Studies on the functions of *Haemaphysalis longicornis* 2-Cys peroxiredoxin, and its application for tick control

フタトゲチマダニ由来 2-Cys ペルオキシレドキシンの機能とマダニ制御への応用に関する研究

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

ABBREVIATIONS

ROS: reactive oxygen species
H_2O_2: hydrogen peroxide
Prx: peroxiredoxin
Prxs: peroxiredoxins
HlPrx2: *Haemaphysalis longicornis* 2-Cys peroxiredoxin
EST: expressed sequenced tag(s)
His: histidine
PBS: phosphate buffered saline
IPTG: isopropyl β-D-1-thiogalactopyranoside
LB: Luria-bertani
rHlPrx2: the recombinant HlPrx2 protein
WT: the wild-type of the recombinant HlPrx2 protein
C51S: the mutant of the recombinant HlPrx2 converting from Cys51 to Ser
C172S: the mutant of the recombinant HlPrx2 converting from Cys172 to Ser
DM: the double mutant of the recombinant HlPrx2 cited above (C51S and C172S)
SDS-PAGE: sodium dodecyl sulfate -polyacrylamide gel electrophoresis
2-ME: 2-mercaptoethanol
MFO: mixed-function oxidation
FBS: fetal bovine serum
NADPH: nicotinamide adenine dinucleotide phosphate
SEC: size-exclusion chromatography
HlPrx: *Haemaphysalis longicornis* 1-Cys peroxiredoxin
qPCR: quantitative polymerase chain reaction
IFAT: indirect immunofluorescent antibody test
RNAi: RNA interference
Luc: firefly *luciferase* gene
ds-: the double-stranded RNA of -
mRNA: messenger RNA
ORF: open reading frame
IFA: incomplete Freund's adjuvant

GENERAL INTRODUCTION

Reactive oxygen species (ROS) that include the superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radicals are produced by the incomplete reduction of oxygen. Among the ROS, hydroxyl radicals are the most reactive and short lived (10^{-9} sec half-life at 37°C) [1]. In addition, through the Fenton reaction, hydroxyl radicals are produced from H_2O_2, the most stable ROS. But controlling hydroxyl radicals is very difficult for organisms because of their short half-lives. Therefore, the removal of H_2O_2 is biologically advantageous [2].

In high concentrations, H_2O_2 is known to be a chemical compound harmful to aerobic organisms due to its ability to seriously damage membrane lipids, nucleic acids, and proteins [3]. To protect against H_2O_2 toxicity, aerobic organisms have evolved antioxidant enzymes, such as catalases, peroxidases, and peroxiredoxins (Prxs) [4]. Prxs are ubiquitous antioxidant enzymes that have been investigated in various organisms [5]. Particularly, high levels of Prxs are produced in mammalian cells, including erythrocytes [6]. Erythrocytes are exposed to more oxidative stress than any other mammalian cells, due to the abundance of heme iron and oxygen, which can generate H_2O_2 [7]. These phenomena indicate that Prxs may play important roles in peroxide detoxification in cells.

Ticks are obligate hematophagous arthropods that need blood feeding at all developmental stages. Blood feeding and the digestion of blood provide nutrition and energy for development, molting, and embryogenesis in ticks [8]. Ticks feed on vertebrate blood that contains high levels of iron, such as ferrous iron, heme, and iron-bound transferrin [9]. Ticks also concentrate iron-containing host blood; this concentration of the blood leads to high levels of iron in ticks. Host-derived iron may react with oxygen in the tick body, resulting in high concentrations of ROS, including H_2O_2 [10,11]. In addition, ticks have genes of antioxidant enzymes, such as *catalases* [12] and *Prxs* [13-15], to scavenge H_2O_2. Although the gene silencing of *Prxs* in ticks affects ticks' blood feeding and oviposition [13], the gene silencing of *catalase* in ticks is not effective [12]. Therefore, Prxs could be considered to play important roles in ticks' blood feeding and oviposition through the regulation of the H_2O_2 concentration in ticks.

In this dissertation, I focused on the 2-Cys Prx in ticks and its application for tick control.

Research Objectives

With following objectives, this dissurtation besides the studies on the functions of

Haemaphysalis longicornis

2-Cys Prx (HlPrx2), and its application for tick control.

AIM 1.

To identify *HlPrx2* gene and characterize recombinant HlPrx2 protein

AIM 2.

To characterize expression profiles and biological functions of HlPrx2 in the ticks

AIM 3.

To clarify the importance of Prxs for antioxidant response using the tick cell line

AIM 4.

To evaluate the vaccine potential of rHlPrx2 against the ticks

CHAPTER 1

Functional analysis of recombinant 2-Cys peroxiredoxin from the hard tick Haemaphysalis longicornis

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1.1 Introduction

Almost all aerobic organisms have developed defense systems to scavenge H_2O_2. Catalases, peroxidases, and peroxiredoxins (Prxs) are scavengers of H_2O_2 [4]. Prxs are ubiquitous antioxidant enzymes investigated in various organisms [5]. Particularly, Prxs are produced at high levels in mammalian cells, including erythrocytes [6,16]. The rate constants of catalases, peroxidases, and Prxs were considered to have almost the same rate (10^7 - 10^8 M^{-1} S^{-1}) [17]. These results indicate that Prxs may have important roles in peroxide detoxification in cells [16].

Prxs can be divided into two groups according to the presence of one or two highly conserved cysteines in organisms, 1-Cys or 2-Cys Prx [5]. Structural and mechanistic data showed the further division of the 2-Cys Prxs into two classes, typical and atypical 2-Cys Prxs [16]. Typical 2-Cys Prxs are identified by two conserved cysteines, peroxidatic and resolving cysteines [18]. Deprotonated peroxidatic cysteine reacts with H_2O_2 via a nucleophilic attack to form cysteine sulfenic acid with the release of water. Then, the resolving cysteine in another Prx molecule will react with the cysteine sulfenic acid to form homodimers via intermolecular disulfide bonds. These disulfide bonds are reduced by oxidoreductases to revert to the active form [19]. In addition, studies of typical 2-Cys Prxs have showed that dramatic changes in the

oligomeric state (dimers and decamers) are associated with changes in oxidative stress, like those occurring during the catalytic cycle [16]. Atypical 2-Cys Prxs have the same mechanism as do typical 2-Cys Prxs but are functionally monomeric [20]. The 1-Cys Prxs conserve only the peroxidatic cysteine and do not contain a resolving cysteine [21]. The mechanism of the 1-Cys Prxs is considered to be one donor thiol that probably forms a transient mixed disulfide bond with the enzyme, followed by its reduction by a second donor thiol, thus recycling the enzyme [16]. However, there has been only one report on tick Prxs focusing on *Haemaphysalis longicornis* 1-Cys Prx until now [15]. Thus, the function of tick Prxs is not completely known.

In this study, I identified a 2-Cys *Prx* gene from *H. longicornis* (*HlPrx2*) and prepared some kinds of recombinant proteins of HlPrx2, including three mutants, to clarify the role of HlPrx2 on antioxidant activity produced by H_2O_2 in ticks.

1.2 Materials and Methods

1.2.1 Identification and characterization of the cDNA encoding the HlPrx2 protein

The putative *HlPrx2* gene was identified using an expressed sequence tag (EST) database constructed from the cDNA library of the fat body from *H. longicornis* as previously described by Boldbaatar *et al*., [22]. A pGCAP1 plasmid containing an

HlPrx2 gene insert was extracted using a Qiagen® Plasmid Mini Kit (Qiagen, Hilden, Germany). The insert was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and the Applied Biosystems® 3500 XL Genetic Analyzer.

The deduced amino acid translation of the *HlPrx2* gene sequence was determined using GENETYX version 7.0 software (GENETYX, Tokyo, Japan). To search homologous genes from GenBank (http://www.ncbi.nlm.nih.gov/genbank), a BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. The domain structure was determined using the SMART program (http://smart.embl-heidelberg.de/). To predict the three-dimensional structure of HlPrx2, the Phyre2 Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/phyre2/) was used [23]. The theoretical molecular mass and isoelectric point were calculated using a ProtParam tool (http://web.expasy.org/protparam/). Putative signal peptide cleavage sites and N-linked glycosylation sites were checked by the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. An alignment analysis was performed using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

1.2.2 Expression and purification of wild-type recombinant HlPrx2 and HlPrx2 mutants

Wild-type (WT) recombinant HlPrx2 (rHlPrx2) was expressed as an N-terminus 6-histidine (His) -tagged protein using the expression vector pRSET C (Invitrogen, Carlsbad, CA, USA). The WT open reading frame sequence was amplified by a polymerase chain reaction (PCR) using a forward primer (HlPrx2 F-*Xho*I) containing a *Xho*I recognition site and a reverse primer (HlPrx2 R-*Eco*RI) containing an *Eco*RI recognition site (Table 1.1). The amplified PCR product was then purified using a GENECLEAN® II KIT (MP Biomedical, Solon, OH, USA) and subcloned into the frame of the pRSET C. The WT was expressed in *Escherichia coli* BL21 (DE3) strain and purified as described previously [24].

To prepare the mutated proteins, the inverse PCR of the plasmid DNA (pRSET C containing HlPrx2 WT) was performed by using mutation primers and KOD-Plus-Neo (Toyobo, Osaka, Japan) following the manufacturer's protocol for a single nucleotide substitution (Table 1.1). The self-ligation of PCR products was performed using DNA Ligation Kit (Takara Bio Inc., Shiga, Japan). The self-ligated PCR products were digested by *Dpn*I at 37°C for 4 hrs. The mutated plasmids, HlPrx2 Cys51Ser, HlPrx2 Cys172Ser, and HlPrx2 Cys51Ser/Cys172Ser; double mutant, named

C51S, C172S, and DM, respectively, were transformed into *E. coli* Stellar strain and subcloned. The mutated plasmids were extracted from *E. coli*, and the confirmation of mutations was analyzed by DNA sequencing. Finally, the mutant rHlPrx2 proteins were expressed in *E. coli* BL21 (DE3) strain and purified similarly to the WT protein.

1.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

500 ng/lane WT and mutant recombinant proteins were mixed with the same volume of sample buffer with or without 10% 2-mercaptoethanol (2-ME). Consequently, the samples with 2-ME were boiled. The prepared protein samples were resolved in a 12% SDS-PAGE gel under reducing (with 2-ME) or non-reducing (without 2-ME) conditions. After SDS-PAGE, the gel was stained by Coomassie Brilliant Blue and viewed using Gel Doc (Bio-Rad, Hercules, CA, USA).

1.2.4. Mixed-function oxidation assay

Mixed-function oxidation (MFO) assay [25] was performed in accordance with the methods of Masatani *et al*. [26]. Briefly, a reaction mixture containing 40 μM ferric chloride ($FeCl_3$), 10 mM dithiothreitol (DTT), 20 mM EDTA, and 25 mM Hepes-NaOH (pH 7.0) was pre-incubated with or without 3.125-400 μg/ml recombinant proteins at

37°C for 1 hr. After pre-incubation, 0.5 μg of pcDNA^{TM} 3.1 (+) plasmid DNA (Invitrogen) was added, and the reaction mixture was incubated for another 3 hrs. Nicking of the supercoiled plasmids by MFO assay was evaluated by running electrophoresis on 1% agarose gel stained with ethidium bromide for visualization. Bovine serum albumin (BSA) and recombinant *H. longicornis* Ferritin 2 (rFer2) were used for negative controls of protein and His-tagged protein, respectively.

1.2.5. Hydrogen peroxide (H_2O_2)-scavenging assay

H_2O_2 consumption was measured using the ferrous oxidation of xylenol orange assay [27], adapted for low peroxide concentrations. The assay reagent consisted of 125 μM xylenol orange, 250 μM ammonium iron (II) sulfate, 100 mM sorbitol, and 25 mM sulfuric acid. After treatment of 50 μg/ml recombinant and mutant proteins with 5 μM H_2O_2, 100-μl sample solutions were added to a 1-ml assay reagent. The mixture was vortexed immediately, left at room temperature for 35 min, and measured at 560 nm using spectrophotometer (Ultrospec 2100 pro, GE Healthcare, Pittsburgh, PA, USA).

1.2.6. Peroxiredoxin activity assay

Peroxiredoxin activity assay was performed in accordance with the methods of Kim *et al*., [28]. A prereaction cocktail that contains 50 mM Hepes-NaOH buffer (pH

7.0), 1 mM EDTA, 200 μM nicotinamide adenine dinucleotide phosphate (NADPH), 0.8 μM thioredoxin reductase, 1.5 μM thioredoxin, and 1 μg rHlPrx2 proteins in 100 μl total volume, was prepared. Then, 100 μM H_2O_2 was added into each well in the 96-well plate. The reaction was started by adding H_2O_2, and NADPH oxidation was monitored for 20 min at 30°C by following absorbance reduction at 340 nm in a microplate reader (SH-9000Lab, Corona Electric, Ibaraki, Japan).

1.2.7. Size-exclusion chromatography

The molecular sizes of the purified WT, C51S, C172S, and DM recombinant proteins were measured by size-exclusion chromatography (SEC). The protein samples were 0.2 μm-filtered before using for the SEC. The 170 μg/ml WT, C51S, C172S, or DM recombinant proteins were loaded into a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) at a flow rate of 0.8 ml/min using the ÄKTAprime plus chromatography system (GE Healthcare) [29]. Phosphate buffered saline (pH 7.4) containing 137 mM NaCl, 27 mM KCl, 81 mM $Na_2HPO_4 \cdot 12H_2O$, and 14.7 mM KH_2PO_4 was used throughout all procedures of the SEC.

1.3 Results

1.3.1 Identification of the 2-Cys peroxiredoxin gene of Haemaphysalis longicornis

The *2-Cys Prx* gene of *H. longicornis* (HlPrx2: accession no. LC049075) was isolated from EST clones from the fat body cDNA libraries of the tick. The full-length *HlPrx2* cDNA is 797 bp, with an open reading frame extending from position 97 to 691, encoding for 197 amino acids (Fig. 1.1). A polyadenylation consensus signal sequence was identified upstream of the poly A tail. The predicted molecular mass of HlPrx2 is 22.3 kDa, and the theoretical isoelectric point is 6.8. The signal peptide and glycoxylation sites were not found by *in silico* analysis. HlPrx2 has an alkyl hydroperoxide reductase- thiol specific antioxidant (AphC-TSA) domain from positions 8 to 141 and a C-terminal domain of 1-Cys peroxiredoxin (1-cysPrx C) domain from 161 to 196. In addition, BLAST analysis of the deduced amino acid sequences and 2-Cys Prx of other organisms, such as *Ixodes scapularis*, *Ornithodoros parkeri*, *Drosophila willistoni*, *Aedes aegypti*, and *Homo sapiens* (Prx2 and Prx4), revealed that HlPrx2 shows high similarities of 88%, 86%, 79%, 78%, 77%, and 76%, respectively. Furthermore, the alignment analysis demonstrated that HlPrx2 has two conserved cysteines at positions 51 and 172 and has an YF motif at positions 193 to 194 (Fig. 1.2).

1.3.2 Confirmation of dimer formation by the disulfide bonds via the two conserved cysteines

The scheme of the reaction mechanism of 2-Cys Prx was considered according to Lu & Holmgren [19]. Briefly, deprotonated peroxidatic cysteine reacts with H_2O_2 to form cysteine sulfenic acid. Then, the resolving cysteine in another molecule will react with the cysteine sulfenic acid and form homodimers via intermolecular disulfide bonds. To determine whether the wild-type protein of rHlPrx2 (WT) can form homodimers by disulfide bonds via the two conserved cysteines, I performed SDS-PAGE analysis with or without 2-mercaptoethanol. SDS-PAGE analysis demonstrated that WT formed a dimer by disulfide bonds under non-reducing conditions (Fig. 1.3). In addition, to investigate whether the conserved cysteines play an important role for the dimerization of rHlPrx2, I carried out the same experiment using mutant proteins (rHlPrx2 Cys51Ser, rHlPrx2 Cys172Ser, and rHlPrx2 Cys51Ser/Cys172Ser; double mutant, named C51S, C172S, and DM, respectively). However, the mutant proteins of rHlPrx2, C51S, C172S, and DM could not form a dimer under non-reducing conditions. These results indicate that either Cys51 or Cys172 is necessary for the dimerization of rHlPrx2.

1.3.3 Measurement of the antioxidant, hydrogen peroxide-scavenging, and peroxiredoxin activity of the wild-type and mutant proteins of rHlPrx2

To demonstrate the antioxidant activity of the WT protein, I performed an mixed-function oxidation (MFO) assay using recombinant proteins [25]. Through agarose gel electrophoresis using plasmid DNA, a band of nicked plasmid DNA, injured by ROS, appears in a higher position as compared to a normal coiled plasmid DNA. In other words, the nicked and coiled forms indicate oxidized and anti-oxidized forms, respectively. The MFO assay showed that the WT had antioxidant activity from a concentration of 25 μg/ml (Fig. 1.4A). Additionally, to investigate the roles of the conserved cysteines in the antioxidant activity of rHlPrx2, I performed an MFO assay using the mutant proteins of rHlPrx2, C51S, C172S, and DM. C51S and DM mutants did not show antioxidant activity at any concentration. Nevertheless, the C172S mutant retained its antioxidant activity from a concentration of 12.5 μg/ml. Generally, Prxs can scavenge H_2O_2 [19]. Accordingly, I performed an H_2O_2-scavenging assay for the WT rHlPrx2. These results showed that the WT has antioxidant activity, indicating that it can scavenge H_2O_2 (Fig. 1.4B). Moreover, the H_2O_2-removal ratio of WT was 67% when comparing 0 and 30 min of the calculated H_2O_2 concentration. In contrast, the control BSA was not able to remove H_2O_2. Additionally, I carried out an

H_2O_2-scavenging assay for the mutant proteins C51S, C172S, and DM to check whether the conserved cysteines contribute to H_2O_2 scavenging. The assay revealed that C51S and DM mutants could not scavenge H_2O_2. Importantly, the C172S mutant was able to scavenge H_2O_2, and the H_2O_2-removal ratio was 79%, higher than the WT's ratio. The 2-Cys Prxs are known to be reduced by the thioredoxin system including thioredoxin and thioredoxin reductase [19]. Thus, to clarify whether rHlPrx2 protein can scavenge H_2O_2 with the thioredoxin system, I performed a peroxiredoxin activity assay. The assay revealed that the WT rHlPrx2 with the thioredoxin system catalyzed the H_2O_2-dependent oxidation of NADPH (Fig. 1.4C), however, mutant proteins of rHlPrx2, including C172S mutant, and BSA did not. These results suggest that Cys51 of rHlPrx2 is necessary for antioxidant activity, and also, both Cys51 and Cys172 are essential for thioredoxin-dependent activity.

1.3.4 Determination of the recombinant HlPrx2 multimer's molecular weight using gel-filtration chromatography

Previous studies show that 2-Cys Prxs form an oligomer [16,30]. To investigate the exact molecular size of the rHlPrx2, gel-filtration chromatography was performed. The molecular weight of the rHlPrx2, WT, C51S, C172S, and DM were 399 kDa, 388

kDa, 448 kDa and 377 kDa, respectively. Notably, only the WT retained the other peak of the 57 kDa molecular mass (Fig. 1.5). This result paralleled that in Fig. 2, strongly suggesting that either Cys51 or Cy172 is essential for the dimerization of rHlPrx2.

1.4 Discussion

In the present study, I identified the *HlPrx2* gene, and the sequence analysis of the HlPrx2 amino acids revealed that HlPrx2 has the AphC-TSA domain and the 1-cysPrx C domain. The AphC-TSA domain is conserved in the Prxs family [16]. In addition, the 1-cysPrx C domain is recognized as a member of the Prxs superfamily that protects cells from membrane oxidation [21]. Alignment analysis of the deduced amino acid sequences of the HlPrx2 and other organisms' 2-Cys Prxs revealed that HlPrx2 is similar to *Homo sapiens'* 2-Cys Prxs, and their similarities with HsPrx2 and HsPrx4 are 77% and 76%, respectively. HsPrx2 is located in the cytosol or cell membrane of erythrocytes [31]. Using an indirect immunofluorescence antibody test, I found HlPrx2 along the basal lamina or cell membrane of some *H. longicornis* tick tissues, including the midgut and ovary, as well as in the cell membrane of hemocytes (unpublished data). These results suggest the possibility that HlPrx2 functions to protect cells against membrane oxidation at the midgut, ovary, or hemocyte of the *H. longicornis* tick. Some

reports showed that the decamers of some peroxiredoxins associated with membrane [32,33]. HsPrx2, that is similar to HlPrx2 with amino acid sequence similarity of 77%, is also lacks a signal peptide and associated with plasma membrane of erythrocytes, however, in the case of patient lack of band 3 gene, HsPrx2 cannot be associated with the plasma membrane in the erythrocytes [31]. In addition, HsPrx2 does not have the large hydrophobic region as well as HlPrx2. These results suggest that HsPrx2 needs band 3 to be bound with erythrocytes' plasma membrane [31] without the signal peptide and the large hydrophobic region. Therefore, HlPrx2 may also have some partners to associate with plasma membrane without the signal peptide and the large hydrophobic region.

