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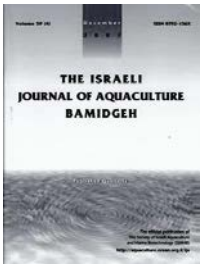

Identification and mRNA Expression of Antioxidant Enzyme Genes in the Mud Crab (*Scylla paramamosain*) in Response to Acute Ammonia and Nitrite Exposure

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Keywords: *Scylla paramamosain*; antioxidant enzyme genes; thioredoxin reductase; ambient stressors

Abstract

Thioredoxin reductase (TrxR) is a conserved protein that is involved in protecting organisms against various oxidative stresses. In this study, a thioredoxin reductase gene was cloned from the mud crab *Scylla paramamosain* (SpTrxR). The full-length cDNA of SpTrxR is comprised of 2724 bp with a 1791 bp open reading frame that encodes a putative protein of 596 amino acids. The deduced amino acid sequence of SpTrxR contains the typical TrxR domain. Quantitative real-time PCR analysis revealed that the SpTrxR mRNA was distributed abundantly in mud crabs, while strong expression was observed mainly in the gills. The expression of antioxidant enzyme genes (SpTrxR, SpTrx, SpSOD, and SpCAT) was measured using quantitative real-time PCR after acute ammonia and nitrite exposure. The results show that antioxidant enzyme genes (SpTrxR, SpTrx, SpSOD, and SpCAT) were modulated by acute ammonia and nitrite exposure. These results suggest that antioxidant enzyme genes play an important role in protecting organisms against oxidative stress.

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Introduction

In aquaculture, organisms are often exposed to temperature changes, environmental pollutants, and invasion of bacteria and viruses. All these factors have adverse effects on the immune system. Ambient stressors can enhance endogenous reactive oxygen species (ROS) in aquatic organisms. ROS play a dual role in signalling pathways (Schreck et al., 1991; Galloway and Depledge, 2001). However, excessive ROS can lead to DNA damage, lipid peroxidation, and induction of apoptosis (Stohs and Bagchi, 1995; Stadtman and Levine, 2003). The cellular antioxidative defense system plays a key role in preventing oxidative stress by reducing ROS production (Selvaraj et al., 2012). Endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), play an important role in preventing oxidative stress.

Thioredoxin is another key antioxidant defense system which protects cells through redox homeostasis against oxidative stress. The thioredoxin system comprised of NADPH, thioredoxin reductase (TrxR), and thioredoxin (Trx) is the major protein-disulfide reductase of the cells (Holmgren and Liu, 2010; Sengupta and Holmgren, 2013). It is also related to a variety of cellular processes including apoptosis, DNA synthesis, and nitric oxide signaling (Holmgren, 1985; Lu and Holmgren, 2014). Trx is a type of small protein, ubiquitously distributed in all organisms. It can also use redox-active cysteine residues in their active site to catalyze thiol-disulfide oxidoreductions (Schurmann and Buchanan, 2008; Sengupta and Holmgren, 2013). Trx can scavenge ROS and reactivate damaged proteins by oxidative stress (Pacitti et al., 2014). It is also known to be one of the main intracellular redox regulatory agents participating in the defense against oxidative stress (Kanzok, et al., 2002). TrxR is an antioxidant enzyme belonging to the pyridine nucleotide-disulfide oxidoreductase family, which also includes several enzymes involved in cellular oxidation and reduction (Mustacich and Powis, 2000). TrxR functions mostly relate to their basic role in Trx reduction by using NADH and NADPH, which are indirectly important in thioredoxin recycling (Elvitigala et al., 2015). TrxR plays important roles in a wide range of cellular processes, such as cell growth, DNA synthesis, apoptosis regulation, and antioxidative defense in organisms (Mustacich and Powis, 2000; Lu and Holmgren, 2014).

The mud crab (*Scylla paramamosain*) is widely distributed along the South China coast. Mud crab cultivation has become increasingly popular in South China, with production exceeding 14 thousand tons. However, mud crabs in aquaculture suffer from ammonia and nitrite stress which threaten their survival in culture. In this study, we investigated effects of ammonia and nitrite exposure on the expression of antioxidant enzyme genes (SOD, CAT, Trx and TrxR). This study may aid in understanding the molecular mechanism of the antioxidative defense system of mud crab in response to ammonia and nitrite stress. It also contributes to the development of strategies for long-term sustainability of mud crab in aquaculture.

Materials and methods

Mud crabs (50±3g) were obtained from farms in Taishan, Guangdong providence. They were held in tanks maintained at 10‰ salinity and 25°C for two weeks and fed twice a day with oyster meat up to 24 h before beginning the experimental treatments.

The crabs were exposed to 0, 15 mg/L ammonia-N, and 15 mg/L nitrite-N for 72 hours. Nitrite test solutions were prepared by adding $NaNO_2$ to 10‰ saltwater until the desired concentration was reached. A stock solution of high purity NH_4Cl (10g/L) was used as a source of ammonia-N, which was subsequently diluted to the desired concentrations of total ammonia-N. Test solutions were renewed every 12 h. After exposure for 0, 12, 24, 48, and 72 h, six mud crabs from each group were randomly sampled. Hepatopancreas was collected and immediately frozen in liquid nitrogen before storage at -80°C.

