Fish & Shellfish Immunology 30 (2011) 430–433

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Molecular characterization and gene expression of ferritin in red abalone (*Haliotis rufescens*)

Karina Salinas-Clarot, Alejandro P. Gutiérrez, Gustavo Núñez-Acuña, Cristian Gallardo-Escárate*

Laboratorio de Genética y Biotecnología Acuícola, Departamento de Oceanografía, Facultad de Ciencias Naturales y Oceanográficas, Centro de Biotecnología, Universidad de Concepción. P.O. Box 160-C, Concepción, Chile

ARTICLE INFO

Article history:
Received 25 June 2010
Received in revised form
24 August 2010
Accepted 19 September 2010
Available online 1 October 2010

Keywords:
Haliotis rufescens
Ferritin
Temperature challenge
RT-PCR
Gene expression

ABSTRACT

Ferritin is the principal iron storage protein in the majority of living organisms. Its capacity to capture the toxic cellular iron in excess in a compact and safe manner, gives to this protein a key role in detoxification and iron storage. It has a main role in cellular homeostasis and in cellular defense against oxidative stress produced by the reactive oxygen species (ROS). In this research, the cDNA coding sequence of ferritin for Red abalone (*Haliotis rufescens*) was obtained, which had an open reading frame (ORF) of 516 bp. The deduced amino acid sequence was consisted of 171 residues with a calculated molecular weight of 19.77 kDa. In addition, tissue expression profiles of ferritin in Red abalone were induced by thermal stress showed an expression peak from 16 °C to 22 °C. The transcriptional level of ferritin was mainly achieved in muscle, digestive gland, gills, foot, mantle and gonad respectively. This research providing more information to better understands the structural and functional properties of this protein in *Haliotis*.

1. Introduction

Ferritin is the major iron storage protein at the cellular and organismal level. Its molecular structure is composed of 24 subunits, which form a hollow capable for storing up to 4500 iron atoms (Fe^{+3}) [1–3]. Its capacity to capture the toxic cellular iron in excess in a compact and safe manner gives to this protein a key role in detoxification and cellular homeostasis [3]. In vertebrates, ferritin is a protein composed of two subunits called Heavy (H) and Light (L). The H subunit has been studied in a variety of species including vertebrate and invertebrate animals, plants and bacteria. In contrast, the L subunit has been only found in vertebrate organisms [2,4,5]. The H subunit has 7 conserved residues among different species which confer ferroxidase activity to the protein, converting Fe^{+2} to Fe^{+3} for a rapid detoxification of iron [6–9] and the L subunit does not has ferroxidase activity but salt bridges that stabilizes ferritin structure, playing a role in iron nucleation and long-term storage [10]. Furthermore, ferritins have been reported from lower vertebrates contain a third subunit type named M subunit [11], and also Abf1 ferritin was characterized from the disk abalone *Haliotis discus discus*, which does not contain any features related to the subunit H or L [12].

The ferritin expression is regulated at both transcriptional and translational levels [2]. The iron levels in the cell regulate the ferritin transcription, removing the interaction between iron regulatory proteins (IRP) and iron response elements (IRE), which is located 28 bp upstream in the 5′-UTR [13]. In addition, translational regulation of ferritin have been reported by oxidative stress [14], oncogenes [15], cytokines [1] and hormones [16]. Gene expression of ferritin has been related to specific tissues or organs, and also to particular environment [17]. Herein, aquatic invertebrates represent an excellent model to understand the iron bioaccumulation in marine systems.

Cellular stress derived from metal or thermal environmental conditions have been reported as factors capable of modifying the innate immune response in several invertebrate species [18–21]. The pivotal importance of reactive oxygen species (ROS) during phagocytosis in all animals becomes the main defense mechanism in invertebrate organisms [22], and also an important role in metabolic processes such as stimulation of signal transduction pathways, cellular growth and apoptosis [23]. However, excessive ROS levels produce oxidative stress, which can be generated by different stressful conditions, such as critical temperatures, causing lipid peroxidation, enzyme deactivation and nucleic acid degradation [24,25]. In response to oxidative stress, the cells activate a defense strategy using ferritin to restrict the metal availability, specifically free iron excess to suppress Fenton's reaction and thereby avoid the formation of •OH which produces damage to

* Corresponding author.
E-mail address: crisgallardo@udec.cl (C. Gallardo-Escárate).