According to Lu & Holmgren [19], the deprotonated peroxidatic cysteine (Cys51 of HlPrx2) reacts with H_2O_2 to form cysteine sulfenic acid with the release of H_2O. Then, the resolving cysteine (Cys172 of HlPrx2) of another HlPrx2 molecule will react with the cysteine sulfenic acid to form homodimers via intermolecular disulfide bonds (Fig. 1.6). To demonstrate the dimerization of rHlPrx2, I performed SDS-PAGE analysis and found that only the WT formed a dimer under a non-reducing conditions but C51S, C172S, and DM mutants did not. Additionally, I performed MFO and H_2O_2-scavenging assays to demonstrate the antioxidant activity for scavenging H_2O_2 of

the recombinant and mutant proteins of HlPrx2; the assays demonstrated that WT and C172S possessed antioxidant activity. In the MFO assay, ROS such as superoxide, H_2O_2, and hydroxyl radicals may be generated [34]. Hydroxyl radicals are primary oxidants that injure the plasmid DNA. However, hydroxyl radicals may be generated by a Fenton reaction. These results indicate that rHlPrx2 has almost the same molecular dynamism of the catalytic cycle of typical 2-Cys Prxs as mentioned by Lu & Holmgren [19], and may function as the antioxidant enzyme for the H_2O_2 scavenger in the tick body.

There have been some reports about a mutation of the resolving cysteine in 2-Cys Prxs, indicating that the mutation results in lowered catalytic rates of the wild type [35,36]. Additionally, reports of truncating several C-terminal residues, including the YF motif of 2-Cys Prxs (see in Fig. 1.2, indicated by right and left arrow), suggested that the antioxidant activity of the mutant was increased [30,37]. The YF motif covers the peroxidatic cysteine of another 2-Cys Prx to protect from overoxidation [5] and limits the dynamics of active site including the peroxidatic cysteine [37]. Based on MFO and H_2O_2-scavenging assays, the WT and the C172S mutant had antioxidant activity from concentrations of 25 and 12.5 μg/ml, respectively, and an H_2O_2-removal ratio of the WT and the C172S mutant of 67% and 79%, respectively. In my study, when the resolving cysteine of rHlPrx2 was mutated, the antioxidant activity seems to

be increased. Same observation was seen when the YF motif was truncated [30,37]. Additionally, the Prx activity assay demonstrated that WT with the thioredoxin system could scavenge H_2O_2 but C172S mutant could not. These results indicate that a positional clash between Cys51 and the YF motif of the C172S mutant of rHlPrx2 may occur, leading to the malfunction of the YF motif. This hypothesis suggests that the YF motif of rHlPrx2 might be strongly related to Cys172.

Prxs are known to form oligomers as shown in some reports [16,30]. Through gel-filtration chromatography of the WT and mutant proteins of rHlPrx2, I obtained high molecular weight fractions of the WT, C51S, C172S, and DM. However, mutants of rHlPrx2 could not form a dimer in SDS-PAGE or gel-filtration chromatography. In some papers, the function of Prxs' oligomerization is considered a molecular chaperone [5,30,38]. Although the antioxidant activity was decreased conversely, the chaperone functions of the native HsPrx2 increased significantly upon exposure to increasing concentrations of H_2O_2 [30]. In addition, the HsPrx2 mutants retained their chaperon activity [30]. These results and a large amount of H_2O_2 that can be generated in the tick's body during blood feeding [11] might indicate that HlPrx2 is important to the function as a chaperone protecting the stability of the basal lamina or cell membrane of the midgut and ovary in tick. Moreover, I estimated the three-dimensional structures of

the wild-type and mutant proteins of HlPrx2 by using the Phyre2 Protein Fold Recognition Server. As predicted by using HsPrx4 [PDB ID: 3TKP [37]] as template, the Phyre2 server revealed a three-dimensional structure of HlPrx2 WT and prepared HlPrx2 decamer and dimer structures by using WinCoot software [39]. The three-dimensional structures of mutant HlPrx2 proteins, C51S, C172S, and DM, were prepared by the same method as for the WT. However, the three-dimensional structures of those proteins are almost the same as the structures of the decamer and dimer forms (Fig. 1.6). These results indicate that Cys51 and Cys172 may not influence the interface of decamers for chaperon activity.

In summary, I identified a 2-Cys *Prx* gene from *H. longicornis* (*HlPrx2*) and prepared some kinds of rHlPrx2 proteins, including three mutants. I determined that Cys51 is necessary for the antioxidant activity of rHlPrx2 for removing H_2O_2, and both Cys51 and Cys172 are essential for the thioredoxin-dependent activity. Moreover, either Cys51 or Cys172 is critical for the dimerization of rHlPrx2 but not for oligomerization. Therefore, HlPrx2 may play an important role in the antioxidant activity, including chaperone activity, in ticks.

Table and Figures in CHAPTER 1

Table. 1.1. Primers for the preparation of recombinant and mutant proteins of HlPrx2

HlPrx2 F-*Xho*I	5’-CCGCTCGAGTAATGGACGTGGTTATG-3’
HlPrx2 R-*Eco*RI	5’-CGGAATTCCAGATCTATTGTTTGGCG-3’
HlPrx2 Cys51Ser F	5’-CGTCTCCCCGACGGAAATCATCGCC-3’
HlPrx2 Cys51Ser R	5’-GGGAGACGAAAGTGAAGTCAAGCGGATAG-3’
HlPrx2 Cys172Ser F	5’-TGGCGAAGTGTCCCCTGCTAACTGGAAGCCTGGC-3’
HlPrx2 Cys172Ser R	5’-AGTTAGCAGGGGACACTTCGCCATGCTTGTCAGTG-3’

Single underline indicates *Xho*I recognition site. Broken underline indicates *Eco*RI recognition site. Serine codons converting from cysteine codons are highlighted in black, and double underlines indicate that the nucleotides are changed by a single nucleotide substitution.

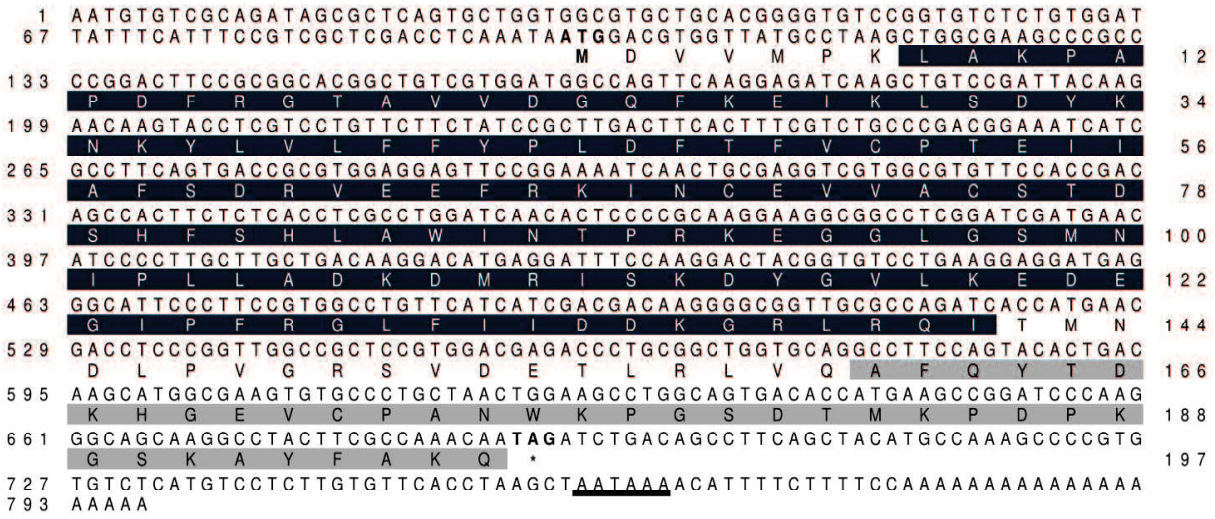

Fig. 1.1. Full-length cDNA and deduced amino acid sequences of HlPrx2 from *H. longicornis*. AphC-TSA and 1-cysPrx C domains are highlighted in black and gray, respectively (AphC-TSA domain: 8 to 141, 1-cysPrx C domain: 161 to 196). The putative polyadenylation signal after the stop codon (TAG) is underlined. The start and stop codons are boldfaced.

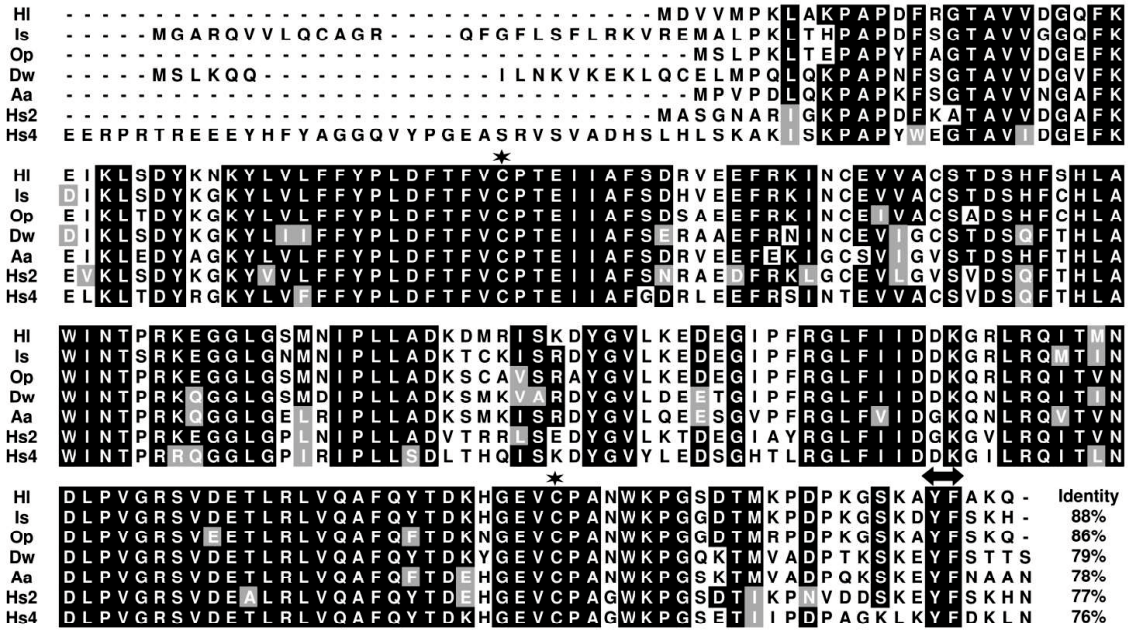

Fig. 1.2. Multiple alignment sequence analysis of the deduced amino acid sequence of HlPrx2 and other organisms' 2-Cys peroxiredoxin. Asterisks indicate the position of conserved cysteines (amino acid numbers 51 and 172) of 2-Cys peroxiredoxins. The two-directional arrow indicates the YF motif of typical 2-Cys Prxs (amino acid number 193 to 194). Similar and homologous amino acids are highlighted in black and gray, respectively. HlPrx2 (Hl), alkyl hydroperoxide reductase of *Ixodes scapularis* (Is; XP_002405466.1), thioredoxin peroxidase of *Ornithodoros parkeri* (Op; ABR23404.1), peroxiredoxin of *Drosophila willistoni* (Dw; XP_002071309.1), 2-Cys thioredoxin peroxidase of *Aedes aegypti* (Aa; AAL37254.1), peroxiredoxin-2 of *Homo sapiens* (Hs2; NP_005800.3), and peroxiredoxin-4 of *H. sapiens* (Hs4; CAG46469.1).

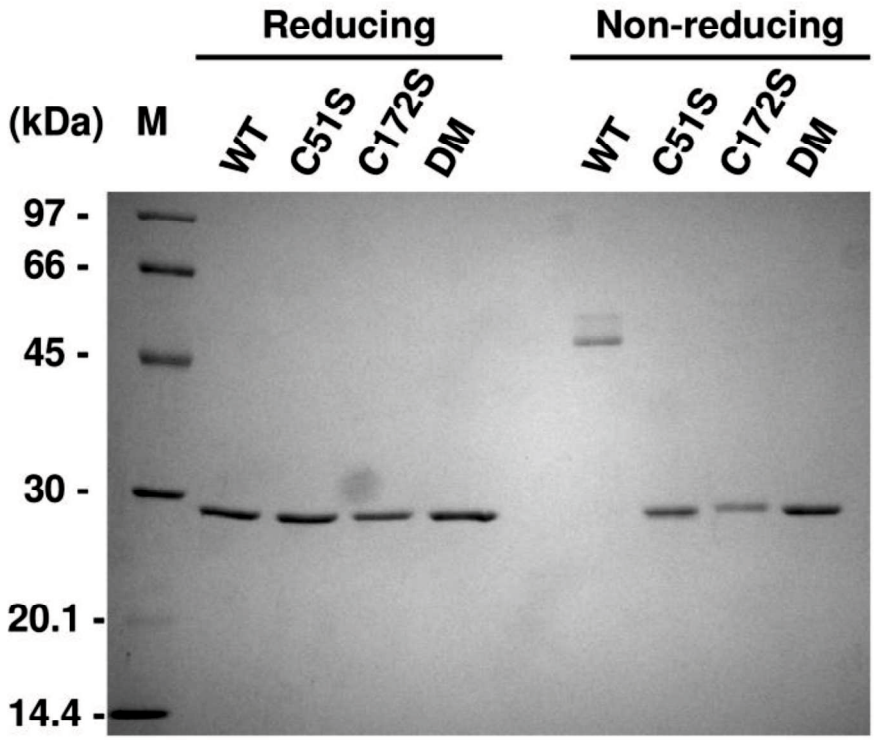

Fig. 1.3. Confirmation of the dimmerizetion by disulfide bonds in wild-type, C51S, C172S, and DM rHlPrx2 prepared using SDS-PAGE analysis. 500 ng of rHlPrx2 (WT, C51S, C172S, and DM) were loaded per lane. WT, wild type; C51S, Cys51Ser; C172S, Cys172Ser; DM, double mutant, Cys51Ser/Cys172Ser; M, marker.

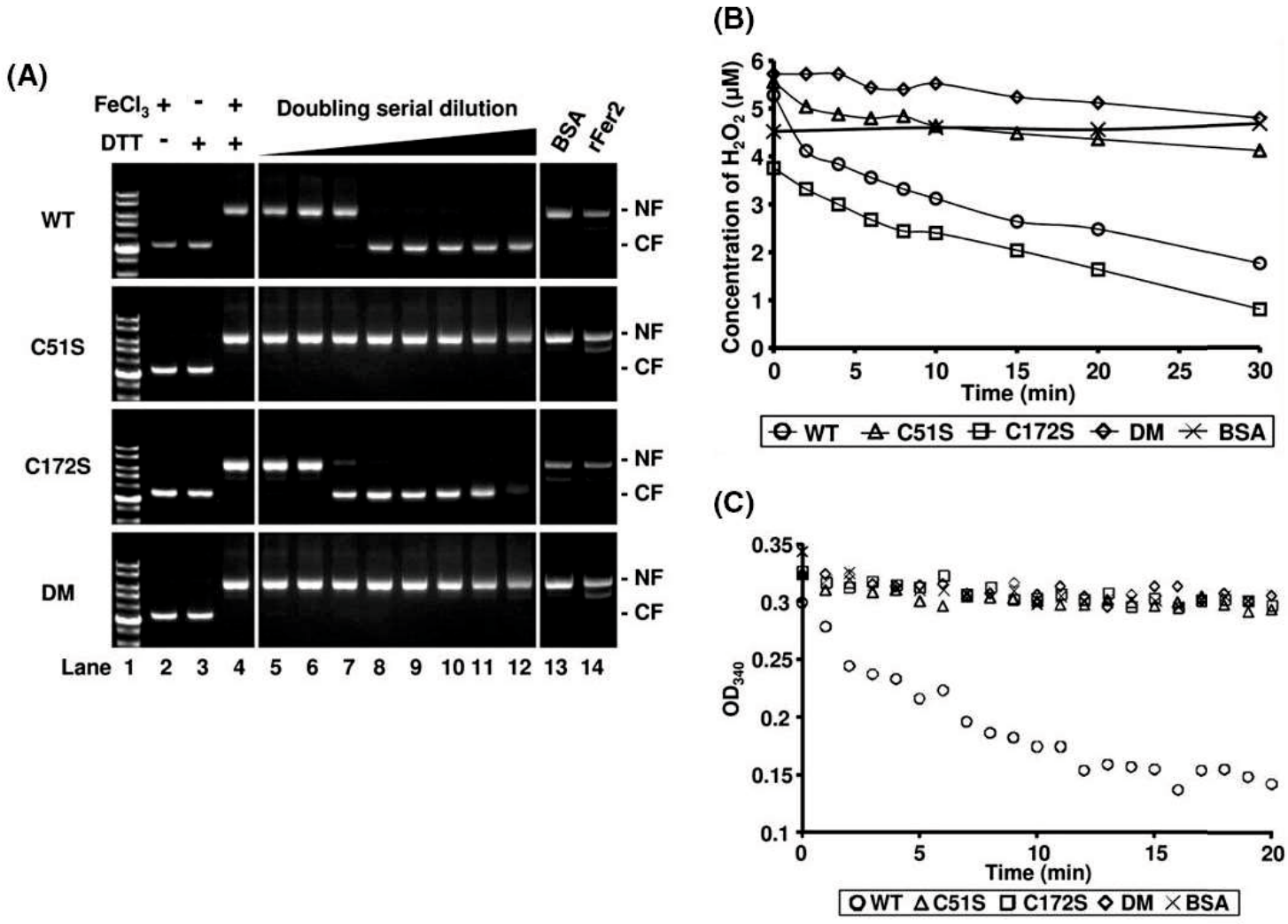
(A)
FeCl3 + - +
DTT - + +
Doubling serial dilution
BSA
rFer2
WT
C51S
C172S
DM
- NF
- CF
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(B)
Concentration of H2O2 (μM)
Time (min)
WT C51S C172S DM BSA
(C)
OD340
Time (min)
WT C51S C172S DM BSA

Fig. 1.4.

Fig. 1.4. (A) Measurement of the antioxidant activity of the rHlPrx2 proteins using MFO assay. The left column indicates the names of recombinant proteins. Lane 1 is the 1,000 b DNA ladder marker. Lanes 2 to 4 represent treatments with or without $FeCl_3$ or DTT. Lanes 5 to 12 are plasmids with doubling serial dilution from 3.125 to 400 μg/ml of recombinant proteins. Lanes 13 and 14 are bovine serum albumin (BSA) and recombinant *H. longicornis* ferritin 2 (rFer2), respectively, at 400 μg/ml. The right column indicates the status of plasmid, the nicked form (NF), and the coiled form (CF). NF and CF indicate oxidized and non-oxidized status, respectively. BSA and rFer2 are negative controls for proteins and His-tagged proteins, respectively. WT, wild type; C51S, Cys51Ser; C172S, Cys172Ser; DM, double mutant, Cys51Ser/Cys172Ser. **(B)** Hydrogen peroxide-scavenging assay for the rHlPrx2 proteins treated with 5 μM H_2O_2. The concentration of H_2O_2 was measured at 0, 2, 4, 8, 10, 15, 20, and 30 min after adding H_2O_2. BSA indicates bovine serum albumin as negative control and sampling was done at 0, 10, 20 and 30 min after adding H_2O_2. **(C)** The peroxiredoxin activity assay for the rHlPrx2 proteins and the thioredoxin system with 100 μM H_2O_2. The absorbance of 340 nm was measured at every minute until 20 min after adding H_2O_2. BSA indicates bovine serum albumin as negative control.

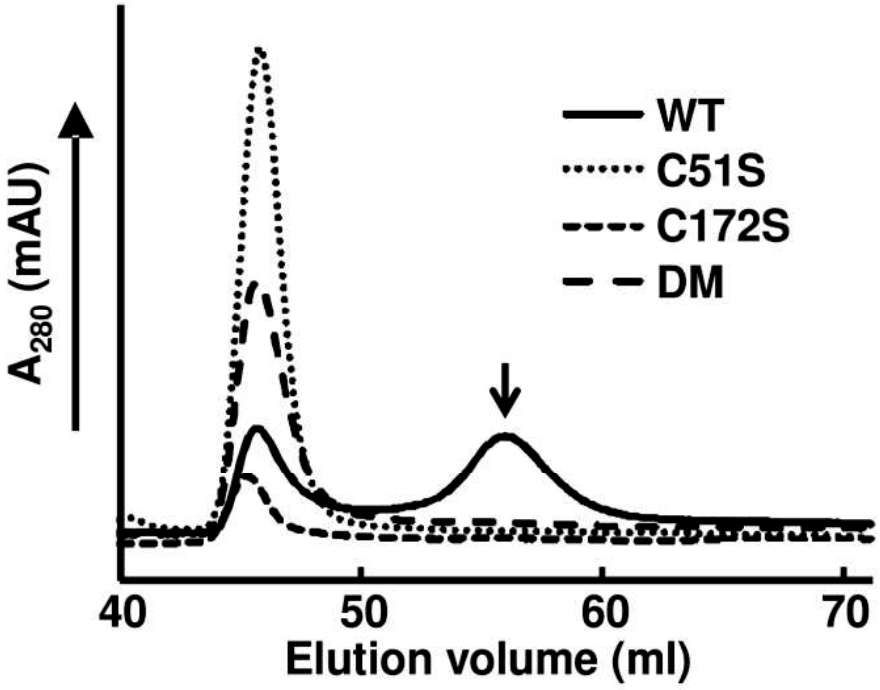

Fig. 1.5. Determination of the multimeric recombinant HlPrx2 protein's molecular weight using gel-filtration chromatography. 170 μg/ml rHlPrx2s (WT, C51S, C172S, and DM) were loaded. The molecular weight of WT, C51S, C172S, and DM are 399 kDa, 388 kDa, 448 kDa and 377 kDa, respectively. The second peak of rHlPrx2 WT shown with the arrow indicates a 57 kDa molecular mass. WT, wild type; C51S, Cys51Ser; C172S, Cys172Ser; DM, double mutant, Cys51Ser/Cys172Ser.

