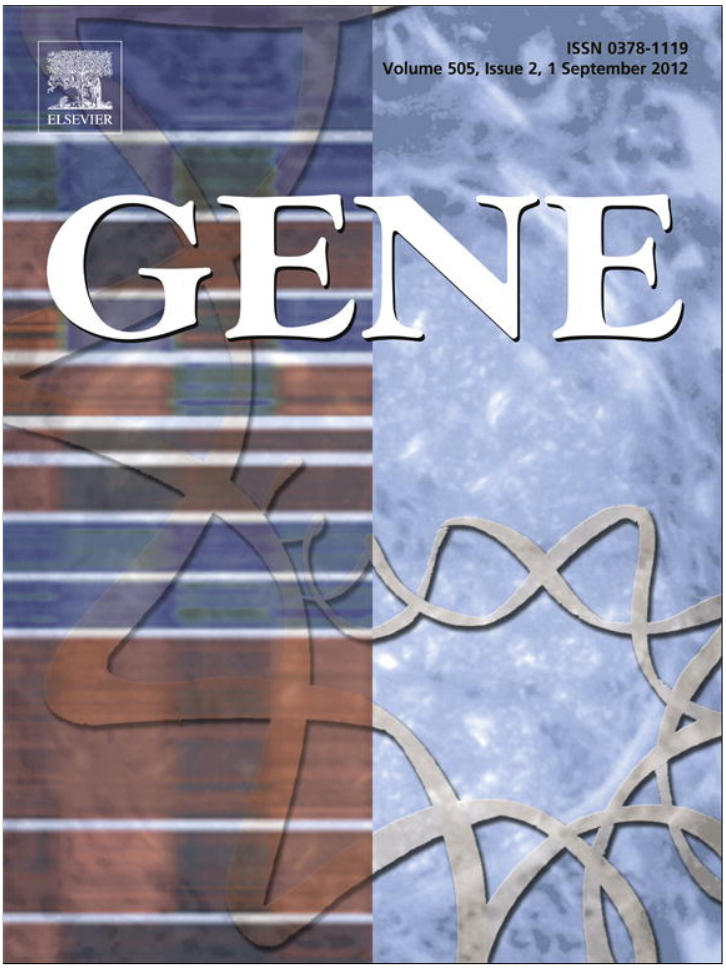

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Molecular cloning and expression patterns of copper/zinc superoxide dismutase and manganese superoxide dismutase in *Musca domestica*

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ABSTRACT

Superoxide dismutases (SODs) are metalloenzymes that represent one important line of defense against reactive oxygen species (ROS). In this paper, two novel *SOD* genes, *MdSOD1* and *MdSOD2*, which putatively encode 261 and 214 amino acid residues respectively were identified and characterized from the housefly *Musca domestica*. The high similarity of *MdSOD1* and *MdSOD2* with SODs from other organisms indicated that they should be two new members of the SOD family. qPCR exhibited a universal expression of *MdSOD1* and *MdSOD2* detected in various tissues of housefly larva, including the fat body, gut, hemocyte and epidermis. Expression profiling reveals that *MdSOD1* and *MdSOD2* can be induced significantly via not only heat shock and cadmium (Cd) stress but also *Escherichia coli* and *Staphylococcus aureus* challenge. The two genes were cloned into the prokaryotic expression vector pET-28a to obtain the fusion proteins rMdSOD1 and rMdSOD2. Between them, the activity of rMdSOD2 was found by visual assay methods. ESI-LC–MS/MS analysis showed that three peptide fragments of the protein rMdSOD2 were identical to the corresponding sequence of *M. domestica* MdSOD2. *MdSOD1* and *MdSOD2* in housefly larvae were abrogated by feeding bacteria expressing dsRNA. High mortalities were observed in the larvae treated with dsRNA of *SODs* at heat shock, Cd stress and bacterial invasion. This phenomenon indicated that *MdSOD1* and *MdSOD2* are related to the survival of *M. domestica* under stress. This may provide new insights into the role of the two SOD genes in protecting *M. domestica* against both stress and bacterial invasion.

1. Introduction

ROS are highly reactive oxygen derived molecules that include free radicals and peroxides. ROS play a significant role in the innate immunity system of insects (Yamamoto et al., 2005). They can stimulate signal transduction and mediate different responses such as cell growth and apoptosis (Suzuki et al., 1997). ROS are formed as natural byproducts of mitochondrial respiration and it is estimated that as much as 0.1% to 5% of all oxygen consumed by mitochondria is converted into ROS. Generation of ROS is an inevitable consequence of oxygen metabolism in most aerobic organisms. Under unstressed conditions, organisms have well-developed defense systems against ROS, and the removal of ROS is in balance. However, when presented with increased ROS under stress conditions, the defense system can be overwhelmed (Zhang et al., 2011). The excess of ROS produced in living organisms has the potential to damage key cellular components including lipids, proteins and DNA (Sinnis et al., 2010). A large amount of ROS can lead to oxidation, loss of cell function (Halliwell and Gutteridge, 1984; Nordberg and Arner, 2001) and damage the cell membranes (Iiyama et al., 2007). In order to balance the harmful and positive effects of ROS production, organisms evolved to use antioxidant systems to maintain oxygen radicals at fitting concentrations (Arenas-Rios et al., 2007; Park et al., 2004).

SODs are the main antioxidant enzyme families in organisms, they are considered as the first defense line against oxidative stresses by catalyzing the dismutation reaction of ROS into hydrogen peroxide and water (den Hartog et al., 2003; Mruk et al., 2002; Vaughan, 1997). Importantly, the SODs were also demonstrated to play a role in immune response induced by bacteria (Cheng et al., 2006; Jung et al., 2005), virus infection (H. Zhang et al., 2007; Q. Zhang et al., 2007a), toxic chemical exposure and thermal stress (Kim et al., 2007).

Abbreviations: *β-actin*, beta-actin; Cd, cadmium; Ct, cycle threshold; FTICR, Fourier transform ion cyclotron resonance; GFP, green fluorescent protein; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; LB, Luria broth; MS, matrix science; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitro blue tetrazolium; NJ, neighbor-joining; ORF, open reading frame; RNAi, RNA interfere; ROS, reactive oxygen species; RT, reverse transcription; SOD, superoxide dismutase; WSSV, white spot syndrome virus.

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According to the metal ion cofactor identified in their active site, they were classified into distinct forms of *SOD*, mainly including *Cu/Zn-SOD*, *Mn-SOD* and *Fe-SOD* (Ni et al., 2007; Zhang et al., 2011). Although *SOD* have been identified from some bacteria, trematode, abalone, oyster, silkworm and shrimp, the studies regarding molecular features and function of *SOD* from insects *Musca domestica* are rarely investigated to our knowledge. The main objectives of the present study are: 1) to clone the full-length cDNA of *SOD* from *M. domestica*; 2) to investigate the tissue expression profile of *SOD*; 3) to examine the transcript expression profile of *SOD* in the housefly larva after thermal stress, heavy metal exposure and bacterial infection; 4) to express recombinant protein in *Escherichia coli* and investigate its biological activity; and 5) to investigate the roles of *SOD* in the housefly by RNAi-mediated gene silencing.

2. Experimental procedures

2.1. Housefly strain

The strain of houseflies was provided by Fengqin He, Institute of Zoology, Chinese Academy of Sciences. Larvae *M. domestica* were raised in a climate room at 25 °C, relative humidity 75% and fed on a medium made of bran (55 g), heat-inactivated yeast (3 g), water (150 mL) and antimycotic nipagin (0.35 g) until pupation. After eclosion, adult flies were fed on water, sugar and milk powder. Flies were maintained at 25 °C under 12 h light/12 h dark cycles (LD12:12) (Codd et al., 2007).

