Molecular Immunology 107 (2019) 115–122

Contents lists available at ScienceDirect

Molecular Immunology

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

MdRDH1, a HSP67B2-like rhodanese homologue plays a positive role in maintaining redox balance in *Musca domestica*

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ARTICLE INFO

Keywords:
Musca domestica
Rhodanese-homology domain protein
Innate immunity
Sulfurtransferaes activity
Oxidative stress

ABSTRACT

Rhodanese homology domains (RHODs) are the structural modules of ubiquitous tertiary that occur in three major evolutionary phyla. Despite the versatile and important physiological functions of RHODs containing proteins, little is known about their invertebrate counterparts. A novel HSP67B2-like single-domain rhodanese homologue, *MdRDH*1 from *Musca domestica*, whose expression can be induced by bacterial infection or oxidative stress. Silencing *MdRDH*1 through RNAi causes important accumulations of reactive oxygen species (ROS) and malondialdehyde (MDA), and increases mortality in the larvae treated with bacterial invasion. The *E. coli* with MdRDH1 and the mutant MdRDH1^{C135A} are transformed, with significant rhodanese activity of the recombinant protein of MdRDH1 in vitro found, without no detection of enzyme activity of the mutant MdRDH1^{C135A}, revealing that catalytic Cys135 in the active-site loop is essential in the sulfurtransferase activity of MdRDH1. When oxidative stress is insulted by phenazine methosulfate (PMS), the *MdRDH*1 transformed *E. coli* shows enhanced survival rates compared with those bacteria transformed with MdRDH1^{C135A}. Our research indicates that MdRDH1 confers oxidative stress tolerance, thus rendering evidence for the idea that rhodanese family genes play a critical role in antioxidant defenses. This paper yields novel insights into the potential antioxidative and immune functions of HSP67B2-like rhodanese homologues in invertebrate.

1. Introduction

Pathogens and environmental stresses are important source of mortality, with animals having developed the ability to sense and fight for such stresses. A common feature in response to both abiotic and biotic stresses is the triggering of oxidative stress via generating reactive oxygen species (ROS). ROS is deleterious in excess, although there is increasing evidence that ROS, as a signaling molecule, plays a significant role in regulating the physiological process. Oxidative stress is supposed to be related to aging and various diseases including Cancer, Diabetes, Parkinson's disease, Alzheimer's disease, Huntington's disease, Depression, and Multiple sclerosis (Haider et al., 2011; Patel and Chu, 2011). For animals, a tight control of ROS homeostasis requires a delicate redox equilibrium system involving low-molecule substances or enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and Glutathione S-transferases (Halliwell and Gutteridge, 1999; Ketterer et al., 1983; Limon-Pacheco and Gonsebatt, 2009; Meng et al., 2013). Recently, several works have reported that rhodaneses, members of the sulfurtransferase superfamily, have potential anti-oxidative functions in mammal (Nakajima et al., 2008), crustacean (Tang et al., 2017, 2018), and bacteria (Cereda et al., 2009; Remelli et al., 2012).

Rhodanese, also called thiosulfate transferase (TST), is the major enzyme of sulfur metabolism for cyanide detoxification. *in vitro*, rhodanese can catalyze the transfer of a sulfur atom from $S_2O_3^{2-}$ to CN- with the concomitant formation of sulfite and thiocyanate (Westley et al., 1983). Sulfur metabolism is significant both in detoxification and in anti-oxidative stress systems (Nakajima, 2015). Rhodanese-domain-containing proteins are widespread in the three life kingdoms and are featured by the presence of a tertiary structure module, namely the rhodanese homology domain (RHOD) (Cipollone et al., 2007). RHODs containing proteins can exist as single units, in tandem repeats, or can be fused to domains with other activities (Bordoa and Bork, 2002). In addition to cyanide detoxification, a range of functions of rhodanese and its homologues have been recognized, including sulfur metabolism, Fe-S cluster formation, and selenium metabolism (Libiad et al., 2014; Ogasawara et al., 2001; Yadav et al., 2013). Emerging evidence has demonstrated the roles of their versatile molecules in antioxidant defense (Nagahara and Katayama, 2005; Nagahara et al., 2007; Remelli et al., 2010).

Despite the versatile and important physiological functions of RHODs containing proteins, little is known about their insect

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https://doi.org/10.1016/j.molimm.2019.01.016
Received 21 December 2018; Received in revised form 25 January 2019; Accepted 25 January 2019
Available online 01 February 2019
0161-5890/

counterparts. In this paper, a single rhodanese domain protein encoding gene is identified from housefly, expressed as *MdRDH*1 (*Musca domestica* rhodanese homologues 1). Besides, the sulfurtransferase activity of rMdRDH1 is verified *in vitro*. The following research in this paper highlights its roles in the modulation of immune response and the maintenance of redox balance in *M. domestica*.

2. Material and methods

2.1. Fly maintenance and sample

Musca larvae were raised on a medium composed of bran, heat inactivated yeast, milk, and the antimycotic nipagin until pupation. After eclosion, adult flies were fed on water, sugar and milk powder. Flies were maintained at a temperature of 25℃, 70% relative humidity, and LD12:12 cycles (Gao et al., 2015).