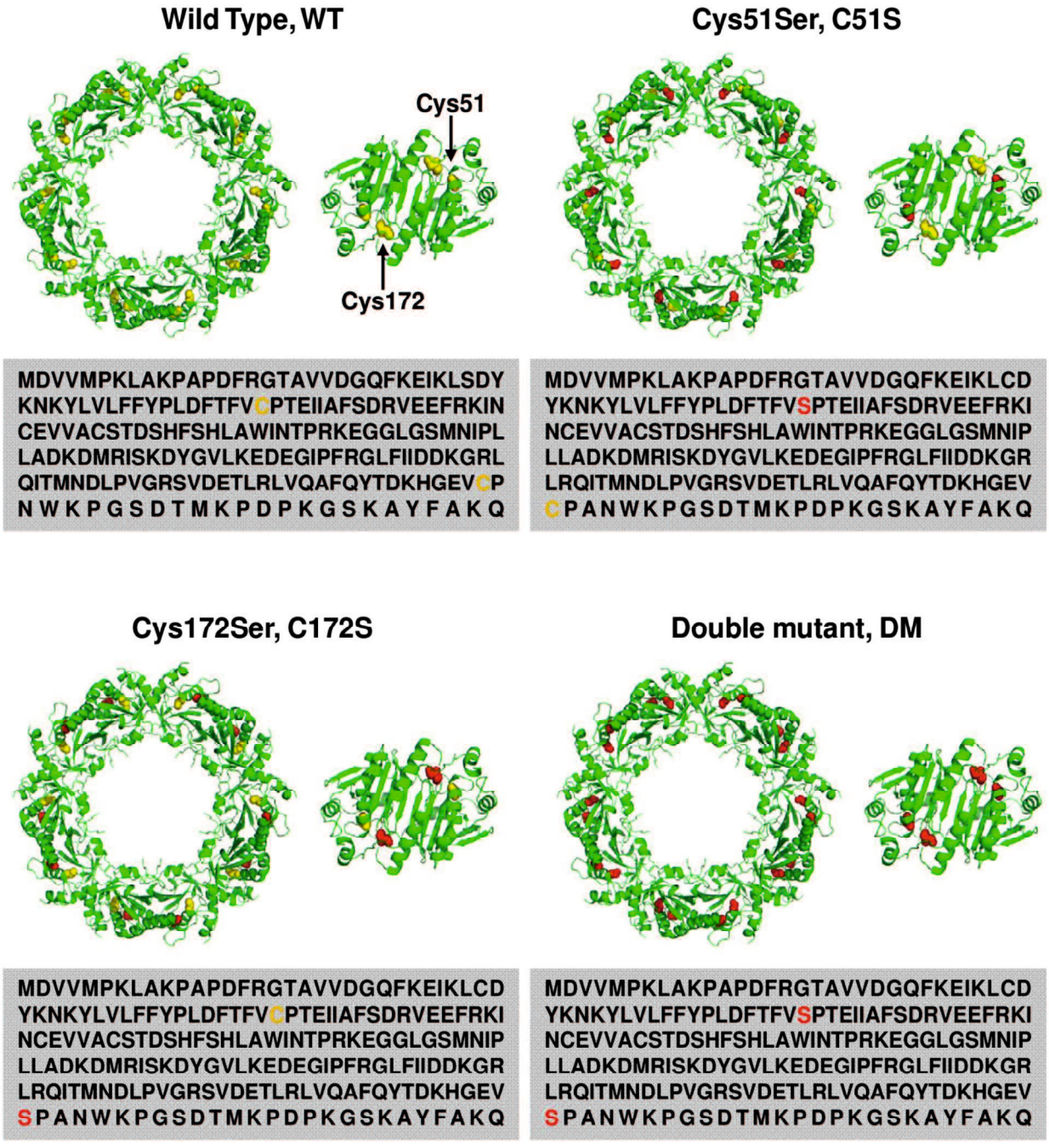
Wild Type, WT
Cys51
Cys172
MDVVMPKLAKPAPDFRGTAVVDGQFKEIKLSDY
KNKYLVLFFYPLDFTFVCPTEIIAFSDRVEEFRKIN
CEVVACSTDSHFSHLAWINTPRKEGGLGSMNIPL
LADKDMRISKDYGVLKEDEGIPFRGLFIIDDKGRL
QITMNDLPVGRSVDETLRLVQAFQYTDKHGEVCP
NWKPGSDTMKPDPKGSKAYFAKQ
Cys51Ser, C51S
MDVVMPKLAKPAPDFRGTAVVDGQFKEIKLCD
YKNKYLVLFFYPLDFTFVSPTEIIAFSDRVEEFRKI
NCEVVACSTDSHFSHLAWINTPRKEGGLGSMNIP
LLADKDMRISKDYGVLKEDEGIPFRGLFIIDDKGR
LRQITMNDLPVGRSVDETLRLVQAFQYTDKHGEV
CPANWKPGSDTMKPDPKGSKAYFAKQ
Cys172Ser, C172S
MDVVMPKLAKPAPDFRGTAVVDGQFKEIKLCD
YKNKYLVLFFYPLDFTFVCPTEIIAFSDRVEEFRKI
NCEVVACSTDSHFSHLAWINTPRKEGGLGSMNIP
LLADKDMRISKDYGVLKEDEGIPFRGLFIIDDKGR
LRQITMNDLPVGRSVDETLRLVQAFQYTDKHGEV
SPANWKPGSDTMKPDPKGSKAYFAKQ
Double mutant, DM
MDVVMPKLAKPAPDFRGTAVVDGQFKEIKLCD
YKNKYLVLFFYPLDFTFVSPTEIIAFSDRVEEFRKI
NCEVVACSTDSHFSHLAWINTPRKEGGLGSMNIP
LLADKDMRISKDYGVLKEDEGIPFRGLFIIDDKGR
LRQITMNDLPVGRSVDETLRLVQAFQYTDKHGEV
SPANWKPGSDTMKPDPKGSKAYFAKQ

Fig. 1.6.

Fig. 1.6. One or both of the conserved cysteines replaced by serines that generate three mutant HlPrx2s (C51S, C172S, and DM) are shown in three-dimensional structure {model based on template 3TKB [37]}, constructed using Phyre2 software [23]. Moreover, the decamer and dimer structures of HlPrx2 are prepared by using WinCoot software [39]. Yellow and red spheres indicate cysteines and serines, respectively. For each protein, the model on the left is the predicted decamer, while the model on the right is the predicted dimer model. The amino acid sequences are enclosed in gray boxes.

CHAPTER 2

2-Cys peroxiredoxin is required in successful blood-feeding, reproduction and antioxidant response in the hard tick *Haemaphysalis longicornis*

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2.1 Introduction

Prxs can be divided into two groups according to the presence of one or two highly conserved cysteines in organisms, 1-Cys or 2-Cys Prx [5]. 2-Cys Prxs are identified by two conserved cysteines, peroxidatic and resolving cysteines [18]. On the other hand, the 1-Cys Prxs have a conserved peroxidatic cysteine and do not contain a resolving cysteine [21]. Enzymes of the Prx family exhibit antioxidant activity that catalyzes the reduction of H_2O_2 into water (H_2O), with thioredoxin as an immediate hydrogen donor or donor thiol, respectively [21].

In some endoparasites, such as *Plasmodium* and *Fasciola* parasites, Prxs have been characterized as antigens or secreted proteins, suggesting that endoparasite Prxs may participate in interactions between the parasites and their hosts [40,41]. Therefore, to evaluate the efficacy of antigens for these endoparasites, basic biological and bio-histological analyses such as mRNA and protein expression profiles, and the localization of proteins in these parasites have been studied.

Ticks need blood meals to develop from one stage to the next and for reproduction. Blood-feeding and the digestion of blood provide nutrition and energy for molting, development, and the vitellogenesis of ticks [8]. Ticks feed on vertebrate blood that contains iron, such as heme, ferrous iron, and other pro-oxidants. Ticks also

concentrate host blood with iron; this concentration of the blood leads to high levels of iron in ticks. Host-derived iron may react with oxygen in the tick body, and then high levels of reactive oxygen species (ROS), including H_2O_2, may be generated [11]. *Haemaphysalis longicornis* 1-Cys Prx (HlPrx) has been reported previously [15]; however, there is still little knowledge about the biological functions of Prxs in ticks.

In Chapter 2, I analyzed mRNA and protein expression profiles and the localization of proteins in tick tissues of *H. longicornis* 2-Cys Prx (HlPrx2). Moreover, *HlPrx* and/or *HlPrx2* gene silencing was performed to clarify their functions in ticks using RNA interference. Finally, I demonstrated that the double knockdown of *HlPrx* and *HlPrx2* led to increased oxidative stress in ticks.

2.2 Materials and Methods

2.2.1 Ticks and animals

The parthenogenetic Okayama strain of *H. longicornis* has been maintained by blood-feeding on the ears of Japanese white rabbits (KBT Oriental Co. Ltd, Saga, Japan) in the Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University [42]. Rabbits were cared for in accordance with the guidelines

approved by the Animal Care and Use Committee of Kagoshima University (Approval no. VM13007) and maintained under regulated conditions throughout the experiments.

2.2.2 Total RNA extraction and cDNA synthesis

To extract total RNA, whole ticks were homogenized using an Automill (Tokken, Chiba, Japan), while dissected organs were disrupted using a pellet pestle motor (Sigma-Aldrich, St. Louis, MO, USA). The extracted RNA was purified using TRI® Reagent (Sigma-Aldrich), and then treated with an RQ1 RNase-Free DNase (Promega, Madison, WI, USA). cDNA synthesis was performed with ReverTra Ace-α-® (Toyobo, Osaka, Japan) following the manufacturer's protocol using 1 μg of total RNA.

2.2.3 Expression analysis of HlPrx2 *mRNA*

The expression analysis of the *HlPrx2* mRNA was performed with real-time PCR using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed to target *HlPrx2* and the internal control genes, as shown in Table 2.1. Standard curves were made from four-fold serial dilutions of the cDNA of adult ticks fed for three days. The PCR cycle profile was as follows: initial denaturation at 95°C for 10 min, 40 cycles of a denaturation step at 95°C for 15 s, and an annealing/extension

step at 60°C for 60 s. The data was analyzed with 7300 system SDS software (Applied Biosystems). At the first step of real-time PCR, *actin*, *tubulin*, *P0*, and *L23* genes were evaluated for standardization and *L23* was selected as the tick reference in the current study.

2.2.4 Production of an antiserum against recombinant HlPrx2

To prepare mouse anti-HlPrx2 sera, 100 μg of recombinant HlPrx2 (rHlPrx2; WT rHlPrx2 in Chapter 1 [14]) and completely mixed with Freund's complete adjuvant (Sigma-Aldrich) and intraperitoneally injected into ddY female mice (four weeks old, Kyudo, Saga, Japan). After two weeks, these mice were injected with 100 μg of rHlPrx2 with Freund's incomplete adjuvant (Sigma-Aldrich) twice at two-week intervals to boost the generation of antibodies against rHlPrx2. Their blood was collected two weeks after the third immunization to obtain specific antisera to rHlPrx2.

2.2.5 Protein extraction and Western blot analysis

Homogenized ticks were suspended in phosphate buffered saline (PBS) and ultrasonicated three times, two minutes each (Vibra-Cell^{TM}; Sonics and Materials, Newtown, CT, USA) on ice and finally centrifuged at 500×*g*. The supernatant was

resolved in a 12% SDS-PAGE gel under reducing conditions. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon®-P; Millipore, Danvers, MA, USA). The membrane was blocked overnight at 4°C with 3% skim milk in PBS (pH 7.4) (blocking solution); it was incubated with a 1:500 dilution of anti-rHlPrx2 mouse sera in blocking solution at 37°C for 1 h. For a loading control, tubulin was detected using antiserum against recombinant *H. longicornis* tubulin [43]. After washing five times in PBS containing 0.05% Tween 20 (PBS-T), the membrane was incubated with a 1:50,000 dilution of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Dako, Glostrup, Denmark) in blocking solution at 37°C for 1 h. After washing five times in PBS-T, bands were detected using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and viewed using FluorChem®FC2 software (Alpha Innotech, San Leandro, CA, USA). To accurately determine differences in the protein expression, band densitometry analysis was performed using Alpha View Software (Alpha Innotech). The band densitometry analysis results shown in this study represent the mean of three trials of Western blot analysis.

2.2.6 Immunostaining

To confirm the localization of HlPrx2 in tick tissues, indirect immunofluorescent antibody tests (IFAT) were performed. Engorged ticks were dissected under a stereomicroscope (SZX10, Olympus, Tokyo, Japan) for collecting tick internal organs. Dissected organs were fixed in a 4% paraformaldehyde phosphate buffer solution (pH 7.4) at 4°C overnight. After washing with a sucrose series, organs were embedded in a Tissue-Tec® O.C.T Compound (Sakura Finetek, Torrance, CA, USA). Frozen sections from each internal organ were cut to a thickness of 10 μm using Kawamoto's film method (Leica Microsystems, Tokyo, Japan) and a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany). The films were blocked with 5 % skim milk in PBS (pH 7.4) (blocking solution) at 37°C for 1 hr, and then incubated with 1:50 dilution in a blocking solution of anti-HlPrx2 mouse serum at 37°C for 1 hr. For the negative control, normal mouse serum (1:50) was used. After washing three times in PBS, the slides were incubated at 37°C for 1 h with Alexa Fluor® 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) with 1:1,000 dilution in blocking solution. After removing the antibody by washing three times with PBS, the films placed on a slide glass and mounted with DAPI (VECTASHIELD®; Vector Laboratories, Burlingame, CA, USA), and then covered with a cover glass. The images were recorded using a

confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany). Hemocytes were prepared using a slide glass instead of film as described previously by Galay *et al.* (2016) [44]. Briefly, hemolymph collected from ticks by amputating the legs was smeared directly on glass slides and air-dried. After drying, hemocyte smears were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. Thereafter, the same method of the internal organs' IFAT was performed.

2.2.7 RNA interference (RNAi)

Two separate PCR reactions of approximately 469 bp with a single T7 promoter were generated using the following primer sets: a T7-attached gene-specific forward primer (HlPrx2 T7-F) and gene-specific reverse primer (HlPrx2 RNAi-R) and a T7-attached gene-specific reverse primer (HlPrx2 T7-R) and gene-specific forward primer (HlPrx2 RNAi-F) (Table 2.1). After gel purification of PCR products using a GENECLEAN® II KIT (MP Biomedicals, Irvine, CA, USA), double-stranded RNA of *H. longicornis* 2-Cys peroxiredoxin (*dsHlPrx2*) was synthesized using the T7 RiboMAX™ Express RNAi System (Promega) with two separate single-promoter templates in accordance with the manufacturer's protocol. Double-stranded RNA of *H. longicornis* 1-Cys peroxiredoxin (*dsHlPrx*) was also synthesized using *HlPrx*

gene-specific primers (Table 2.1). The firefly *luciferase* (*Luc*) gene [45] was used for a control (*dsLuc* group). One microgram of *dsLuc*, *dsHlPrx*, *dsHlPrx2*, or *dsDouble* (*dsHlPrx* and *dsHlPrx2* were mixed at 1 μg concentration each) was injected into 30 unfed adult female ticks in each experimental group through the fourth coxae into the hemocoel. Injected ticks were observed for one day at 25°C and subsequently transferred to rabbits with each group infesting separate ears. Three to four days after attachment, three ticks were manually detached to confirm gene silencing using RT-PCR. The remaining ticks were allowed to feed until engorgement, and the total number of engorged ticks, the engorged body weight, the oviposition, and the hatching rate were assessed.

2.2.8 Detection of hydrogen peroxide (H_2O_2) in adult female ticks during blood-feeding

The H_2O_2 concentration in ticks was measured using the ferrous oxidation of xylenol orange assay [27]. Briefly, homogenized unfed and partially fed ticks were suspended in 200 μl of Milli-Q H_2O, while homogenized engorged ticks were suspended in 900 μl of Milli-Q H_2O. The samples were centrifuged at 500×*g*, and the supernatant was collected. The supernatant from the engorged ticks was further diluted 10 times in Milli-Q H_2O. Ninety microliters of the supernatant from unfed and partially

fed ticks or the diluted supernatant from engorged ticks was used for a sample solution as described later. The assay reagent consisted of 125 μM xylenol orange, 250 μM ammonium iron (II) sulfate, 100 mM sorbitol, and 25 mM sulfuric acid. One hundred microliters of the sample solutions was added to a 1-ml assay reagent. The mixture was vortexed immediately, left at room temperature for 30 min, and measured at 560 nm using a spectrophotometer (Ultrospec 2100 pro; GE Healthcare, Pittsburgh, PA, USA). Finally, the ratio of the H_2O_2 concentration (μM) to the corresponding tick's body weight (mg) was calculated.

2.2.9 Statistical analysis

All experiments were conducted in two or three separate trials. Data except for hatching rate were statistically analyzed using Welch's *t*-test. Hatching rate analysis was done using the chi-square test. $P< 0.05$ and $P< 0.01$ were considered to be statistically significant vs control.

2.3 Results

2.3.1 Transcription profiles of HlPrx2

The mRNA levels of *HlPrx2* in whole female ticks and internal organs during blood-feeding and in different developmental stages (egg, larval, nymphal and adult

stages) were investigated using real-time PCR. *HlPrx2* mRNA was upregulated in whole female ticks, developmental stages, and all internal organs (salivary glands, midgut, ovaries, fat bodies, synganglia and hemocytes) during blood-feeding (Fig. 2.1). In the whole body, mRNA was upregulated at day 1 and, in spite of higher expression levels as compared to those of unfed stage, gradually decreased thereafter (Fig. 2.1A). Upregulation of the mRNA level was also observed in the developmental stages from unfed to engorgement, and the immature stages, including the egg, showed higher expression levels as compared to the adult stage (Fig. 2.1B). In the midgut, mRNA was drastically increased at day 1 and decreased thereafter (Fig. 2.1C, Midgut). In the ovary, the expression level gradually increased until day 2, drastically increased at day 3, and decreased thereafter (Fig. 2.1C, Ovary). In the hemocytes, the expression level increased at day 1 and remained almost the same at day 2, drastically increased from day 3 to day 4, and then slightly decreased at the engorged state (Fig. 2.1C, Hemocytes). The expression levels of *HlPrx2* gene in ovaries and hemocytes were higher than those of other internal organs. In other tissues, such as the salivary glands, fat bodies and synganglia, mRNA was upregulated from unfed to day 1 and remained at a high level until engorgement (Fig. 2.1C). These results indicate that the mRNA of *HlPrx2* gene was upregulated in ticks by blood-feeding. The high levels of mRNA expression in the

ovaries and hemocytes suggest that *HlPrx2* gene may be related to the reproduction and immune response of ticks.

2.3.2 Protein expression profiles of HlPrx2

The protein expression levels of HlPrx2 in whole female ticks and internal organs during blood-feeding and in different developmental stages were investigated by Western blot analysis using HlPrx2-specific antisera. The predicted molecular mass of HlPrx2 protein is approximately 22 kDa, and the theoretical isoelectric point (pI) is 6.8; the signal peptide and glycoxylation sites were not found *in silico* analysis [14]. However, the calculated molecular mass in Western blot analysis was approximately 26 kDa. The mobility of native HlPrx2 protein in Western blot analysis decreased because the pI= 6.8 is slightly low. HlPrx2 expression was generally upregulated during blood-feeding in the whole body, the developmental stages, and the midgut (Fig. 2.2). In the whole body and the developmental stages, the HlPrx2 expression level was upregulated from unfed to engorgement (Fig. 2.2A, B). Notably, in the developmental stages, protein expression levels seemed to be almost the same, although immature stages, including the egg, showed higher mRNA expression levels as compared to those of the adult stage (Figs. 2.1B and 2.2B). In Fig. 2.2B, other bands under HlPrx2 band at

the engorged state of all stages can be seen. These bands are considered to be non-specific bands derived from the blood of the host rabbit (Fig. 2.3). These non-specific bands in the rabbit blood are cross-reacted with rHlPrx2 antisera; thus, these are considered to be a candidate for the cross-reacted protein related to 2-Cys peroxiredoxin of rabbit (Figs. 2.3 and 2.4). Moreover, in the knockdown ticks, the band of HlPrx2 protein was decreased as compared to control group (Fig. 2.4). Therefore, the anti-rHlPrx2 mouse serum used in this study was considered as specifically working.