2.2. Exposure to stress and sampling

To investigate the expressions of *SOD*s in various tissues, hemocytes, fat body, gut and epidermis were performed according to the methods described previously (Dong et al., 2011) for RNA extraction.

The heat resistance and Cd tolerance assays were performed according to the methods described previously (Tang et al., 2011). For heat shock, the 3rd instar larvae were exposed at 42 °C for 1 h and untreated larvae were sampled as control simultaneously. After treatment at 42 °C, larvae were transferred to normal condition at 25 °C for a continuous post-stress recovery. Samples were randomly collected at 15 min, 30 min, and 1 h during the heat shock treatment at 42 °C and 1 h, 2 h, and 4 h during the recovery time separately.

In order to learn the response of the housefly to Cd stress, the 3rd instar larvae were reared on a medium which was replaced with water with a concentration of $CdCl_2$ solution that ranged from 0 mM to 30 mM for 24 h. Control was achieved using normal medium. When maximum induced concentrations were measured within a 24-hour period, samples were collected at 3 h, 6 h, 12 h and 24 h.

For septic injury infection experiments, the 2nd instar larvae were challenged according to the method described previously (Dong et al., 2011). Septic injury was produced by pricking the abdomen of the larvae with a needle previously dipped into a concentrated culture of *E. coli* or *Staphylococcus aureus*. Larvae were sampled for RNA extraction at 0, 3, 6, 12, 24, 36, 48 and 72 h after infection.

2.3. Total RNA extraction and cDNA preparation

Total RNAs were immediately extracted from whole bodies or larval tissues using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA purity was estimated using spectrophotometric measurements at $A_{260/280}$ absorbance, and integrity was checked by agarose gel electrophoresis. The first-strand cDNA was made using the universal forward primer AOLP (Liu et al., 2009). A reverse transcription (RT) reaction was performed with 2 μg RNA as a template in RT buffer containing 10 mM deoxy-ribonucleotide triphosphate, 10 mM of each primer, 1 mM dithiothreitol and one unit of M-MLV in 25 μg total volume. After an initial 5 min denaturation at 70 °C, 1 h extension at 42 °C, followed by cooling to 4 °C. The sequences of primers are listed in Table 1.

Table 1
Primers used in PCR of two genes in the housefly.

Primers	Sequences (5′–3′)
AOLP	GGCCACGCGTCGACTAGTAC$(T)_{16}$(A/C/G)
MdSOD1-F1	ATGGTCGGGCTACATTCCGC
MdSOD2-F1	GACCGCTCTTACCGTTGCTG
AP	GGCCACGCGTCGACTAGTAC
MdSOD1-F2	TCCACAAAAATATGGCAGAA
MdSOD1-R2	GCAGCTATAACTTTTGCGCC
MdSOD2-F2	CGAAAGAAACATGTTTGCTC
MdSOD2-R2	GCGCTGCTTCTTACTTGGCG
MdSOD1-F3	TGACGCCAGGTTTACATGGC
MdSOD1-R3	GCCCGACCATTGTCATCAGC
MdSOD2-F3	ACCAGACCTATGTCAACAAC
MdSOD2-R3	CTTCAAATCAGCAGAAGGTT
Actin-F	GAGAAATCCTATGAACTTCCCGACG
Actin-R	GGATACCGCAAGATTCCATACCCAA
MdSOD1-F4	GCGCTAGCATGGCAGAAATTAAGGTTGAAT
MdSOD1-R4	GCAAGCTTTTATAACTTTTGCGCCCTTGTA
MdSOD2-F4	GCGCTAGCATGTTTGCTCTCAGCCGTAATG
MdSOD2-R4	GCAAGCTTTTACTTGGCGTCGGCGAAACGT
MdSOD1-F5	GCGAATTCGCAGAAATTAAGGTTGAATT
MdSOD1-R5	GCCTCGAGTTATAACTTTTGCGCCCTTG
MdSOD2-F5	GCGAATTCTTTGCTCTCAGCCGTAATGT
MdSOD2-R5	GCCTCGAGTTACTTGGCGTCGGCGAAAC
GFP-F1	CGGAATTCATGGTGAGCAAGGGCGAGGA
GFP-R1	CGCTCGAGCTTGTACAGCTCGTCCATGC

2.4. Cloning and analysis of MdSOD1 and MdSOD2 genes

Two ESTs of suspected *SOD* genes were obtained by searching our transcriptomic database for *M. domestica* (Liu et al., 2012). They were named *MdSOD1* and *MdSOD2*. The 3′ end of *MdSOD1* and *MdSOD2* cDNAs were amplified using the primers MdSOD1-F1, MdSOD2-F1 and AP respectively. To confirm cDNA sequence, two gene-specific primer pairs, MdSOD1-F2 and MdSOD1-R2, MdSOD2-F2 and MdSOD2-R2 were designed basing on the sequence of the assembled contig described above.

The putative *MdSOD1* and *MdSOD2* ORFs were found by Bioedit program. Sequence identity and similarity of the two *SOD* genes were compared with other species by using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein analyses were performed using ExPASy online tools (http://us.expasy.org/tools). Overall similarity was visualized using neighbor-joining (NJ) trees constructed using Mega 4.1 from nucleic acid sequences aligned with ClustalW. One thousand bootstraps were performed to assess nodal supports.

2.5. qPCR and statistical analysis

To establish two *SOD* genes' expression, we cloned beta-actin (*β-actin*) mRNA partial sequences from *M. domestica* as the reference gene for internal standardization.

The mRNA expression levels of *MdSOD1* and *MdSOD2* were measured by qPCR. Two pairs of gene-specific primers MdSOD1-F3 and MdSOD1-R3, MdSOD2-F3 and MdSOD2-R3 were used to quantify transcripts of SODs. Two *β-actin* primers, Actin-F and Actin-R were used to amplify a 115 bp fragment as endogenous control to calibrate the cDNA template for corresponding samples.

Expression level of the target gene was calculated by comparing the cycle threshold value (Ct) to the reference gene *β-actin*. The relative quantification (comparative method) was calculated using the ΔΔCt method (Farcy et al., 2007). All samples were normalized to the ΔCt value of a reference gene to obtain a ΔΔCt value (ΔCt target − ΔCt reference). The final relative expression was calculated using the following formula: $F = 2^{-(\Delta Ct\ target - \Delta Ct\ reference)}$. A randomly selected untreated control sample was used as the calibrator for calculating relative expression ratios. All statistics were determined by one-way ANOVA post-test, Tukey's multiple comparison tests

using SPSS 13.0 and a two-tailed, homoscedastic, Student's *t*-test using Excel (Microsoft). In all cases, differences of $P<0.05$ were considered to be significant. All experiments were repeated at least three times. All means and standard errors were calculated using raw (untransformed) data. Prior to regression analyses, all data were checked for normality using probability plots, and data not conforming to a normal distribution were log-transformed using the Johnson transformation.

2.6. Expression of rMdSOD1 and rMdSOD2 in E. coli

The DNA fragments encoding *MdSOD1* and *MdSOD2* were obtained by PCR amplification with the primers MdSOD1-F4 and MdSOD1-R4, MdSOD2-F4 and MdSOD2-R4, which encompassed the Nhe I and Hind III sites, respectively. The amplified fragments were digested with the endonucleases, and subcloned into a pET-28a expression vector digested with the same enzymes. The constructed plasmid, denoted as rMdSOD1 and rMdSOD2, were transformed into competent cells of *E. coli* BL21 (DE3) for fusion expression. A parent vector without inserts was used as a control. rMdSOD1 and rMdSOD2 fusion proteins were expressed in *E. coli* and induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C for 4 h. Luria broth (LB) was used for bacterial culture medium. Expression efficiency of different transformants was assessed by analysis of the target protein band in SDS-PAGE.