The total RNA was extracted from selected tissues of mud crabs, using Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. The concentration of each extracted RNA sample was measured using a Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was checked by electrophoresis on 1% agarose gels. Subsequently, total RNA was dissolved using Rnase-free water and was then stored at -80°C. Single-stranded cDNA was then synthesized from 1μg total RNA using PrimeScript RT reagent Kit With gDNA Eraser (Takara, Dalian,

China) following manufacturer's instructions. cDNA templates were then stored at -80°C for further analysis.

To determinate the full-length cDNA of SpTrxR, two specific primers, R1 and R2 (Table 1), were designed based on the sequence data from a cDNA library constructed previously by a next-generation sequencing technology. The RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions. The forward and reverse primers were designed based on the partial cDNA sequence obtained above (Table 1). The nested PCR program for 3´ and 5´RACE were as follows : 5 cycles at 94°C for 30 s, 72°C for 2min; 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 2 min; 25 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 2 min, and a 10 min final extension at 72°C. The nested PCR products were then cloned and sequenced as described above.

The nucleotide sequence and deduced amino acid sequence of SpTrxR cDNA were performed using the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The open reading frame (ORF) was predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf). Translation and protein analysis were performed by ExPaSy tools (http://www.expasy.org/tools/). The conserved domains of SpTrxR were predicted by the SMART program available from the EMBL website (http://smart.embl-heidelberg.de/). The phylogenetic tree was constructed based on Molecular Evolution Genetics Analysis (MEGA) software version 4.1 by the neighbor-joining method and 1000 replications of bootstrap.

The mRNA levels of SpTrxR in different tissues were checked with quantitative real-time RT-PCR. Tissues (hemocytes, gill, muscle, heart, stomach, hepatopancreas, and intestine) were collected separately from six healthy fish. The relative mRNA level was compared with muscle expression. Total RNA extraction, DNase I treatment and cDNA synthesis were conducted according to the method described above.

To further investigate the expression profiles of antioxidant enzyme genes in response to ammonia and nitrite stress, SOD, CAT, Trx, and TrxR were measured by RT-PCR. Specific primer pairs of SOD, CAT, and Trx were designed based on published mud crab mRNA sequences available in NCBI using Primer Premier 5 (Table 1). The specificity of the primer was examined by conventional PCR and melting curve sequence analysis.

Table 1. The sequences of primers in this experiment.

Primers name	*Nucleotide sequence (5´-3´)*
TrxR-F	ATGAAGTCACGGTTAGAGG
TrxR-R	CCAGGACTCGATTATTCTCC
TrxR-3gsp	GGGCTGTCGGAGGAGGTGGCTGTA
TrxR-5gsp	GTTGGAGGCCCGCAGTGCTAATAG
RT-TrxR-F	GAGGCGATTCAGAACCACATAGG
RT-TrxR-R	GTAATACAATGCTCCTTGGCTCC
RT-18S-F	CCTCGTTCATGGGAGACAAT
RT-18S-R	CTAGTCGACGGATCTCCAGC
RT-Trx-F	CAAGGATGACTTTGACAAGCAGC
RT-Trx-R	AACTGTCCACTTTCTTTCCCTCC
RT-CAT-F	CGGGCAGCCGAGCAAC
RT-CAT-R	GATGTCCTGTAGCAGAATGGGTC
RT-SOD-F	ATCCTCAGGTGGCTGCTATGTT
RT-SOD-R	CCAGAAGATGGTATGGTTCAAATG

Real-time PCR was amplified in an ABI 7500 real-time PCR machine (Applied Biosystems, USA) using SYBR Premix Ex TaqTM (Takara, Dalian, China) following the manufacturer's recommendations. Before the qRT-PCR experiments, specificity and efficiency of the primers above were determined. Reaction mixtures were 20 μL, containing 2 μL diluted cDNA sample (50 ng/μL), 0.4 μL ROX, 10 μL 2× SYBR Premix Ex Taq, 0.4 μL each of primer (10μM), and 6.8 μL dH_2O. The real-time PCR conditions were as follows: 94°C for 10 min, then 45 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by 10 min at 72°C. The standard equation and correlation coefficients

were determined by constructing a standard curve using a serial dilution of cDNA. cDNA of each sample isolated from six fish in each treatment was detected by qRT-PCR analysis. Each sample was amplified in triplicate. At the end of this process, the threshold cycle (Ct) values were determined from each sample. Relative gene expression levels were evaluated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

All data were expressed as means ± standard deviation. Significant differences were evaluated by a one-way ANOVA followed by Duncan's multiple range tests. Statistical analysis was performed using SPSS 18.0 software (SPSS, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