In the midgut, although the protein expression level was very low in unfed stage, it significantly increased from unfed to partially fed states and significantly decreased to engorged state (Fig. 2.2C, Midgut). In the salivary glands, ovaries, and fat bodies, the protein expression levels of HlPrx2 were constant during blood-feeding (Fig. 2.2C). These results indicate that the protein expression of HlPrx2 is strongly upregulated in the whole body, especially the midgut, by blood-feeding; however, the expression levels of HlPrx2 protein in the other tissues, such as the salivary glands, ovaries and fat bodies, were constant during blood-feeding. The drastic increase of HlPrx2 protein expression in the midgut during blood-feeding suggests that HlPrx2 protein could be related to the antioxidant response in this tissue because ticks' midgut may be exposed to high concentrations of ROS during blood-feeding.

2.3.3 Localization of HlPrx2 in the salivary glands, midgut, ovaries and hemocytes from engorged adult female ticks

Western blot analysis showed the high expression of HlPrx2 protein in the whole body and internal organs. To determine localization in the cells of internal organs, IFAT was performed using some internal organs of engorged female ticks. In the salivary glands, positive fluorescence was detected in the cell membrane of the acinar cells (SA) and granular cells (SGG) and in the basal lamina of the salivary duct (SD) (Fig. 2.5 Salivary glands). In the midgut, positive fluorescence was detected in the basal lamina of the digestive cells (Fig. 2.5 Midgut). In the ovary, positive fluorescence was detected in the cell membrane of the oocytes and basal lamina of the oviduct (Fig. 2.5, Ovary), whereas in the hemocytes, positive fluorescence was detected in the cell membrane (Fig. 2.5, Hemocytes). These results demonstrate that the HlPrx2 protein was associated to the tissue membranes.

2.3.4 Effects of HlPrx *and/or* HlPrx2 *gene silencing on the blood-feeding and reproduction of female ticks*

To clarify the functions of the *HlPrx* and *HlPrx2* genes, gene silencing using the RNAi method was conducted. Gene silencing was confirmed by semi-quantitative RT-PCR and Western blot analysis (Fig. 2.6A). The knockdown of *HlPrx* and/or

HlPrx2 caused significant differences in the ticks' engorged body weight, egg weight and hatching rate (Table 2.2). The ticks' engorged body weight and egg weight significantly decreased (Fig. 2.6B, C). Notably, double knockdowns, wherein both *HlPrx* and *HlPrx2* were silenced, showed almost the same results as *HlPrx* silencing. *HlPrx2* silencing resulted in a greater decrease in engorged body weight and egg weight when compared to those of *dsDouble* and *dsHlPrx* silencing. However, the hatching rates of *dsHlPrx* and *dsHlPrx2* groups were similar (Table 2.2). These results suggest that the knockdown of *HlPrx* and/or *HlPrx2* genes significantly decreased engorged body weight, egg weight and hatching rate as compared to the *dsLuc* group.

2.3.5 Increasing the concentration of H_2O_2 by the double knockdown of HlPrx *genes before and after blood-feeding*

To elucidate the observed effects of *HlPrx* and/or *HlPrx2* gene silencing during blood-feeding, H_2O_2 concentrations were measured in female ticks. Gene silencing was also confirmed by semi-quantitative RT-PCR (data not shown). In the unfed and engorged states, the *dsDouble* group showed significantly higher concentrations of H_2O_2 as compared to the *dsLuc* group (Fig. 2.7, Unfed and Engorged). *HlPrx* or *HlPrx2* gene-silenced groups only showed slightly higher concentrations of H_2O_2 as compared to the *dsLuc*-injected group in the unfed state (Fig. 2.7, Unfed). On

the other hand, in the engorged state, the *HlPrx2* gene-silenced group showed a slightly higher concentration of H_2O_2, whereas the *HlPrx* gene-silenced group showed a slightly lower concentration of H_2O_2 as compared to the *dsLuc*-injected group (Fig. 2.5, Engorged). These results demonstrate that the knockdown of both *HlPrx* and *HlPrx2* genes leads to a high concentration of H_2O_2 in ticks before and after blood-feeding.

2.4 Discussion

To protect against the toxicity of H_2O_2, aerobic organisms have evolved antioxidant enzymes, such as catalases, peroxidases and Prxs [17]. Moreover, ticks lack heme synthesis and catabolism pathways because they are unable to prepare σ-aminolevulinic acid, a heme precursor, even at genomic levels [46,47]. Therefore, they rely on heme from their host and store heme in hemosomes of the midgut without digestion [11,48]. These facts suggest that ticks might face difficulties in producing proteins that contain heme, such as catalase and peroxidase, which are both H_2O_2-scavenging enzymes [16]. Moreover, ticks must acquire nutrients from the host blood meal and metabolize these nutrients via catabolism and anabolism [49]. *Plasmodium* parasites also take in nourishment from host blood and are likely to utilize members of the Prx family as the principal enzymes for reducing peroxides, including

H_2O_2, because they lack catalase and peroxidase [40]. Therefore, Prxs might be similarly essential to the regulation of the H_2O_2 concentration for ticks.

In this study, I found that *HlPrx2* mRNA expression was upregulated by blood-feeding (Fig. 2.1). On the other hand, HlPrx2 protein expression was almost stable during blood-feeding, except in the midgut (Fig. 2.2). In the whole body, although mRNA expression was upregulated by blood-feeding when compared to the unfed state (Fig. 2.1A, B), protein expression seemed to be constant in all states of blood-feeding except for the engorged state, where it showed an increased expression level (Fig. 2.2A, B). *Fasciola* parasites secrete Prxs into their hosts to regulate their environment for survival in the host body [41]. My results suggest that ticks may also secrete HlPrx2 protein into hosts as *Fasciola* parasites do, and the inconsistency of protein expression in comparison with mRNA expression may be due to the release of HlPrx2 proteins. Protein expression in the whole body increased according to the state of engorgement (Fig. 2.2C). This drastic change seems to be related to body size, because tick body weight notably increases from day 4 to engorgement, and the increase in body weight is about 100-fold compared to unfed ticks [50]. It may be also in response to the very large amounts of blood ingested during the rapid engorgement stage, which may expose ticks to higher levels of ROS. Although other developmental

stages (larval and nymphal stages) also showed similar tendencies in HlPrx2 protein expression (Fig. 2.2B), *HlPrx2* mRNA expression in larval and nymphal stages was higher than in the adult stage (Fig. 2.1B). This result suggests that HlPrx2 protein might have an important role in the molting and survival of immature stages during blood-feeding and after engorgement.

In the internal organs, especially the midgut, HlPrx2 mRNA and protein expression was consistent (Figs. 2.1C and 2.2C). The mRNA and protein expression levels were negligible in the unfed midgut (Figs. 2.1C and 2.2C). Blood-feeding acts as a trigger, inducing the upregulation of HlPrx2 mRNA and protein expression. In IFAT examination of the midgut, HlPrx2-specific fluorescence was detected in the basal lamina (Fig. 2.5). There have been some reports that the multimer of 2-Cys Prxs are associated with membranes, such as red blood cells [32,33]. My results, along with those of previous reports, suggest that HlPrx2 protects digestive cells against membrane oxidation and suppresses unnecessary diffusion of H_2O_2 from midgut lumen and digestive cells. On the other hand, the midgut, ovaries, and fat bodies are known to produce vitellogenin, a phospholipoglycoprotein and a member of the lipid transfer protein superfamily that is the precursor of major yolk proteins in all oviparous organisms [22,51]. During blood-feeding, the expression patterns of tick vitellogenin

are upregulated from day 3 to engorgement; the highest expression of mRNA and protein is observed upon engorgement [22]. Vitellogenin also has a positive effect on oxidative stress resistance in bees and is a preferred target of oxidative carbonylation in comparison with hemolymph proteins in adult bees [52]. In addition, in the ovaries and fat bodies, HlPrx2 mRNA expression was upregulated from around day 3, and protein expression was present stably (Figs. 2.1C, 2.2C). This indicates HlPrx2 protein could protect vitellogenin and the organs synthesizing vitellogenin, such as the midgut, the fat bodies, and the ovaries, from the oxidative stress that occurs during blood-feeding. In the salivary glands, HlPrx2 mRNA expression was upregulated during blood-feeding (Fig. 2.1C), while protein expression was upregulated from unfed to partially fed states (Fig. 2.2C). Moreover, in the case of *HlPrx*, the other known peroxiredoxin of *H. longicornis*, mRNA is upregulated in the salivary glands, and HlPrx protein is also highly expressed in the salivary glands [15]. Anti-HlPrx antibodies were detected in the host serum after several repeated tick infestations [15], suggesting that the HlPrx was released from ticks into the host eliciting to produce anti-HlPrx on immune response. In *Fasciola* parasites, infective parasites excyst from a dormant state following ingestion and penetrate the intestinal wall before migrating to the liver; in this nutrient- and oxygen-rich environment, the parasites undergo rapid growth and development, and

energy is supplied by aerobic respiration [53]. This developmental situation of *Fasciola* parasites is similar to the development of ticks during blood-feeding. In addition, *Fasciola* parasites secrete Prxs into their host to regulate their environment for survival in the host body [41]. These findings strongly suggest that tick Prxs may be also secreted into the host's body in a way similar to that of *Fasciola* parasites.

In hemocytes, *HlPrx2* mRNA expression was upregulated during blood-feeding, and a specific fluorescence was also detected in cell membranes of the hemocytes (Figs. 2.1C and 2.3). In *Ixodes ricinus*, two *Prx* homologous genes (Accession nos. AY333958 and AY333959) were strongly induced in the hemolymph after *Borrelia burgdorferi* infection [54]. Furthermore, *Borrelia* exploits the salivary Salp25D, a protein homologous to Prx in *Ixodes scapularis*, for protection against reactive oxygen intermediates generated by the mammalian neutrophils at the vector-host interface [55]. These results indicate that HlPrx2 might be related to immune response, e.g. digestion of foreign bodies such as *Borrelia* and *Babesia* parasites in hemocytes. In the mosquito *Anopheles stephensi*, 2-Cys Prx (AsPrx-4783) expression induced in the midgut was two to seven times higher in malaria parasite-infected insects than in uninfected mosquitoes [56]. Two *Prx* genes of *I. ricinus*

were also induced in the midgut by *B. burgdorferi* infection [54]. HlPrx2 in the midgut may also be involved in immune response; however, further investigation is necessary.

Knockdown experiments of *HlPrx* and/or *HlPrx2* genes were also performed and the H_2O_2 concentrations after the knockdown of these genes were measured (Table 2.2 and Figs. 2.6 and 2.7). The H_2O_2 concentration of no injection group in the unfed state was about 3 μM (data not shown). In comparison to insects, the H_2O_2 concentration in normal state silkworms was also reported at about 3 μM [57]. These observations may suggest that at a normal state, tick and silkworm H_2O_2 concentrations might have the same range. Therefore, this detection method of H_2O_2 concentration was considered as functionally acceptable. In the unfed and engorged states, the *dsDouble* group showed significantly higher concentrations of H_2O_2 as compared to the *dsLuc* group. These results suggest a synergistic regulation of H_2O_2 by both HlPrx and HlPrx2. In addition, phenotype evaluation after the knockdown of *HlPrx* and/or *HlPrx2* demonstrated significant decreases in the engorged body weight, egg weight and hatching rate, particularly after *HlPrx2* knockdown. The antioxidant activities evaluated by a metal-catalyzed oxidation system seemed to be almost the same comparing 1-Cys Prx and 2-Cys Prx from the bumblebee *Bombus ignites* [58]. The donors of 1-Cys Prxs and 2-Cys Prxs are thiol and thioredoxin, respectively [21]. Thioredoxin is a major

disulfide reductase system which can provide electrons to a large range of enzymes and is found to be critical for DNA synthesis and defense against oxidative stress [19]. Taken together, the 1-Cys and 2-Cys Prxs seemed to have almost the same antioxidant activity and their donors are different. These data indicate that 2-Cys Prx is more related to cell metabolism through the antioxidant activity because of its utilization of thioredoxin as donor, thus, HlPrx2 knockdown in the ticks led to the significant decrease in engorged body weight, egg weight, and hatching rate in spite of no significant effect to H_2O_2 concentrations in the knockdowned ticks. Therefore, these findings suggest that HlPrxs play an important role in successful blood-feeding and reproduction, with HlPrx2 being apparently more significant. Additionally, the observed effects in the *dsDouble* group were milder than those of the *dsHlPrx2* group. H_2O_2 can activate signaling pathways to stimulate cell proliferation, differentiation and migration in multicellular organisms [59]. These results suggest that the *dsDouble* group, but not the *dsHlPrx2* group, was exposed to a high concentration of H_2O_2, leading to higher engorged body weight, egg weight and hatching rate as compared to the *dsHlPrx2* group.

In endoparasites, Prx has been shown to be the most important detoxifying enzyme for their survival [40,41] making it a candidate for use in vaccine development

and a therapeutic target in treating endoparasitic infectious diseases [60-63]. In ticks, there have been a few reports on Prxs. However, anti-HlPrx antibodies were detected in the host serum after several repeated tick infestations [15], suggesting that HlPrx was released from ticks into the host and the amount of released HlPrx protein was quite small since several infestations of ticks were done to detect the anti-HlPrx antibody. In addition, ticks ingest and concentrate large amounts of the host-derived blood [50], it can be suggested that the anti-HlPrx antibody would be concentrated in tick's body. In the present study, anti-HlPrx2 antibody cross-reacted with some rabbit Prx from normal rabbit blood (Fig. 2.3), giving some concerns whether HlPrx2 can be a good vaccine candidate. However, the knockdown of *HlPrx* and/or *HlPrx2* genes significantly affected tick blood-feeding, reproduction and antioxidant activity (Table 2.2 and Figs. 2.6 and 2.7). Therefore, tick Prx can be a potential target for tick control and provide further understanding of the oxidative stress coping mechanisms in ticks during blood-feeding.

In summary, mRNA and protein expression profiles of HlPrx2 and the localization of this protein in tick tissues were investigated. Real-time PCR showed that *HlPrx2* gene expression in whole bodies and internal organs was significantly upregulated during blood-feeding. However, protein expression was constant

throughout blood-feeding. Moreover, a knockdown experiment of *HlPrx2* was performed using RNAi to evaluate its function in ticks. The knockdown of the *HlPrx2* gene caused significant differences in body weight, egg weight and hatching rate in engorged ticks as compared to those of the control group. Finally, the detection of H_2O_2 after the double knockdown of *HlPrxs* in ticks showed that H_2O_2 concentration increased before and after blood-feeding. Therefore, HlPrx2 can be considered important for successful blood-feeding and reproduction through the regulation of H_2O_2 concentrations in ticks during blood-feeding.

Tables and Figures in CHAPTER 2

Table 2.1. Gene-specific primers used in Chapter 2

Primers	Sequence (5' → 3')
HlPrx2 RT-F	TATGCCTAAGCTGGCGAAGC
HlPrx2 RT-R	CAGGCGAGGTGAGAGAAGTG
HlPrx1 RT-F	ATGAGGTCCTCCGTGCTACT
HlPrx1 RT-R	TGCCACACCGTCATAAGCAT
HlPrx2 real time-F	GTGTGCCCTGCTAACTGGAA
HlPrx2 real time-R	ATGAGACACACGGGGCTTTG
HlPrx2 T7-F	TAATACGACTCACTATAGGGATCAAGCTGTCCGATTACAAGAAC
HlPrx2 T7-R	TAATACGACTCACTATAGGTTCCAGTTAGCAGGGCACACT
HlPrx2 RNAi-F	GATCAAGCTGTCCGATTACAAGAAC
HlPrx2 RNAi-R	TTCCAGTTAGCAGGGCACACT
HlPrx1 T7-F	TAATACGACTCACTATAGGCACCACGGTTGGATCAAGGA
HlPrx1 T7-R	TAATACGACTCACTATAGGTTTGCAGAGCCACCACTCAA
HlPrx1 RNAi-F	CACCACGGTTGGATCAAGGA
HlPrx1 RNAi-R	TTTGCAGAGCCACCACTCAA
Actin RT-F	CCAACAGGGAGAAGATGACG
Actin RT-R	ACAGGTCCTTACGGATGTCC
Actin real time-F	ATCCTGCGTCTCGACTTGG
Actin real time-R	GCCGTGGTGGTGAAAGAGTAG
Tubulin real time-F	TTCAGGGGCCGTATGAGTAT
Tubulin real time-R	TGTTGCAGACATCTTGAGGC
P0 real time-F	CTCCATTGTCAACGGTCTCA
P0 real time-R	TCAGCCTCCTTGAAGGTGAT
L23 real time-F	CACACTCGTGTTCATCGTCC
L23 real time-R	ATGAGTGTGTTCACGTTGGC

Underlines denote T7 RNA polymerase promoter sequences

Table 2.2. Effects of *HlPrx2* and *HlPrx1* genes silencing in ticks

Knockdown groups	Infest No.	Drop No.	Engorged body weight (mg)	Egg weight (mg)	Ratio of egg weight / engorged body weight	Hatching rate (%)
dsLuc	30	25	263.7 ± 58.9	162.1 ± 38.9	0.61 ± 0.03	100
dsDouble	30	15	218.8 ± 66.2 *	130.3 ± 46.7 *	0.59 ± 0.08	87
dsHlPrx2	30	22	204.4 ± 56.3 **	116.7 ± 45.3 **	0.55 ± 0.09 **	77 ***
dsHlPrx1	30	28	210.0 ± 59.8 **	124.8 ± 43.4 **	0.58 ± 0.06 *	78 ***

* shows the significant difference as compared with the *dsLuc* group by Welch's *t*-test ($P < 0.05$).

** shows the significant difference as compared with the *dsLuc* group by Welch's *t*-test ($P < 0.01$).

*** shows the significant difference as compared with the *dsLuc* group by chi-square test ($P < 0.05$).

Table 2.3. Candidates for non-specific bands from Japanese white rabbit blood in western blot analysis

Candidate protein*	Predicted molecular weight (kDa)**	Isoelectric point**	Calculated molecular weight (kDa)***	Identity with HlPrx2*	Accession no.*
Peroxiredoxin 1	22	8.2	23 (Non-specific 1)	77%	XP_002715184
Thioredoxin-dependent peroxide reductase	28	8.3	24 (Non-specific 2)	67%	XP_002718732
HlPrx2	22	6.8	26	-	LC049075

* The deduced amino acid translation of the *HlPrx2* gene sequence was determined using GENETYX version 7.0 software (GENETYX, Tokyo, Japan). A BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search homologous genes from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

** The theoretical molecular mass and isoelectric points were calculated using a ProtParam tool (http://web.expasy.org/protparam/).

*** The calculated molecular weight was assessed using the FluorChem®FC2 software (Alpha Innotech Hessisch Oldendorf, Germany) band analysis tool.

