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# 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

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ABSTRACT

A novel selenium-dependent glutathione peroxidase (Se-GPX) was cloned from abalone *Haliotis discus hannai* Ino (HdhGPx) by homology cloning with degenerate primers and RACE techniques. The full length of HdhGPx cDNA was 963 bp with a 669 bp open reading frame (ORF) encoding 222 amino acids and a 101 bp eukaryotic selenocysteine insertion sequence (SECIS) in 3′ untranslated region (UTR). It was showed that HdhGPx has a characteristic codon at $^{235}$TGA$^{237}$ that corresponds to selenocysteine (SeC) as $U_{72}$. Sequence characterization revealed that HdhGPx contains a characteristic GPx signature motif 2 ($_{96}$LGLPCNQF$_{103}$), an active site motif ($_{179}$WNFEKF$_{184}$). In addition, two potential N-glycosylation sites ($_{112}$NGTE$_{115}$ and $_{132}$NLTQ$_{135}$) were identified in HdhGPx. 3D modeling analysis showed that the overall structure of HdhGPx monomer had more similarity to human GPx3 than human GPx1. Relatively higher-level mRNA expression was detected in hepatopancreas, mantle and gonad by real-time PCR assays. The relative expression levels of HdhGPx mRNA in hepatopancreas and haemocytes were detected by real-time PCR in abalone fed with nine different diets containing graded levels of selenium (0.15, 1.32 and 48.7 mg kg$^{-1}$), zinc (6.69, 33.85 and 710.63 mg kg$^{-1}$) and iron (29.17, 65.7 and 1267.2 mg kg$^{-1}$) for 20 weeks, respectively. The results showed that the expressions of HdhGPx mRNA were statistically higher at adequate dietary selenium (1.32 mg kg$^{-1}$), zinc (33.85 mg kg$^{-1}$) and iron (65.7 mg kg$^{-1}$) than those in low dietary minerals, respectively. But HdhGPx mRNA expression levels were down-regulated by high contents of dietary selenium (48.7 mg kg$^{-1}$), zinc (710.63 mg kg$^{-1}$) and iron (1267.2 mg kg$^{-1}$), respectively. These results indicated that adequate dietary minerals could increase the mRNA expression of HdhGPx, and then to increase the total antioxidant capacities in abalone.



## 1. Introduction

During the aerobic cellular metabolisms, mass of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH·) are generated in all animals (Genestra, 2007). ROS can stimulate signal transduction pathways, mediate cell growth and apoptosis, and be responsible for killing invading microorganisms (Roch, 1999; Bogdan et al., 2000). However, the excessive accumulation of these toxic by-products causes serious damage to lipids, proteins and nucleic acids when oxidative stresses are induced by radiation, high temperature, malnutrition, chemicals or pathogens (Schwarz, 1996; Fang et al., 2002; Murphy and DeCoursey, 2006; Cabreiro et al., 2009). In order to protect cells against the toxicity caused by ROS, animals have evolved protective antioxidant enzymatic systems, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Yu, 1994; Thorpe et al., 2004).

Among these, glutathione peroxidase (GPx, EC.1.11.1.9) is a family of proteins ubiquitously found in aerobic organisms (Ursini et al., 1995). Glutathione peroxidase protects biomembranes and other cellular components from oxidative damage by catalyzing the reduction of a variety of ROS which can regulate the intracellular signal transduction and play important roles in innate immune responses (Owuor and Kong, 2002). According to the presence of selenocysteine (SeC) encoded by a TGA, these GPxs are classified into two subgroups including selenium-dependent glutathione peroxidase (Se-GPx) and non-selenium glutathione peroxidase (non-Se-GPx) (Arthur, 2000). Se-GPx catalyze the reduction of organic and inorganic peroxides like organic hydroperoxide (ROOH) and hydrogen peroxide ($H_2O_2$) to lipid alcohol and/or water, while non-Se-GPx reduce only organic peroxide using glutathione (GSH) as the reducing substrate which is recycled by glutathione reductase (Almar et al., 1998;