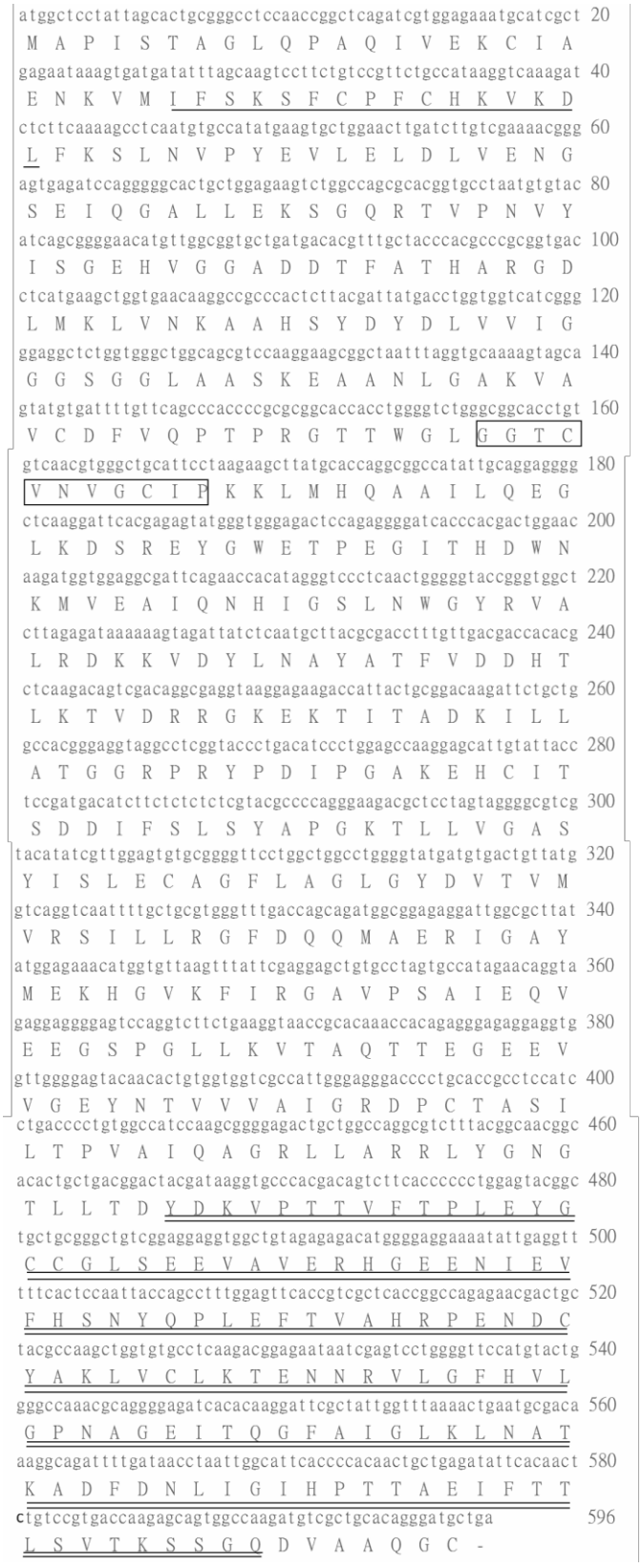

Fig.1. Nucleotide sequence of SpTrxR cDNA and the deduced amino acid sequence. Glutaredoxin active site was underlined. Pyridine nucleotidedisulfide oxidoreductase class-1 active site was boxed. Nucleotide-disulfide oxidoreductase dimerization domain was double underlined.

Results

Full length sequence of SpHSP40 cDNA was 2724 bp. It contained an open reading frame of 1791 bp. There was a 446 bp of 5´untranslated region (UTR) upstream of the start codon of a messenger RNA (mRNA) and a 487 bp 3´UTR with a poly (A) tail following the stop codon. ORF encoded a polypeptide of 596 amino acids with a theoretical isoelectric point of 5.52 and predicted molecular weight of 64.3 kDa. The domain and motif analysis showed that SpTrxR1 protein contained typical TrxR features including a proximal glutaredoxin domain (residues 13-108), glutaredoxin active site (residues 26-41), pyridine nucleotidedisulfide oxidoreductase class-1 active site (PYRIDINE_REDOX_1; residues 157-167), FAD/NAD(P)-binding domain(residues 111-458), and a pyridine nucleotide-disulfide oxidoreductase dimerization domain (residues 466-591) (Fig.1).