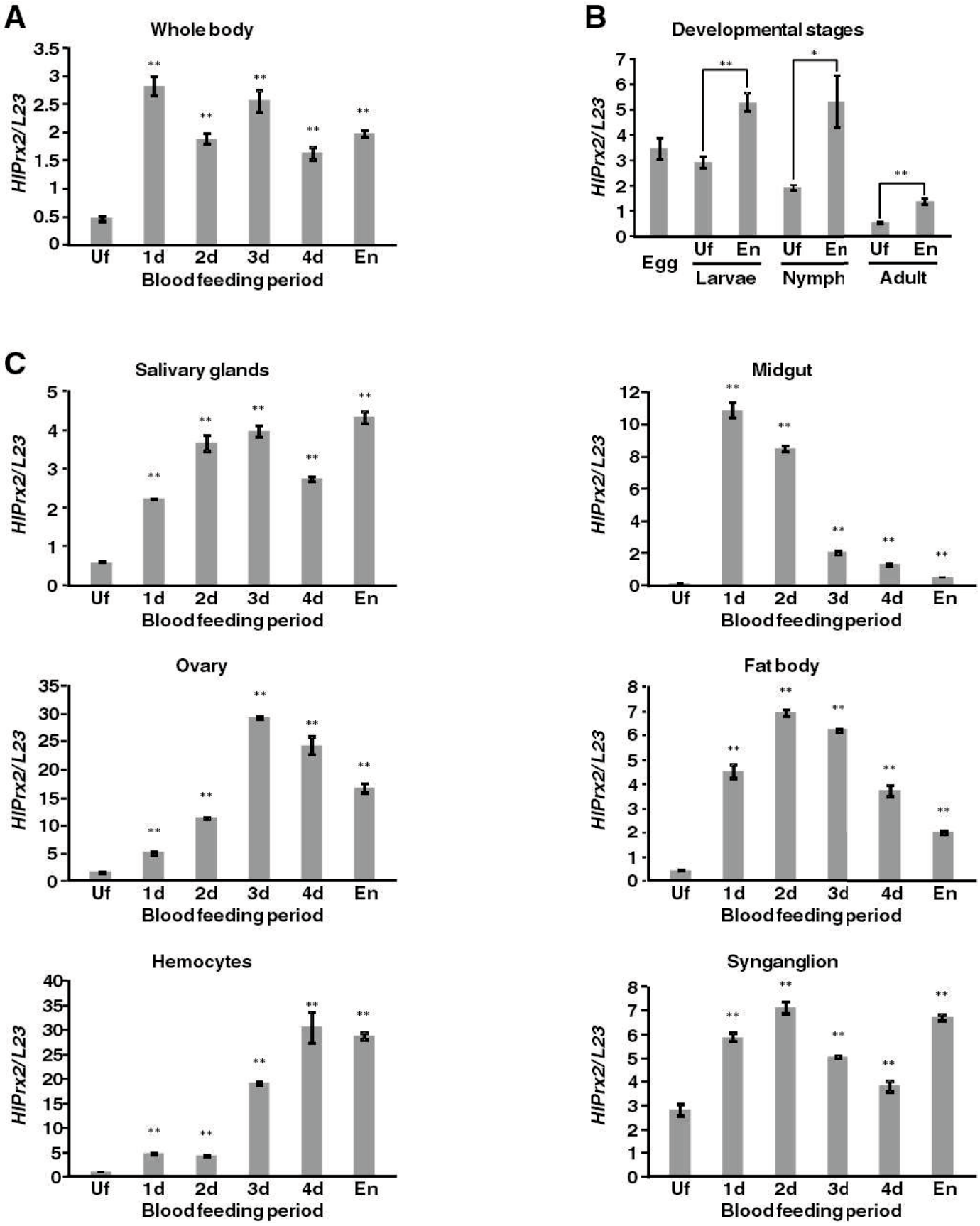
A
Whole body
HlPrx2/L23
Uf
1d
2d
3d
4d
En
Blood feeding period
B
Developmental stages
HlPrx2/L23
Egg
Uf
En
Larvae
Nymph
Adult
C
Salivary glands
Midgut
Ovary
Fat body
Hemocytes
Synganglion

Fig. 2.1.

Fig. 2.1. (A) Transcription profiles of *HlPx2* in whole ticks during blood-feeding analyzed by real-time PCR (Uf, unfed females; 1d-4d, adults partially fed for 1–4 days). **(B)** Transcription profiles of *HlPrx2* in unfed and engorged tick developmental stages. **(C)** Transcription profiles of *HlPrx2* in the internal organs: salivary glands, midgut, ovary, fat body, hemocytes, synganglion). *L23* was used as the internal control. Data are presented as the mean ± standard deviation (SD). $^{*}P < 0.05$; $^{**}P < 0.01$, significant differences *vs dsLuc* by Welch's *t*-test. *Abbreviations*: Uf, unfed ticks; En, engorged ticks.

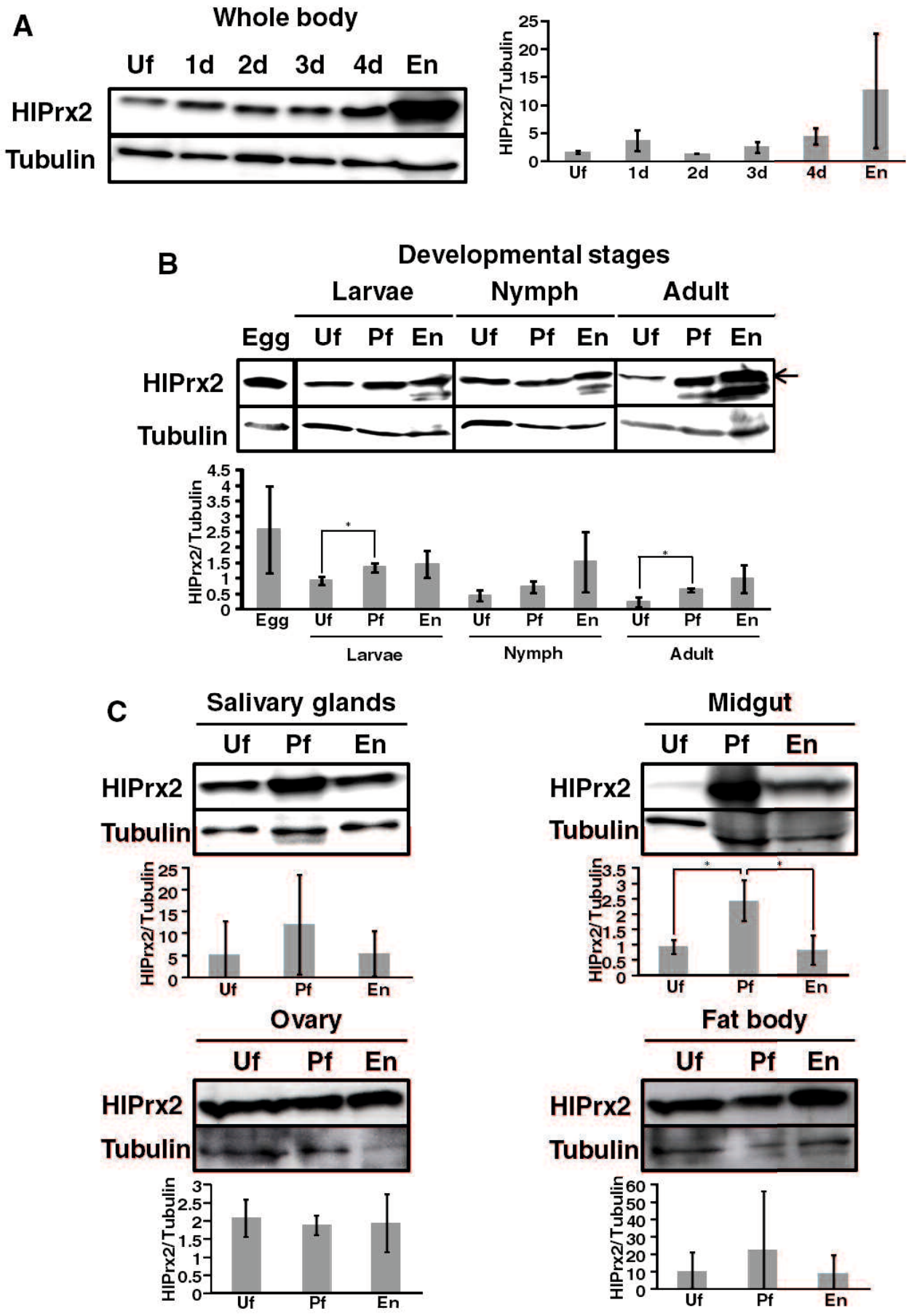
A
Whole body
Uf 1d 2d 3d 4d En
HIPrx2
Tubulin
HIPrx2/Tubulin
B
Developmental stages
Larvae Nymph Adult
Egg Uf Pf En Uf Pf En Uf Pf En
HIPrx2
Tubulin
HIPrx2/Tubulin
Larvae
Nymph
Adult
C
Salivary glands
Midgut
Ovary
Fat body
Uf Pf En
HIPrx2
Tubulin
HIPrx2/Tubulin

Fig. 2.2.

Fig. 2.2. (A) Protein expression profiles of *HlPx2* in whole ticks during blood-feeding as analyzed by Western blot analysis. **(B)** Protein expression profiles of *HlPrx2* in developmental stages. *Arrow* indicates native HlPrx2 protein as distinguished from the non-specific bands below. **(C)** Protein expression profiles of *HlPrx2* in the internal organs (salivary glands, midgut, ovary, fat body, hemocytes, and synganglion). For a loading control, tubulin was detected. The bars show the results of band densitometry analysis for HlPrx2. The relative expression was calculated based on tubulin. Data are presented as the mean ± standard deviation (SD). $^{*}P < 0.05$, significant differences by Welch's *t*-test. *Abbreviations*: Uf, unfed adults; Pf, partially fed adults at day 3; En, engorged adults.

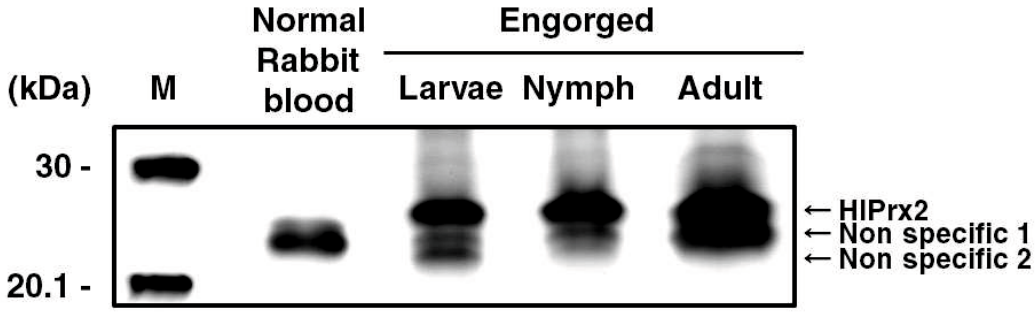

Fig. 2.3. Comparison of normal rabbit blood and engorged-state samples in developmental stages using Western blot analysis. The top arrow indicates native HlPrx2 protein, the middle arrow indicates non-specific band 1, and the bottom arrow indicates non-specific band 2. M, marker.

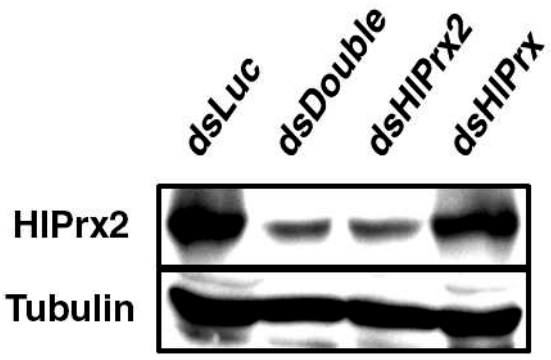

Fig. 2.4. Confirmation of antibody's specificity in *HlPrx* and/or *HlPrx2* genes-silencing partially fed adult ticks. Each tick's total protein was extracted from 3 ticks pooled. The left column indicates the specific anti-serum. For loading control, tubulin was detected.

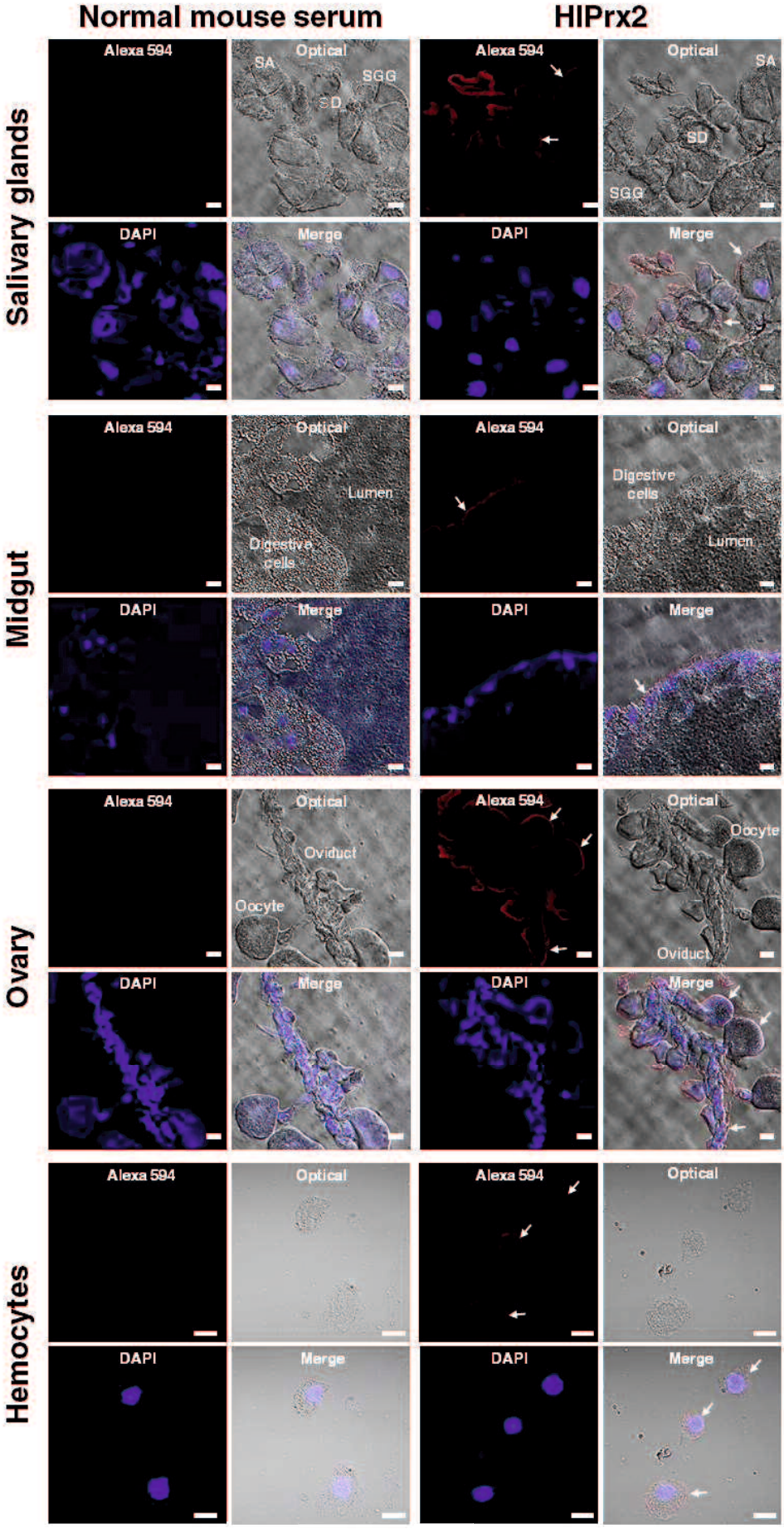
Normal mouse serum
HIPrx2
Salivary glands
Midgut
Ovary
Hemocytes
Alexa 594
Optical
DAPI
Merge
SA
SGG
SD
Lumen
Digestive cells
Oviduct
Oocyte

Fig. 2.5.

Fig. 2.5. Localization of HlPrx2 protein in the salivary glands, midgut, ovary and hemocytes from engorged adult ticks using IFAT under a confocal laser scanning microscope. Anti-HlPrx2 mouse serum was used as a primary antibody. Anti-mouse IgG conjugated with Alexa Fluor 594 was used as a secondary antibody and nuclei were visualized using DAPI. Normal mouse serum was used for a control. *Arrows* indicate the specific fluorescence. *Abbreviations*: SA, salivary gland acinar cells; SGG, salivary gland granular cells; SD, salivary duct. Scale-bars: 20 μm (salivary glands, midgut and ovary) and 10 μm (hemocytes).

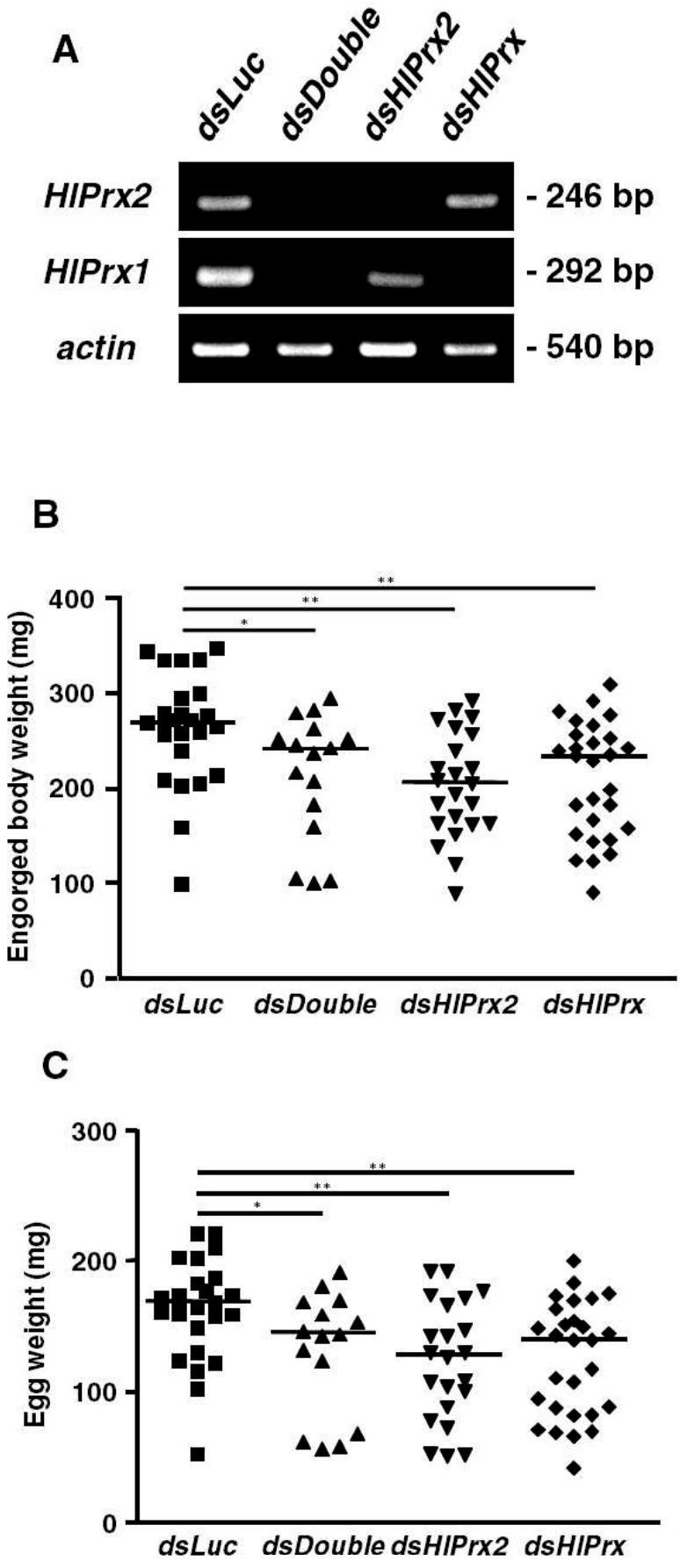
A
dsLuc
dsDouble
dsHlPrx2
dsHlPrx
HlPrx2
HlPrx1
actin
- 246 bp
- 292 bp
- 540 bp
B
Engorged body weight (mg)
400
300
200
100
0
**
**
*
dsLuc
dsDouble
dsHlPrx2
dsHlPrx
C
Egg weight (mg)
300
200
100
0
**
**
*
dsLuc
dsDouble
dsHlPrx2
dsHlPrx

Fig. 2.6.

Fig. 2.6. (A) Knockdown confirmation of *HlPrx* and/or *HlPrx2* genes in partially fed adult ticks. Each tick total RNA was extracted from 3 ticks pooled. The left column indicates the detection primer set; *actin* was used as a control. The right column indicates the size of the PCR products. **(B)** Column graph for engorged body weight in the knockdown experiment. **(C)** Column graph for egg weight after finishing oviposition by engorged adult ticks in the knockdown experiment. Horizontal lines indicate the median values. *Abbreviations*: *dsLuc*, double-stranded *Luciferase*-injected group; *dsHlPrx2*, double-stranded *HlPrx2*-injected group; *dsHlPrx*, double-stranded *HlPrx*-injected group; *dsDouble*, both double-stranded *HlPrx*- and *HlPrx2*-injected group. $^{*}P < 0.05$; $^{**}P < 0.01$, significant differences *vs dsLuc* by Welch's *t*-test.