1050-4648/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2010.09.009

biomolecules as proteins, lipids and DNA [26–28]. Despite the studies carried out, there is still a need to understand the relationship between cellular stress and the transcriptional level of genes that involves the innate immune response of marine invertebrates. In this research, the coding sequence of ferritin in Red abalone (*Haliotis rufescens*) was described and its gene expression was measured in different tissues and after thermal stress treatments.

2. Materials and methods

2.1. Samples

Red abalone individuals were collected from the abalone experimental harvesting center located in the Estación de Biología Marina de Dichato of the Universidad de Concepción, Chile. These individuals were maintained at 16 °C (basal temperature) and samples of epipodium tissue from abalones exposed to thermal stress were extracted (50 mg), fixed in RNA later Stabilization Reagent (Ambion) and stored at −80 °C to subsequently perform the RNA purification.

2.2. Total RNA extraction

The total RNA was extracted from the epipodium tissue using the TRIZOL reagent (Ambion) according to manufacturer's instructions and was stored at −80 °C. The RNA purity was established with a ND-1000 spectrophotometer (NanoDrop Technologies, US) and its quality was observed using electrophoresis in agarose gels at 1%. Subsequently, the cDNA was obtained using RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions with random hexamer primers.

2.3. Cloning and sequencing

The ORF (open reading frame) obtained from ferritin was amplified with primers described by De Zoysa and Lee [12] for *Haliotis discus discus* (Abf2F 5′-ATGGCCCAAACTCAA-CCC; Abf2R 5′-TCACGTGCGACTATGCCC). The PCR reactions were carried out with a final volume of 12.5 μL in a Veriti thermocycler (Applied Biosystem®) with a PCR program at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 40 s with a final extension at 72 °C for 5 min. The amplified products were analyzed using electrophoresis in agarose gels at 1.0% with a 100 bp ladder (New England BioLabs®). Then PCR products was purified with Gel Extraction Kit (Fermentas) and inserted into plasmids pCR2.1 TOPO® (Invitrogen). Subsequently, competent cells *E. coli JM-109* were transformed with the plasmid using electroporation, and then cultured in agar plates LB/amp/IPTG/Xgal overnight at 37 °C. The plasmids that contained the insert were purified with plasmid mini kit II (E.Z.N.A), and then sequenced by Macrogen Inc.

2.4. DNA sequences and structure analysis

The sequences obtained with the Abf2 primers of ferritin were analyzed with the software Geneious Pro v5.0.2 software (©Biomatters Ltd.) in order to obtain a consensus sequence, which was compared with other sequences of ferritin published in NCBI using BLAST-N. In addition, putative amino acid sequence alignment was also performed to obtain a consensus sequence of ferritin from Red abalone, and to identify conserved regions and secondary structure. Finally, a phylogenetic analysis was performed with MEGA software v4.0 using the Neighbor-joining method.

2.5. Expression profile analysis of ferritin gene

To analyze the expression profiles of ferritin gene under thermal stress, Red abalones were maintained at different temperatures (18, 20, 22, 24 y 26 °C) using 300 W heaters. A total of fifteen individuals were subjected to these temperatures for 1 h, and then epipodium were sampled at each temperature treatment. In addition, various tissues, including gills, digestive gland, muscle, mantle, gonad and foot were collected from three abalones not exposed to thermal stress. For each experiment, equal amount of tissue was used to isolate RNA as described above. Then, RT-PCR were performed using Abf2F and Abf2R primers [12], and a positive control of β-actin [29] was amplified to normalize the concentration of each cDNA template. Electrophoretic images and the integrated optical densities (IOD) of amplified bands were analyzed using the Image pro plus software (Media Cybernetics). Statistical analysis was performed with a one-way ANOVA followed by the Student-Newman-Keuls test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Sequence analysis

The Red abalone ferritin open reading frame was 516 bp in length coding a protein of 171 amino acids (Fig. 1). The amino acid sequence of this unit contains the iron binding regions IBRS 1 (^{61}REHAEKLMKYQNTRGGR^{77}) and IBRS 2 (^{124}DAQMCDFLESEY-LEEQVKA-IK^{144}). It also has the 7 characteristic amino acids that have ferroxidase function on the ferritin in H subunits present in mammals, which are: Glu 25 (GAG), Tyr 32 (TAC), Glu 59 (GAG), Glu 60 (GAG), His 63 (CAT), Glu 105 (GAG) and Gln 139 (CAG). Furthermore, two cysteine residuals (Cys 12 and 18) that have an important function in iron incorporation and oxidation were observed. Finally, a glycosylation site composed of Asn-Gln-Ser-Leu ^{109}NQSL^{112} was found, and also a Tyr 27 residual that is considered responsible for fast biomineralization in the vertebrate H subunits. The molecular mass and isoelectric points calculated were 19.77 kDa and 4.85 respectively.