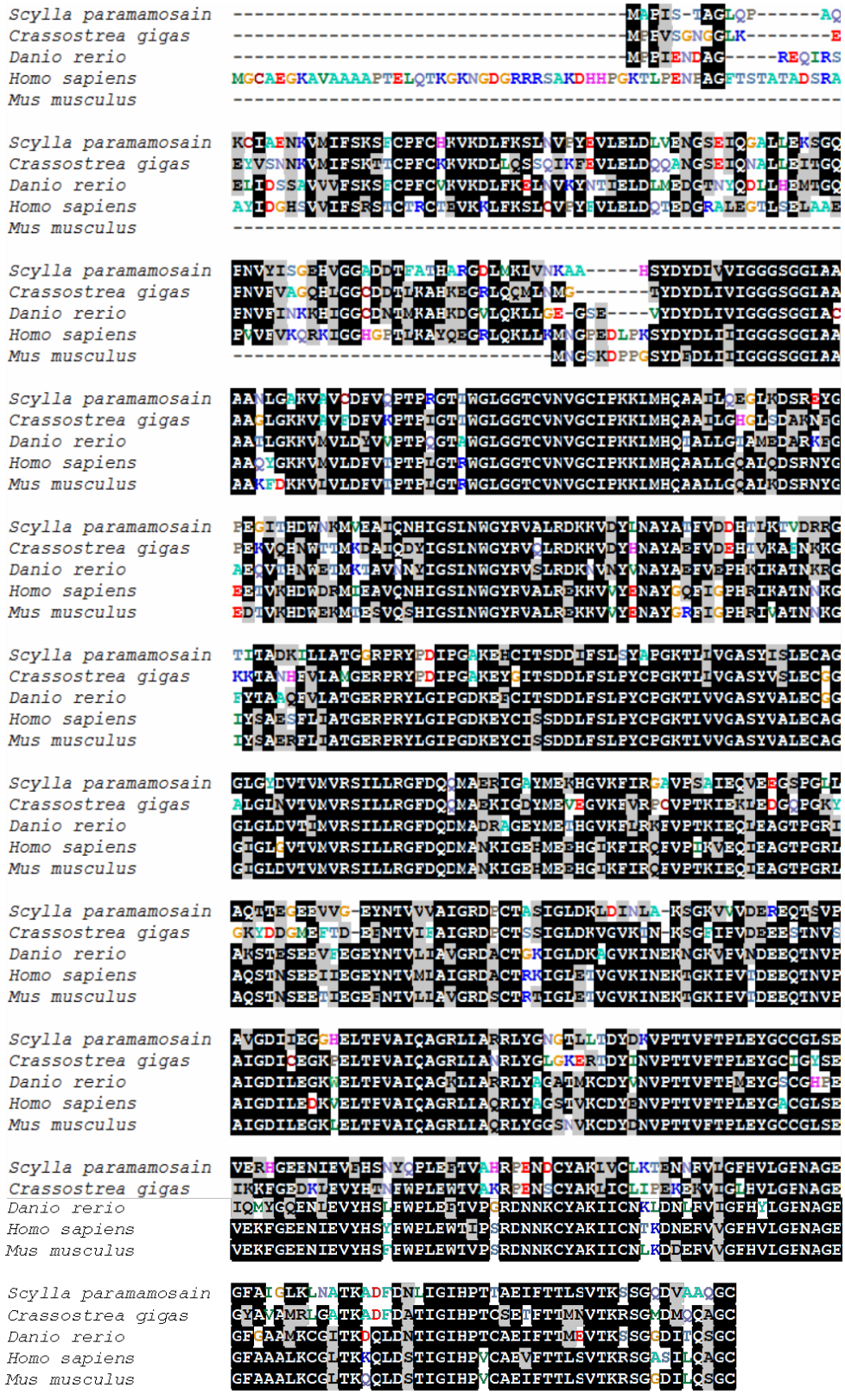

Fig.2. Multiple alignments of deduced amino acid sequence of SpTrxR with other TrxR using Clustal X.

The deduced amino acid sequence of SpTrxR was compared with its homologs from other organisms. The results showed that SpTrxR shared 69%, 65%, and 63% homology with the TrxR1s from *Hyalella azteca*, *Crassostrea gigas*, and *Danio rerio*, respectively. Multiple sequences alignment revealed that SpTrxR shared a high degree of identity of TrxR (Fig.2).

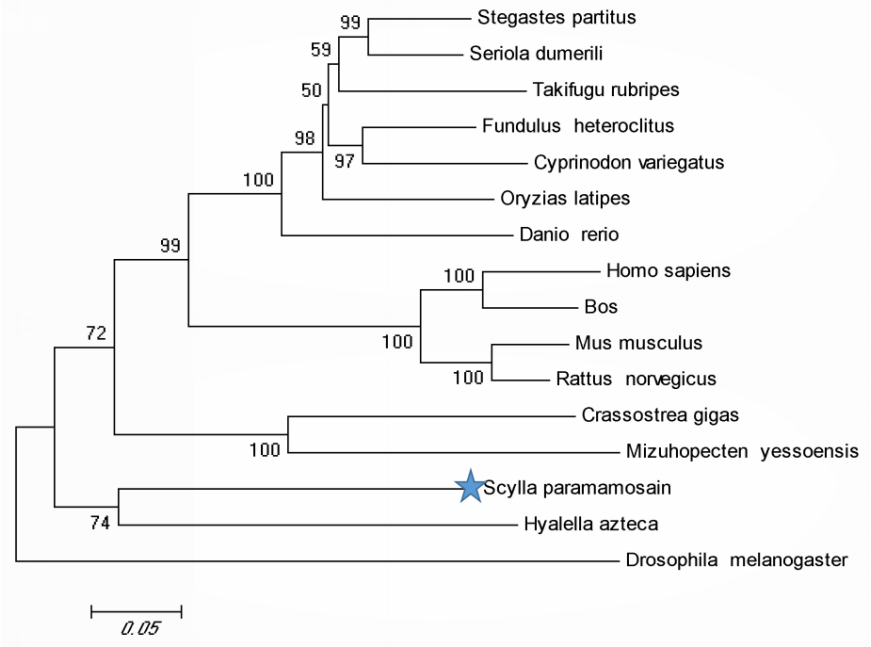

Fig.3. Phylogenetic analysis of SpTrxR with other members of the TrxR. The phylogenetic tree was constructed using MEGA software 4.1 by the Neighbor-joining method and 1000 replications of bootstrap.

