Pakistan J. Zool., vol. 47(4), pp. 933-942, 2015.

Molecular Cloning, Characterization and Expression Analysis of Mitochondrial Manganese Superoxide Dismutase of an Oriental River Prawn, *Macrobrachium nipponense*

Wenfeng Wang,[1] Lingmin Mu,[1] Xinsheng Wu,[2] Hong Yang[3] and Qianji Ning[3]*
[1]Department of Life Sciences and Technology, Xinxiang Medical University, Xinxiang 453004, P. R.China
[2]Department of Vasculocardiology, the 371 Central Hospital of PLA, Xinxiang 453004, P. R.China
[3]College of Life Sciences, Henan Normal University, Xinxiang 453002, P. R.China

Abstract.- A mitochondrial manganese superoxide dismutase (mtMnSOD) gene was cloned from the hepatopancreas of *Macrobrachium nipponense* using the rapid amplification of cDNA ends (RACE) method. The full-length cDNA of *mtMnSOD* was 1113 bp with a 654 bp of open reading frame encoding a 218 amino acid (aa) protein with a 21 aa signal peptide sequence in the N-terminus. The calculated molecular mass of the translated protein of mtMnSOD was 31.33 kDa with an estimated pI of 7.12. The deduced amino acid sequence of mtMnSOD had similarity of 97%, 88% and 86% to the *mtMnSOD* of the *Macrobrachium rosenbergii*, *Marsupenaeus japonicus and Fenneropenaeus chinensis*, respectively. The mMtMnSOD contained a manganese superoxide dismutase domain (DVWEHAYY), four conserved amino acids responsible for binding manganese, and two N-glycosylation sites. Real-time RT-PCR analysis showed that the *mtMnSOD* mRNA from *M. nipponense* was expressed in hepatopancreas, muscle, haemocytes, ovary, mandibular organ and gill, but relatively higher in the hepatopancreas. The highest levels of *mtMnSOD* transcripts in hepatopancreas were in stage C among the molt cycle. Injection of pathogenic bacteria *Aeromonas hydrophila* resulted in the significant increase in *mtMnSOD* expressions 3 h after treatment, indicating that MnSOD may act as an important molecule involved in immune defense against *A. hydrophila.*

Key Words: *Macrobrachium nipponense,* mtMnSOD, real-time RT-PCR, expression analysis.

INTRODUCTION

The oriental river prawn, *Macrobrachium nipponense* is an economically important cultured species in China, Japan and Vietnam. Over the last few decades, disease outbreaks caused by various pathogens have been major threats to the prawn farming industry, which has led to huge economic losses. Therefore, how to prevent disease outbreaks is of primary concern. Recently, enhancing the immunity of the prawn become a focus of disease outbreak prevention (Anchalee *et al*., 2013). However, unlike vertebrates, shrimps are believed to lack adaptive immunity and completely depend on their innate immunity, including both cellular and humoral components. The cellular response involves hemocyte phagocytosis, which generates reactive oxygen species (ROS) to eliminate pathogens (Roch, 1999; Munoz *et al*., 2000; Warner, 1994). However, excessive ROS, due to its low reaction specificity, can not only kill the invading pathogenic microorganisms, but also cause serious damage to host organs, tissues and cells, further leading to the destruction of the body physiological functions and the immune system (Holmblad and Soderhall, 1999). Some antioxidant enzymes including superoxide dismutase (SOD) can eliminate the excessive ROS in the pathogen-infected body in a timely manner to maintain normal cell metabolisms (Hung *et al*., 2014; Sun *et al*., 2014; Zhang *et al*., 2007). Therefore, SOD has been an extremely important enzyme in the antioxidant defense pathways in biological immune system (Campa-Cordova *et al*., 2002; Fink and Scandalios, 2002; Zhang *et al*., 2014). SODs including cytosolic manganese superoxide dismutase (cytMnSOD) and mitochondrial MnSOD (mtMnSOD) are widely distributed, and almost exist in all aerobic organisms (Lin *et al*., 2008; Gomez-Anduro *et al*., 2006). The *cytMnSOD* and *mtMnSOD* genes have been cloned from some crustaceans, such as *Fenneropenaeus chinensis*, *Carcinus maenas*, *Macrobrachium rosenbergii* and *Marsupenaeus japonicus* (Cheng *et al*., 2006; Lin *et al*., 2010b; Zhang *et al*., 2007; Marius *et al*., 2003; Imtiaz *et al*., 2012). Among

* Corresponding author: ningqianji1964@163.com.
0030-9923/2015/0004-0933 $ 8.00/0
Copyright 2015 Zoological Society of Pakistan

these species, SOD have been shown to be involved in the innate immune responses of invertebrates, as evidenced by the rapid modulation of SOD transcription during challenges with bacteria (Gomez-Anduro *et al*., 2006), or viruses (Cheng *et al*., 2006). However, little information is available for the *M. nipponense* SOD genes and their immune response against pathogen infection.