The phylogenetic analyses show that the ferritin subunit was highly similar to other ferritins belonging to invertebrates. (Fig. 2). A significant grade of similarity was observed among ferritin sequences from *Haliotis discus hannai* (Genbank accession number DQ845482) and with the Abf2 subunit of *Haliotis discus discus* (Genbank accession number DQ821494). The ferritin sequences of ostreids and other aquatic invertebrates present less similarity.

3.2. Ferritin gene expression analysis in different tissues and response to thermal stress

The ferritin gene was amplified in all the tissues analyzed and a higher gene expression was found in muscle, followed by digestive gland, gills, foot and mantle. The lowest ferritin gene expression was observed in gonad tissue, being nearly 2-fold lower than in muscle. However, no significant differences ($p < 0.05$) among gills, foot and digestive gland were found (Fig. 3A). The gene expression of β-actin was detected in all tissues and no gene expression differences were archived. The results obtained by RT-PCR in response to thermal stress showed that ferritin levels increased significantly when the temperature was raised, whereas the expression levels of the internal control (β-actin) did not vary significantly with the different heat shock treatments. The ferritin gene expression increased up to reach the highest expression peak at 22 °C (Tpeak), then decreased in the treatments with temperatures over the Tpeak. This was confirmed by the statistical analysis

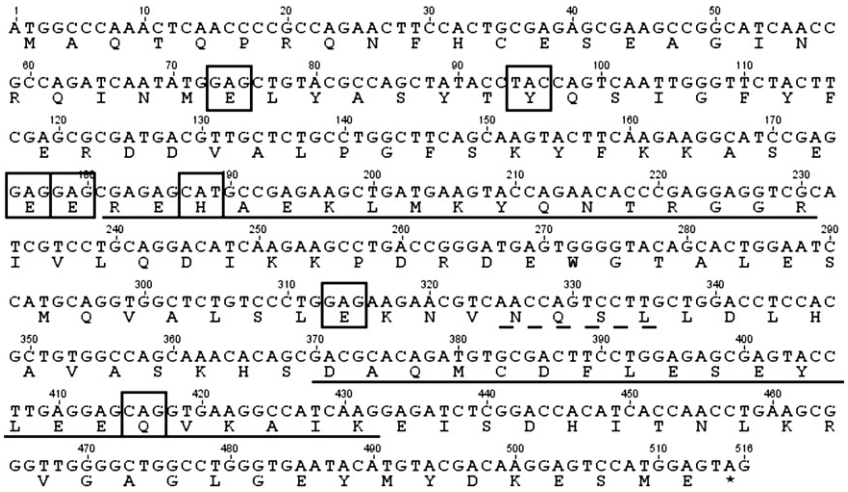

Fig. 1. The nucleotide sequence of the Abf2 ferritin coding region in Red abalone and the amino acid sequence with its initial codon (AUG) and its terminal codon TAG (asterisk). The cDNA sequence and deducible amino acid sequence have been subjected in the Genbank database with the accession number GU191936 and ACZ73270 respectively. The iron binding regions 1 and 2 (IBRS) are underlined. The seven amino acids corresponding to the ferroxidase center are enclosed in boxes and the putative glycosilation site (NQLS) is underlined with a broken line.

of the normalized IOD data, which found significant differences between each thermal stress treatment (Fig. 3B).

4. Discussion

In this study, we report the coding cDNA sequence of ferritin Abf2 subunit in Red abalone, which displayed high sequence similarity with known *ferritin* H subunit genes from other vertebrate organisms. The results showed that the genes encoding 7 residues corresponding to the ferroxidase site were highly similar to those found by Durand et al. [6] in *Crassostrea gigas*, by Zhang et al. [9] in *Pinctada fucata* and by Wang et al. [8] in *Meretrix meretrix*. A Tyrosine residual participating in iron biomineralization and two cysteine residuals involved in iron oxidation was also found. The percentage of similarity of this subunit found was higher in the H subunit (64%) than in the L subunit (49%) of *Homo sapiens*. It also has over 98% of similarity with the ferritin sequences found in *H. discus hannai* and *H. discus discus*. All this evidence suggests that the Abf2 subunit found in red abalone can be categorized as a type H subunit. Consequently, the phylogenetic analysis of ferritin is in agreement with previously phylogenetic relationships reported for this protein in mollusks.