Hemocyte and three tissues including cuticle, gut, and fat body were collected from the 3rd instar larvae for tissue distribution analysis by the methods available (Dong et al., 2011). For experiments on septic injury infection, the 2nd instar larvae found got a challenge based on previous the methods. Briefly, with septic injury produced by pricking the abdomen of the larvae by using a needle dipped into a concentrated culture of *Escherichia coli* or *Staphylococcus aureus* previously (Tang et al., 2012). Larvae were sampled for RNA extraction after infection for 3, 6, 12, 24, 36, 48 and 60 h, respectively. Oxidative stress was induced by injecting 0.1 μg/μl of doxorubicin (DOX) solution into the 2nd instar larvae. Then larvae were sampled after injection for 3, 6, 12, 24, 48 and 60 h for *MdRDH*1 qRT-PCR analysis and superoxide dismutase (SOD) activity test. The SOD activity was measured spectrophotometrically by the nitro blue tetrazolium (NBT) method (Sun et al., 1988). All samples were reserved in liquid nitrogen before the extraction, and all experiments were repeated three times.

2.2. Cloning and analysis of the MdRDH1 gene

Total RNA was extracted from the 3rd instar larvae using Trizol reagent (Invitrogen, USA) by protocol. 2 μg of total RNA was used to make cDNA by M-MLV reverse transcriptase (Promega, USA) with primer AOLP. A gene encoding for a RHOD containing protein was found in the *M. domestica* transcriptome database. Primers MdRDH-F and MdRDH-R were designed to clone and verify this gene sequence. The PCR reaction was initially a 4-minute predenaturation at the temperature of 94℃, 94℃ for 90 s, 58℃ for 30 s, 72℃ for 60 s. After 35 amplification cycles, the last extension step was extended to 10 min at a temperature of 72℃. The PCR products were purified and ligated into pMD18-T easy vector (TaKaRa, China). Randomly selected positive clones were sequenced.

The putative *MdRDH*1 open reading frame was found by ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Multiple sequence alignment analysis was performed via the NCBI blast and CLC main workbench program. The signal peptide of MdRDH1 was predicted by the SignalP 4.1 server program (http://www.cbs.dtu.dk/services/SignalP/). A multiple protein coding RHOD sequence was obtained from the GenBank. The protein domains of a total of 140 RHODs from insects, crustaceans, digenea, mammalia, monocots, dicotyledoneae, yeast, bacteria, fish, reptilia, actinopterygii, aves, ulvophyceae and arachnoidea were searched via web CD-search tools, including SMART for Ensembl database (http://smart.embl-heidelberg.de) and Batch for NCBI database (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) (Tang et al., 2018). The GenBank protein sequences that can be significantly aligned with RHODs conservative domain were collected to do multiple sequences alignment by ClustalW, then constructed a Neighbor-Joining tree with 1000 bootstraps by virtue of MEGA 10 program (Kumar et al., 2018).

2.3. Quantitative analysis of MdRDH1 transcript expression

Total RNA was freshly extracted from various tissues or bacteria challenged larvae using TRizol reagent. cDNA was synthesized and subjected to qRT-PCR which was conducted on a LightCycler system (Roche) using 2 × Fast Super EvaGreen qRT-PCR Master Mix (S2008, US Everbright Inc., China). Expression level of the *MdRDH*1 gene was calculated by comparing the Ct value to the reference gene *β-actin*. The primers RtF1 and RtR1, RtF2 and RtR2 were used for amplifying *MdRDH*1 and *β-actin* fragments respectively. The relative quantification was calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). All experiments were repeated three times. The significance at $p < 0.05$ was analyzed using one-way ANOVA.

2.4. Silencing of MdRDH1 by RNAi

Function analyses were performed by RNA interference-mediated gene silencing, with the RNAi experiment performed by a method described previously (Gao et al., 2015). Fragments of *MdRDH*1 and *GFP* control were PCR amplified with T7 promoter primers RNAiF1/RNAiR1 and RNAiF2/RNAiR2. The transcription experiments were conducted using the T7 High Efficiency Transcription Kit (Transgen Biotech, China) according to the instructions. Larvae were injected with either MdRDH1 dsRNA or GFP dsRNA. Six larvae of each group (dsMdRDH1 or dsGFP) were selected at the 12^{th} and 24^{th} hour after the dsRNA injection. Then, the efficiency of RNAi was measured by qRT-PCR and western blot. The purified protein rMdRDH1 was used as an antigen to produce rabbit polyclonal antibody by the traditional method. The RNAi efficiency was determined by western blot analysis. Total Proteins of dsRNA injection (12 and 24 h) were extracted with a KEYGEN protein extraction kit (Keygen, China) and analyzed by SDS-PAGE. After electrophoresis, the peptides were transferred to nitrocellulose membranes with a Mini Trans-Blot system (Bio-Rad, USA). The rabbit anti-MdRDH1 polyclonal antibody (1:100 dilution) was used as the primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) (1:300) was used as the secondary antibody. β-actin was used as the reference. The experiments containing three biological replications and each including three technical replications.