The aim of the present work was to clone *M. nipponense mtMnSOD* gene and analyze its expression. In the present study, we first cloned, sequenced and analyzed the *mtMnSOD* gene from the hepatopancreas of *M. nipponense*, and studied its expression at transcript level by Real-time RT-PCR in various tissues and in hepatopancreas from different molt-staged prawns, and analyzed *mtMnSOD* transcripts when *M. nipponense* was injected with *Aeromonas hydrophila*—a widespread representative of *Aeromonas* found in water and water habitants. Our data will not only provide more information for further investigation into the structure and function of the *MnSOD* gene, but also lay the theoretical foundation for the study of resistant mechanisms in crustaceans.

MATERIALS AND METHODS

Experimental animal

Prawns (*M. nipponense*) were obtained from a Fishing Ground in Yuanyang, Henan Province, China, and acclimated at 27±1°C in running-water tanks in the laboratory and fed twice daily for 2 weeks before the experiments. Only healthy prawns with body length of 4.5±0.5 cm in the intermolt stage were used for the study, except for the molt cycle test.

The molt cycle is divided into several substages according to the degree of hardness of the exoskeleton and the retraction of the epithelium within the setae of antennal scales (Cheng *et al*., 2006). They are postmolt (A, B), intermolt (C) and premolt (D0, D1, D2, and D3). Five molt stages (A, B, C, D0-1, and D2-3) were used to examine *mtMnSOD* expression in the hepatopancreas.

Total RNA isolation and reverse transcription (RT)

Total RNA was extracted from *M. nipponense* in various tissues (hepatopancreas, muscle, haemocytes, mandibular organ, ovarian and gill) using TRIzol (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. The quality of RNA was monitored by 1.2% agarose gel electrophoresis (Invitrogen). First-strand cDNAs were synthesized using M-MLV First-Strand cDNA synthesis Kit (Takara, Japan) according to the manufacturer′s instructions.

PCR and subcloning of mtMnSOD cDNA

The hepatopancreas cDNA was used as the template for PCR reaction. Degenerate primers (mtSOD P-1 and mtSOD P-2) were designed based on the highly conserved nucleotides of known *mtMnSOD* of arthropods. The PCR product was subcloned into the pMDT-19 (Takara, Japan) and sequenced from both directions by commercial sequencing company (Invitrogen).

RACE-PCR was performed to obtain the full-length cDNA sequence of *mtMnSOD*. From the partial cDNA sequences, specific primers designed were used to characterize the 5′ and 3′ regions of the *mtMnSOD* cDNA by RACE-PCR (Takara, Japan) according to the manufacturer′ s protocol. 5′ Race outer primer and mtSOD P5-1 were used for the first-round PCR of 5′ RACE, 3′ Race outer primer and mtSOD P3-1 for the first-round PCR of 3′ RACE, respectively. Subsequently, the first-round PCR products were used as the template to perform the nested PCR using Race inner primers（5′inner, 3′inner) included in the kit, and gene specific primers mtSOD P5-2, mtSOD P3-2. The 5′ RACE and 3′ RACE PCR products were gel purified, cloned, and sequenced as above described. The sequences of primers used above were listed in Table I.

Sequence analysis

The *mtMnSOD* gene sequence was analyzed and compared using the BLASTX and BLASTP with a GenBank database search. The potential N-glycosylation sites and signal peptide were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and SignalP program (http://www.cbs.dtu.dk/services/SignalP), respectively. The multiple sequence alignment was created using the CLUSTAL W analysis program. Phylogenetic trees were constructed by the

Neighborjoining method using MEGA4.0 software. The reliability of the tree obtained was assessed by bootstrapping, using 1000 bootstrap replications.

Table I.- Primers used in the experiments.

Name of the primer	Sequences (5′-3′)
mtSOD P-1	CTYCAYCACTCAAAGCATCACC
mtSOD P-2	CTCCCACACATCRATTCCAAA
mtSOD P-F	AGGTTCAATGGAGGAGGTCACA
mtSOD P-R	AGTGCCCCCTTTTGCTTGTTA
mtSOD P3-1	AGGTTCAATGGAGGAGGTCACA
mtSOD P3-2	GGGCTCAGGATGGGGTTGGTTA
mtSOD P5-1	AGTGCCCCCTTTTGCTTGTTA
mtSOD P5-2	TGTGACCTCCTCCATTGAACCT
18S RNA-F	TGTTACGGGTGACGGAGAA
18S RNA-R	CATTCCAATTACGCAGACTCGG

Temporal and spatial expression of mtMnSOD

The mRNA expressions of *M.nipponense mtMnSOD* in various tissues, and that in hepatopancreas from different molt-staged prawns were measured by quantitative real-time PCR following the manufacture′s instructions of SYBR *Premix Ex Taq*™ (TaKaRa, Japan). The proPO primers (mtSOD P-F, mtSOD P-R) and the internal standard 18S rRNA primers (18S RNA-F, 18S RNA-R) were designed based on the sequences of the prawn (*M. nipponense*) (GenBank ID: HQ852225.1, DQ531769.1) (Table I). The PCR reactions were performed as previously described by Wang *et al*. (2013). The real-time quantitative PCR data were analyzed by $2^{-\Delta\Delta C}$.