Our study did not found the iron response element sequence (IRE) at 5′UTR position, because the ferritin gene was partially sequenced.

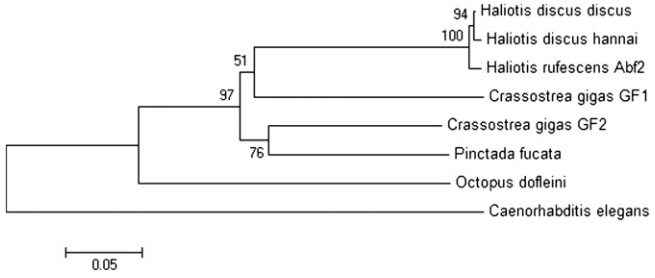

Fig. 2. Phylogenetic analysis of the Red abalone Abf2 ferritin. The tree is based on the alignment of the nucleotide sequences of the ferritin coding regions. The accession numbers of the sequences used to create the phylogenetic tree are: *H. discus discus* Abf2 (N° DQ821494), *H. discus discus* Abf1 (N° DQ821493), *H. discus hannai* (N° DQ845482.1), *C. gigas* GF1(N° CAD91440), *C. gigas* GF2 (N° AAP83794), *Pinctada fucata* (N° AF547223), *Asterias forbesii* (N° AF001984), *C. elegans* (N° CN072543) and *Octopus dofleini* (N° AAD29639).

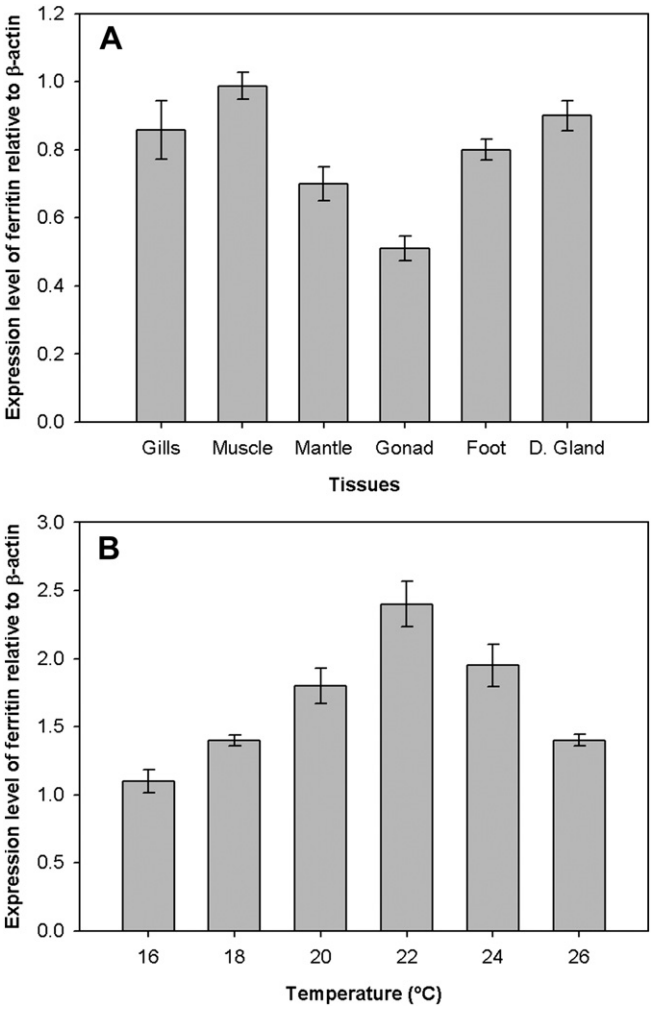

Fig. 3. (A) Expression of ferritin in gills, muscle, mantle, gonad, foot and digestive gland in Red abalone. (B) Expression levels of ferritin in response to thermal stress treatments from 16 °C to 26 °C. The normalised IOD data are graphed with mean (mean) ± standard error (SE). $P = 0.00001$.