After the injection of dsRNA for each group, the total proteins were extracted by virtue of a KEYGEN protein extraction kit (Keygen, China) and were employed to test oxidative stress biomarkers ROS and MDA. Specifically, the ROS levels were quantified according to reactive oxygen species assay kit instructions (Nanjing Jiancheng Bioengineering Institute, China). A MDA detection kit was chosen to figure out the MDA content as a marker of lipid peroxidation according to the instructions (Nanjing Jiancheng Bioengineering Institute, China). The processed RNAi and control larvae were subjected to immune experiment. After that, larvae found a challenge with *E. coli* and *S. aureus* presented above and reared on a normal medium for 24 h. Survival rates of larvae could be noted in the treatment experiments, with all experiments repeated for three times.

2.5. Recombinant expression and western blot

The DNA fragment encoding *MdRDH*1 was achieved by the PCR amplification with the primers ExF1 and ExR1 containing *Eco*RI and *Xho*I restriction site overhanging. The amplified fragment and pET-30a vector were digested with the restriction enzymes *Eco*RI and *Xho*I, and then ligated together. Meanwhile, a mutant $MdRDH1^{C135A}$ was constructed. Primers ExF1 and MuR1 were employed to amplify fragment I, with MuF1 and ExR1 used to amplify fragment II. The two fragments being amplified, the full length of mutant $MdRDH1^{C135A}$ DNA fragment was obtained by overlapping PCR using the two purified DNA fragments as the templates; it was digested and ligated to pET-30a. After that, the recombinant plasmids (pET30a-MdRDH1 and pET30a-$MdRDH1^{C135A}$) were transferred into *E. coli* BL21 (DE3) for expression;

bacteria cultures were incubated in LB medium (containing 30 mg/ml of kanamycin) at a temperature of 37°C when shaking at 220 rpm. The culture mediums reaching OD_{600} of 0.8, fusion proteins were induced with 1 mM of IPTG for 4 h at a temperature of 25°C when shaking at 160 rpm. The recombinant proteins rMdRDH1 and rMdRDH1^{C135A} were purified by using Ni-NTA Resin (GenScript, China) on the basis of the instructions.

The purified protein rMdRDH1 was employed as an antigen in the production of rabbit polyclonal antibody using the traditional method. The purified rMdRDH1 and total protein of the housefly larvae extracted with a KEYGEN protein extraction kit (Keygen, China) were analyzed by SDS-PAGE; after electrophoresis, the peptides were transformed into nitrocellulose membranes. Western blot performed with anti-rMdRDH1 polyclonal antibody at 1:150 dilution as the primary antibody Table 1.

Table 1
PCR primers used in this study.

Primer	Sequence
AOLP	GGCCACGCGTCGACTAGTAC(T)$_{16}$(A/C/G)
MdRDH-F	CCATCAAGCTTCTTGTACAGTCGAG
MdRDH-R	TTTCACATAGATAATTATTTTTATT
RtF1	ATGAGTTTGTGTCGATTTGCCTTTG
RtR1	TTCGCCGTATCTTTATGACTGTTGT
RtF2	GAGAAATCCTATGAACTTCCCGACG
RtR2	GGATACCGCAAGATTCCATACCCAA
RNAiF1	TAATACGACTCACTATAGGGCGCAACAACTGACAAGCGGC
RNAiR1	TAATACGACTCACTATAGGGCG
RNAiF2	TAATACGACTCACTATAGGGCGAATGGTGAGCAAGGGCGAGGA
RNAiR2	TAATACGACTCACTATAGGGCGACTTGTACAGCTCGTCCATGC
ExF1	ATGAATTCCAACAACTGACAAGCGGCGG
ExR1	ATCTCGAGTTACTGTGGCAATCCCTCAT
MuF1	CCAATTGTTTTCTATGCCCGCTCGGGCAGAAGA
MuR1	TCTTCTGCCCGAGCGGGCATAGAAAACAATTGG

2.6. Enzyme assay and kinetic studies

The rhodanese activity of rMdRDH1 and rMdRDH1^{C135A} was measured by the reported method (Tang et al., 2017; Tayefi-Nasrabadi and Rahmani, 2012). The reaction velocity was determined from linear slopes of absorbance-time curve, with one unit of rhodanese activity defined as micromoles of thiocyanate developed per minute at pH 9.2 and a temperature of 37°C. The Michaelis constant (Km) for cyanide ion was figured out by varying the concentrations of NaCN between 10 mM and 50 mM at 50 mM of $Na_2S_2O_3$. Km for sodium thiosulphate was calculated by varying the $Na_2S_2O_3$ concentration from 10 mM to 50 mM at 50 mM of NaCN. Then the kinetic parameters were worked out from the double reciprocal plots, with data being representative for the verification of the three independent experiments.

2.7. Oxidative stress tolerance assay of MdRDH1-expressing E. coli

The oxidative stress tolerance assay was tested using previously method (Tang et al., 2017). In brief, *E. coli* BL21 (DE3) containing pET30a-MdRDH1 or pET30a-MdRDH1^{C135A} was developed in LB medium at a temperature of 37°C till OD_{600} reached 0.6, with protein expression induced for 1 h considering IPTG at the final concentration of 1 mM. To explore the antioxidant activity of rMdRDH1, phenazine methosulfate (PMS) were applied to the culture at final concentrations of 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, and 0.14 mg/ml respectively, with bacteria incubated when shaked very gentle at a temperature of 37°C for 1 h. 50 ml of 100-fold diluted culture in fresh LB medium was placed on the LB agar medium plate and was incubated at a temperature of 37°C for 12 h. Colonies counted, the mean values of logarithm CFU (lgCFU) on plates were figured out and employed for the generation of survival plots. The logarithm value was expressed by 0 with the CFU being 0, with the experiments repeated three times.