Expression of mtMnSOD in A. hydrophila*-injected prawn*

A. hydrophila, a ubiquitous bacterium, is a widespread representative of *Aeromonas* found in water and water habitants (Longshaw, 2011), incidence of *A. hydrophila* in fish and shellfish including *M. nipponensehas* been reported (Sahoo *et al*., 2007). Therefore, we injected 20μL bacterial suspension of *A. hydrophila* into the ventral sinus of *M. nipponense* in the stage C, resulting in 1.0×10^5 cfu $prawn^{-1}$. After injection, the prawns were returned to the tanks. Before the injection and at 3, 6, 12, and 24 h post-injection, respectively, three prawns were randomly sampled from the tanks, and hepatopancreas were sampled for *mtMnSOD* expression. Each treatment was performed in three replicates. Gene expression of *mtMnSOD* was determined by quantitative real-time RT-PCP as described above.

Statistical analysis

All data were representative of three independent experiments, and expressed as mean ± SE. All statistical calculations were performed using SPSS13.0 software. One-way ANOVA and Duncan′s multiple comparison test were used to compare the significant differences between groups. Significant difference was accepted at $P < 0.05$.

RESULTS

Cloning and characterization of mtMnSOD gene

The full-length cDNA of *mtMnSOD* was 1113 bp with a 654-bp open reading frame encoding a protein of 218 amino acids. The cDNA included a 31 bp of 5′UTR, a 428 bp of 3′UTR which contained a stop codon, a polyadenylation signal site (AATAAA) and poly(A) tail (Fig. 1). The predicted molecular mass of the mature protein is 24.05 kDa with an estimated pI of 7.12. The cDNA sequence and deduced amino acid sequence have been submitted to the NCBI database (GenBank ID: HQ852225.1).

The deduced amino acid sequence has a 21 aa signal peptide sequence in the N-terminus, and contained a manganese superoxide dismutase domain (DVWEHAYY), four conserved amino acids responsible for binding manganese H48, H96, D180 and H184 and two N-glycosylation sites NHT and, NLS. The deduced amino acid sequence of *M. nipponense mtMnSOD* showed similarity of 40% to that of cytMnSOD, and had similarity of 97%, 88%, 86% and 84% to the *mtMnSOD* of the *M. rosenbergii*, *M. japonicus*, *F. chinensis*, *and C. maenas,* respectively (Fig. 2).

By comparing a full-length multiple alignment sequence of mtMnSOD with that of other arthropods, a phylogenetic tree was constructed by using the Neighbor-joining method (Fig. 3). The phylogenetic analysis indicated that arthropod MnSODs could be classified into two distinct branches, namely cytMnSODs and mtMnSODs. The MN-mtMnSOD (*M. nipponense* mtMnSOD) groups

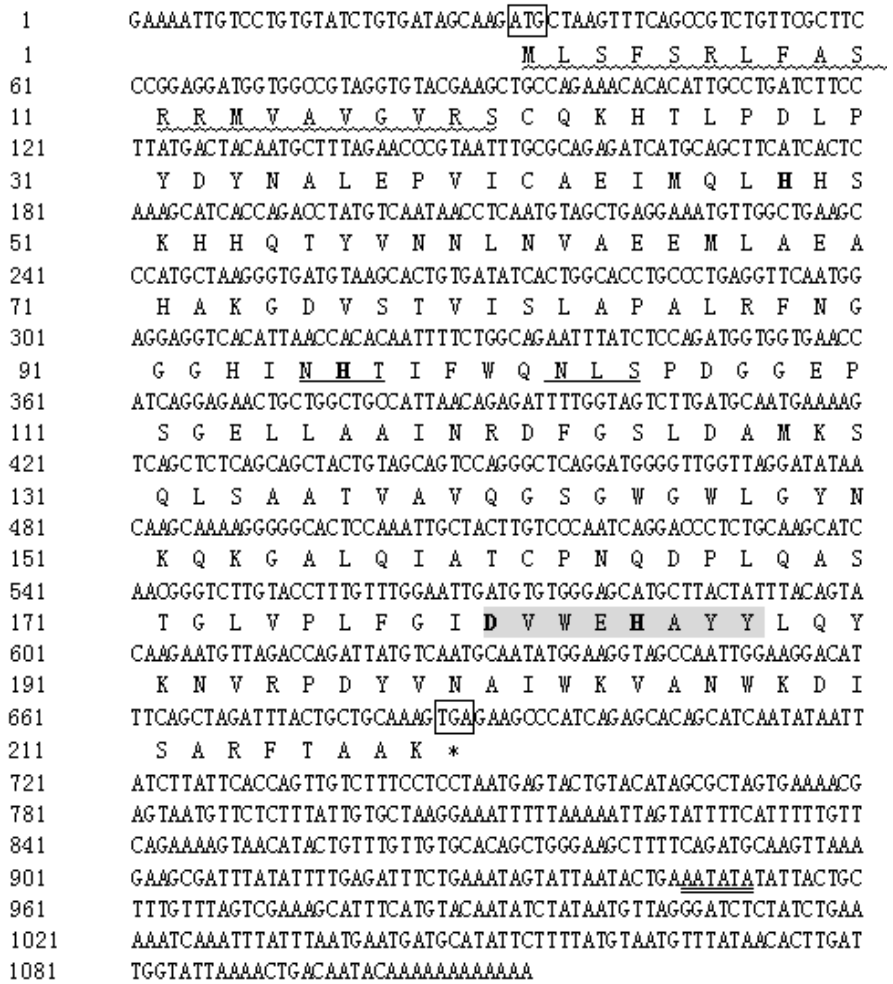

Fig. 1. Nucleotide sequence and deduced amino acid of *mtMnSOD* from *M. nipponense.* □: The start and stop codon. The polyadenylation signal is marked with a wavy underline Potential N-glycosylation sites are underlined. The putative manganese binding sites are bold (H110, H158, D243 and H247). The putative mtMnSOD signature is shown in the shadow.

together with MR-mtMnSOD (*M. rosenbergii* mtMnSOD), but located far away from other cytMnSODs.