To understand the evolutionary relationship, the phylogenetic tree of different TrxR was constructed by MEGA4.1. As shown in Fig.3, the SpTrxR proteins from vertebrates and invertebrates clustered according to their corresponding subgroup. In the phylogenetic tree, SpTrxR was similar to the other invertebrates, which is in agreement with the concept of traditional taxonomy.

As shown in Fig.4, the SpTrxR mRNA transcript is widely expressed in all selected tissues (hemocytes, gill, muscle, heart, stomach hepatopancreas, and intestine). The highest expression level of SpTrxR transcript was observed in the gills, followed by expression in the hemocytes and hepatopancreas; the lowest expression levels of SpTrxR transcript were observed in the muscle, intestine, stomach, and heart.

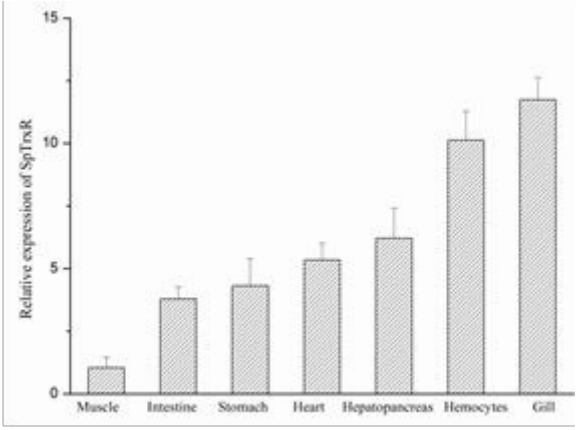

Fig.4. Tissue-specific mRNA expression of the SpTrxR determined by quantitative real-time PCR. The relative SpTrxR mRNA expression of each tissue was calculated by the $2^{-\Delta\Delta CT}$ method using 18S rRNA as a reference gene. Data are presented as mean ± SD (N= 6).

The hepatopancreas is known to play an important role in host defense. It is an important organ with a number of functions. The expression profile of antioxidant enzyme genes (SpTrx, SpTrxR, SpCAT and SpSOD) in hepatopancreas under ammonia and nitrite exposure was investigated using RT-PCR. In response to nitrite stress, SpTrxR mRNA transcript in hepatopancreas increased significantly at 12 h, peaked at 24 h (4.04-fold), declined gradually from 48 h and returned to the original level at 72 h (Fig. 5a). As shown in Fig. 5b, significantly increased expression of SpTrxR was observed 48 h after ammonia exposure.

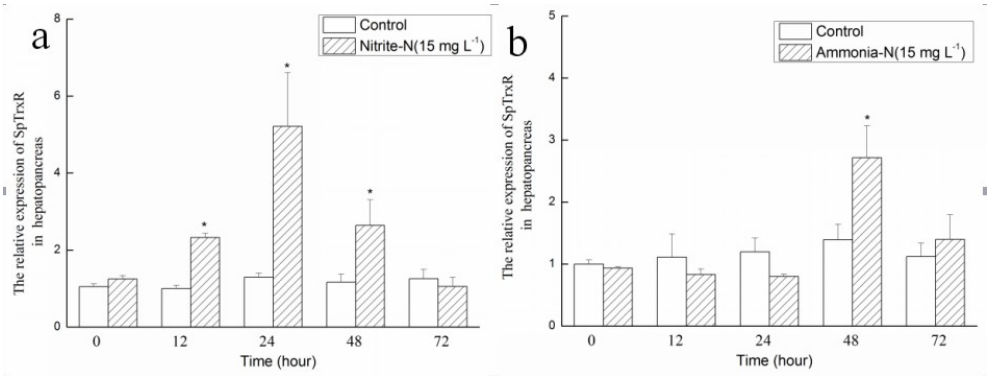

Fig.5. Relative expression levels of SpTrxR in hepatopancreas in response to **(a)** nitrite, and **(b)** ammonia, stress. Data are presented as mean±SD (N=6). Asterisks indicated results that are significantly different from the control (P<0.05).

As shown in Fig. 6a, significantly increased expression of SpTrx was observed from 24 h to 72 h after nitrite exposure. The expression level of SpTrx mRNA transcript increased significantly at 24 h and increased further to a stable high level at 48 h, but then returned to control levels at 72 h after ammonia exposure (Fig. 6b).