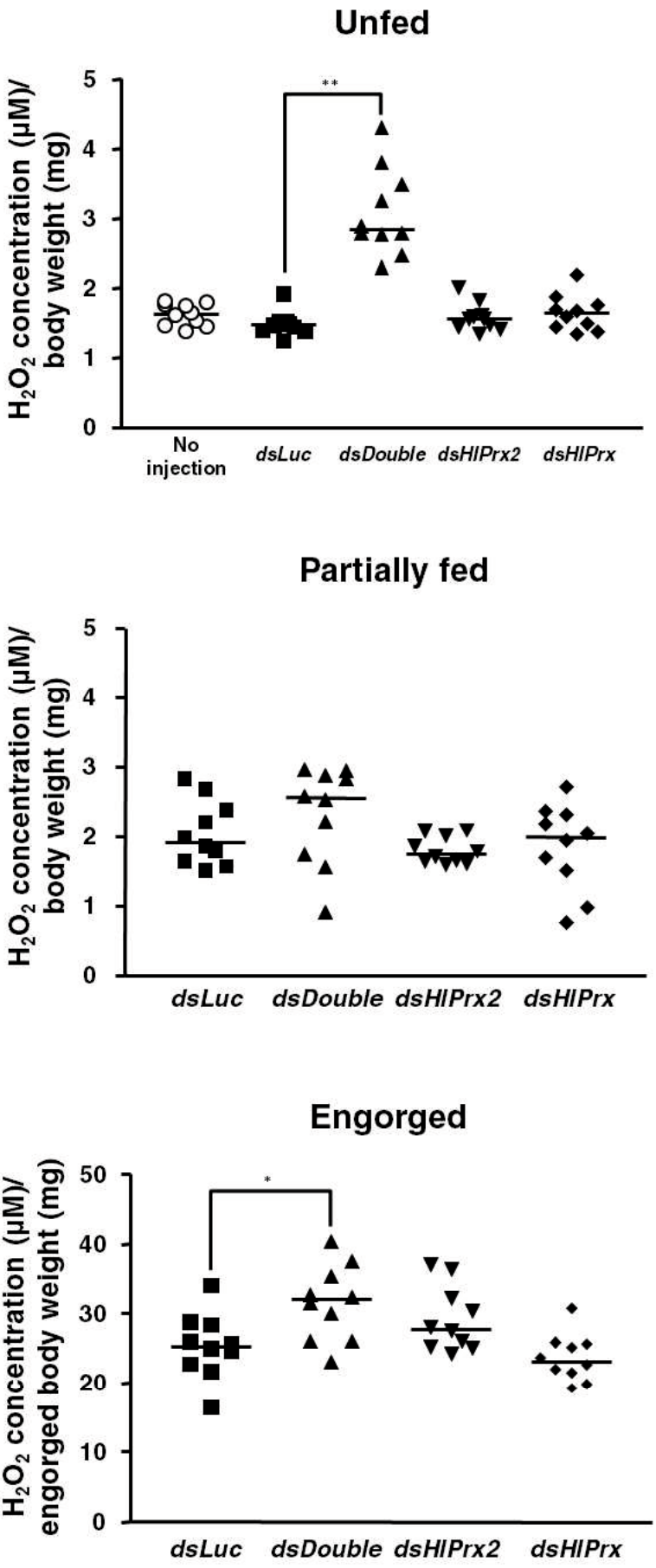
Unfed
H_2O_2 concentration (µM)/ body weight (mg)
5
4
3
2
1
0
**
No injection
dsLuc
dsDouble
dsHlPrx2
dsHlPrx
Partially fed
H_2O_2 concentration (µM)/ body weight (mg)
5
4
3
2
1
0
dsLuc
dsDouble
dsHlPrx2
dsHlPrx
Engorged
H_2O_2 concentration (µM)/ engorged body weight (mg)
50
40
30
20
10
0
*
dsLuc
dsDouble
dsHlPrx2
dsHlPrx

Fig. 2.7.

Fig. 2.7. Concentrations of hydrogen peroxide (H_2O_2) from *HlPrx* and/or *HlPrx2* knockdown ticks during blood-feeding. Data are presented as the ratio of H_2O_2 concentration (μM) to tick body weight or engorged body weight (mg). Horizontal bars indicate the median value. *Abbreviations*: *dsLuc*, double-stranded *Luciferase*-injected group; *dsHlPrx2*, double-stranded *HlPrx2*-injected group; *dsHlPrx*, double-stranded *HlPrx*-injected group; *dsDouble*, both double-stranded *HlPrx*- and *HlPrx2*-injected group. $^{*}P < 0.05$; $^{**}P < 0.01$, significant differences *vs dsLuc* by Welch's *t*-test.

CHAPTER 3

Peroxiredoxins are important for the regulation of hydrogen peroxide concentration induced by paraquat in *Ixodes scapularis* embryo-derived cell line (ISE6)

3.1 Introduction

In my previous study, a tick Prx, *Haemaphysalis longicornis* 2-Cys Prx (HlPrx2), was well characterized [13,14]. Knockdown of the *HlPrx2* gene caused significant decreases in the engorged body weight, egg weight, and hatching rate for larvae as compared to the control group [13]. In addition, the detection of H_2O_2 after the knockdown of *HlPrx2* and a 1-Cys type of *H. longicornis* Prx (HlPrx) [15] in ticks showed that the concentration of H_2O_2 significantly increased as compared with control before and after blood feeding. The expression levels of the *HlPrx2* gene in ovaries were higher than in other internal organs, such as the salivary glands, midguts, and fat bodies [13]. These results indicate that the regulation of H_2O_2 concentrations in ticks by their Prxs might be important for ovaries to succeed at reproduction.

To observe the interaction among tick Prxs, H_2O_2 generation, and tick ovaries, a cell line of embryos derived from *Ixodes scapularis* (ISE6) and an intracellular specific chemical fluorescent probe (BES-H_2O_2-Ac) were used. In the present study, to evaluate the antioxidant activity of Prxs against H_2O_2 in ticks, the H_2O_2-detection method using a BES-H_2O_2-Ac probe was established in ISE6 cells *in vitro*. I evaluated the effects on the generation of H_2O_2 in ISE6 cells of paraquat which is known to induce oxidative stress in mammalian cells.

3.2 Materials and methods

3.2.1 Culture of cells

The ISE6 cell line from the embryo of *I. scapularis* was grown at 34°C in L-15B medium (pH 6.4–6.6) with 10% fetal bovine serum (FBS), 5% tryptose phosphate broth, and antibiotics [64,65].

3.2.2 Detection of hydrogen peroxide using BES-H_2O_2-Ac in ISE6 cells

ISE6 cells were seeded in a 48-well plate at 300 µl/well of 1.0×10^6 cells/ml and incubated overnight at 34°C. After removing the supernatants, 500 µl of phosphate-buffered saline (PBS) was added to each well and suspended. After the cells were transferred to a 1.5-ml tube, the cells were centrifuged at 630×*g* for 3 min. The supernatants were replaced to 0, 1, 5, and 10 µM BES-H_2O_2-Ac (Wako, Osaka, Japan) in a culture medium without FBS. The cells were incubated at 34°C for 1 hr with 0.1 µM Hoechst 33342 (Dojindo, Kumamoto, Japan) for 30 min. The cells were washed with PBS 2 times, and then 120 µl of the culture medium was added. The cells were transferred to a 96-well black plate at 100 µl/well to measure fluorescence. Fluorescence was detected using a microplate reader (SH-9000Lab, Corona Electric,

Ibaraki, Japan) with excitation at 480 nm and emission at 535 nm for the BES-H_2O_2-Ac, and with excitation at 352 nm and emission at 461 nm for the Hoechst 33342.

3.2.3 Paraquat treatment

ISE6 cells were seeded in a 48-well plate at 300 µl of 1.0×10^6 cells/ml and incubated overnight at 34°C. After removing the culture medium, the cells were treated with several concentrations (0, 0.1, 1, 5, 10, and 20 mM) of paraquat in the culture medium at 34°C for 24 hrs. After the cells were washed with PBS twice after 24 hrs, the cells were used for *in vitro* cell proliferation and survival assays, as mentioned below.

3.2.4 RNA interference (RNAi) using double-stranded RNA

The Prxs in ticks have been well studied in *Haemaphysalis longicornis*; thus, I compared *H. longicornis* Prxs and *I. scapularis* Prxs using nucleotide BLAST. The analysis of the nucleotide BLAST revealed that *H. longicornis* 1-Cys Prx (Accession No. AB038382 [15]) and *I. scapularis* 1-Cys Prx (Accession No. AF209911) have high identity of 91%. On the other hand, both *I. scapularis* 2-Cys Prxs (Accession Nos. XM_002405422 and DQ065943) have high identity with *H. longicornis* 2-Cys Prx (Accession No. LC049075 [14]) at 88% and 76%, respectively. In this study, I focused

on one *I. scapularis* 1-Cys Prx (Accession No. AF209911, *IsPrx1*) and two *I. scapularis* 2-Cys Prxs, 1 and 2 (Accession No. XM_002405422, *IsPrx2-1*; and Accession No. DQ065943, *IsPrx2-2*).

Two separate PCR reactions of approximately 564 bp with a single T7 promoter were generated using the following primer sets: a T7-attached gene-specific forward primer (IsPrx1 T7-F) and gene-specific reverse primer (IsPrx1 RNAi-R); and a T7-attached gene-specific reverse primer (IsPrx1 T7-R) and gene-specific forward primer (IsPrx1 RNAi-F) (Table 3.1). After the gel purification of PCR products using a GENECLEAN® II KIT (MP Biomedicals, Irvine, CA, USA), double-stranded RNA of *I. scapularis* 1-Cys Prx (*dsIsPrx1*) was synthesized using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA) with two separate single-promoter templates in accordance with the manufacturer's protocol. Double-stranded RNA of *I. scapularis* 2-Cys Prx-1 and -2 (*dsIsPrx2-1* and *dsIsPrx2-2*) was also synthesized using *IsPrx2-1* and *IsPrx2-2* gene-specific primers (Table 3.1). The *enhanced green fluorescent protein* (*EGFP*) gene was used for control (*dsEGFP* group). *dsEGFP* groups included 0.5- and 1.5-µg groups. The 0.5-µg *dsEGFP* group was the control for the single knockdown groups, such as the *dsIsPrx1*, *dsIsPrx2-1*, and *dsIsPrx2-2* groups.

The 1.5-µg *dsEGFP* group was the control for the triple knockdown group, *dsIsPrxs-all* containing *dsIsPrx1*, *dsIsPrx2-1*, and *dsIsPrx2-2*.

ISE6 cells were seeded in a 48-well plate at 300 µl of 1.0×10^6 cells/ml and incubated overnight at 34°C. The dsRNA (0.5 µg/well), 18 µl of Opti-MEM (Gibco, Grand Island, NY, USA), and 3.5 µl of HilyMax (Dojindo) were mixed and incubated at room temperature for 15 min. Cells in the plates were washed twice with PBS, and 300 µl of the culture medium without FBS was added. Then the incubated mixture was added to the medium in each well, and the cells were incubated at 34°C for 16 hrs. Three hundred microliters of the culture medium with FBS was added, and the cells were incubated at 34°C for another 32 hrs. The transfected cells were harvested, and the knockdown was checked using RT-PCR. The PCR cycle profile was as follows: initial denaturation at 94°C for 2 min, 30 cycles of a denaturation step at 94°C for 30 s, an annealing step at 65°C for 60 s, and an extension step at 72°C for 90 s. The primer sets used in this study are shown in Table 3.1. The gene-silenced cells were used in *in vitro* cell proliferation and survival assays, as mentioned below.

3.2.5 Total RNA extraction and cDNA synthesis

To extract total RNA, one well of ISE6 cells was harvested from a 48-well plate and transferred to a 1.5-ml tube. The harvested cells were centrifuged at 630×*g* for 3 min, and the supernatants were removed. The extracted RNA was purified using TRI® Reagent (Molecular Research Center, Cincinnati, OH, USA), and then treated with an RQ1 RNase-Free DNase (Promega). cDNA synthesis was performed with ReverTra Ace-α-® (Toyobo, Osaka, Japan), following the manufacturer's protocol, using 1 μg of total RNA.

3.2.6 In vitro *cell proliferation and survival assays*

After paraquat treatment or RNAi, the ISE6 cells were used for *in vitro* cell proliferation and survival assays. After washing with PBS, the cells were diluted in 120 μl of culture medium. One hundred μl of cells was transferred to a 96-well plate for cell proliferation assay (MTT assay, Promega), and the remaining 20 μl of cells was transferred to a 1.5-ml tube for cell survival assay (Trypan blue assay).

The cells in a 96-well plate for MTT assay were incubated for 20 hrs at 34°C, and 15 μl of the dye solution was added to each well. The plate was incubated for another 4 hrs. Then 100 μl of solubilization solution/stop mix was added to each well

and incubated overnight at 34°C. The absorbance of OD_{570nm} was detected using the microplate reader (SH-9000Lab).

Ten µl of the cells in the 1.5-ml tube for the Trypan blue assay was mixed with the same volume of Trypan blue. Then, dead Trypan blue-stained cells were counted using a hemocytometer.

3.2.7 Detection of hydrogen peroxide in peroxiredoxin-silenced ISE6 cells after treatment with 1-mM paraquat using the BES-H_2O_2-Ac probe with Hoechst 33342

ISE6 cells were seeded in a 48-well plate at 300 µl of 1.0×10^6 cells/ml and incubated overnight at 34°C. The cells in the plates were washed twice with PBS. The washed cells were incubated with the dsRNA as previously described. After *IsPrx* knockdown, the cells were treated with 1-mM paraquat for 24 hrs at 34°C. The cells were washed twice with PBS and incubated with 5 µM of BES-H_2O_2-Ac for 1 hr and with 0.1 µM of Hoechst 33342 for 30 min in the culture medium without FBS in 1.5-ml tubes at 34°C. Then, the cells were washed twice with PBS and concentrated 2.5 times. One hundred microliters of concentrated cells was transferred to the 96-well black plate. Fluorescence was monitored using a fluorescence microscope and detected using the microplate reader as previously described.

3.2.8 Statistical analysis

All experiments were conducted in three or four wells. Data were statistically analyzed using Welch's *t*-test. Knockdown experiments also were done using Welch's *t*-test vs. each control group (single knockdown group vs. *dsEGFP* 0.5-µg group and triple knockdown group vs. *dsEGFP* 1.5-µg group). The data presented the mean ± standard deviation (SD). $P< 0.05$ and $P< 0.01$ were considered to be statistically significant differences.

3.3 Results

3.3.1 Comparison of the concentration of the BES-H_2O_2-Ac probe in ISE6 cells

To decide the concentration of the BES-H_2O_2-Ac probe to be used in this study, fluorescence intensities were compared among several concentrations, such as 0, 1, 5, and 10 µM in ISE6 cells. Visual fluorescence under the microscope appeared to have almost the same intensity between 5- and 10-µM probe concentrations (Fig. 3.1A). However, the fluorescence microplate reader showed that the fluorescence intensity significantly increased in a concentration-dependent manner from 1 to 10 µM (Fig. 3.1B). These results indicated that 5 µM of the BES-H_2O_2-Ac probe was appropriate for the present study.

3.3.2 In vitro *cell proliferation assay (MTT assay) and cell survival assay (Trypan blue assay) for ISE6 cells after treatment with several concentrations of paraquat*

To determine the experimental concentration of paraquat treatment for the ISE6 cells, MTT and Trypan blue assays were conducted. The cells were treated with several concentrations of paraquat (0, 0.1, 1, 5, 10, and 20 mM). MTT assay showed that the proliferation of ISE6 cells was affected by 0.1 mM of paraquat treatment. Cell proliferation significantly decreased in a concentration-dependent manner from 0.1 to 20 mM (Fig. 3.2A). In addition, the proliferation of cells drastically decreased in ISE6 cells treated with between 1- and 5-mM paraquat (Fig. 3.2A). On the other hand, the Trypan blue assay, a survival assay for cells, showed that paraquat treatment of 5, 10, and 20 mM significantly affected the ISE6 cell survival rate. There were no significant differences among 0-, 0.1-, and 1-mM paraquat treatments of ISE6 cells (Fig. 3.2B). In the untreated ISE6 cells, the mRNA expression levels of *IsPrxs* seemed to show no RT-PCR differences after exposure to 1-mM paraquat for 24 hrs (data not shown). These results demonstrated that 1-mM paraquat treatment was an acceptable experimental condition for ISE6 cells in this study.

3.3.3 MTT assay and Trypan blue assay for ISE6 cells after the gene silencing of IsPrxs *using the RNAi method*

To evaluate the effects of *Prx* gene silencing on ISE6 cells, MTT assay and Trypan blue assay were performed. First, the gene silencing of *IsPrxs* in each knockdown group was confirmed by semi-quantitative RT-PCR (Fig. 3.3A). The genes of each knockdown group were silenced by specific dsRNA of *IsPrxs*. After the confirmation of *IsPrx* gene silencing in the ISE6 cells, MTT and Trypan blue assays were conducted. The MTT assay demonstrated almost the same level of cell proliferation among *dsEGFP* 0.5 μg, *dsIsPrx1*, *dsIsPrx2-1*, and *dsIsPrx2-2* groups (Fig. 3.3B). No significant difference in cell proliferation between *dsEGFP* 1.5-μg and *IsPrxs-all* groups was observed (Fig. 3.3B). Moreover, the Trypan blue assay showed no significant differences among all knockdown groups (Fig. 3.3C). These results demonstrated that there were no significant differences among all knockdown groups, such as *dsEGFP* 0.5 μg, *dsEGFP* 1.5 μg, *dsIsPrx1*, *dsIsPrx2-1*, *dsIsPrx2-2*, and *dsIsPrxs-all*. Therefore, *IsPrx* gene silencing has little or no effect on cell proliferation and the survival of ISE6 cells.

3.3.4 Detection of BES-H_2O_2-Ac/Hoechst 33342 in IsPrx *gene-silenced ISE6 cells after treatment with 1-mM paraquat for 24 hrs*

Assays were conducted to evaluate the H_2O_2 generation in ISE6 cells on silencing of *IsPrxs* with 1-mM paraquat treatment. The *IsPrx* gene-silenced ISE6 cells were treated with 1-mM paraquat for 24 hrs at 34°C. After paraquat treatment, the cells were incubated with a 5-µM BES-H_2O_2-Ac probe and 0.1-µM Hoechst 33342. The fluorescence microscopy analysis demonstrated that *IsPrxs*-silenced cells manifested high concentrations of H_2O_2, as shown by the high intensity of fluorescence within the cytoplasm (Fig. 3.4A). To quantify the intensities of H_2O_2 fluorescence from probe-treated ISE6 cells, fluorescence microplate reader analysis was performed. The fluorescence microplate reader revealed that *IsPrx1*, *IsPrx2-1*, and *IsPrx2-2* or *IsPrxs-all* knockdown led to increasing H_2O_2 intensity, as compared with the *dsEGFP* 0.5-µg or *dsEGFP* 1.5-µg groups, respectively (Fig. 3.4B). These results suggest that the knockdown of *IsPrx1*, *IsPrx2-1*, and *IsPrx2-2* genes leads to an increased concentration of H_2O_2 in ISE6 cells after treatment with 1-mM paraquat.

3.4 Discussion

Prxs are a family of six isoenzymes in mammalian cells [66]. In addition, Prxs are ubiquitous antioxidant enzymes produced at high levels in mammalian cells [5,6]. Kinetic measurements imply that Prxs reduce cellular peroxides by more than 90% [67,68]. Therefore, Prxs are considered to be among the most important antioxidant enzymes, known to balance the production of cellular H_2O_2 which is essential for cell signaling and metabolism in mammalian cells [69]. On the other hand, ticks lack heme synthesis and catabolism pathways, as they are unable to produce σ-aminolevulinic acid, a heme precursor [46], even at genomic levels [47]. Therefore, they rely on heme from their host and heme stored in hemosomes of the midgut without digestion [48]. These facts suggest that ticks might have difficulty producing proteins that contain heme, such as catalase and peroxidase, which are both H_2O_2-scavenging enzymes. Moreover, the double knockdown of *HlPrx* and *HlPrx2* led to significantly higher concentrations of H_2O_2 in unfed adult ticks as compared to the control group in *H. longicornis* [13]. These results suggest that Prxs also are important antioxidant enzymes for ticks to balance the production of cellular H_2O_2 in mammalian cells.

Recently, H_2O_2-scavenging enzymes, such as catalases and Prxs, have been studied in ticks to understand ticks' antioxidant mechanisms for tick control [12,13] or

the relationship between ticks and their pathogens [55,70]. These results demonstrated that the knockdown of *Prxs* has a greater effect on the ticks' blood feeding and reproduction than does the gene silencing of catalases, although only the knockdown of multiple *Prxs* (*HlPrx* and *HlPrx2*) affects the concentration of H_2O_2 in ticks before and after blood feeding [12,13]. On the other hand, *Borrelia* exploits salivary Salp25D, a protein homologous to 1-Cys Prx in *I. scapularis*, for protection against reactive oxygen intermediates generated by mammalian neutrophils at the vector-host interface [55]. The inhibition of the catalase gene and protein also leads to the low transmission of *Rickettsia parkeri* to tick eggs in the Gulf-coast tick *Amblyomma maculatum* [70]. Therefore, the enzymes, such as catalases and Prxs, that control the H_2O_2 concentration in ticks can be considered to be important in tick blood feeding and reproduction through the regulation of the H_2O_2 concentration and the relationship between ticks and their pathogens.

In the present study, paraquat was used as the H_2O_2 inducer in ISE6 cells. After exposure to high concentration of paraquat, ISE6 cells manifested distinctive low cell proliferation and survival in MTT and Trypan blue assays. The MTT assay is based on the cellular conversion of a tetrazolium salt into a formazan product [71]. The metabolism of tetrazolium salt into formazan is dependent on the active mitochondria of

live cells. Thus, this assay can be reworded as a mitochondrial activity assay. Although mitochondria in live cells are exposed to high oxidative stress, the oxidative stress is controlled using nicotinamide adenine dinucleotide (NADH). Paraquat would react with NADH or nicotinamide adenine dinucleotide phosphate (NADPH), and then the paraquat becomes paraquat-radical (PQ·). PQ· occurs as a superoxide, and this superoxide would cause oxidative stress to organisms. Therefore, paraquat can be used as an oxidative stress inducer in ISE6 cells in which the target of paraquat-derived oxidative stress might be the mitochondria of the cells.