Temporal and spatial expression of mtMnSOD

The expression levels of *mtMnSOD* mRNA were measured in different tissues using real-time PCR. The results indicated that the gene was expressed in the hepatopancreas, haemocytes, muscle, mandibular organ, ovarian and gill, with the high expressions in hepatopancreas and low expressions in mandibular organ (Fig. 4).

Considering the high expressions of the *MnSOD* gene in the hepatopancreas, we selected only the hepatopancreas of prawn for further investigation. The fluctuations in the expression level of *mtMnSOD* gene in hepatopancreas were affected by the molt cycle. The *mtMnSOD* mRNA expression was significantly up-regulated in the stage B, and achieved the highest level in the stage C, and was then sharply down-regulated in the stage D0-1 and reached the lowest in the stage D2-3 till stage A. However, no significant differences were observed in *mtMnSOD* mRNA transcription between the stages A, B, D0-1 and D2-3 of prawns (Fig.5).

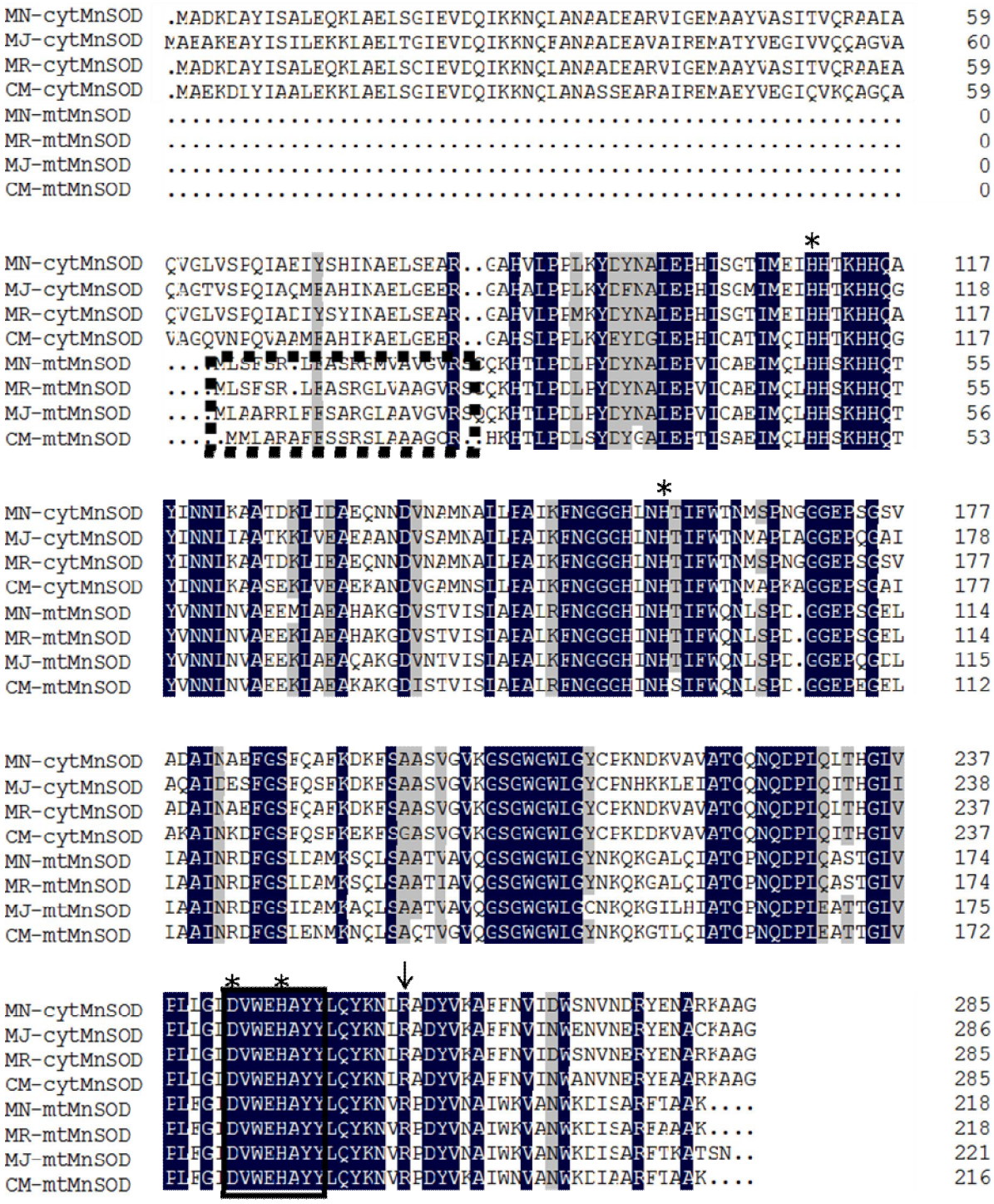

Fig. 2. Alignment of the amino acid sequences of MnSOD from *M. nipponense* and other crustaceans. The dotted line box shows the signal sequence peptide of mtMnSOD. The full line box shows manganese superoxide dismutase domain or signature. The putative manganese binding sites for MnSOD are shown with asterisks. The arrow indicates the R residual involved in the structural stability of MnSOD.