However, this element is highly conserved among ferritins of vertebrate and invertebrate species, except for a few species as *Lymnaea stagnalis* in which it has not been found [7]. In reference to the results of ferritin gene expression in different tissues, the highest levels were found in the muscle, followed by gills, foot and digestive gland. Possibly, this occurs because these tissues participate in iron storage and also in metabolic process as gaseous exchange, incorporating dissolved metals and macromolecules degradation [30–32]. Regarding to the mantle, the ferritin expression was lower than other tissues except for gonad. However, this tissue has an important role in the incorporation of metals such as Ca, Mn, P and Fe during the formation of the mollusk shell [33]. In pearl oysters (*Pinctada fucata*) Zhang et al. [9], performed studies of hybridization with digoxigenin markers generated from cDNA that codes for ferritin. Strong signs of hybridization were detected in the mantle, which shows that the ferritin would be involved in the formation of the shell. Our results could be explained because the abalones analyzed were adults and the shell growth rate decreases at this stage.

The ferritin gene expression levels in Red abalone vary significantly at higher temperatures, doubling at 22 °C and 24 °C in relation to the basal temperature (16 °C), which shows that the ferritin expression in red abalone is induced by thermal stress, over-expressing at higher temperatures. A hypothesis for this response could be related to the fact that when temperatures increase, ROS increases to excessive levels, which activate ferritin transcription in order to suppress Fenton's reaction. However, increase in temperature results in variation in whole metabolism of the organism, which might provide other sources for ferritin expression changes. In the other hand, Zhou et al. [34] induced ferritin over-expression in Pacific white shrimp using stress caused by changes in the ambient pH. Additionally, Larade and Storey [35] exposed the common periwinkle to anoxia periods, doubling the ferritin levels in the cell. Like these studies, our research shows that ferritin is apparently involved in the cytoprotector mechanisms of cells. However, additional studies are needed to understand the changes of ferritin expression in different tissues under thermal stress. Future studies will be performed to obtain the full-length sequence of ferritin in Red abalone, and how its expression is linked to innate immunity and oxidative activity. This is the first study to partially characterize the ferritin mRNA of *Haliotis rufescens*.

Acknowledgments

This work was supported by the National Commission for Scientific and Technological Research of Chile-CONICYT (FONDEF D07I1085).