2.7. Recombinant protein purification and refolding

In order to obtain the recombinant protein, the culture volume was increased to 100 mL and the culture condition was optimized. The transformed *E. coli* were incubated at 200 rpm, 37 °C for 4 h after inducing with 1 mM IPTG, cells were harvested by centrifugation at 4 °C and used for the purification of recombinant protein through High Affinity Ni-Charged Resin according to the User Manual. The purified protein was diluted to 1 mg/mL with buffer A (10 mM dithiothreitol and 8 M urea), then 1:100 (purified protein: buffer B) refolded by stepwise dialysis through 8.0, 6.0, 4.0, 3.0, 2.0, 1.0, 0.5 and 0 M urea buffer B (50 mM Hepes, 20 mM KCl and 5 mM dithiothreitol, pH7.4) at 4 °C for 16 h respectively (Thomas et al., 1997; Q. Zhang et al., 2007b).

2.8. Determination of protein concentration and in vitro SOD activity

Protein concentration measurement was conducted according to Bradford (1976) using bovine serum albumin (BSA) as the standard. In Native-PAGE analysis, rMdSOD1 and rMdSOD2 applied concentrations were about 0.6 and 0.8 mg/mL respectively. Visualization of the SOD activity on Native-PAGE gels (7.5% separating gel, 3.75% stacking gel and Tris-glycine buffer pH8.0, experimental system did not contain SDS) was performed by the nitro blue tetrazolium (NBT) illumination method (Beauchamp and Fridovich, 1971). The gel was soaked in an NBT solution for 30 min, and then the NBT solution was poured off and replaced by a riboflavin solution for 20 min. Subsequently, the gel was illuminated until it became uniformly blue.

2.9. ESI-LC–MS/MS identification of the active protein

The target bands were excised from the gel with a sterile scalpel and digested with sequencing-grade trypsin according to Shevchenko's method (Shevchenko et al., 1996). The digested samples were analyzed by LC–MS/MS using Dionex Ultimate 3000 chromatography system with nano-spray and Bruker Apex-Ultra 7.0 T Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. In brief, the LC buffers were as follows: buffer A (0.1% formic acid in Milli-Q water at v/v) and buffer B (90% acetonitrile and 0.1% formic acid in Milli-Q water at v/v). Then, 5 μL of sample was loaded at 500 nL/min onto the C18 column (75 μm × 15 mm, PepMap C18 column, 3 μm, 100 Å, LC-Packings). The peptides were eluted from the column at a constant flow rate of 200 nL/min with a linear gradient from 10% buffer A to 40% buffer B in 90 min, and then to 100% buffer B in 2 min. The HPLC was interfaced to the mass spectrometer with FS360-20-10-D-20 PicoTip (New Objectives) fitted to an On-line NanoElectrospray Source (Bruker Daltonik, Germany). The ultra apex FTICR mass spectrometry was operated in the positive mode in multiple-charged mode with analyte and source dependent parameters optimized for full scan mode from m/z 100 to 2000.

MS signal was searched by MASCOT v. 2.1 (Matrix Science) using the local NCBIr database. Searches were performed without variable oxidation and fixed modifications of residues. The maximum of missed cleavage was set to 1 to limit the trypsin cleavage sites. Precursor mass tolerance and fragment mass tolerance were set to 5 ppm and 0.01 Da, respectively.

2.10. Vector construction and dsRNA preparation

In order to construct the plasmid expressing dsRNA, the DNA fragments encoding *MdSOD1* and *MdSOD2* were obtained by PCR amplification with the primers MdSOD1-F5 and MdSOD1-R5, MdSOD2-F5 and MdSOD2-R5. Green fluorescent protein (GFP) fragment which was used as control was amplified from pEGFP-N1 with the primers GFP-F and GFP-R. The primers encompassed EcoR I and Xho I sites respectively. A PCR reaction was an initial 4 min predenaturation at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 55 °C, 60 s at 72 °C; 10 min at 72 °C. The amplified fragment was digested with endonucleases, and subcloned into a L4440 vector (gift of Dr. Jennifer Aiello, Cold Spring Harbor Laboratory, USA) digested with the same enzymes. The constructed plasmid, denoted as L-SOD1, L-SOD2 and L-GFP, were then transformed into the RNase III deficient *E. coli* strain HT115, which is unable to degrade dsRNA.

To produce dsRNA, single colonies of HT115 bacteria containing L-SOD1, L-SOD2 and L-GFP were grown to $OD_{600}=0.5$ and induced by the addition of 1 mM IPTG for 4 h at 37 °C. Before feeding bioassays, bacterial cells were collected from 100 ml culture by centrifugation at 4000 rpm for 10 min, resuspended in 150 ml sterile water, and then used for *M. domestica* feeding. 10 mg eggs were collected and hatched. These larvae were fed with HT115 bacteria containing recombinant plasmid following the procedure reported by Paschka et al. (2003).

2.11. Feeding bioassays

A randomized block design ($n=100$) was used for these feeding experiments. Before stress experiments, the 1st instar larvae were reared on diet containing bacteria expressing dsRNA for *MdSOD1*, *MdSOD2*, GFP and normal diet. The 3rd larvae RNAi treated and control were subjected to various stress tests. For heat shock test, larvae were incubated in an artificial climate incubator T: 42 °C, RH: 75%. For Cd stress test, larvae were reared on a medium made of 30 mM $CdCl_2$ for 24 h. For immune experiments, larvae were challenged with *E. coli* and *S. aureus* as described above, and then reared on a normal medium for 24 h. Survival rates of larvae were observed in all stress experiments.

3. Results

3.1. Cloning and analysis of full-length cDNAs of MdSOD1 and MdSOD2

The nucleotide sequences for *MdSOD1* and *MdSOD2* were submitted to GenBank under accession numbers JF919738 and JF919739. *MdSOD1* is comprised of 931 bp, containing a 786 bp open reading frame (ORF) which encodes 261 amino acids. *MdSOD2* is comprised of 845 bp, containing a 645 bp ORF which encodes 214 amino acids. The residues required for copper (His-121, -123, -138), zinc (His-138, -146, -157 and Asp-160) and manganese (His-48, -92, -176, -179) binding were well conserved in insect, mammalian and mollusk. The full-length nucleotide sequences and the deduced amino acid sequences are shown