In mammalian cells, 2-Cys Prxs localize in the cytoplasm, mitochondria, and endoplasmic reticulum (ER), and 1-Cys Prxs localize in the cytoplasm and mitochondria [72]. In this study, *I. scapularis* Prx homologs of *H. longicornis* 1-Cys [15] and 2-Cys [13,14] Prxs were identified using nucleotide BLAST. *H. longicornis* 2-Cys Prx (HlPrx2) is a cytosolic protein. *IsPrx2-1* and *HlPrx2* genes have high homology at 88%. These results indicate that the IsPrx2-1 protein could be considered a cytosolic protein. Meanwhile, *IsPrx2-2* and *HlPrx2* genes have high homology at 76%. Moreover, the IsPrx2-2 amino acid sequence has a signal peptide. Among the human Prx family, human Prx4 (hPrx4) is the only known secretory form located not only intracellularly but also in the extracellular space [73]. hPrx4 is also known to exert a

protective function against oxidative damage by scavenging ROS in the extracellular space [74]. These reports and our data suggest that IsPrx2-2 is related to controlling oxidative stress, not only at the intracellular level but also in the extracellular space; thus, *IsPrx2-2* gene silencing led to a high concentration of H_2O_2 within ISE6 cells. On the other hand, *IsPrx1* gene silencing in ISE6 cells had little effect on the H_2O_2 concentration as compared with *IsPrx2s* gene silencing. In the *H. longicornis* tick, the knockdown experiment of 1-Cys and/or 2-Cys *Prxs* revealed that 2-Cys *Prx* gene silencing was more effective in reference to ticks' blood feeding and reproduction, although the H_2O_2 concentration in ticks during blood feeding was slightly higher than that of a control group [13]. These results demonstrated that tick 1-Cys Prx might play a minor role in controlling H_2O_2 in ticks; thus, 1-Cys Prx might be important in the interaction between ticks and their pathogens.

In summary, I observed that paraquat could induce H_2O_2 in ISE6 cells as an oxidative stress inducer. In addition, I established the H_2O_2 detection method in ISE6 cells using an intracellular H_2O_2 probe (BES-H_2O_2-Ac). Moreover, the gene silencing of *IsPrxs* led to high levels of H_2O_2 in ISE6 cells under the microplate reader and the fluorescence microscope. These results suggest that paraquat acts as an H_2O_2 inducer, and *Prx* genes are important for the regulation of the H_2O_2 concentration in ISE6 cells.

Table and Figures in CHAPTER 3

Table 3.1. Gene-specific primers used in Chapter 3

Primers	Sequence (5' → 3')
IsPrx1 RT-F	AGCTCATCGCTCTCTCCTGTG
IsPrx1 RT-R	TGGTTGTTCGGAGGTAGTTCTTG
IsPrx2-1 RT-F	TGTGCCGGTCGTCAATTTGG
IsPrx2-1 RT-R	TGCCCTCGTCTTCCTTGAGG
IsPrx2-2 RT-F	GCTTCAGGGCACAACATTCAC
IsPrx2-2 RT-R	ACCCAGTCCTCCTTGCTTCC
IsPrx2-3 RT-F	TGTCGAGCACCTCATACACTCTC
IsPrx2-3 RT-R	CCAGACCTCCTTGCTTCCTC
IsPrx1 T7-F	TAATACGACTCACTATAGGGGATCCTTTCCCCAACTTCACC
IsPrx1 T7-R	TAATACGACTCACTATAGGGTGCCCTTCTGCCAACCA
IsPrx1 RNAi-F	GATCCTTTCCCCAACTTCACC
IsPrx1 RNAi-R	TGCCCTTCTGCCAACCA
IsPrx2-1 T7-F	TAATACGACTCACTATAGGACTTCCCAAGCTGACCCACC
IsPrx2-1 T7-R	TAATACGACTCACTATAGGGTTGGCAGGGCACACTTCAC
IsPrx2-1 RNAi-F	ACTTCCCAAGCTGACCCACC
IsPrx2-1 RNAi-R	GTTGGCAGGGCACACTTCAC
IsPrx2-2 T7-F	TAATACGACTCACTATAGGGATCTCTAAACCTGCTCCCGACTT
IsPrx2-2 T7-R	TAATACGACTCACTATAGGGGACATACTTCGCCGTGCTTG
IsPrx2-2 RNAi-F	ATCTCTAAACCTGCTCCCGACTT
IsPrx2-2 RNAi-R	GACATACTTCGCCGTGCTTG
Tick mt-rrs F	CTGCTCAATGATTTTTTAAATTGCTGTGG
Tick mt-rrs R	CCGGTCTGAACTCAGATCAAGTA

Underlines denote T7 RNA polymerase promoter sequences.

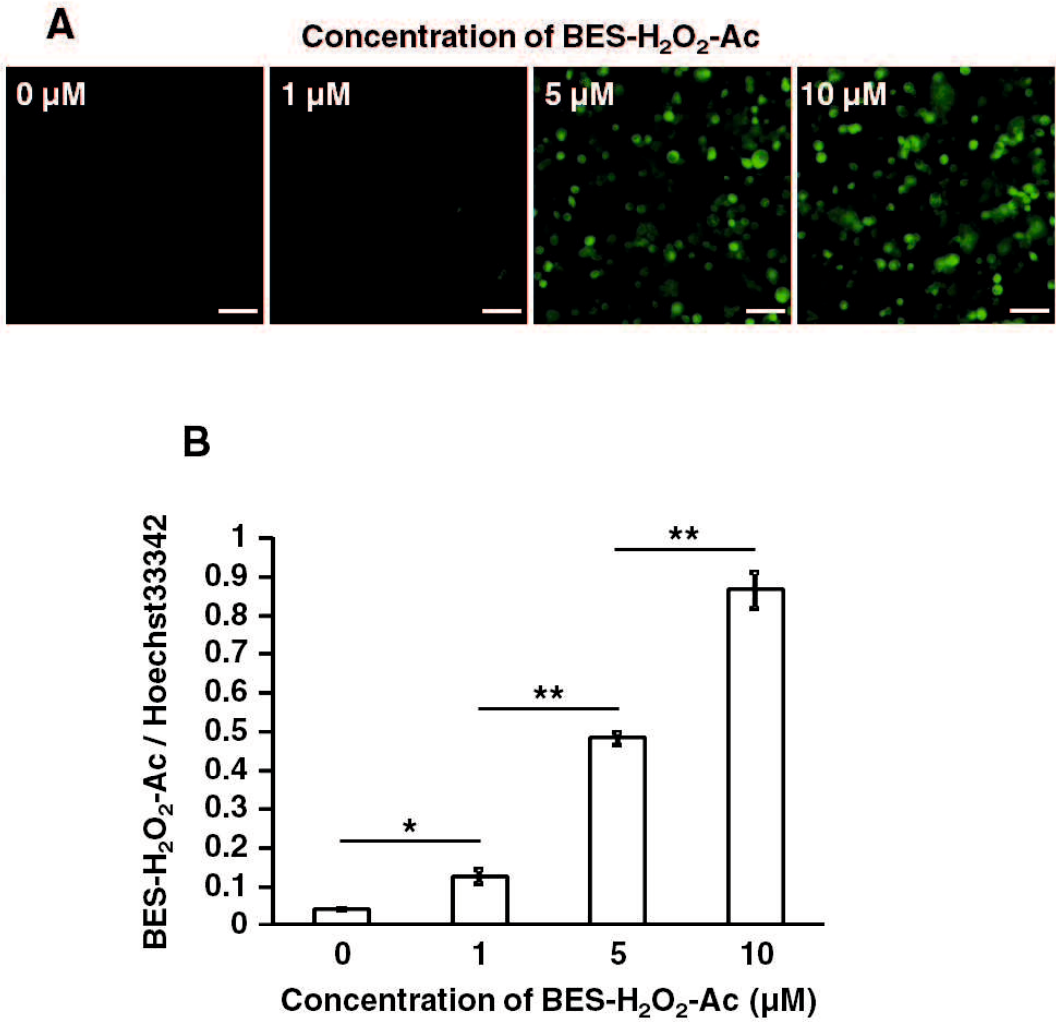

Fig. 3.1. Comparison of the fluorescence of several concentrations of the BES-H_2O_2-Ac probe in ISE6 cells. **(A)** The fluorescence of the BES-H_2O_2-Ac probe in ISE6 cells. Scale bars: 50 μm. **(B)** Graph of the fluorescence intensity of the BES-H_2O_2-Ac probe in ISE6 cells after exposure for 1 hr and in Hoechst 33342 for 30 min. Intensities are shown as the ratios of BES-H_2O_2-Ac/Hoechst 33342 intensities. Probe concentrations are 0, 1, 5, and 10 μM. $^{*}P< 0.05$; $^{**}P< 0.01$ indicate significant differences by Welch's *t*-test.

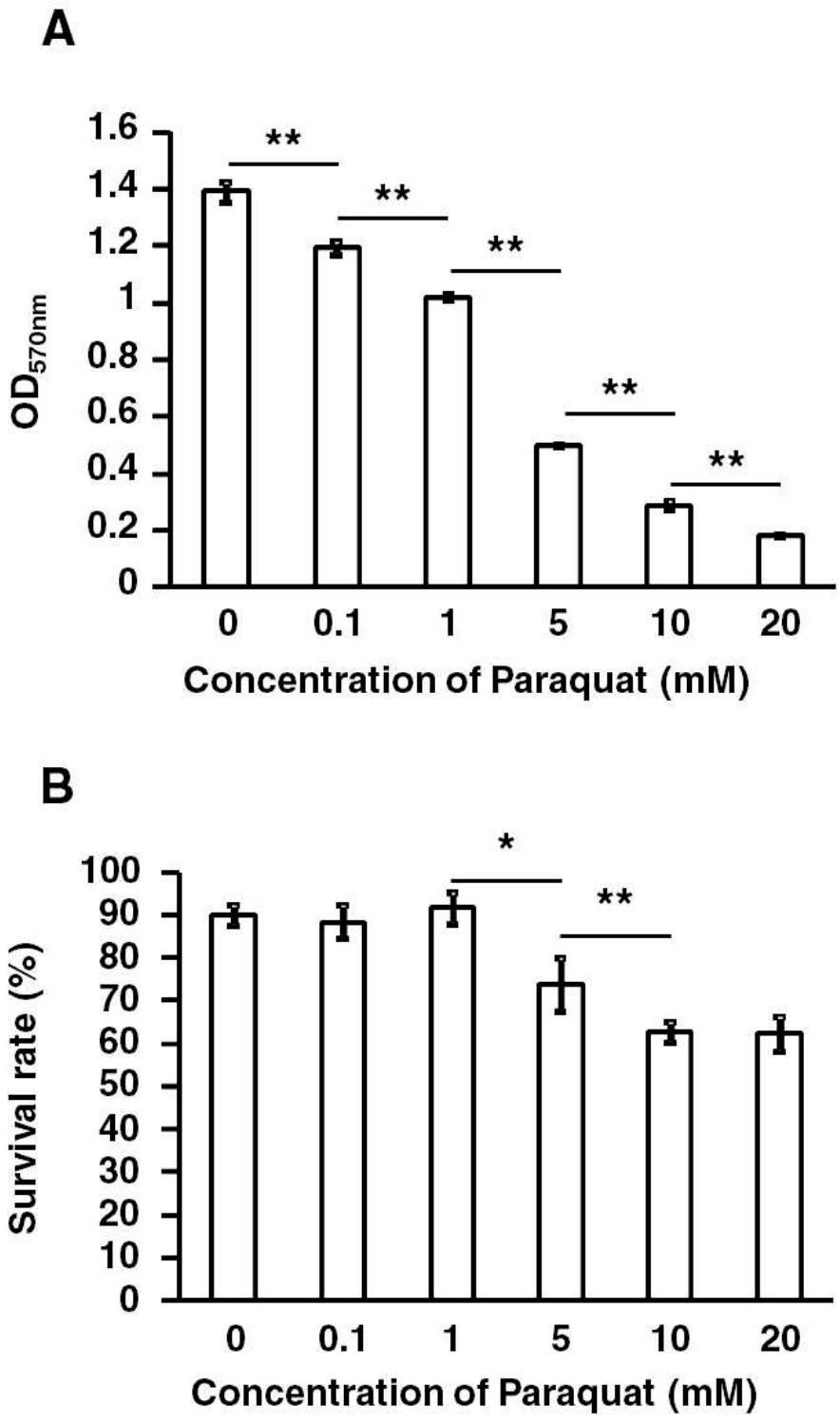
A
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD570nm
**
**
**
**
**
0
0.1
1
5
10
20
Concentration of Paraquat (mM)
B
100
90
80
70
60
50
40
30
20
10
0
Survival rate (%)
*
**
0
0.1
1
5
10
20
Concentration of Paraquat (mM)

Fig. 3.2.

Fig. 3.2. Effects of several concentrations of paraquat on ISE6 cells. **(A)** *In vitro* cell proliferation (MTT) assay for ISE6 cells after exposure to several concentrations of paraquat. Paraquat was used at concentrations of 0, 0.1, 1, 5, 10, and 20 mM. The absorbance at OD_{570nm} was detected using a microplate reader. **(B)** Cell survival (Trypan blue) assay for ISE6 cells after treatment with several concentrations of paraquat. Dead cells were counted using a hemocytometer. Survival rates were calculated as the surviving cells/total counted cells. $^{*}P< 0.05$; $^{**}P< 0.01$ indicate significant differences by Welch's *t*-test.

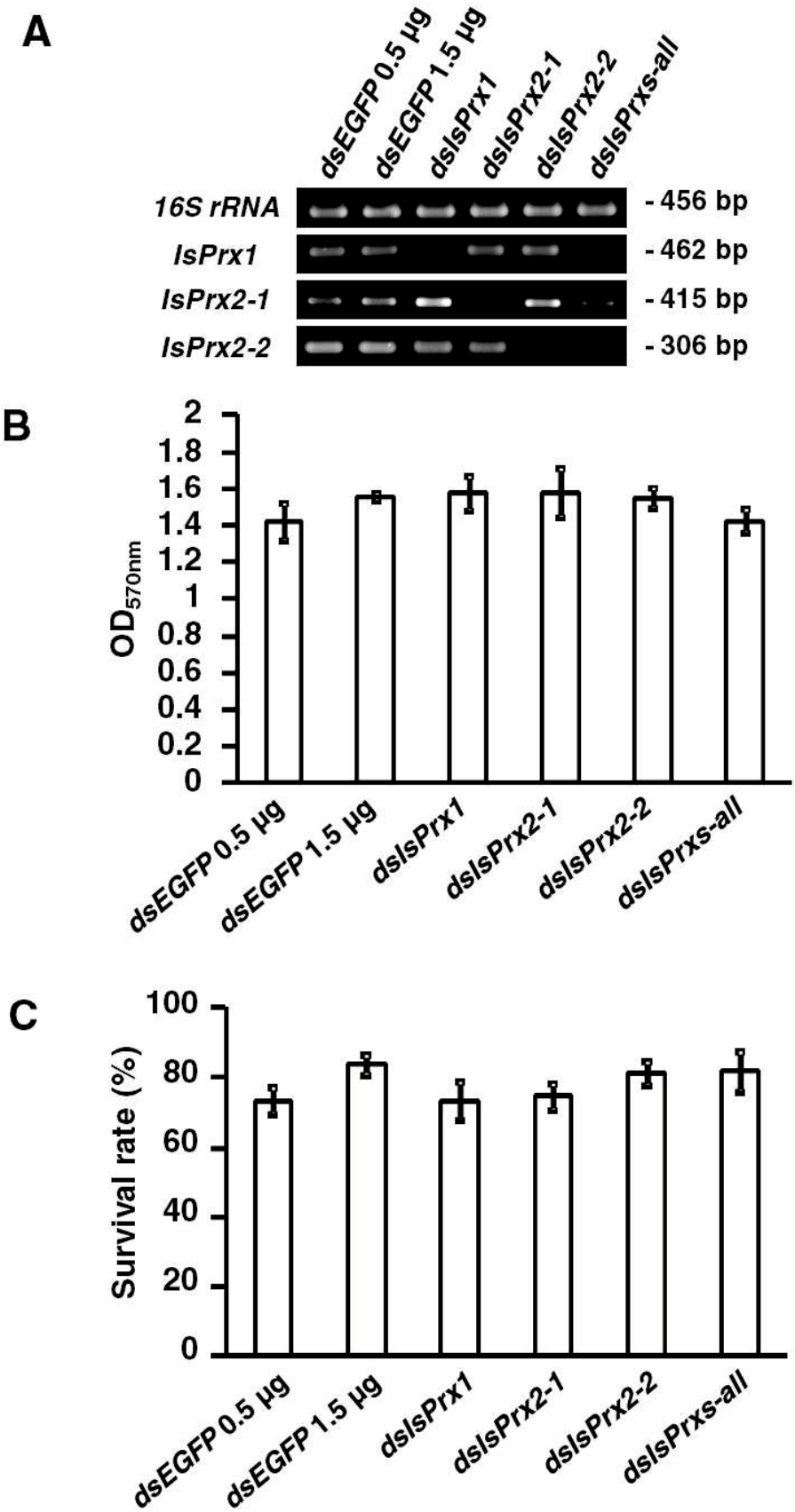
A
dsEGFP 0.5 µg
dsEGFP 1.5 µg
dsIsPrx1
dsIsPrx2-1
dsIsPrx2-2
dsIsPrxs-all
16S rRNA
IsPrx1
IsPrx2-1
IsPrx2-2
- 456 bp
- 462 bp
- 415 bp
- 306 bp
B
OD570nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C
Survival rate (%)
100
80
60
40
20
0

Fig. 3.3.

Fig. 3.3. Effects of *IsPrxs* gene silencing on ISE6 cells. **(A)** Knockdown confirmation of *IsPrx* gene silencing in ISE6 cells. Total RNA was extracted from knockdown cells, and then cDNA was synthesized. After RT-PCR, the products were analyzed in 1.5% agarose gel. The left and right columns indicate target genes and their product sizes, respectively. **(B)** *In vitro* cell proliferation (MTT) assay for ISE6 cells after knockdown of *IsPrx* genes; the absorbance at OD570nm detected using the microplate reader. **(C)** Cell survival (Trypan blue) assay for ISE6 cells after the knockdown of *IsPrx* genes. Dead cells were counted using a hemocytometer. Survival rates were calculated as the surviving cells/total counted cells. *dsEGFP* 0.5-µg and *dsEGFP* 1.5-µg groups are controls for single gene silencing (*dsIsPrx1*, *dsIsPrx2-1*, and *dsIsPrx2-2*) and triple gene silencing (*dsIsPrxs-all*) groups, respectively. Data were analyzed using Welch's *t*-test.

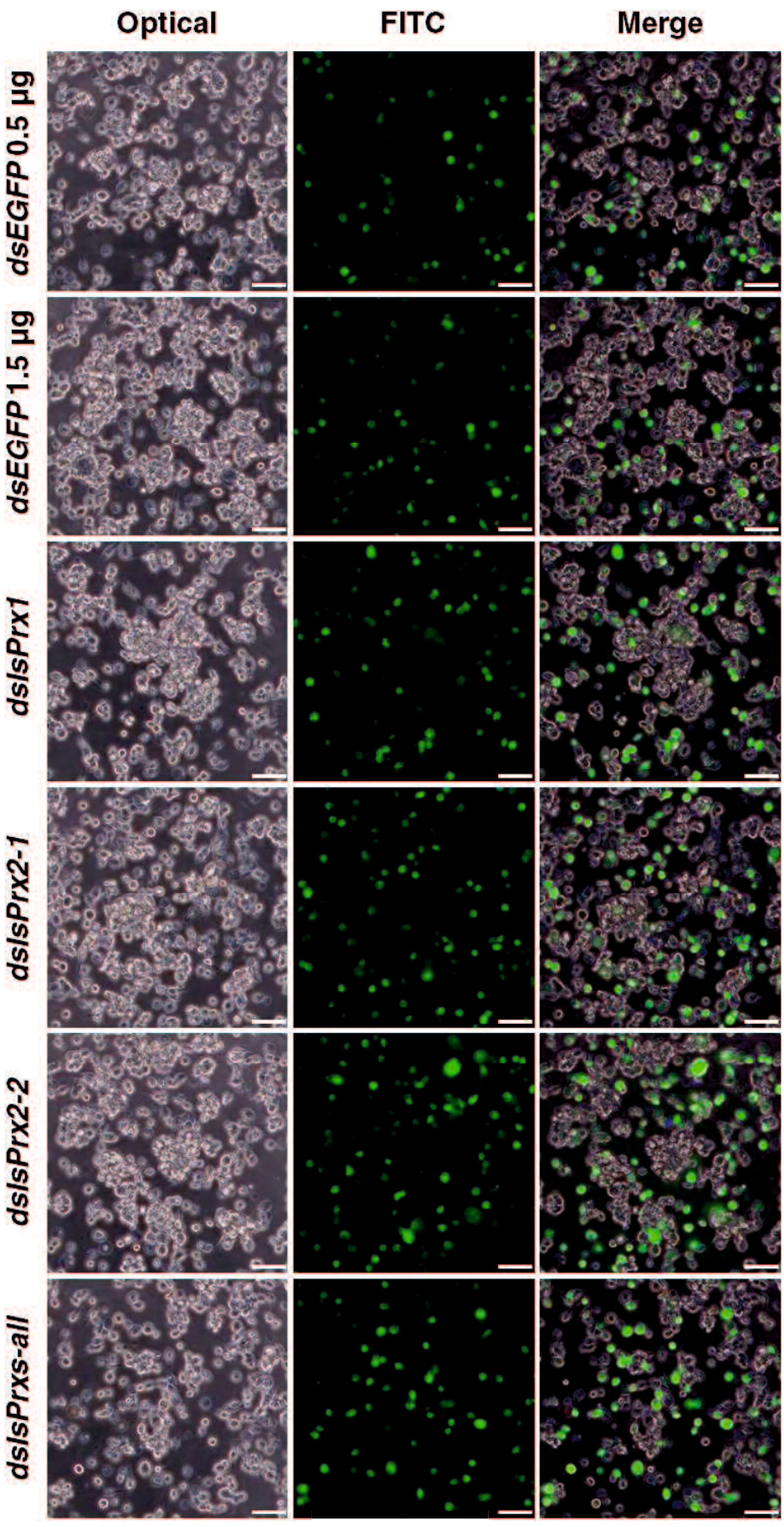
Optical
FITC
Merge
dsEGFP 0.5 µg
dsEGFP 1.5 µg
dsIsPrx1
dsIsPrx2-1
dsIsPrx2-2
dsIsPrxs-all

Fig. 3.4A.