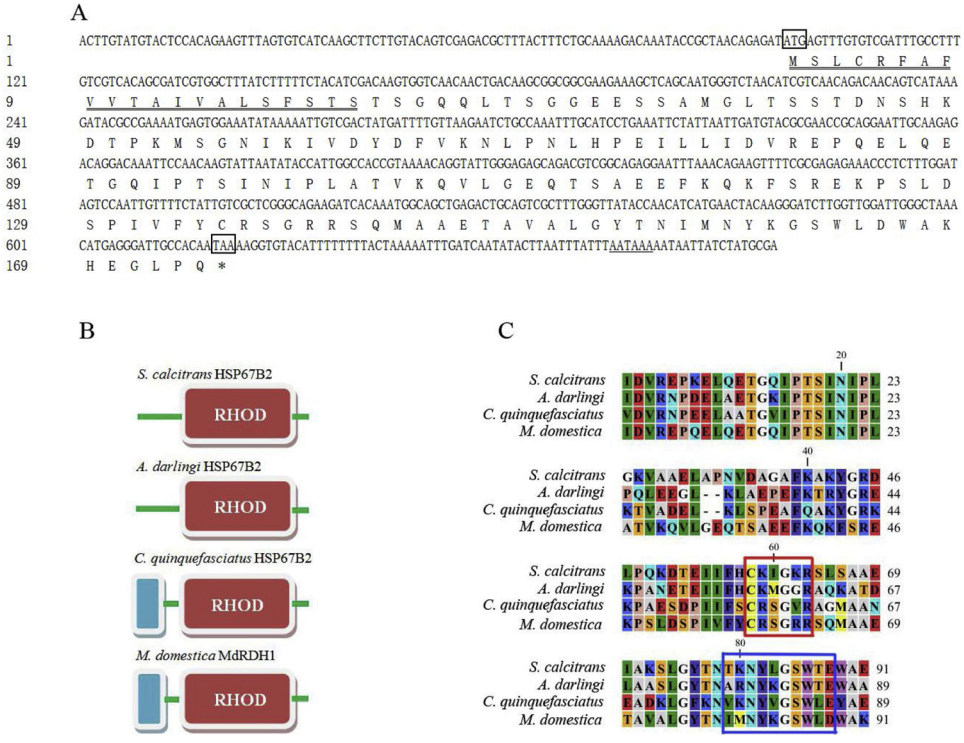

Fig. 1. Organization and sequence alignment of MdRDH1 with rhodanese homology domains of representative HSP67B2 homologues from insects. (A) The nucleotide sequences of MdRDH1 cDNA from M. domestica. The deduced amino acid sequence was shown below the nucleotide sequence. ATG and TAA indicated the start site and the stop codon, respectively. The AATAAA letters corresponded to the polyadenylation signal, and the signal peptide at the N-termini was underlined. (B) Domain organization of MdRDH1 and HSP67B2 homologues from *Stomoxys calcitrans* (XP_013110580.1), *Anopheles darling* (ETN62322.1), and *Culex quinquefasciatus* (XP_001864125.1). : signal peptides; RHOD: putative rhodanese domain. (C) Sequence alignment of the rhodanese homology domains of insect HSP67B2 homologues. The conserved active site cysteine was highlighted in yellow. Residues forming the six-amino acid active-site loop (CRSGRR) of the putative catalytic domain and rhodanese signature (IMNYKGSW-LDW) in C-terminal were respectively marked in boxes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 2. Phylogenetic tree based on rhodanese homology domain. Group I was composed mainly of multi-domain rhodanese homologues including Cdc25 and MOCS; Group II was composed mainly of genes with tandem domain rhodanese homologous including TST and MPST; Group III was made up of single domain including TSTD1, TSTD3 and HSP67B2. MdRDH1 (▲▲) was in Group III, sharing common evolutionary origin with insect HSP67B2. One thousand bootstraps were performed on the Neighbor-Joining tree to check the repeatability of the results.

3. Results

3.1. Sequence analysis of housefly MdRDH1

Based on the *M. domestica* transcriptomic database search, an expression sequence tags of RHOD gene was identified. This gene was cloned from *M. domestica* and sequence analysis showed that this gene had a rhodanese homologue domain so named *MdRDH*1 (GenBank accession number: XM_005189395.2). It had a 5′UTR sequence of 96 bp and a 3′UTR sequence of 74 bp. The open reading frame of MdRDH1 was 525 bp long, encoding a protein of 174 amino acids residues with the putative signal peptides of 21 residues (Fig. 1A). The amino acid sequence of MdRDH1 shared the highest identity with heat shock protein 67B2-like homologue of *Stomoxys calcitrans* (KNC32295.1; 68% identity). Protein domain analysis indicated that MdRDH1 protein contained a putative rhodanese domain (from Ile 78 to Lys 168) (Fig. 1B). Further, it was found that the residue of 58–63 (CRSGRR) by extremely conservative region spanning was conformed to the six-amino acid active-site loop “CRXGXR/T” found in the catalytic domains of rhodaneses. Besides, a recognizable rhodanese signature (IMNYKGSWLDW) appeared in the C-terminal end of the protein (Fig. 1C).