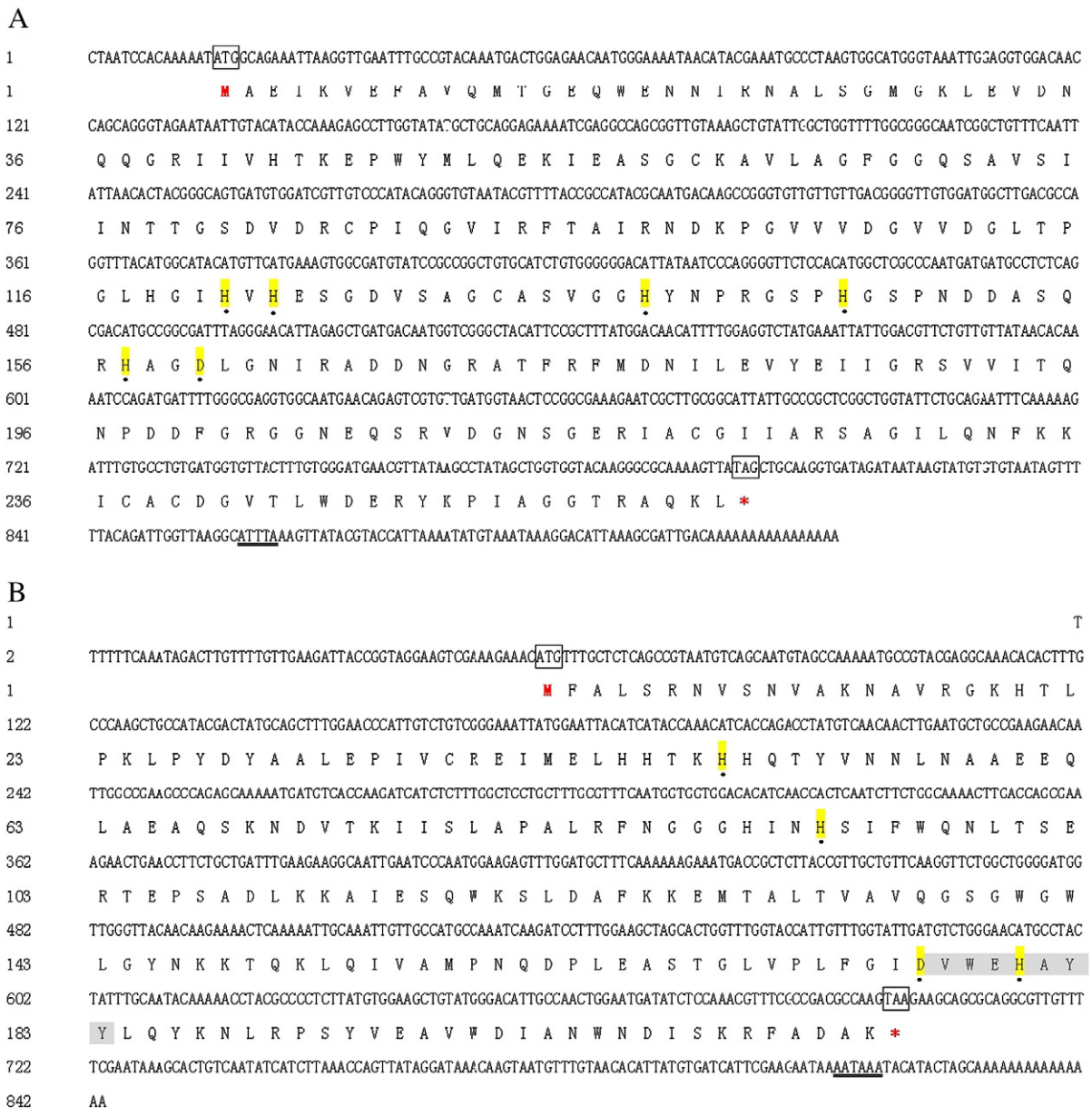

Fig. 1. The nucleotide sequences of MdSOD1 (A) and MdSOD2 (B) cDNA from Musca domestica. The deduced amino acid sequences are shown below the nucleotide sequence. The putative polyadenylation signal sequences (ATTTA/AATAAA) are indicated by a double line. Letters in the box indicate the start codon ATG, and an asterisk * indicates the stop codon. The amino acids required for copper (His-121, -123, -138) and zinc (His-138, -146, -157 and Asp-160) binding were shaded and marked with emphasis (A). MnSOD signatures (-DVWEHAYY-) were shaded, and the amino acids responsible for combination with the metal (His-48, -92, -176 and 179) were shaded and marked with emphasis (B).

in Fig. 1. The calculated molecular mass of the deduced *MdSOD1* and *MdSOD2* is 27.98 and 24.23 kDa respectively.

3.2. Alignment and phylogenetic analysis

Sequence comparison shows that the MdSOD1 and MdSOD2 of *M. domestica* share 46–81% identity with those from other species (Fig. 2). Phylogenetic relationship analysis reveals that the deduced amino acid sequence of MdSOD1 and MdSOD2 are separately clustered. MdSOD1 and MdSOD2 branch are in good agreement with Cu/ZnSOD and Mn-SOD branch respectively. The MdSOD1 and MdSOD2 from *M. domestica* are in the different subgroup as the Cu/ZnSOD and Mn-SOD subgroup (Fig. 3). This fact suggests that MdSOD1 and MdSOD2 belong to the Cu/ZnSOD and MnSOD families respectively.

3.3. Tissue-specific expression patterns of MdSOD1 and MdSOD2

In qPCR study, the primer pairs MdSOD1-F3 with MdSOD1-R3, and MdSOD2-F3 with MdSOD2-R3 were used to amplify a 100 and a 184 bp for *MdSOD1* and *MdSOD2* fragments with cDNAs from the fat body, gut, hemocyte, and epidermis tissue of larvae using *β-actin* as a positive control. The qPCR shows that both *MdSOD1* and *MdSOD2* are detected in all tissues tested (Fig. 4).

3.4. Quantification of MdSOD1 and MdSOD2 mRNA expressions under heat shock stress

Exposure to 42 °C for 1 h following the recovery at their acclimation temperature (25 °C) significantly affected the expression of *MdSOD1* and *MdSOD2* (Fig. 5). *MdSOD1* and *MdSOD2* relative expression levels