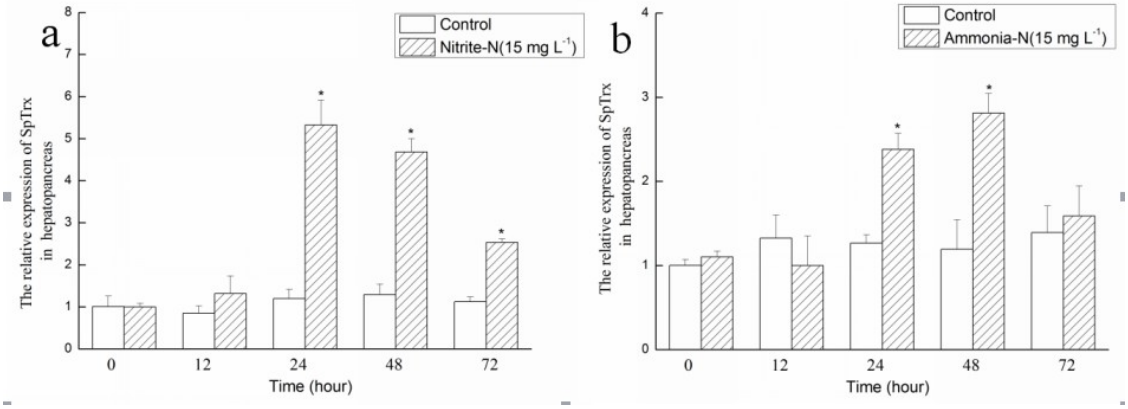

Fig.6. Relative expression levels of SpTrx in hepatopancreas in response to **(a)** nitrite, and **(b)** ammonia, stress. Data are presented as mean±SD (N=6).
Asterisks indicate results that are significantly different from the control (P<0.05).

The transcripts of SpCAT remained at control levels during the first 12 h and then increased significantly from 24 h to 72 h after nitrite exposure (Fig. 7a). The expression levels of SpCAT were up-regulated at 12 and 24 h after ammonia exposure (Fig. 7b).

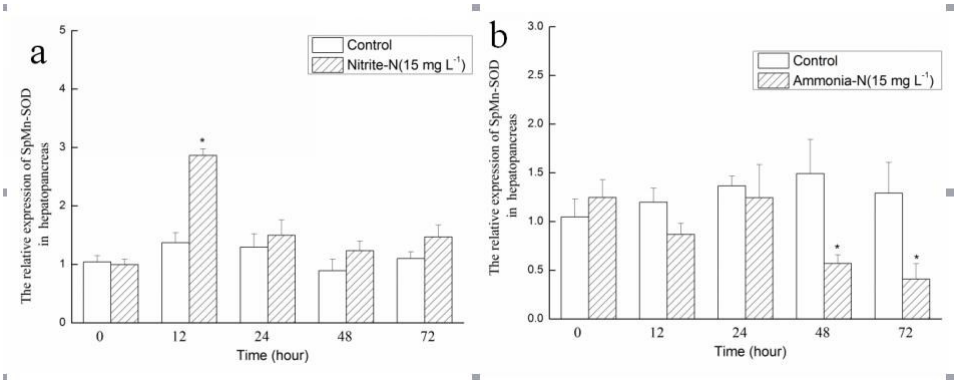

Fig.7. Relative expression levels of SpCAT in hepatopancreas in response to **(a)**nitrite and **(b)** ammonia stress. Data are presented as mean±SD (N=6). Asterisks indicate results that are significantly different from the control (P<0.05).

The expression level of SpSOD mRNA transcript was significantly up-regulated at 12 h and returned to its original level from 24 h to 72 h after nitrite exposure (Fig.8a). The expression level of SpSOD mRNA was not significantly different at 12 h and 24 h after ammonia exposure. However, a significant decrease of SpSOD transcript was observed at 48 h and 72 h after ammonia exposure (Fig. 8b)

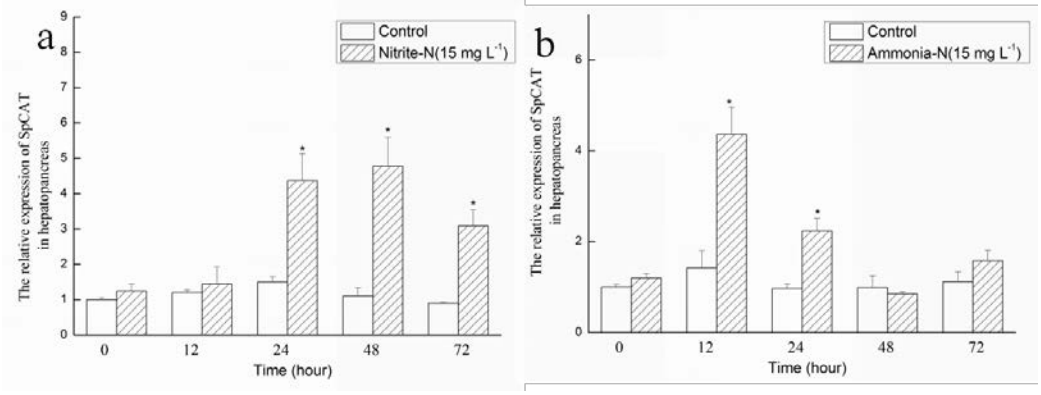

Fig.8. Relative expression levels of SpSOD in hepatopancreas in response to **(a)** nitrite, and **(b)** ammonia, stress. Data are presented as mean±SD (N=6). Asterisks indicate results that are significantly different from the control (P<0.05).