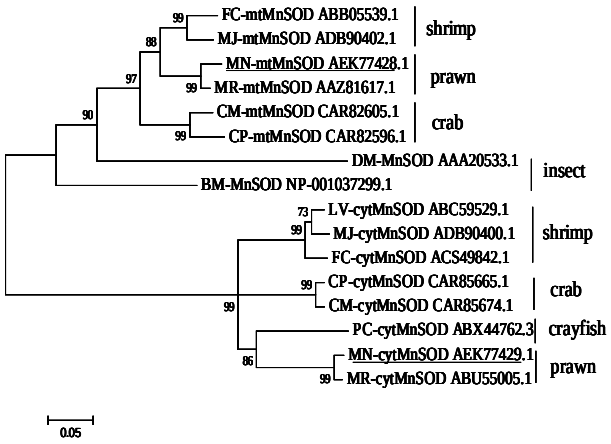

Fig. 3. Neighbor-joining phylogenetic tree of cytMnSOD and mtMnSOD amino acid sequence from different arthropod species. The two MnSODs from *M.nipponense* were underlined.

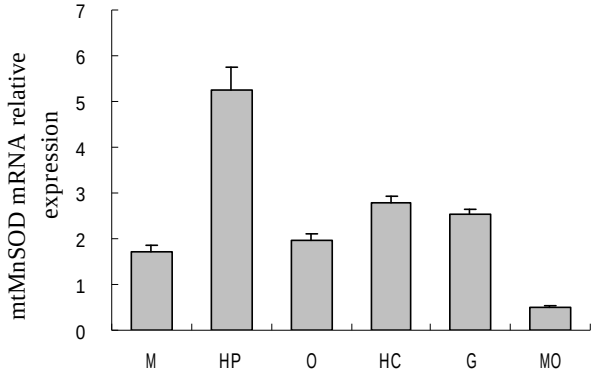

Fig. 4. Real-time PCR analysis of *mtMnSOD* transcripts from different tissues. M, muscle; HP, hepatopancreas; O, ovarian; HC, haemocytes; G, gill and MO, mandibular organ.

Expression of mtMnSOD in A. hydrophila*-injected prawn*

To investigate the transcriptional response of *mtMnSOD* gene *against bacterial challenge*, we inject the bacterium *A. hydrophila* into the ventral sinus of *M. nipponense* in the intermoult stage. Following bacterial challenge, we found that the expression of *mtMnSOD* was modulated by bacterial challenge. The *mtMnSOD* transcripts in hepatopancreas increased significantly 3 h after injection of *A. hydrophila* (P<0.05), reaching the highest level at 3 h, which was approximately 5-fold higher than that before injection, suggesting that the bacterial stimulation can up-regulate the transcripts of *mtMnSOD*. Subsequently, the transcripts of *mtMnSOD* were down-regulated at 6 h post-injection, and then declined to original level at 12-24 h post-injection till the end of the test (Fig. 6).

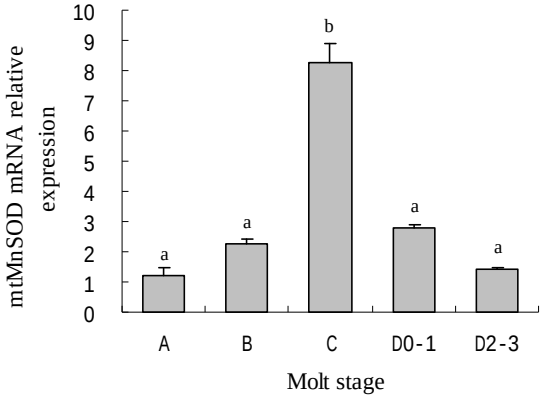

Fig. 5. Changes in *mtMnSOD* mRNA transcripts in hepatopancreas during the molt cycle. Different letters indicate statistical significant difference (p < 0.05).

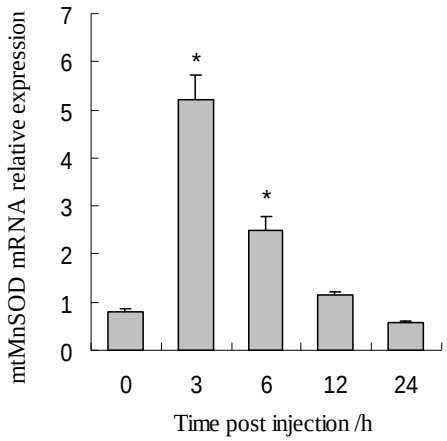

Fig. 6. The induction of *mtMnSOD* expressions by *A. hydrophila.* *P < 0.05, vs 0 h.