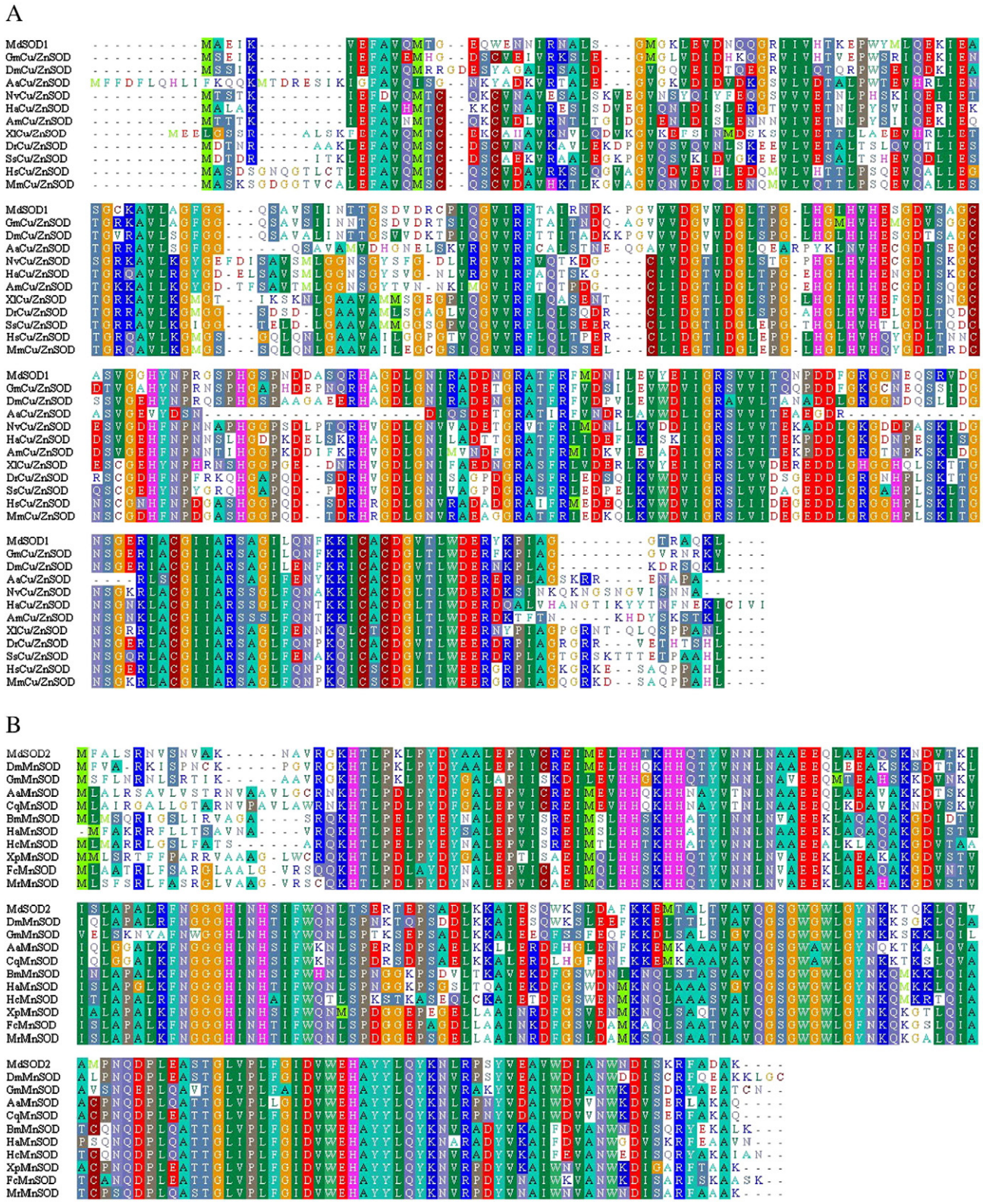

Fig. 2. Multiple alignment of Musca domestica MdSOD1 (A) and MdSOD2 (B) with other known species homologues. Amino acid residues that are conserved in at least 50% of sequences are shaded in color.

increased rapidly after the heat shock treatment at 42 °C for 30 min in comparison to that of the control. *MdSOD1* and *MdSOD2* expressions continued rising even during recovery 1 h after heat shock. However, when the larvae were placed at 25 °C to recover 1 h after heat shock treatment, decline of *MdSOD1* and *MdSOD2* expressions was observed.

3.5. Transcriptional responses of MdSOD1 and MdSOD2 to heavy metal exposure

During metal treatments, *MdSOD1* and *MdSOD2* mRNA expressions significantly increased and reached the highest level at 5 mM

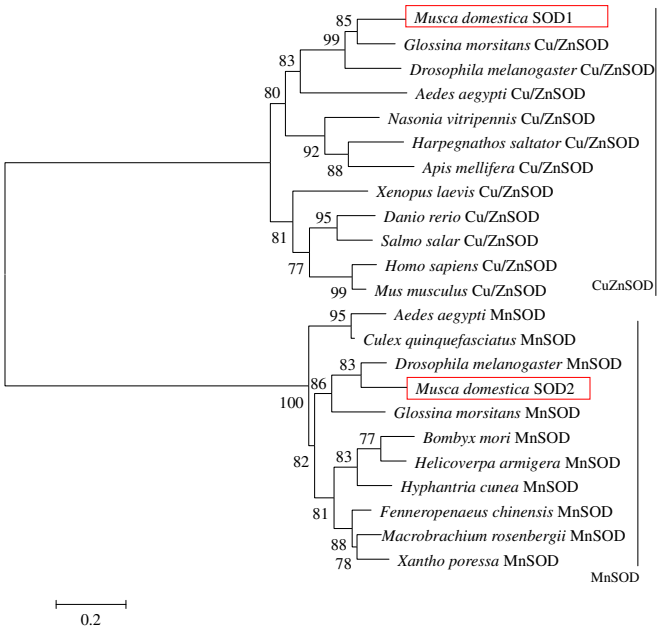

Fig. 3. A NJ tree summarizing overall similarity of the SOD genes MdSOD1 and MdSOD2. Numbers at the nodes indicate bootstrap proportions. GenBank Accession numbers are provided with the species' names. For Cu/ZnSOD: Glossina morsitans ADD19143, Drosophila melanogaster AAK07691, Aedes aegypti XP_001660067, Nasonia vitripennis XP_001607380, Harpegnathos saltator EFN83935, Apis mellifera XP_625006, Xenopus laevis NP_001086811, Danio rerio NP_001191151, Salmo salar NP_001133786, Homo sapiens AAM50090, and Mus musculus AAD23832. For MnSOD: Aedes aegypti XP_001649850, Culex quinquefasciatus EDS44530, Drosophila melanogaster AAA20533, Glossina morsitans ADD18846, Bombyx mori NP_001037299, Helicoverpa armigera ACY70995, Hyphantria cunea ABL63640, Fenneropenaeus chinensis ABB05539, Macrobrachium rosenbergii ABU55004, and Xantho poressa CAR82602.

(Fig. 6). After peaking at 5 mM, stable expressions were observed at 10, 20 and 30 mM.

To investigate the involvement of the two *SOD* genes during prolonged Cd^{2+} exposure, 5 mM Cd^{2+} concentration were provided to the larvae as food for times ranging from 0 h up to 24 h, mRNA expression of the two *SOD* genes increased with time and reached the highest level at the end of the experiment (Fig. 7).

3.6. Expression profiles of MdSOD1 and MdSOD2 after E. coli and S. aureus infection

The transcript level of *MdSOD1* in larvae increased after infection for 6 h. However, it decreased after 12–24 h. From 36 h post-infection, the transcript level recovered and significantly increased till 72 h post-infection at the end of the experiment (Fig. 8).

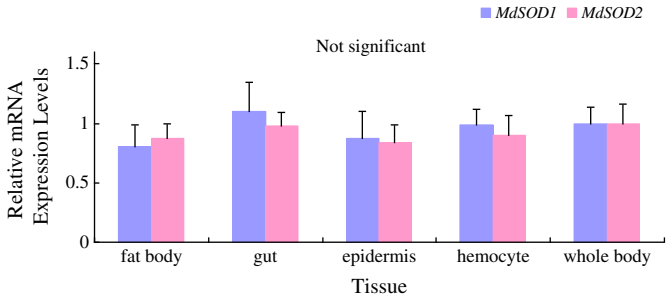

Fig. 4. MdSOD1 and MdSOD2 transcripts in different tissues from the 3rd instar larvae of Musca domestica. MdSOD1 and MdSOD2 transcript levels in the fat body, gut, hemocyte, and epidermis tissue are normalized to that in the whole body. All the data are analyzed from six individuals. Vertical bars represent the mean ± S.D. (N = 6.)

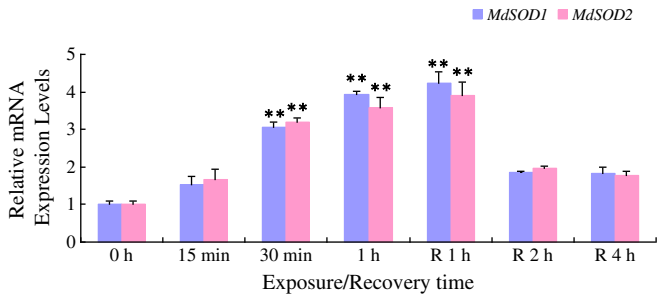

Fig. 5. MdSOD1 and MdSOD2 mRNA expression profiles in the 3rd instar larvae of Musca domestica after 42 °C challenge as measured by qPCR. β-Actin gene is used as an internal control to calibrate the cDNA template for all the samples. R = recovery time. Difference across blank group is indicated with two asterisks (P<0.01).