Discussion

In this study, we reported the characterization and expression analysis of cDNA of TrxR gene from *Scylla paramamosain.* The SpTrxR cDNA sequence was predicted to have a 1791 bp ORF encoding 596 amino acid residues. The domain and motif analysis showed that SpTrxR protein contained a typical TrxR domain with glutaredoxin domain, FAD/NAD(P)-binding domain, and pyridine nucleotide-disulfide oxidoreductase dimerization domain. SpTrxR contained the highly conserved pyridine nucleotidedisulfide oxidoreductase class-1 active site in FAD/NAD(P)-binding domain. Blastp analysis suggested that the deduced amino acid of SpTrxR shared a high degree of identity of TrxR1 with *yalella azteca* (69%), *Crassostrea gigas* (65%), and *Danio rerio* (63%). The phylogenetic tree revealed that SpTrxR was an evolutionarily conserved protein. All these results demonstrated the SpTrxR is a novel member of thioredoxin reductase superfamily.

SpTrxR transcript expression in different tissues can be helpful to further understand its function. In our study, the level of SpTrxR mRNA was constitutively expressed in all examined tissues, which further supported its important role in physiological processes. The level of SpTrxR was high in the gills, hemocytesɲ and hepatopancreas. A high level of TrxR transcript expression was observed in *Oplegnathus fasciatus* blood, gillɲ and liver (Elvitigala *et al*, 2015). These results may relate to the function of TrxR. The gills of aquatic organisms are a multi-functional organ for excretion, osmoregulation, and respiration, and are exposed to the aquatic environment. To prevent harmful effects of oxidative stress, antioxidative defense mechanisms must be activated in host organisms (Elvitigala et al., 2015). Therefore, abundant mRNA expression of SpTrxR can be expected in gills.

Hemocytes can produce excessive amounts of ROS in response to invading pathogens. The high level of TrxR transcript in hemocytes maintains an optimum redox balance. The hepatopancreas is an important tissue for immune defense (Gao et al., 2008). Furthermore, the hepatopancreas is the main metabolic center for ROS production. All these results indicated that SpTrxR was dominantly expressed in these tissues and might therefore play an important role in the immune system in mud crabs.

Ammonia and nitrite are the most common environmentally harmful factors in the aquatic environment and are physiologically harmful to aquatic animals (Benli et al., 2008). Previous studies showed that oxidative stress in aquatic organisms was caused by toxicity from ammonia and nitrite (Guo et al., 2013; Cheng et al., 2015). Antioxidant defense systems play an important role against oxidative stress damage. In this study, the mRNA expression of SOD and CAT changed after environmental ammonia and nitrite exposure. Chronic ammonia exposure can induce oxidative stress in liver and white muscle of Nile tilapia juveniles, which in turn alters antioxidant enzymes which prevent

oxidative damage (Hegazi et al. 2010). Antioxidant enzymes (CAT and SOD) in *Litopenaeus vannamei* were up-regulated to protect hemocytes against nitrite stress (Guo et al. 2013). All these results suggest that SOD and CAT play an important role in the antioxidant defense system during ammonia and nitrite exposure.

In our study, SpTrx expression was up-regulated by acute ammonia and nitrite stress. Increased SpTrx expression after acute ammonia and nitrite exposure may be attributed to protection of the cells against oxidative stress. The thioredoxin system is another key antioxidant system to protect against oxidative stress. TRx is involved in an array of cellular functions, including regeneration of oxidative damage proteins, scavenging ROS, DNA synthesis, activation of transcription factors such as p53, NF-kB and activation protein-1 (Nakamura et al., 1997; Tanaka et al., 2002). A variety of oxidative stresses induce Trx. Trx mRNA levels in orange-spotted grouper were up-regulated after viral challenge with Singapore grouper iridovirus infection (Wei et al. 2012). TrxR is also generally considered to have antioxidant activity. Lung TrxR was specifically up-regulated at birth by O_2 (Kumuda et al. 1999). TrxR mRNA was up-regulated after environmental pollutants in *Chironomus riparius* (Nair and Choi (2012). TrxR played an important role in preventing cell damage from oxidative and pathogenic stress in *Oplegnathus fasciatus* (Elvitigala et al., 2015). In our study, we investigated the expression of SpTrxR after ammonia and nitrite exposure. We found that mRNA expression level of SpTrxR was modulated by ammonia and nitrite exposure. Based on the key roles of TrxR in protection against oxidant stress, we predicted that SpTrxR might also play a key role in protection against oxidative stress.