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Swiergosz-Kowalewska et al., 2006). Many studies have demonstrated the importance of GPxs to the immune or anti-oxidative response as well as the role in protecting cells against bacteria (Liu et al., 2007; De Zoysa et al., 2008; Ren et al., 2009; Yeh et al., 2009), physical and chemical challenges (Doyen et al., 2006; De Zoysa et al., 2008). GPx has been one of the potential indicators to address the health of aquatic organisms (Dandapat et al., 2000; Fu et al., 2006, 2007) and/or to detect environmental problems (Almar et al., 1998; Valavanidis et al., 2006; Woo et al., 2009).

Abalone *Haliotis discus hannai* Ino are large algivorous marine gastropods, and the most commercially important specie of gastropods in aquaculture for Asia (Mai et al., 1995; Chen et al., 2005). However, abalone 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; De Zoysa et al., 2008; Doyen et al., 2008). It is of important to alleviate stresses and enhance the animal 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 on the anti-oxidation responses at post-translational level. It was showed that optimal micronutrients (e.g., selenium, vitamin A and vitamin E) could significantly elevate the activities of GPx and could protect cells of abalone from peroxidation damage in order to maintain maximum growth and antioxidant system (Wan et al., 2004; Fu et al., 2006, 2007). Furthermore, it has been shown that abalone *H. discus hannai* could maintain better growth after feeding with adequate dietary Se (1 mg $kg^{-1}$), Zn (35 mg $kg^{-1}$) and Fe (65 mg/kg), respectively (Mai and Tan, 2000; Tan and Mai, 2001; Wan et al., 2004). However, there is no published literature on the interaction mechanisms between GPx and dietary minerals (Se, Zn or Fe) in abalone at transcriptional levels. Furthermore, identification and cloning of Se-GPx gene will further help us to better understand the molecular basis of anti-oxidative responses to the nutritional states of abalone *H. discus hannai*.

Therefore, the aims of the present study were to clone and characterize the full-length cDNA sequence of a selenium-dependent glutathione peroxidase from abalone *H. discus hannai* (HdhGPx). Furthermore, to evaluate the expression levels of HdhGPx mRNA in various tissues of abalone adult without any treatments, as well as HdhGPx expression levels in abalone fed with dietary Se, Zn or Fe, respectively.

## 2. Materials and methods

### 2.1. Abalone, experiment designs diets, and sample collection

Abalone *H. discus hannai* were obtained from a spawning in October 2008 at Jimo Fisheries Co., Shandong, China. Four abalone adult (average weight: 35 ± 0.34 g; average shell length: 65.01 ± 0.45 mm) were used for HdhGPx gene isolation and tissue-specificity expression detection. After being anesthetized with ice, haemocytes, gonad, hepatopancreas, kidney, mantle, adductor muscle and gill of abalone adult were collected and immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation and subsequent analyses.

Abalone juveniles (initial shell length: 13.05 ± 0.25 mm; initial body weight: 0.3 ± 0.04 g) were used to evaluate transcriptional expression levels of HdhGPx after the abalone were fed with graded levels of dietary Se, Zn or Fe, respectively. Nine artificial diets with graded levels of supplemented minerals were formulated to feed abalone, including three levels of dietary Se (low: 0 mg/kg, adequate: 1 mg/kg and high: 50 mg/kg), three levels of dietary Zn (low: 0 mg/kg, adequate: 35 mg/kg and high: 700 mg/kg) and three levels of dietary Fe (low: 0 mg/kg, adequate: 65 mg/kg and high: 1300 mg/kg), respectively. The compositions of the experimental diets are presented in Table 1. Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$), iron sulfate ($FeSO_4 \cdot 7H_2O$) and zinc sulfate ($ZnSO_4 \cdot 7H_2O$) were used as the sources of dietary Se, Fe and Zn, respectively. Procedures of diet preparation were modified from previous studies on abalone (Mai and Tan, 2000; Tan and Mai, 2001; Wan et al., 2004). Final Se concentrations in the diets of three graded levels of Se were found to be 0.15 mg/kg, 1.32 mg/kg and 48.7 mg/kg, respectively. Final Fe concentrations in the diets of three graded levels of Fe were 29.19 mg/kg, 65.7 mg/kg and 1267.2 mg/kg, respectively. And those for Zn in the diets of three graded levels of Zn were 6.69 mg/kg, 33.85 mg/kg and 710.63 mg/kg, respectively, as determined by inductively coupled plasma-atomic emission spectrophotometer (ICP-OES; VISTA-MPX, VARIAN) (Zhang et al., 2003). All the dietary flakes were sealed in sample bags and stored at −20 °C until use.