References

[1] Wei Y, Miller SC, Tsuji Y, Torti SV, Torti FM. Interleukin-1 induces ferritin heavy chain in human muscle cells. Biochemical and Biophysical Research Communications 1990;169:289–96.
[2] Orino K, Watanabe K. Molecular, physiological and clinical aspects of the iron storage protein ferritin. The Veterinary Journal 2008;178:191–201.
[3] Harrison PM, Arosio P. Ferritins: molecular properties, iron storage function and cellular regulation. Biochimica Biophysica Acta-Bioenergitics 1996;1275:161–203.
[4] Kim SR, Lee KS, Yoon HJ, Park NS, Lee SM, Kim I, et al. Molecular cloning, expression and characterization of cDNAs encoding the ferritin subunits from the beetle, *Apriona germari*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 2004;138:423–33.
[5] Theil EC. Ferritin: at the crossroads of iron and oxygen metabolism. Journal of Nutrition 2003;133:1549S–53S.
[6] Durand JP, Goudard F, Pieri J, Escoubas JM, Schreiber N, Cadoret JP. *Crassostrea gigas* ferritin: cDNA sequence analysis for two heavy chain type subunits and protein purification. Gene 2004;338:187–95.
[7] Vondarl M, Harrison PM, Bottke W. cDNA cloning and deduces amino-acid sequence of 2 ferritins: soma ferritin and yolk ferritin, from the snail *Lymnaea stagnalis*. European Journal of Biochemistry 1994;222:353–66.
[8] Wang XM, Liu BZ, Xiang JH. Cloning, characterization and expression of ferritin subunit from clam *Meretrix meretrix* in different larval stages. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 2009;154:12–6.
[9] Zhang Y, Meng QX, Jiang TM, Wang HZ, Xie LP, Zhang RQ. A novel ferritin subunit involved in shell formation from the pearl oyster (*Pinctada fucata*). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 2003;135:43–54.
[10] Santambrogio P, Levi S, Arosio P, Palagi L, Vecchio G, Lawson DM, et al. Evidence that a salt bridge in the light chain contributes to the physical stability difference between heavy and light human ferritins. Journal of Biological Chemistry 1992;267:14077–83.
[11] Giorgi A, Mignogna G, Bellapadrona G, Gattoni M, Chiaraluce R, Consalvi V, et al. The unusual co-assembly of H- and M-chains in the ferritin molecule from the Antarctic teleosts *Trematomus bernacchii* and *Trematomus newnesi*. Archives of Biochemistry and Biophysics 2008;478:69–74.
[12] De Zoysa M, Lee J. Two ferritin subunits from disk abalone (*Haliotis discus discus*): cloning, characterization and expression analysis. Fish & Shellfish Immunology 2007;23:624–35.
[13] Torti FM, Torti SV. Regulation of ferritin genes and protein. Blood 2002;99:3505–16.
[14] Tsuji Y, Ayaki H, Whitman SP, Morrow CS, Torti SV, Torti FM. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. Molecular and Cellular Biology 2000;20:5818–27.
[15] Tsuji Y, Kwak E, Saika T, Torti SV, Torti FM. Preferential repression of the H-subunit of ferritin by adenovirus E1A in NIH-3T3 mouse fibroblasts. Journal of Biological Chemistry 1993;268:7270–5.
[16] Leedman PJ, Stein AR, Chin WW, Rogers JT. Thyroid hormone modulates the interaction between iron regulatory proteins and the ferritin mRNA iron-responsive element. Journal of Biological Chemistry 1996;271:12017–23.
[17] Connell DW. Bioaccumulation of xenobiotic compunds. CRC Press; 1990.
[18] Wang FY, Yang HS, Gao F, Liu GB. Effects of acute temperature or salinity stress on the immune response in sea cucumber, *Apostichopus japonicus*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 2008;151:491–8.
[19] Gagnaire B, Frouin H, Moreau K, Thomas-Guyon H, Renault T. Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish & Shellfish Immunology 2006;20:536–47.
[20] Morley NJ. Interactive effects of infectious diseases and pollution in aquatic molluscs. Aquatic Toxicology 2010;96:27–36.
[21] Cheng WT, Wang LU, Chen JC. Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*. Aquaculture 2005;250:592–601.
[22] Hooper C, Day R, Slocombe R, Handlinger J, Benkendorff K. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. Fish & Shellfish Immunology 2007;22:363–79.
[23] Li CH, Ni DJ, Song LS, Zhao J, Zhang H, Li L. Molecular cloning and characterization of a catalase gene from Zhikong scallop *Chlamys farreri*. Fish & Shellfish Immunology 2008;24:26–34.
[24] Lesser MP. Oxidative stress in marine environments: biochemistry and physiological ecology. Annual Review of Physiology 2006;68:253–78.
[25] Ekanayake PM, De Zoysa M, Kang HS, Wan Q, Jee Y, Lee YH, et al. Cloning, characterization and tissue expression of disk abalone (*Haliotis discus discus*) catalase. Fish & Shellfish Immunology 2008;24:267–78.
[26] De Zoysa M, Whang I, Lee Y, Lee S, Lee JS, Lee J. Transcriptional analysis of antioxidant and immune defense genes in disk abalone (*Haliotis discus discus*) during thermal, low-salinity and hypoxic stress. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 2009;154:387–95.
[27] Kleinveld HA, Swaak AJG, Hack CE, Koster JF. Interactions between oxygen free radicals and proteins. Scandinavian Journal of Rheumatology 1989;18:341–52.
[28] Storey KB. Oxidative stress: animal adaptations in nature. Brazilian J Med Biol Res. 1996;29:1715–33.
[29] Bryant M, Flint H, Sin F. Isolation, characterization, and expression analysis of three actin genes in the New Zealand black-footed abalone, *Haliotis iris*. Marine Biotechnology 2006;8:110–9.
[30] Walsh AR, O'Halloran J. The accumulation of chromium by mussels *Mytilus edulis* (L.) as a function of valency, solubility and ligation. Marine Environmental Research 1999;43:41–53.
[31] Carefoot TH, Taylor BE, Land S. Use of isolated digestive-gland cells in the study of biochemical and physiological processes in gastropod molluscs. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology 2000;125:497–502.
[32] Ragg NLC, Taylor HH. Oxygen uptake, diffusion limitation, and diffusing capacity of the bipectinate gills of the abalone, *Haliotis iris* (Mollusca: Prosobranchia). Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology 2006;143:299–306.
[33] Marin F, Luquet G, Marie B, Medakovic D. Molluscan shell proteins: primary structure, origin, and evolution. p. 209–276. In: Current topics in developmental biology, vol 80. San Diego: Elsevier Academic Press Inc; 2008.
[34] Zhou J, Wang WN, Ma GZ, Wang AL, He WY, Wang P, et al. Gene expression of ferritin in tissue of the Pacific white shrimp, *Litopenaeus vannamei* after exposure topH stress. Aquaculture 2008;275:356–60.
[35] Larade K, Storey KB. Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate. Journal of Experimental Biology 2004;207:1353–60.