Based on the multiple sequence alignment of the RHOD domains including a representative subset of the entries resulting from the GenBank, with an unrooted phylogenetic tree constructed by the Neighbor Joining method (Fig. 2). Three distinct groups in the tree could be identified. Group I was composed mainly of multi-domain rhodanese homologues including Cdc25 and MOCS, and the catalytic rhodanese domain of group I was often combined with other characterized protein domains. Group II mainly consisted of proteins with tandem domain (or double domain), including TST and MPST. Group III was made up of single domain, including TSTD1, TSTD3 and HSP67B2. Phylogenetic analysis revealed that MdRDH1 was in the group III and that it was close to the HSP67B2 from insects.

3.2. The expression of MdRDH1 mRNA in different tissues and in response to bacteria stimulation

The transcription levels of *MdRDH*1 in different tissues of the 3rd instar larvae, including fat body, cuticle, gut and hemocyte, were

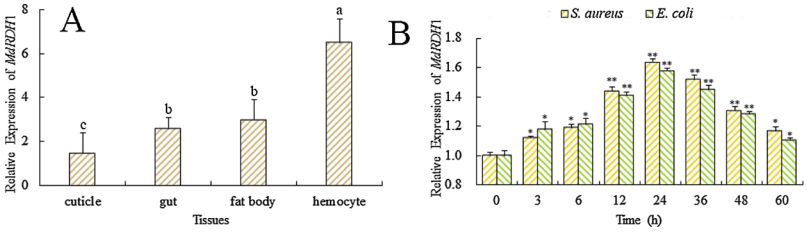

Fig. 3. Expression profiles of *MdRDH*1 in various tissues (A) and *E. coli* or *S. aureus* challenged larvae (B). *β-actin* gene was used as an internal control to calibrate the cDNA template for all the samples. Each bar represented the mean value of the three determinations with the standard deviation (SD) of the means (mean ± SD, n = 3). Means with different letters (a, b, c) are significantly different at $P < 0.05$. ** denoted highly significant difference ($p < 0.01$) and * denoted significant difference ($p < 0.05$) between the treatment and the control.

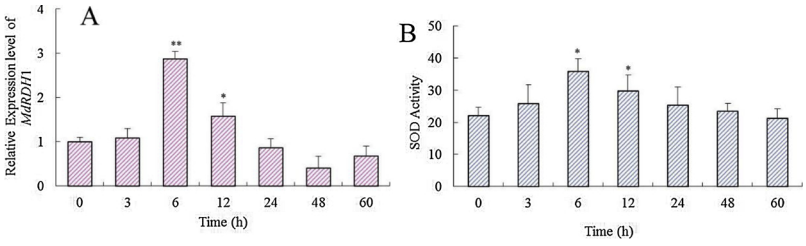

Fig. 4. *MdRDH1* expression and SOD activity induced by doxorubicin. (A) Temporal expression of *MdRDH1* mRNA after doxorubicin injection. (B) SOD activity change after doxorubicin injection. Vertical bars represented the mean ± SE (N = 6). ** denoted highly significant difference ($p < 0.01$) and * denoted significant difference ($p < 0.05$) between the treatment and the control.

shown in Fig. 3A. *MdRDH1* RNA was examined in all the tissues with the highest expression level appearing in hemocyte, which was 4.3-fold ($p < 0.01$) in cuticle, intermediate expression in fat body and gut. Upon either *S. aureus* or *E. coli* challenge, the expression of *MdRDH1* was enhanced and the maximum level appeared at the 24th hour after infection (Fig. 3B). However, *MdRDH1* expression declined gradually from the 24th hour to 60th hour after infection, though the levels were still greater than those in untreated controls.

3.3. Changes of MdRDH1 expression and SOD activity under DOX stress

The effect of DOX injection on the expression of *MdRDH1* gene were explored via qRT-PCR. With DOX injected, the expression level of *MdRDH1* mRNA increased greatly and got to its peak at the 6th hour (2.9-fold, $p < 0.01$) (Fig. 4A). the DOX-induced oxidative stress was determined by the measurement of SOD activity (Fig. 4B). A significant increase of the SOD activity was observed at the 6th hour and 12th hour after DOX injection (1.5 and 1.2-fold, $p < 0.05$).