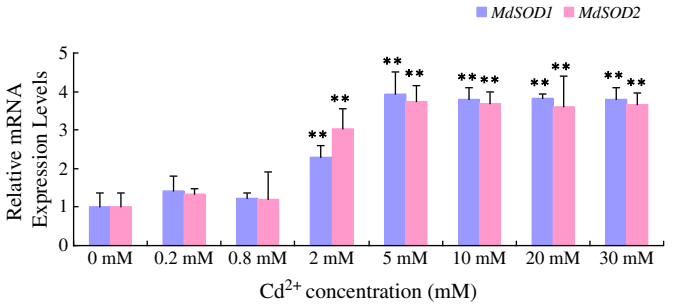

Fig. 6. Relative expression level of MdSOD1 and MdSOD2 in the 3rd instar larvae of Musca domestica exposed to different concentrations of Cd for 24 h. Significant differences from the control were indicated with two asterisks at P<0.01.

The expression of *MdSOD2* showed a slightly different trend contrasting to that of *MdSOD1*. The level of *MdSOD2* gradually increased after challenge (Fig. 9), and decreased slightly at 3 h, then increased at 6 h post-challenge. After 24 h post-challenge, transcript level increased gradually until the end of the experiment.

3.7. Expression and purification of SOD recombinant protein

The BL21 (DE3) pLysS strain, transformed with the recombinant expression vector pET-28a accumulated a high amount of recombinant protein after IPTG induction for 4 h. SDS-PAGE analysis reveals that rMdSOD1 and rMdSOD1 proteins accounted for about 37% and 30% of the total *E. coli* protein as determined by Bio-Rad gel scanning system (Fig. 10). The two SOD proteins were purified using High Affinity Ni Charged Resin. The yield of the purified rMdSOD1 and rMdSOD2 protein was about 2.5 and 1.5 mg/mL in 100 mL culture media respectively.

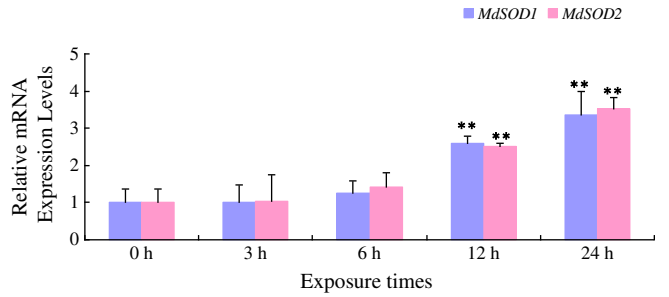

Fig. 7. Relative expression levels of MdSOD1 and MdSOD2 in the 3rd instar larvae of Musca domestica after treatment with 5 mM $CdCl_2$. Significant differences with respect to controls are marked by asterisks (**P<0.01).

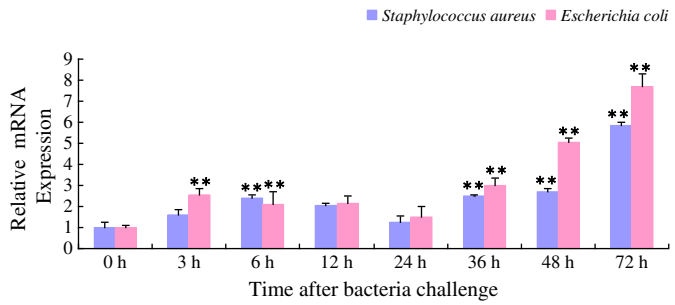

Fig. 8. qPCR analysis of MdSOD1 gene expression in the 2nd instar larvae of Musca domestica challenged by Staphylococcus aureus and Escherichia coli. Significant differences between the challenged and the blank group are indicated with two asterisks at $P<0.01$.

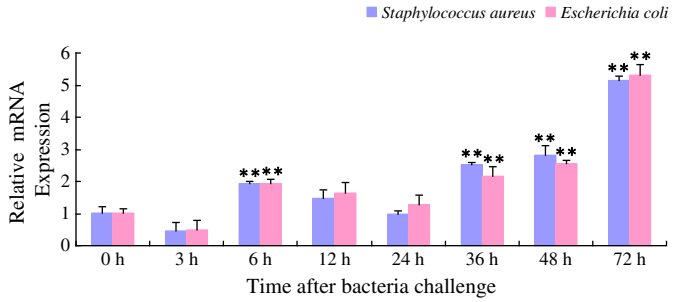

Fig. 9. qPCR analysis of MdSOD2 gene expression in the 2nd instar larvae of Musca domestica challenged by Staphylococcus aureus and Escherichia coli. Significant differences between the challenged and the blank group are indicated with two asterisks at $P<0.01$.

3.8. Refolding and enzyme assay of the recombinant protein

The purified recombinant proteins were inactive, but after refolding by stepwise dialysis, two SODs' activity was demonstrated by Native-PAGE analysis. Electrophoresis was carried out using 7.5% gel in Tris-glycine buffer (pH 8.0). We cut a Native-PAGE gal into two halves, one half stained by Coomassie blue, another half stained by NBT. The rMdSOD2 active bands are indicated by arrows (Fig. 11B). Unfortunately, rMdSOD1 activity was absent.

3.9. Identification of the active protein

The target band (Fig. 11A) was excised from the gel with a sterile scalpel and digested with trypsin. After in-gel digestion and identification

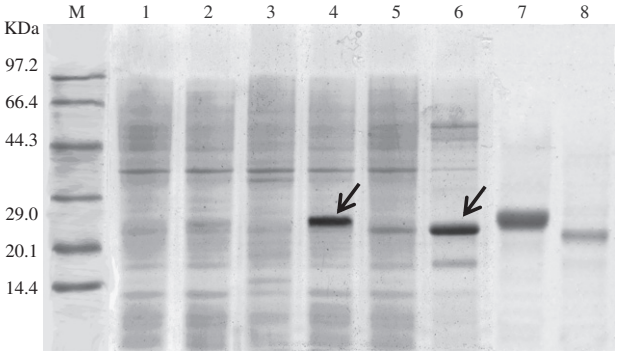

Fig. 10. Expression of the rMdSOD1 and rMdSOD2 fusion proteins. Protein samples are separated by SDS-PAGE. M: protein marker; lane 1: non-induced cells with pET-28a; lane 2: induced cells with pET-28a; lane 3: non-induced cells with rMdSOD1; lane 4: induced cells with rMdSOD1. Lane 5: non-induced cells with rMdSOD2; lane 6: induced cells with rMdSOD2; lane 7: purified protein rMdSOD1; lane 8: purified protein rMdSOD2.

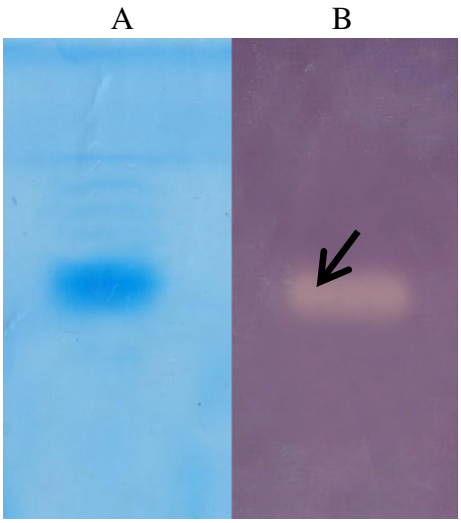

Fig. 11. Native-PAGE analysis of purification protein of rMdSOD2. Electrophoresis is carried out using 7.5% gel in Tris-glycine buffer (pH 8.0) and the gel was stained by NBT method. A: the gel of rMdSOD2 was stained by Coomassie blue; B: the gel of rMdSOD2 was stained by NBT.

using ESI-LC–MS/MS, three peptide fragments of rMdSOD2 were matched with the amino acid sequence of MdSOD2 from *M. domestica* (Fig. 12). The first matched peptide fragment (EIMELHHTKHHQT YVNNLNAAEEQLAEAQSKNDVTKIISLAPALR), the second fragment (TEPSADLKK) and the last fragment (LQIVAMPNQDPLEASTGLV PLFGIDVWEHAYYLQYKNLRPSYVEAVWDIANWNDISKR) could be located at the positions 39–83, 104–112 and 152–209 in deduced amino acid sequences of the MdSOD2 from housefly. So, the results demonstrate that the recombinant protein is MdSOD2 from *M. domestica*.