Prior to initiation of the feeding experiment, abalone were acclimated to laboratory conditions for 2 weeks. During the acclimation period, abalone in the treats of dietary Se were fed with Se-deficient diet. Abalone in the treats of dietary Fe were fed with Fe-deficient diet and abalone in the treats of dietary Zn were fed with Zn-deficient diet (Table 1). Then after measuring and recording the shell length and body weight, abalone juveniles were assigned to a flow-through system using a completely randomized design with nine triplicate treatments. Each replicate was stocked with 30 abalone juveniles in acrylic tank (100 L) containing a black waved board as a shelter. Each diet was fed to satiation to abalone once daily (17:00) for 20 weeks. Every morning, feces and excess diets were removed to maintain water quality. During the experimental period, water temperature ranged from 12.5 to 21.0 °C, salinity of 30–34, pH 7.6–7.9, and dissolved oxygen was not less than 7.0 mg/L. Selenium, zinc and iron concentrations in the seawater flowing into the rearing system were 1.46 μg/L, 4.0 μg/L and 2.0 μg/L determined by ICP-OES ($n = 3$), respectively. There were negligible levels of free ammonia and nitrite. At the termination of the feeding experiment, animals were not fed for 3 days. Hepatopancreas and haemocytes from three abalone in each replicate were randomly collected. And these samples were also immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation and subsequent analyses.

### 2.2. RNA extraction, cDNA synthesis and cloning of HdhGPx gene

Total RNA was extracted from tissues using Trizol Reagent (Invitrogen, USA), spectrophotometrically quantified, and electrophoresed on a 1% denaturing agarose gel to test the integrity. For each reverse transcription (RT) reaction, 3 μg of total RNA was firstly treated with RQI RNase-Free DNase (Takara, Japan) to remove DNA contaminant, and then subjected to cDNA synthesis by SuperScript™ II RT reverse transcriptase (Takara, Japan) in 25 μL volume according to reagent's instructions. Generally, an Oligo (dT)-adaptor primer (5′-AAGCAGTGGTATCAACGCAGAGTAC$T_{25}$-3′) was used as RT primer to introduce an adaptor. The degenerate primer pair of HdhGPx 01 F (5′-GTGAAYGTDGCCACVTACT-GAGG-3′) and HdhGPx 01 R (5′-CGGGVCGTACRWVGATMGGMT-3′) was designed based on GPx nucleotide sequences of freshwater mussel *Dreissena polymorpha* (EF194204), river mussel *Unio tumidus* (DQ830766) and asiatic clam *Corbicula fluminea* (EF547366) by using the Clustal W 1.83 multiple sequence alignments program (http://www.ebi.ac.uk/clustalw/). The polymerase chain reaction (PCR) to get the fragment of HdhGPx was conducted on Eppendorf Mastercycler gradient (Eppendorf, German) to amplify HdhGPx cDNA fragment using *Ex Taq*™ (5 U $\mu L^{-1}$) (Takara, Japan). The PCR program was followed by 35 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min followed by a 10 min extension at 72 °C. The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel for length difference and cloned into the pMD-18 T vector (Takara, Japan). After transforming into the competent cells of *Escherichia coli* DH5α, the recombinants were identified through blue-white color selection in ampicillin-containing LB plates and confirmed by PCR. Three positive clones in each PCR fragment were