3.4. Knockdown of MdRDH1in housefly induced oxidative stress and increased sensitivity to bacterial infection

RNA interference-mediated gene silencing was conducted to analyze *MdRDH1* in a functional way. Results of qRT-PCR and western blot showed that injection of *MdRDH1* dsRNA could greatly reduce the level of MdRDH1 at the 24th hour (Fig. 5A and B). With *MdRDH1* knocked down, an analysis was made of the redox status of housefly, and the oxidant-sensitive probe DCFH-DA was employed to examine the ROS levels in control and *MdRDH1* RNAi groups. The results indicated that the level of ROS endogenously generated in *MdRDH1* RNAi group was higher than that in control group. The concentration of MDA was also quantified for ROS production can elicit MDA accumulation by the lipid peroxidation. As shown in Fig. 5C, silencing *MdRDH1* resulted in a 1.5-fold increase in MDA content by comparing *GFP* dsRNA control. For the role of *MdRDH1* to be characterized in the immune system of the housefly, dsMdRDH1 RNAi larvae found a challenge with *E. coli* or *S. aureus* with the survival rates observed at the 24th hour after the challenge (Fig. 5D). Upon *S. aureus* or *E. coli* infection, the cumulative survival rates of the *MdRDH1*-depleted housefly dropped to 20% and 30%, while those of dsGFP RNAi groups were 60% and 57%, respectively. The cumulative survival flies challenged with *E. coli* or *S. aureus* were investigated at 24 h. The survival rates of larvae after *S. aureus* and *E. coli* challenge were 63% and 60% respectively, which were not differ significantly between the untreated controls and treated with dsGFP groups.

3.5. Recombinant expression, sulfurtransferase activity and its roles in oxidation resistance of MdRDH1

For further enzyme activity analyses, the mature MdRDH1 and its mutant MdRDH1^{C135A} were subjected to recombinant expression in *E. coli* BL21 (DE3). The molecular sizes and expression quantities of the recombinant proteins were confirmed via SDS-PAGE (Fig. 6A). The recombinant his-tagged proteins that are soluble were purified using the immobilized nickel-affinity chromatography. The antiserum against MdRDH1 was prepared using the recombinant protein. After that, thiosulfate: cyanide sulfurtransferase activity was affirmed by the modified Eskandarzade's method using thiosulfate as sulfane-sulfur donor and cyanide as sulfur acceptor. The rhodanese activity of rMdRDH1 was estimated to be 39.54 U/mg (N = 3), but no activity was detected for rMdRDH1^{C135A}. Fig. 6 B and C indicated that the Lineweaver-Burk plots were to determine the kinetic parameters of rMdRDH1. The Km values of $Na_2S_2O_3$ and NaCN were 89.64 and 50.31 mM, respectively.

To demonstrate the involvement of MdRDH1 in triggering protective processes against oxidative events, the response of *E. coli* bacteria expressing rMdRDH1or rMdRDH1^{C135A} to PMS induced oxidative stress (Fig. 6D) was analyzed. The cumulative death rate of expressing rMdRDH1 was significantly lower than that mutant rMdRDH1^{C135A}. When the concentration of PMS was increased to 0.14 mg/ml, no bacterial clone of the mutant group was detected, but an average of 167 clones expressing MdRDH1 appeared in plate. It showed that the resistance of *E. coli* to PMS exposure was strongly strengthened due to the presence of rMdRDH1. A higher survival rate of bacteria expressing rMdRDH1 than that of mutant rMdRDH1^{C135A} was observed with the concentration of PMS being 0.02-0.14 mg/ml.

4. Discussion

Two major sulfurtransferases involved in sulfide metabolism, namely MST and rhodanese, have been relatively well studied in mammals. The rhodanese family is enigmatic. A large amount of genes encoding rhodanese homology domain (RHOD) proteins has been identified in the genomic or transcriptomic databases (Tang et al., 2017), but most of them are functionally unknown or solely tentatively classified via phylogenetic relationships (Cipollone et al., 2007).

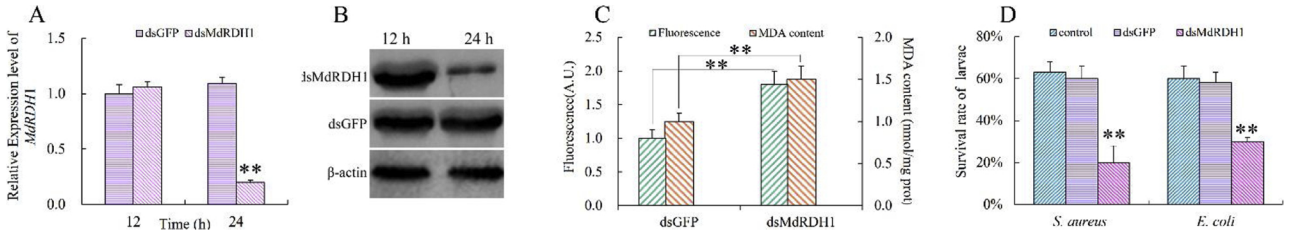

Fig. 5. Gene knockdown analysis of *M. domestica*. RNAi efficiency was evaluated by qRT-PCR (A) and western blot (B). Intracellular levels of ROS and MDA (C) in *MdRDH1* depleted (dsRNA knockdown) the housefly larvae. Knockdown of *MdRDH1* gene led to increased mortalities in the larvae under *S. aureus* or *E. coli* challenge (D). Data shown above were the mean values ± S.D. of the three separate experiments. ** denoted highly significant difference ($p < 0.01$).

