone (*Haliotis discus discus*): cloning, recombinant protein purification, characterization of antioxidant activities and expression analysis. Fish Shellfish Immunol. 24, 294–307.
Woo, S., Yum, S., Park, H.S., Lee, T.K., Ryu, J.C., 2009. Effects of heavy metals on antioxidants and stress-responsive gene expression in Javanese medaka (*Oryzias javanicus*). Comp. Biochem. Physiol. C 149, 289–299.
Wu, C.L., Mai, K.S., Zhang, W.B., Ai, Q.H., Xu, W., Wang, X.J., Ma, H.M., Liufu, Z.G., 2010a. Molecular cloning, characterization and mRNA expression of selenium-dependent glutathione peroxidase from abalone *Haliotis discus hannai* Ino in response to dietary selenium, zinc and iron. Comp. Biochem. Physiol. C 152, 121–132.
Wu, C.L., Zhang, W.B., Mai, K.S., Liang, X.F., Xu, W., Wang, J., Ma, H.M., 2010b. Molecular cloning, characterization and mRNA expression of selenium-binding protein in abalone (*Haliotis discus hannai* Ino): response to dietary selenium, iron and zinc. Fish Shellfish Immunol. 29, 117–125.
Yu, B.P., 1994. Cellular defenses against damage from reactive oxygen species. Physiol. Rev. 74, 139–162.
Zhang, X.Y., Zhang, M.Z., Zheng, C.J., Liu, J., Hu, H.J., 2009. Identification of two hsp90 genes from the marine crab, *Portunus trituberculatus* and their specific expression profiles under different environmental conditions. Comp. Biochem. Physiol. C 150, 465–473.
Zhang, W.B., Mai, K.S., Xu, W., Ma, H.M., 2003. Metabolic responses to dietary cholecalciferol and phosphorus in abalone *Haliotis discus hannai* ino. J. Exp. Zool. 299A, 110–117.
Zhang, W.B., Wu, C.L., Mai, K.S., Chen, Q.Y., Xu, W., 2011. Molecular cloning, characterization and expression analysis of heat shock protein 90 from Pacific abalone, *Haliotis discus hannai* Ino in response to dietary selenium. Fish Shellfish Immunol. 30, 280–286.
Ye, L., Yu, K.K., Wang, R.C., 1997. The study on the pathogenic bacteria of the fester disease of cultured juvenile discus abalone. J. Fish Sci. China 4, 43–48 (in Chinese with English abstract).
Zou, J., Guo, Y., Guettouche, T., Smith, D.F., Voellmy, R., 1998. Repression of heat shock transcription factor HSF1 by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94, 471–480.
Zhang, J.F., Wang, X.R., Guo, H.Y., Wu, J.C., Xue, Y.Q., 2004. Effects of water-soluble fractions of diesel oil on the antioxidant defenses of the gold fish, *Carassius auratus*. Ecotoxicol. Environ. Saf. 58, 110–116.