DISCUSSION

Currently, it has been demonstrated that the ubiquitous Cu/ZnSOD in vertebrate cells has been replaced by MnSOD in crustacean cells using the

hemocyanin for oxygen transportation. This replacement of Cu/ZnSOD by MnSOD seemed to be involved in the demands for copper ions during the synthesis of hemocyanin (Marius *et al*., 2003). There are two types of MnSOD, cytMnSOD and mtMnSOD. The latter is found in bacteria, plants, invertebrates and vertebrates, whereas the former is found only in crustaceans (crab, lobster, prawn and shrimp). In this study, the *mtMnSOD* gene was first cloned from *M. nipponense*. At amino acid 194 in the amino acid sequence of mtMnSOD, there existed a conserved arginine residue (R). This R residue is near the characteristic peptide of MnSOD (Fig. 2) and involves the structural stability of MnSOD, which has been demonstrated in other species (Gabbianelli *et al*., 1997). In addition, a polyadenylation signal (AATATA) and ATTTA regions which are known to be involved in the regulation of mRNA stability were observed in the 3'UTR, respectively (Malter and Hong, 1991). The main difference between cytMnSOD and mtMnSOD was at the N-terminus of the protein (Lin *et al*., 2010). All mtMnSODs from crustaceans contain a signal peptide consisting of 20 amino acid residues at their N-terminus, while cytMnSOD doesn't have this signal peptide because it still stays in the cytosol after the synthesis (Brouwer *et al*., 1997). After the removal of the signal peptide sequence in mtMnSOD from *M. nipponense*, the predicted molecular mass of the mature peptide was 21.8 kDa, which is consistent with the molecular mass of MnSOD (21.7 kDa) purified from the muscles from *M.nipponense* by Yao *et al*. (2004). Therefore, this MnSOD purified by Yao *et al*. was speculated to be mtMnSOD (Yao *et al*., 2004).

Our data showed that the *mtMnSOD* transcript was detected in all the examined tissues, which is consistent with the results from other animals (San *et al.,* 2014;Wang *et al.,* 2007). As an important antioxidant enzyme, MnSOD may play an important role in maintaining specific tissue function. The different expression levels of MnSOD in different tissues were speculated to be related to tissue-dependent mitochondrial content and oxidative load, as this antioxidant enzyme is a principal scavenger of ROS generated in mitochondrial respiration (Cho *et al.,* 2009). The hepatopancreas and haemocytes are the main organs involved in immunity, and the gills participate in the filtration and removal of the pathogens, in which MnSOD are required to eliminate the excessive ROS (Sun *et al.,* 2014). This rightly explains our results that the expressions of *mtMnSOD* transcript were higher in these tissues.

Crustacean molting is a complex physiological process, and this cyclic change can affect the expressions of multiple genes in the body (Engel *et al.,* 2001; Rao *et al.,* 2008). A study in *Callinectes sapidus* showed that the activity of mtMnSOD can be detected throughout the whole molt cycle, while the highest activity of cytMnSOD was detected in the hepatopancreas only in stage C among the molt cycle (Marius *et al.,* 2003). However, few studies have focused on the relationship between *MnSOD* gene expression and molting cycle. Our results showed that the fluctuations in the expressions of *mtMnSOD* were affected by the molt cycle, and its highest expression level was observed in stage C among the molt cycle, suggesting that transcription of this gene may be regulated by the ecdysone. Such tremendous changes in the body's oxygen consumption should be one of the factors that affect the expression of the *MnSOD* gene while molting (Engel *et al.,* 2001).

In aerobic organisms, SOD protects cells from superoxide anions by converting superoxide into oxygen and hydrogen peroxide. In crustacean, SODs also function in the innate immune system, which includes both humoral and cellular responses (Hung *et al.,* 2014). Hemocyte phagocytosis, one of the cellular responses, produces ROS to eliminate pathogens. Campa-Cordova *et al.* (2002) found that after challenge with the immunostimulants, the activities of SOD in the haemocytes and muscles from *L. vannamei* were increased, and a significant increase in the survival rate of this *L. vannamei* was also observed after the infection with the pathogenic bacteria, indicating that SOD has a regulatory role in the immune response in prawns. Considering the high expressions of the *MnSOD* gene in stage C among the molt cycle and in the hepatopancreas, we selected the shrimps in stage C among the molt cycle for challenge test. Our data showed that the transcripts of *MnSOD* in hepatopancreas increased significantly at 3 h post challenge with *A. hydrophila,* suggesting that *mtMnSOD* have roles in

the innate immune system of *M. nipponense*, which is consistent with the results in hepatopancreas of WSSV virus-infected Chinese shrimps (Cheng *et al*., 2006a,b). By contrast, Gomez-Anduro *et al*. (2006) found that no significant changes were observed in the expression level of the *mtMnSOD* gene in the hepatopancreas from *M. rosenbergii* 3 h after the injection of *Lactococcus garvieae,* while the expression level of the *cytMnSOD* gene cultures was about three times lower than the normal after the same treat treatment (Gomez-Anduro *et al*., 2006). The differences between these aforementioned results may be due to the different species and different types and concentrations of the pathogens. When the shrimps are stimulated by the lower concentration of pathogens, more ROS will be generated in the shrimp cells. Consequently, the expression of *MnSOD* gene will also be up-regulated to eliminate the ROS. The invasion of the higher concentration of pathogens into the crustaceans can damage their tissues and cells, which results in the reduction in the cells expressing *MnSOD*. In addition, the different oxidative stress in shrimp caused by different types of pathogens and the different abilities to scavenge ROS generated may also lead to the differences in the gene expression of *MnSOD* (Hung *et al*., 2014).