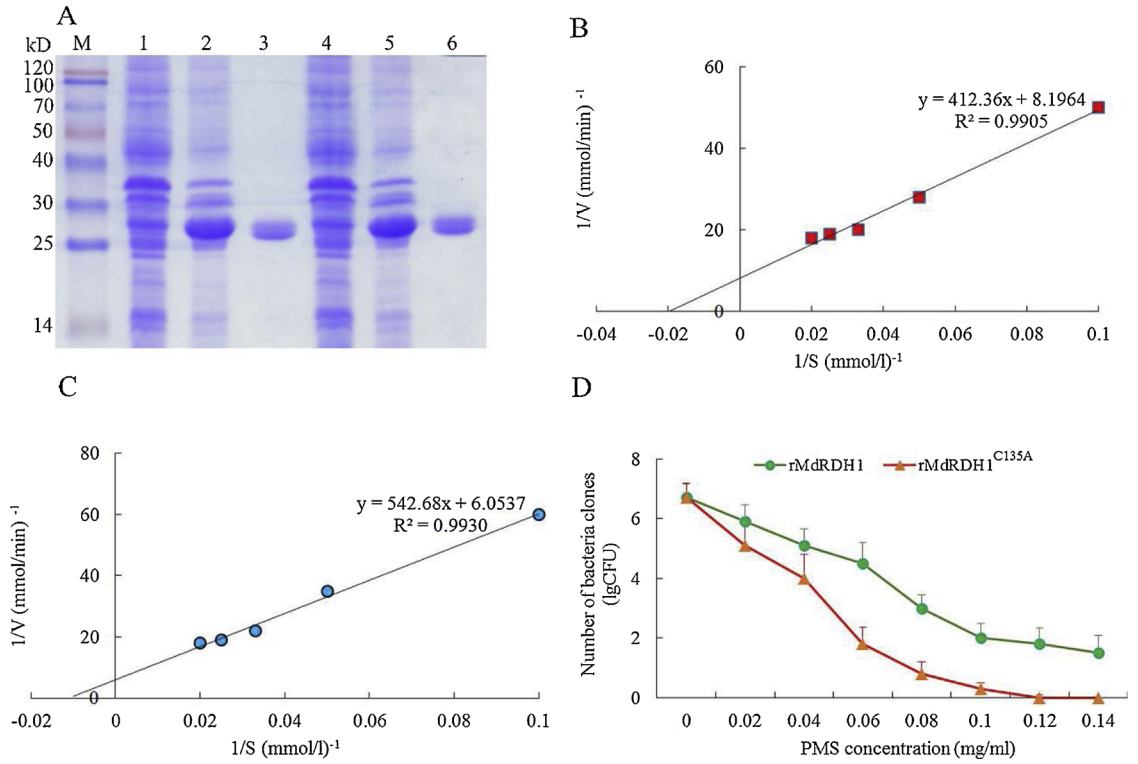

Fig. 6. Recombinant expression, sulfurtransferase activity and its roles in oxidation resistance of rMdRDH1. (A) SDS-PAGE analysis of rMdRDH1 and rMdRDH1^{C135A} proteins. Lane M: protein molecular standard (kDa). Lane 1: negative control of rMdRDH1 (without induction). Lane 2: IPTG induced rMdRDH1. Lane 3: purified rMdRDH1. Lane 4: negative control of rMdRDH1^{C135A} (without induction). Lane 5: IPTG induced rMdRDH1^{C135A}. Lane 6: purified rMdRDH1^{C135A}. (B, C) Lineweavere-Burk plot of the reciprocals of initial rate versus varying the concentration of NaCN or $Na2S_2O_3$ for the determination of kinetic parameters' Km values. The calculated Km values of $Na_2S_2O_3$ and NaCN were 89.64 and 50.31 mM respectively. Each point on the Lineweaver-Burk plots represented average of the three replications. (D) Effect of phenazine methosulfate (PMS) on the growth of *E. coli* expressing rMdRDH1 and rMdRDH1^{C135A}.

Analyses of these sequences have indicated that they are highly heterogeneous in spite of the conservation of the rhodanese signatures (Bordoa and Bork, 2002; Cipollone et al., 2007). The proposed consensus pattern of rhodanese signature is [A/V]-X2-[F/Y]-[D/E/A/P]-G-[G/S/A]-[W/F]-X-E-[F/Y/W], which is conserved in the C-terminal catalytic domain of rhodaneses from eubacteria, archaea, and eukaryotes (Bordoa and Bork, 2002; Cipollone et al., 2007; Tang et al., 2018). In this paper, a gene encoding a HSP67B2-like rhodanese homologue with a single RHOD has been characterized from *M. domestica*, expressed as MdRDH1. Based on the suggested rhodanese signature, a discernable but not exactly conformed rhodanese signature (IMNYKGSWLDW) has been found in the C-terminal end of MdRDH1. In addition to the rhodanese signature, the C-terminal rhodanese domains are characterized by another short active-site loop with the conservative sequence CRXGX[R/T] (Tang et al., 2017; Westley et al., 1983). In MdRDH1, a potential six-amino acid active-site loop (CRSGRR) harboring an invariable catalytic cysteine residue has been recognized. It has been supposed that the catalysis of sulfur-transfer occurs by a double displacement reaction concerning the transient formation of a persulfide-containing intermediate (rhodanese-S) where the transferring sulfur is bound to be the catalytic Cys residue (Bordoa and Bork, 2002). For MnRDH1 and MnRDH2, the rhodanese homologues of *Macrobrachium nipponense* and mutation of the catalytic cysteine lead to a complete loss of sulfurtransferase activities (Tang et al., 2017, 2018). Here, the catalytic activity of MdRDH1 and its mutant MdRDH1^{C135A} have been expressed and detected where the putative catalytic Cys135 in the active-site loop is replaced by Ala residue. The detection of significant thiosulfate: cyanide sulfurtransferase (rhodanese) activity of rMdRDH1 in vitro has declared the first identity of insect rhodanese. Meanwhile, the complete loss of sulfurtransferase activity in rMdRDH1^{C135A} has proved that the catalytic cysteine of MdRDH1 is indispensable for its rhodanese activity. Despite the exact rhodanese activity of MdRDH1, the relatively high Km has revealed weak affinities of MdRDH1 with cyanide and $Na_2S_2O_3$, suggesting that cyanide detoxification could not be the major role of MdRDH1 in housefly. But the nature catalytic substrates and physiological functions of MdRDH1 have been getting confusing.