In conclusion, full-length cDNA encoding SpTrxR was cloned from mud crab. It was constitutively expressed in the tested tissues, while strong expression was observed in the gills. The antioxidant system (SpTrxR, SpTrx, SpSOD, and SpCAT) tried to protect cells from oxidative stress induced by ammonia and nitrite stress. We speculate that improving the ability of antioxidant defense may enhance crustacean tolerance to ammonia and nitrite exposure, via dietary supplemental antioxidant nutrients.

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References

Benli, A.C.K., Köksal, G., Özkul, A., 2008. Sublethal ammonia exposure of Nile tilapia (*Oreochromis niloticus* L.): effects on gill liver and kidney histology. *Chemosphere* 72, 1355–1358.

Cheng, C.H., Yang, F.F., Ling, R.Z., Liao, S.A., Miao, Y.T., Ye, C.X., Wang, A.L., 2015. Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (*Takifugu obscurus*). *Aquat Toxicol*. 164 , 61-71.

Elvitigala, D.A., Whang, I., Lee, J., 2015. Molecular profiling and functional insights of rock bream (*Oplegnathus fasciatus*) thioredoxin reductase 3-like molecule: investigation of its transcriptional modulation in response to live pathogen stress. *Gene* 570,122-131.

Galloway, T.S., Depledge, M.H., 2001. Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicol*. 10,5-23.

Gao, B., Jeong, W.I., Tian, Z., 2008. Liver: an organ with predominant innate immunity. *Hepatology*. 47(2),729-736.

Guo, H., Xian, J.A., Li, B., Ye, C.X., Wang, A.L., Miao, Y.T., Liao, S.A., 2013. Gene expression of apoptosis-related genes, stress protein and antioxidant enzymes in hemocytes of white shrimp *Litopenaeus vannamei* under nitrite stress. *Comp Biochem Physiol C Toxicol Pharmacol*. 157(4):366-71.

Holmgren, A., 1985. Thioredoxin, *Annu. Rev. Biochem*. 54,237–271.

Holmgren, A., Lu, J., 2010. Thioredoxin and thioredoxin reductase: current research with special reference to human disease, *Biochem. Biophys. Res. Commun*. 396,120–124.

Kanzok, S.M. Rahlfs, S. Becker,K. Schirmer,R.H., 2002. Thioredoxin, thioredoxin reductase, and thioredoxin peroxidase of malaria parasite *Plasmodium falciparum*, *Methods Enzymol*. 347, 370–381.

Kumuda, C.D., Guo, X.L., White, C.W., 1999. Induction of thioredoxin and thioredoxin reductase gene expression in lungs of newborn primates by oxygen. *Am. J. Physiol. Lung Cell Mol. Physiol*. 276, 530-539.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2-^{\Delta\Delta CT}$method. Methods 25,402–408.

Lu, J. Holmgren, A., 2014. The thioredoxin antioxidant system. *Free Radic Biol Med*.66,75-87.

Mustacich, D., Powis, G., 2000. Thioredoxin reductase. *Biochem. J.* 346, 1–8.

Nair, P.M., Choi, J., 2012. Characterization and transcriptional regulation of thioredoxin reductase 1 on exposure to oxidative stress inducing environmental pollutants in *Chironomus riparius*. *Comp Biochem Physiol B Biochem Mol Biol.* 161,134-139.

Nakamura, H., Nakamura, K., Yodoi, J., 1997. Redox regulation of cellular activation. *Annu Rev Immunol*.15,351-69.

Pacitti, D., Wang, T., Martin, S.A., Sweetman, J., Secombes, C.J., 2014. Insights into the fish thioredoxin system: expression profile of thioredoxin and thioredoxin reductase in rainbow trout (*Oncorhynchus mykiss*) during infection and in vitro stimulation. *Dev Comp Immunol*.42(2):261-77.

Schreck, R., Rieber, P., Baeuerle, P.A., 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kB transcription factor and HIV-1. *EMBO J* 10,2247-58.

Schurmann, P. Buchanan, B.B., 2008.The ferredoxin/thioredoxin system of oxygenic photosynthesis, *Antioxid. Redox Signal*. 10,1235–1274.

Sengupta, R., Holmgren, A., 2013. The role of thioredoxin in the regulation of cellular processes by S-nitrosylation. *Biochim Biophys Acta*. 1820(6),689-700.

Stadtman, E., Levine, R., 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*. 25,207-218.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. *Biol. Med*. 18, 321–336.

Selvaraj, V., Yeager-Armstead, M., Murray, E., 2012. Protective and antioxidant role of selenium on arsenic trioxideeinduced oxidative stress and genotoxicity in the fish hepatoma cell line PLHC-1, *Environ. Toxicol. Chem*. 31 (12),2861-2869.

Tanaka, T., Hosoi, F., Yamaguchi, Y., Nakamura, H., Masutani, H., Ueda, S., 2002. Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J* 21,1695-703.

Wei, J., Guo, M., Ji, H., Yan, Y., Ouyang, Z., Huang, X., Hang, Y., Qin, Q., 2012. Cloning, characterization, and expression analysis of a thioredoxin from orange-spotted grouper (*Epinephelus coioides*). *Dev Comp Immunol*. 38 ,108–116.