In conclusion, the mtMnSOD cDNA was cloned from hepatopancreas of *M. nipponense*. The *mtMnSOD* cDNA consisted of a 654-bp open reading frame that encoded a sequence of 218 amino acids. MnSOD showed common features including a manganese super oxide dismutase domain, and four amino acids for binding manganese. The mtMnSOD had a 21 aa mitochondrial-targeting sequence in the N-terminus. The deduced amino acid sequence of *M. nipponense mtMnSOD* had similarity of 97%, 88% and 86% to the *mtMnSOD* of the *M. rosenbergii*, *M. japonicus* and *F. chinensis*, respectively. The *mtMnSOD* transcripts were expressed in hepatopancreas, muscle, haemocytes, ovary, mandibular organ and gill, but relatively higher in the hepatopancreas. The highest levels of *mtMnSOD* transcripts in hepatopancreas were in stage C among the molt cycle. The *mtMnSOD* transcripts in hepatopancreas of *M. nipponense* were upregulated 3h after *A.hydrophila* injection, indicating an induction of this SOD system in a short time. Taken together, our results suggest that *mtMnSOD* have roles in the innate immune system of *M. nipponense*.

ACKNOWLEDGMENTS

This work is supported by grants from the National Natural Science Foundation of China (No.30940008), The Henan Scientific and Technological Research Projects (No. 142102310045 and 142300410164), the Natural Science Project of the Education Department of Henan Province (No.14A240003 and 14A180031) and the cultivate Foundation of Xinxiang medical college (No. 2013ZD109).

REFERENCES

ANCHALEE, T., KUNLAYA, S., PREMRUETHAI, S. AND SUREERAT, T., 2013. Discovery of immune molecules and their crucial functions in shrimp immunity. *Fish Shellfish Immunol.*, **34**: 954-967.

BROUWER, M., BROUWER, T.H., GRATER, W., ENGHILD, J.J. AND THOGERSEN, I.B., 1997. The paradigm that all oxygen-respiring eukaryotes have cytosolic CuZn-superoxide dismutase and that Mn-superoxide dismutase is localized to the mitochondrion does not apply to a large group of marine arthropods. *Biochemistry,* **36**:13381-13388.

CAMPA-CORDOVA, A.I., HERNANDEZ-SAAVEDRA, N.Y., DE PHILIPPIS, R. AND ASCENCIO, F., 2002a. Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to β-glucan and sulphated polysaccharide. *Fish Shellfish Immunol.*, **12**:353-366.

CAMPA-CORDOVA, A.I., HERNANDEZ-SAAVEDRA, N.Y. AND ASCENCIO, F., 2002b. Superoxide dismutase as modulator of immune function in American white shrimp (*Litopenaeus vannamei*). *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.*, **133**:557-565.

CHENG, W., TUNG, Y.H., CHIOU, T.T. AND CHEN, J.C., 2006a. Cloning and characterization of mitochiondrial manganese superoxide dismutase (mtMnSOD) from the giant freshwater prawn *Macrobrachium rosenbergii. Fish Shellfish Immunol.*, **21**:453-466.

CHENG, W., TUNG, Y.H., LIU, C.H. AND CHEN, J.C., 2006b. Molecular cloning and characterization of cytosolic manganese superoxide dismutase (cytMn-SOD) from the giant freshwater prawn *Macrobrachium rosenbergii*.

Fish Shellfish Immunol., **20**:438-449.

CHO, Y.S., LEE, S.Y., BANG, I.C., KIM, D.S. AND NAM, Y.K., 2009. Genomic organization and mRNA expression of manganese superoxide dismutase (Mn-SOD) from *Hemibarbus mylodon* (Teleostei, Cypriniformes). *Fish Shellfish Immunol*., **27**:571-576.

ENGEL, D.W., BROUWER, M. AND MERCALDO, A.R., 2001. Effects of molting and environmental factors on trace metal body-burdens and hemocyanin concentrations in the American lobster, *Homarus americanus*. *Mar. environ. Res*., **52**: 257-269.

FINK, R.C. AND SCANDALIOS, J.G., 2002. Molecular evolution and structure-function relation ships of the superoxide dismutase gene families in angiosperms and their relationship to other eukaryotic and prokaryotic superoxide dismutase. *Arch. Biochem. Biophys.*, **399**:19-36.

GABBIANELLI, R., BATTISTONI, A., POLITICELLI, F., MEIER, B., SCHMIDT, M., ROTILIO, G. AND DESIDERI, A., 1997. Effect of lys175 mutation on structure function properties of *Propionibacterium shermanii* superoxide dismutase. *Protein Eng.*, **10**:1067-1070.

GOMEZ-ANDURO, G.A., BARILLAS-MURY, C.V., PEREGRINO-URARTE, A.B., GUPTA, L., GOLLAS-GALVÁN, T., HERNÁNDEZ-LÓPEZ, J. AND YEPIZ-PLASCENCIA, G., 2006. The cytosolic manganese superoxide dismutase from the shrimp *Litopenaeus vannamei*: molecular cloning and expression. *Dev. Comp. Immunol*., **30**:893-900.