Different from the mammalian rhodaneses which are mainly located in the mitochondria (Wrobel et al., 2017), MdRDH1 is a possible secretory protein in view of the presence of a signal peptide at the N terminal. The similar signal peptide pattern has also been observed in the *Culex quinquefasciatus* heat shock protein 67B2 (XP_001864125.1), which showed 54% of sequence identity with MdRDH1. Investigation of tissue distribution has revealed that *MdRDH*1 was detected mainly in hemocytes of normal housefly. The tissue distribution characteristics of *MdRDH*1 is similar with that of *MnRDH*1, another invertebrate rhodanese homologue from freshwater prawn *M. nipponense* (Tang et al., 2018). By contrast, mammalian rhodaneses are usually highly expressed in hepatocytes or epithelial cells (Aminlari and Gilanpour, 1991). These expression features have implied that invertebrate HSP67B2-like rhodanese homologues have some special functions different from their mammalian counterparts.

*Paralichthys olivaceus PoDusp*6 encodes a protein with a conserved rhodanese homology domain, which has been significantly up-regulated post LPS and poly (I: C) stimulations at the later stage in the head kidney macrophages. Findings have suggested that *PoDusp*6 might act as an essential modulator in inflammatory response (Li et al., 2017). In *M. nipponense*, the transcriptions of both *MnRDH*1 and *MnRDH*2 have

been enhanced upon bacteria challenge (Tang et al., 2017, 2018). In this paper, the expression of *MdRDH*1 has been up-regulated significantly in larvae challenged with bacteria, hinting that *MdRDH*1 might participate in the immune process in an unknown way.

The antibacterial immune process has often been accompanied by respiratory burst (also known as oxidative burst), which is the rapid release of ROS from different types of cells, such as neutrophils and macrophages. ROS produced by host are critical components of the antimicrobial repertoire in insects, but excessive ROS could spoil cellular proteins, lipids and DNA, causing fatal lesions in host. Therefore, antioxidant enzymes, such as SOD, have been frequently induced to maintain redox equilibrium during the immune process. And in consideration of the fact that sulfur metabolism is often closely related to both detoxification and antioxidation, the expression of *MdRDH*1 in DOX induced housefly oxidation stress model has been analyzed. DOX is considered as an efficient oxidative agent generating ROS and H_2O_2 *in vivo* (Šimůnek et al., 2009). The activity of SOD and the transcriptional levels of *MdRDH*1 have been enhanced significantly after DOX injection, thus suggesting that *MdRDH*1 might have an adaptive antioxidative defense role in housefly.

Furthermore, results have shown that the levels of ROS and MDA, frequently-used oxidative damage biomarkers, have been significantly increased in *MdRDH*1-depleted housefly, which is consistent with the previous findings that impaired rhodanese expression was associated with increased whole cell ROS in hemodialysis patients (Krueger et al., 2010). *Oryza sativa* calcium-sensing receptor (OsCAS) has been including the rhodanese-like protein domain, and knocking out OsCAS has caused significantly higher MDA contents than those overexpressed (Zhao et al., 2015). Thus, the hypothesis has been proposed that MdRDH1 might help host to maintain redox homeostasis during the course of immune response in housefly.

To confirm the antioxidative function of MdRDH1, oxidative stress resistance assay of *E. coli* expressing *MdRDH*1 has been conducted. Chemical oxidant PMS has been widely used as a superoxide generator provoking oxidative stress in animals or microorganisms (Cereda et al., 2009). And the result in this paper has shown that the *MdRDH*1-transformed *E. coli* has shown greatly enhanced survival rates by comparing those transformed bacteria with *MdRDH*1^{C135A} upon treatment by PMS. It has also further verified its antioxidative roles in a conservative and direct way.

In summary, the rhodanese-identity of a housefly HSP67B2-like protein has been identified by enzymology and its potential role in immunity and oxidation resistance been revealed, and however the detailed molecular mechanism for invertebrate HSP67B2-like rhodanese in keeping the redox balance still remains to be addressed, which should attract more attention.

Contributions of authors

Contributed to the designed experiments: FSL. Performed the experiments and data analyses: TT, YBL, HHS and PRC. Contributed significantly to analysis and manuscript preparation: TT and FSL. Wrote the manuscript: TT and FSL. All authors read and approved the manuscript.

Funding

This work was financially supported by the National Natural Science Foundation of China (31572327, 31830084).

Conflict of interest

We declare that we have no conflict of interest to this work.

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