3.10. Expression level of MdSOD1 and MdSOD2 genes after RNAi feeding

Housefly larvae were exposed to dsRNA by feeding HT115 *E. coli* carrying L4440-derived vectors with sequence specific to MdSOD1 and MdSOD2 that spanned the predicted ORF (786 and 645 bp). Knock-down efficiency of MdSOD1 and MdSOD2 at 60 h post-feeding was monitored using qPCR. The results reveal that the expression levels of MdSOD1 and MdSOD2 fed dsRNA are significantly lower than that of controls. When normalized to the β-actin, MdSOD1 and MdSOD2 mRNA expression levels dropped down to 32% and 24% of the control values (Fig. 13). It indicates that the expression of MdSOD1 and MdSOD2 genes in the housefly larvae is inhibited successfully by feeding RNAi method.

3.11. MdSOD1 and MdSOD2 (RNAi) larvae are sensitive to hyperpyrexia, Cd^{2+}, and bacterial infection

In our experiments, thermotolerance was highly compromised in larvae following RNAi-treatments. In comparison, the control L-GFP RNAi-treated larvae remained alive but all the larvae in the L-SOD1 and L-SOD2 treated groups were not able to survive after heat shock at 42 °C for 6 h.

```
  1 MFALSRNVSN VAKNAVRGKH TLPKLPYDYA ALEPIVCREI MELHHTKHHQ
 51 TYVNNLNAAE EQLAEAQSKN DVTKIISLAP ALRFNGGGHI NHSIFWQNLT
101 SERTEPSADL KKAIESQWKS LDAFKKEMTA LTVAVQGSGW GWLGYNKKTQ
151 KLQIVAMPNQ DPLEASTGLV PLFGIDVWEH AYYLQYKNLR PSYVEAVWDI
201 ANWNDISKRF ADAK
```

Fig. 12. Three matched peptide fragments and their positions in the deduced amino acid sequence of Musca domestica MdSOD2. The three matched peptide fragments are marked in red.

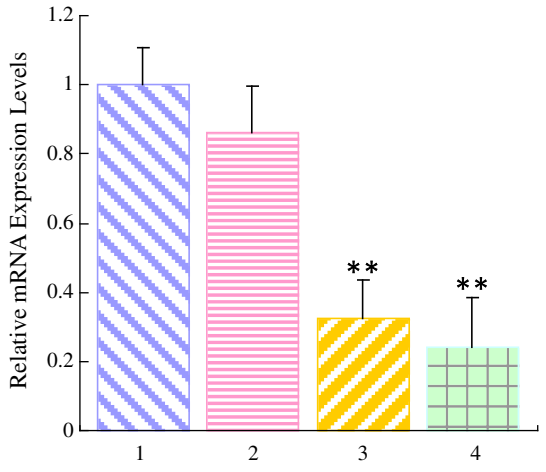

Fig. 13. Knock-down of MdSOD1 and MdSOD2 expressions by ingestion of bacteria expressing dsRNA. The samples of each group are collected at 60 h. Inductive stage 3 points represented 1: normal control; 2: feeding bacteria expressing L-GFP RNAi; 3: feeding bacteria expressing L-SOD1 RNAi; and. 4: feeding bacteria expressing L-SOD2 RNAi. Significant differences from the control were indicated with two asterisks at $P<0.01$.

After stress with Cd^{2+}, the survival rate of larvae in group with L-SOD1 and L-SOD2 significantly decreased. The mortality was up to 76% and 89% at 24 h which was remarkably lower than that in normal and L-GFP groups (0%) (Fig. 14).

To further characterize the role of MdSOD1 and MdSOD2 in the immune system of housefly, larvae were challenged with E. coli and S. aureus respectively. The survival rates were detected at 12 h and 24 h post-challenge. The normal L-GFP RNAi-treated were used as control to exclude non-specific effect of feeding. The L-SOD1 and L-SOD2 RNAi-treated group cumulative mortalities reached to 80% and 82% after challenge with E. coli, and 11% and 18% with S. aureus, which were significantly lower than that in control groups.

4. Discussion

Our group is particularly interested in immunology and stress biology, we screened out dozens of genes that are supposed to play a role in immunity and stress resistance. Some antioxidant enzymes or proteins, such as SOD, metallothioneins, glutathione S-transferases, glutathione peroxidases, and glutathione reductases are identified and proved to be involved in stress resistance of M. domestica. There are at least 6 SODs in our database. According to order in ESTs, they are named as MdSOD1 and MdSOD2. We choose them as Cu/Zn-SOD and Mn-SOD representative to research. The current results about the M. domestica SODs will be very helpful for further work on immunity and stress resistance.

In the study, full-length cDNAs of MdSOD1 and MdSOD2 which apparently belong to two distinct SOD families of Cu/ZnSOD and MnSOD respectively were cloned from housefly based on their sequencing similarities to other known SOD cDNAs. As reported, these two SOD genes with different evolutionary rates were also partly revealed by the identity analysis and genetic distance calculation in the study. According to the mentioned result, MdSOD1 and MdSOD2 were widely expressed in all tested tissues, including hemocyte, fat body, gut, and epidermis of the housefly, while the expression levels were not differently significant. It was indicated that the expressions of SOD in the housefly were non tissue-specific but ubiquitous, which