HOLMBLAD, T. AND SODERHALL, K., 1999. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquaculture*, 172:111-123.

HUNG M.N., SHIOMI, R., NOZAKI, R., KONDO, H. AND HIRONO, I., 2014. Identification of novel copper/zinc superoxide dismutase (Cu/ZnSOD) genes in kuruma shrimp *Marsupenaeus japonicus*. *Fish Shellfish Immunol.*, **40**:472-477.

IMTIAZ, A. AND NAZ, S., 2012. A rapid and cost-effective protocol for screening known genes for autosomal recessive deafness. *Pakistan J. Zool.*, **44**:641-647.

LIN, Y.C., LEE, F.F., WU, C.L. AND CHEN, J.C., 2010. Molecular cloning and characterization of a cytosolic manganese superoxide dismutase (cytMnSOD) and mitochondrial manganese superoxide dismutase (mtMnSOD) from the kuruma shrimp *Marsupenaeus japonicus*. *Fish Shellfish Immunol.*, **28**:143-150.

LIN, Y.C., VASEEHARAN, B. AND CHEN, J.C., 2008. Identification of the extracellular copper-zinc superoxide dismutase (ecCuZnSOD) gene of the mud crab *Scylla serrata* and its expression following β-glucan and peptidoglycan injections. *Mol. Immunol.*, **45**:1346-1355.

LONGSHAW, M. 2011. Diseases of crayfish: a review. *J. Inverteb. Pathol.*, **106**:54-70.

MALTER, J. AND HONG, Y. A., 1991. A redox switch and phosphorylation are involved in the post-translational up-regulation of the adenosine-uridine binding factor by phorbol ester and ionophore. *J. biol. Chem.*, **266**: 3167-3171.

MARIUS, B., THEA, H.B., WALTER, G. AND BROWN-PETERSON, N., 2003. Replacement of a cytosolic copper/zinc superoxide dismutase by a novel cytosolic manganese superoxide dismutase in crustaceans that use copper (haemocyanin) for oxygen transport. *Biochem. J.*, **374**:219-228.

MUNOZ, M., CEDENO, R., RODRGUEZ, J., MIALHE, E. AND BACHÈRE, E., 2000. Measurement of reactive oxygen intermediate production in aemocytes of the penaeid shrimp, *Penaeus vannamei*. *Aquaculture*, **191**:89-107.

RAO, M.S., RAJITHA, B., PAVITRA, E. AND ANJANEYULU, N., 2008. Changes of copper and protein profiles in hepatopancreas and hemolymph tissues during different molt stages of white Shrimp, *Litopenaeus vannamei*. *Biotechnology*, **7**:153-156.

ROCH, P., 1999. Defense mechanisms and disease prevention in farmed invertebrates. *Aquaculture*, **172**:125-145.

SAHOO, P.K., PILLAI, B.R., MOHANTY, J., KUMARI, J., MOHANTY, S. AND MISHRA, B.K., 2007. *In vivo* humoral and cellular reactions, and fate of injected bacteria *Aeromonas hydrophila* in freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol.*, **23**:327-340.

SUN, S.M., ZHU, J., JIANG, X.J., LI, B. AND GE, X.P., 2014. Molecular cloning, tissue distribution and expression analysis of a manganese superoxide dismutase in blunt snout bream *Megalobrama amblycephala*. *Fish Shellfish Immunol.*, **38**:340-347.

WANG, W.F., YANG, H., LIU, F., CHEN, X.L., LV, Y.J. AND NING, Q.J., 2013. A novel effect of imidazole derivative KK-42 on increasing survival of *Aeromonas hydrophila* challenged. *Fish Shellfish Immunol.*, **34**:167-172.

WANG, Y.C., CHANG, P.S. AND CHEN, H.Y., 2007. Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.*, **23**:1161-1177.

WARNER, H.R., 1994. Superoxide dismutase, aging, and degenerative disease. *Free Radic Biol. Med.*, **3**:249-258.

YAO, C.L., WANG, A.L., WANG, W.N. AND SUN, R.Y., 2004. Purification and partial characterization of Mn superoxide dismutase from muscle tissue of the shrimp *Macrobrachium nipponense*. *Aquaculture*, **241**:621-631.

ZHANG, Q.L., LI, F.H., WANG, B., ZHANG, J.Q., LIU, Y.C., ZHOU, Q. AND XIANG, J.H., 2007. The mitochondrial manganese superoxide dismutase gene in Chinese shrimp *Fenneropenaeus chinensis*: Cloning,

distribution and expression. *Dev. Comp. Immunol*., **31**:429-440.

ZHANG, Y.Y., LIU, B., GE, X.P., LIU, W.B., XIE, J., REN, M.C., CUI, Y.T., XIA, S.L., CHEN, R., ZHOU, Q., PAN, L. AND YU, Y., 2014. The influence of various feeding patterns of emodin on growth, non-specific immune responses, and disease resistance to *Aeromonas hydrophilain* juvenile Wuchang bream (*Megalobrama amblycephala*). *Fish Shellfish Immunol.*, **36**: 187-193.

(Received 15 December 2014, revised 7 February 2015)