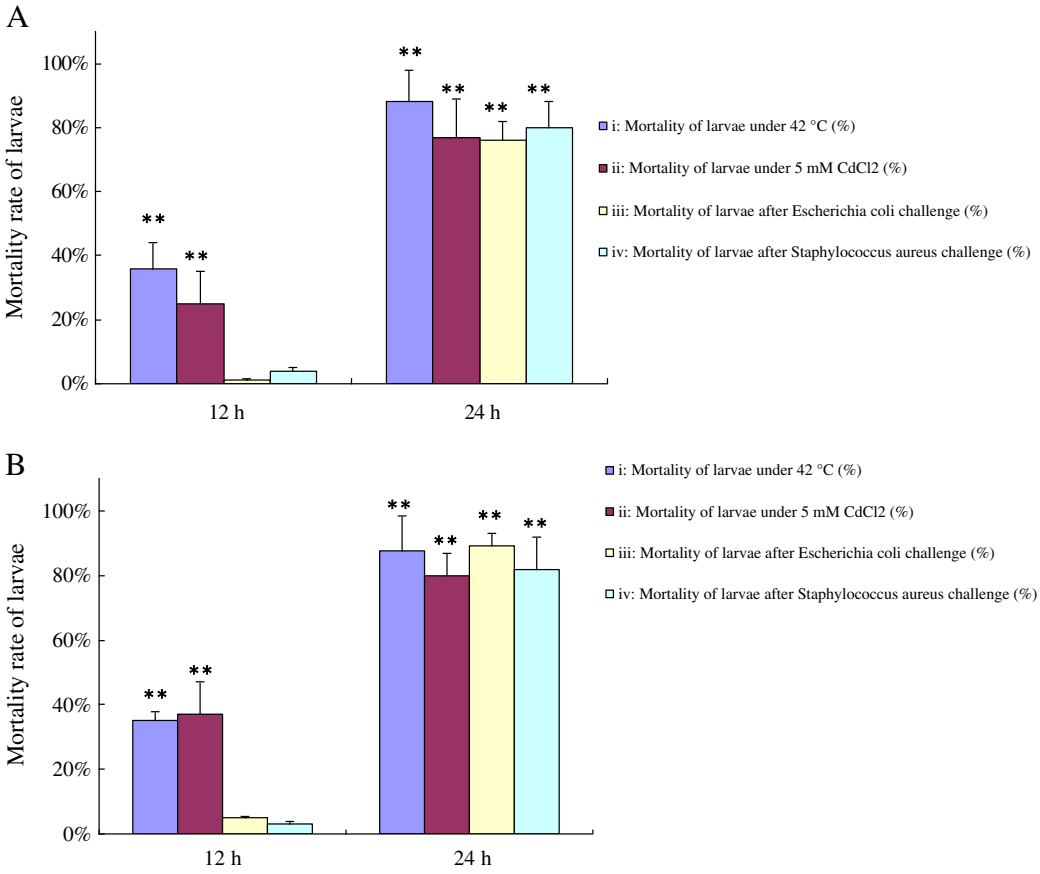

Fig. 14. The profiles of MdSOD1 RNAi (A) and MdSOD2 RNAi (B) mortalities of housefly in four different stressors. i: the larvae under 42 °C; ii: the larvae under 5 mM $CdCl_2$; iii: the larvae after Staphylococcus aureus challenge; and iv: the larvae after Escherichia coli challenge. Significant differences from the control (survival rate of L-GFP dsRNA larvae) are indicated with two asterisks at $P<0.01$.

was not a surprise since Cu/ZnSOD and MnSOD were expressed in a wide range of cell types that have already been reported previously (Zelko et al., 2002).

High temperature, one of the major environmental factors, affected the growth and productivity of the housefly. During heat treatment, transcript level of MdSOD1 and MdSOD2 increased at heat shock for 30 min and reached the highest level during recovery for 1 h after heat shock. The formation of ROS has been identified to be one of the fast kinetic events in host exposed to excessive temperature, which would possibly elevate mitochondrial ROS production (Becker et al., 2011). ROS have potential toxicity on biomolecules and membranes, while antioxidant enzyme SODs which are metalloenzymes that represent one important line of defense against ROS can convert superoxide anion into hydrogen peroxide and water; the increased antioxidant capacity during heat treatment has been reported in plant as well (Sathiyaraj et al., 2011). In the present experiments, the highest expression level of MdSOD1 and MdSOD2 at 1 h recovery after heat shock indicated that the magnitude of environmental temperature change could be a way to transfer information on ROS production to subsequent processes, such as SOD gene expression, avoiding disordering physiological metabolism or damaging ROS balance.

Many heavy metal testes could cause oxidative stress. Cd is known that it can enhance the intracellular formation of ROS and promote cellular oxidative stress. Both Cu/ZnSOD and MnSOD have been considered as general oxidative stress responsive factors whose expression at transcriptional and translational levels might be influenced by a variety of intracellular and environmental cues (Cho et al., 2006; Kim et al., 2007). In addition, Cd can compete with essential metals for protein-binding sites (Pruski and Dixon, 2002), inducing the release of Fe^{2+} and Cu^{2+} ions and increasing the production of ROS (Xu et al., 2011). In general, the Cd^{2+} exposure elevated the lipid peroxidation in subject tissues and modulated the activities of SOD or other antioxidant defense systems as well (Dabas et al., 2011). In our research, the expression level of SOD which was particularly higher in the experimental group than in the control increased with the Cd^{2+} concentration ranging from 0.2 to 5 mM in the medium. The results indicated that low-level Cd^{2+} concentration can induce SOD, but the increasing Cd^{2+} concentration (>5 mM) cannot help to excess SOD. Expression levels of these two SODs slightly declined at 10, 20, 30 mM for 24 h, perhaps because of a decrease in the metabolic capacity of the organisms due to Cd toxicity. Certainly, Cd^{2+} induced expression since levels of these two SOD genes were several folds greater than the control after exposure to 30 mM Cd^{2+} for 3 h and were reached to the highest at 24 h that were consistent with previous findings, in which the clam specimens were exposed to Cu^{2+} and the highest level of TgmMnSOD was reached at 24 h (C. Li et al., 2011). The host could adopt stress strategies to control the ROS at a fitting level by utilization or control of antioxidant enzymes. It was speculated that MdSOD1 and MdSOD2 were acute response proteins involved in heavy metal contaminant challenge in M. domestica.

In bacterial challenged experiments, up-regulation of these two SOD genes' expression from the infected groups at the first 6 h post-challenge showed that the two genes might be involved in a temporal expression because of the physical stimulation by pricking or the invasion of heterogenous bacteria. At 36 h and 72 h post-injection, the transcript level in the challenged group was significantly higher than that in the control group suggesting that during the phagocytosis of bacteria by housefly immune cells, the level of ROS increases and more SODs are needed to balance the effect of ROS. In the present study, live E. coli and S. aureus were used for larvae challenge which would unceasingly reproduce in hosts. And the infection progress brought higher levels of ROS which severely destroyed the normal function of larvae's cells and finally caused the larvae to change into pupae earlier. The significant rise of the transcript level of SOD after bacterial invasion suggested that the gene transcriptions were inducible. As described, Chinese shrimp Fenneropenaeus chinensis MnSOD was activated in hemocytes and hepatopancreas when cells were infected with white spot syndrome virus (WSSV) (Q. Zhang et al., 2007a), while Crassostrea hongkongensis SOD (ChMnSOD and ChCuZnSOD) could be induced after infection by Vibrio alginolyticus (Yu et al., 2011). Therefore, the significant rise of the transcript levels of MdSOD1 and MdSOD2 deduced that the two genes may play an important role in the immune responses against infection.

Abiotic factors (such as heat or Cd stress) and biotic stressors (such as bacterial infection) can cause various disorders. The generation of NADPH-oxidase is always associated with disorders, thereby inducing the production of ROS. A high level of ROS could lead to proteotoxicity in the host cell itself, resulting in a serious damage. MdSOD1 and MdSOD2 as the main antioxidant enzymes, they participated in the process of clearing the excess ROS, avoiding physiological metabolism disorder and ROS balance damage.

By using RNAi, a powerful method to inhibit gene expression in a sequence specific manner in which silencing the target gene through feeding engineered bacteria has been successfully carried out in many insect species (X. Li et al., 2011). A significant decrease of MdSOD1 and MdSOD2 mRNA were observed at 60 h post-feeding. RNAi feeding demonstrated that it was feasible to silence genes by feeding bacteria expressing dsRNA in M. domestica. RNAi knockdown of the MdSOD1 and MdSOD2 genes significantly increased the mortality of larvae under heat shock, Cd treatment, and bacterial invasion. These results further confirmed that MdSOD1 and MdSOD2 were important genes involved in the resilience and immunity of M. domestica.

In conclusion, not only the purification and some characteristics of Cu/ZnSOD and MnSOD have been described for the first time from housefly but also the nucleotide sequence of cDNAs encoded MdSOD1 and MdSOD2 have been determined. Since SODs play an important immuno-modulatory role in protection organisms, this study will provide a valuable and reliable information to research the oxidative stress induced regulation of the housefly during high temperature, Cd stress and bacterial invasion. The expression of MdSOD1 and MdSOD2 genes was silenced using the RNAi technology with a high mortality in RNAi-treated groups. These results implied that MdSOD1 and MdSOD2 were important enzymes involved in the housefly's against tolerance to climatic changes, environmental Cd pollution and immune defense reaction after the bacterial infection.

Acknowledgments

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