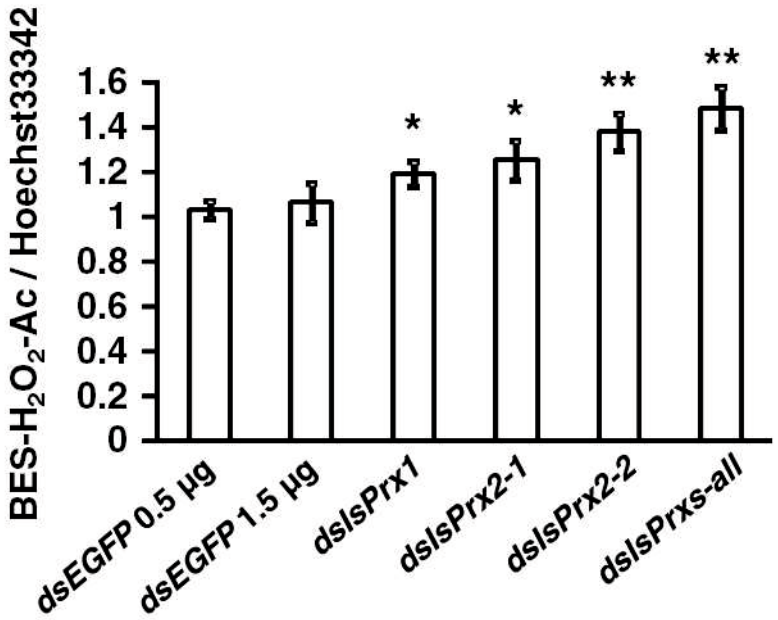

Fig. 3.4B.

Fig. 3.4. Effects of 1-mM paraquat treatment on *IsPrx* gene-silenced ISE6 cells. **(A)** The fluorescence of the BES-H_2O_2-Ac probe in *IsPrx* gene-silenced ISE6 cells was observed under a fluorescence microscope after paraquat treatment. The left column indicates the silenced genes in ISE6 cells. Images on the left are optical, those in the middle are FITC for the BES-H_2O_2-Ac probe, and those on the right are merged optical and FITC images. Scale bars: 50 µm. **(B)** Graph of the fluorescence intensity of the BES-H_2O_2-Ac probe in *IsPrx* gene-silenced ISE6 cells after exposure to 1-mM paraquat for 24 hrs. The intensities are shown as the ratio of BES-H_2O_2-Ac/Hoechst 33342 intensities. *dsEGFP* 0.5-µg and *dsEGFP* 1.5-µg groups are used as controls for single gene silencing (*dsIsPrx1*, *dsIsPrx2-1*, and *dsIsPrx2-2*) and triple gene silencing (*dsIsPrxs-all*) groups, respectively. Data were analyzed using Welch's *t*-test. $^{*}P< 0.05$; $^{**}P< 0.01$ indicate significant differences vs. each control group.

CHAPTER 4

Evaluation of vaccine potential of 2-Cys peroxiredoxin from the hard tick *Haemaphysalis longicornis*

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4.1 Introduction

Peroxiredoxins (Prxs) are considered to be vaccine candidates against *Leishmania donovani* [62] or *Fasciola hepatica* [63]. At the infective stage, *F. hepatica* flukes excyst from a dormant state following ingestion and penetrate the intestinal wall before migrating to the liver; in this nutrient- and oxygen-rich environment, the flukes undergo rapid growth and development, and energy is supplied by aerobic respiration [53]. The developmental situation of *Fasciola* parasites is similar to the development of ticks during blood feeding. In addition, *Fasciola* parasites secrete Prxs into their host to regulate their environment for survival in the host body [41]. These results strongly suggest that tick Prxs could help ticks' successful blood feeding in a way similar to that of *Fasciola* parasites.

In Chapter 4, I considered tick Prxs to be a potential target for tick control that could provide further understanding of oxidative stress coping mechanisms in ticks during blood feeding. In the present study, the efficacy of *Haemaphysalis longicornis* 2-Cys peroxiredoxin (HlPrx2) was evaluated as an antigen candidate for the vaccine.

4.2 Materials and Methods

4.2.1 Ticks and animals

A parthenogenetic *H. longicornis* population (Okayama strain) [42] was maintained for several generations by feeding on the ears of Japanese white rabbits (Kyudo, Saga, Japan) at the Experimental Animal Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan. Four-week-old female BALB/c mice (BALB/cN Sea, Kyudo) were used for vaccination. Animals in my experiments were used in accordance with approved guidelines (approval numbers VM15055 and VM15056) from the Animal Care and Use Committee of Kagoshima University.

4.2.2 Expression and purification of recombinant H. longicornis *2-Cys peroxiredoxin (HlPrx2)*

Recombinant HlPrx2 (rHlPrx2) was expressed as a histidine (His)-tagged protein using the expression vector pRSET C (Invitrogen, Carlsbad, CA, USA) as described previously [14]. Briefly, the open reading frame sequence was amplified using polymerase chain reaction (PCR). The amplified PCR product was then purified using a GENECLEAN® II KIT (MP Biomedical, Solon, OH, USA) and subcloned into the frame of the pRSET C. rHlPrx2 was expressed in *Escherichia coli* BL21 (DE3)

strain and purified using the Biologic DuoFlow™ Chromatography System (Bio-Rad, Hercules, CA, USA) with a HisTrap FF column (GE Healthcare, Pittsburgh, PA, USA); the collected fractions were then dialyzed using a dialysis tube against phosphate buffered saline (PBS) at 4°C. Finally, the resulting rHlPrx2 protein was adjusted to a concentration of 1 mg/ml.

4.2.3 Protein and endotoxin analysis

The *E. coli* lysate and purified rHlPrx2 proteins were resolved in a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After SDS-PAGE, the gel was stained by Coomassie Brilliant Blue and viewed using Gel Doc (Bio-Rad). Endotoxin measurement was performed using a ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA).

4.2.4 Immunization protocol of mice

At three-week intervals, female 4-week-old BALB/c mice (Kyudo) were twice immunized twice subcutaneously with 30 μg of rHlPrx2 alone or rHlPrx2 mixed with an equal volume of incomplete Freund's adjuvant (IFA). Control mice were injected with PBS or PBS+IFA. Six mice for each group were used in this study. Antisera were collected from the orbital sinus under anesthesia before the first administration and one

week after each administration (1st administration=administration; 2nd administration=booster). Antigen-specific antibody titers were evaluated by enzyme-linked immunosorbent assay (ELISA).

4.2.5 Immune response

The specific antibody titers of the immunized mice were determined by ELISA. Ninety-six-well Nunc MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with rHlPrx2 diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) (100 ng in 100 µl/well) overnight at 4ºC. The plates were washed three times with 0.05% PBS-Tween 20 (PBS-T), and the wells were saturated with 150 µl of 1% skim milk in PBS (saturation solution) for one hr at room temperature (RT). The saturation solution was discarded, and the plates were washed four times with PBS-T. The wells were incubated for one hr at RT in the presence of 100 µl of five-time serial dilutions of tested mice sera in a saturation solution. The initial dilution of total IgG and IgG1 began at 1:100, while that of IgG2a started at 1:50. After washing the plates six times with PBS-T, the wells were incubated with 100 µl of different secondary antibody solutions for one hr at RT. To detect specific total IgG, IgG1, and IgG2a, the following antibodies diluted in a saturation solution were used:

IgG—Horseradish peroxidase (HRP)-conjugated Goat Anti-Mouse IgG (1:4000—SouthernBiotech, Birmingham, AL, USA)

IgG1—HRP-conjugated Goat Anti-Mouse IgG1 (1:4000—SouthernBiotech)

IgG2a—HRP-conjugated Goat Anti-Mouse IgG2a (1:4000— SouthernBiotech)

The plates were again washed six times with PBS-T, and SureBlue™ TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was added at 100 µl/well and incubated at 37°C for 30 min. The reaction was stopped by the addition of 50 µl of stop solution (equal volumes of 0.5 N HCl and 0.3 N sulfuric acid mixed). The absorbance at 450 nm was read in a Bio-Rad Novapath ELISA plate reader (Bio-Rad). Antibody titers were defined as the dilution rate ($\log_{10}$) under 0.5 < OD450 nm.

4.2.6 Protein extraction and Western blot analysis

Homogenized partially fed tick midguts were suspended in PBS and sonicated for six min at 45 kHz using a VS-100III ultrasonic cleaner (AS ONE Corporation, Osaka, Japan) and then centrifuged at 500×*g*. The supernatant was resolved in SDS-PAGE gel under reducing conditions. After SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon®-P; Millipore, Danvers, MA, USA). The membranes were blocked for one hr at RT with 0.3% skim

milk in PBS-T (blocking solution); they were incubated with 1:100 dilutions of rHlPrx2-immunized mouse sera (sera containing the highest titers during tick infestation were used in each immunized group) and a 1:500 dilution of anti-rHlPrx2 mouse sera for positive control [13] in a blocking solution at 4^{o}C overnight. For loading control, tubulin was detected using antiserum against recombinant *H. longicornis* tubulin [43]. After washing three times in PBS-T, the membranes were incubated with a 1:50,000 dilution of HRP-conjugated sheep anti-mouse IgG (Dako, Glostrup, Denmark) in a blocking solution at RT for one hr. After washing three times in PBS-T, bands were detected using Clarity™ Western ECL Substrate (Bio-Rad) and viewed using FluorChem®FC2 software (Alpha Innotech, San Leandro, CA, USA).

4.2.7 Tick infestation on rHlPrx2-vaccinated mice

To evaluate the effects of the rHlPrx2 immunization of mice against ticks, 20 nymphal *H. longicornis* ticks were allowed to feed on an immunized mouse, three weeks after the booster, until fully engorged by the feeding capsule method [75]. The total number of ticks that successfully engorged divided by the number of infested ticks (engorged rate), the total number of nymphal ticks molted to adult ticks divided by the number of successfully engorged ticks (molting rate), and the survival rate after molting from nymphs to adult ticks were assessed. Mouse antisera were collected by

exsanguination under anesthesia after tick infestations were finished. Antigen-specific antibody titers were also evaluated by ELISA, using the protocols mentioned above.

4.2.8 Statistical analysis

A one-way ANOVA test was applied to the obtained data, and statistically significant differences ($P < 0.001$) in the antibody titers of the same state's or group's antisera were demonstrated. For pair comparisons within groups, Tukey's test was applied. Data, from tick infestations on the vaccinated mice, except for the engorged body weight, were statistically analyzed using a Chi-square test. Analysis of the engorged body weight was done using Welch's *t*-test. $P < 0.05$ and $P < 0.01$ were considered to be statistically significant differences.

4.3 Result

4.3.1 rHlPrx2 protein expression and purification

rHlPrx2 proteins were expressed in *E. coli* as inclusion bodies and purified using the Biologic DuoFlow™ Chromatography System. The *E. coli* lysate and purified rHlPrx2 proteins were analyzed in SDS-PAGE. SDS-PAGE demonstrated a single band with a molecular mass of approximately 27 kDa including His-tag (Fig. 4.1, arrowhead).

The endotoxin levels of the final purified samples were less than 0.02 endotoxin units (EU) per 30 μg of purified rHlPrx2, which were used for vaccinating mice (Table 4.1). In addition, Brito and Singh (2011) [76] stated that endotoxin levels in the vaccine type of recombinant subunit for preclinical research of less than 20 EU/ml are acceptable. These results suggest that the rHlPrx2 was pure and has an acceptable endotoxin level for vaccinating mice.

4.3.2 Antibody titers after immunization

Titers of rHlPrx2 with or without an IFA group (rHlPrx2+IFA or rHlPrx2) using total IgG secondary antibodies significantly increased after the administration and booster or the administration, respectively. However, the titer post tick infestation was almost the same as the booster (Fig. 4.2A, rHlPrx2 and rHlPrx2+IFA). The total IgG antibody titers of the rHlPrx2 and rHlPrx2+IFA groups were significantly higher than those of the control groups (PBS and PBS+IFA) after the administration, booster, and tick infestation. In addition, the total IgG antibody titers of the rHlPrx2+IFA group showed higher titers as compared with the rHlPrx2 group in all states (Fig. 4.2A). Using the IgG1 isotype as a marker for Th2 lymphocytes, the antibody titers showed almost the same transition with the total IgG transition (Fig. 4.2B). Using the IgG2a isotype as a marker for Th1 lymphocytes, although the antibody titers of rHlPrx2+IFA group

significantly increased after the administration and booster, the titer post tick infestation had almost the same levels as post booster (Fig. 4.2C, rHlPrx2+IFA). Interestingly, the titers of the rHlPrx2 group increased slightly after the booster but had almost the same titer levels post tick infestation (Fig. 4.2C, rHlPrx2). The IgG2a antibody titers of the rHlPrx2+IFA group were significantly higher than those of the control groups after administration, booster, and tick infestation. On the other hand, the IgG2a antibody titers of the rHlPrx2 group showed significantly, but slightly higher titers as compared with those of control groups post booster and tick infestation (Fig. 4.2C).

IgG1 titers were higher than IgG2a titers (Fig. 4.2B, C). In addition, the total IgG and IgG1 antibody titers showed almost the same trend in the rHlPrx2 group (Fig. 4.2A, B). These results suggest that rHlPrx2 could induce especially a Th2—but not a Th1—immune response. Moreover, vaccination using rHlPrx2 alone could induce high antibody titers of total IgG and IgG1 titers as compared with those of control groups, indicating that the rHlPrx2 protein could be highly immunogenic to the host.

4.3.3 The recognition of native HlPrx2 protein in the crude tick protein sample using immunized mouse sera

I also evaluated whether rHlPrx2-immunized mouse sera could recognize native HlPrx2 protein from a crude tick protein sample using Western blot analysis. The post tick infestation state in each group showed the highest antibody titers. For this reason, I used those sera from the same mouse as the first antibody in each immunization state. The partially fed tick midgut proteins were used as crude tick protein samples, since the native HlPrx2 proteins were significantly upregulated in the midgut during the partially fed stage of tick blood feeding, and the HlPrx2 protein expression levels had almost the same level and trend in nymphs and adults [13]. In the rHlPrx2+IFA immunized group, the native HlPrx2 protein could be detected after the administration of the rHlPrx2 protein (Fig. 4.3, rHlPrx2+IFA). In addition, the rHlPrx2-immunized group's sera could detect the native HlPrx2 protein after the booster (Fig. 4.3, rHlPrx2). The control immunized groups' sera could not detect the native HlPrx2 protein (Fig. 4.3, PBS and PBS+IFA). These results suggest that antibodies in the mice induced by rHlPrx2 protein immunization can bind to recognize the native HlPrx2 protein; therefore, the rHlPrx2 proteins used could have retained almost the same structure of the native HlPrx2 protein in mice.

4.3.4 Effects of immunization on H. longicornis *nymphal ticks*

After immunization twice with rHlPrx2, mice were challenged with nymphal ticks. Twenty ticks were infested on each mouse and allowed to feed until engorgement. However, there were no significant differences in the engorged rate, engorged body weight, molting rate, and survival rate after molting among ticks detached from vaccinated mice (Table 4.2). These results suggest that immunization with rHlPrx2 had little or no effect on the blood feeding of nymphal ticks.

4.4 Discussion

In endoparasites, Prxs have been shown to be the most important detoxifying enzyme for their survival [40,41], making it a candidate for use in vaccine development and as a therapeutic target in treating endoparasitic infectious diseases [54,56]. Studies using Prxs as vaccines have been performed with *L. donovani* [62] and *F. hepatica* [60,61,63]. Although there have not been studies reporting on tick Prx vaccination and host immune responses against tick Prxs, *H. longicornis* 1-Cys Prx protein (HlPrx), in the same family as HlPrx2, was detected in host sera infested with several ticks over a short interval [15]. These results suggest that tick Prxs may be secreted into their hosts and could be a candidate for both vaccine and immunotherapeutic development.

Therefore, I performed the vaccine experiment in mice using the recombinant tick Prx protein (rHlPrx2) in the present study.

Prxs have been studied for their role in parasite survival and virulence necessitating the production of efficient defenses against ROS by the host immune system [41,53,77]. In addition, *L. donovani* Prx is an antigen that elicited a high level of IgG1 as a marker of Th2 lymphocytes, but not IgG2a as a marker of Th1 lymphocytes [62]. In the present study, I evaluated the vaccine efficacy of rHlPrx2 in mice using rHlPrx2 with or without IFA. The results demonstrated that the vaccine with or without IFA led to almost the same antibody titer against total IgG and IgG1 as a marker of Th2 lymphocytes. On the other hand, IgG2a as a marker for Th1 lymphocytes was low titer in the rHlPrx2 without IFA vaccinated groups. These phenomena suggest that the induction of the host's Th2 immune response could lead to low levels of the host Th1 immune response; thus, the parasites could escape from the host's Th1 immune response through, for instance, migrations of macrophages, dendritic cells, and neutrophils to eliminate the parasites.

I also evaluated the vaccine efficacy for the challenge of nymphal ticks to the immunized mice. However, the effects of rHlPrx2 vaccination against ticks' engorgement success rate, engorged body weight, molting rate to adult stage, and

survival rate after molting were small or nonexistent. There have been a few reports regarding tick Prxs. Anti-HlPrx antibodies were detected in the host serum after several repeated tick infestations [15], suggesting that tick Prxs might be released from ticks into the host and that the amount of released HlPrx protein was quite small, since several infestations of ticks were needed for the anti-HlPrx antibody to be successfully detected. In addition, another report [62] and the present study (Fig. 4.2) have demonstrated that Prxs without the adjuvant could induce high antibody titers, especially in Th2 immune responses, such as that of IgG1. The IgG1 antibody response is promoted by the Th2 immune response, which is counterbalanced by a Th1 immune response [78]. In the tick challenge experiment, the induction of the Th2 immune response in mice by rHlPrx2 immunization and tick infestations could lead to low levels of the host Th1 immune response; therefore, ticks would escape host Th1 immune responses. These results demonstrated that immunization with the rHlPrx2 protein would have little to no effect on nymphal ticks during and after blood feeding.

Although the effects of rHlPrx2 immunization had little to no effect, I demonstrated that immunization with the rHlPrx2 protein could only induce high and acceptable antibody titers, even as compared with the immunization with the rHlPrx2 protein with IFA. This phenomenon was considered to be the reason that the reduced

2-Cys Prxs are typically in the form of decamers arranged in a ring-like toroid structure. During peroxidatic cycling, decamers dissociate into dimers upon disulfide formation and are regained upon disulfide reduction [5,79]. These results suggest that 2-Cys Prxs without the adjuvant could induce high antibody titers because 2-Cys Prxs can form multimers. Actually, the rHlPrx2 proteins form an oligomer [14]. On the other hand, immunization with rHlPrx2 proteins especially stimulated IgG1 antibodies related to Th2 immune responses (Fig. 4.2). In helminth parasites, helminth infection induces M2 macrophages, related to Th2 immune responses, to the site of the infection, and the helminth recombinant Prx, which is inoculated intraperitoneally to mice, also induces M2 macrophages [60]. Moreover, M2 macrophages can promote differentiation from Th0 to Th2 lymphocytes [80]. Thus, rHlPrx2 proteins might induce M2 macrophages and stimulate the host Th2 lymphocytes in vaccinated mice.

In animal models challenged with pathogens such as *Coccidioides immitis* [81], *Listeria monocytogenes* [82], *Schistosoma mansoni* [83], respiratory syncytial virus [84], and *Candida albicans* [85], vaccines inducing Th1 immune responses have been proven highly effective at preventing infections, whereas vaccines inducing Th2 immune responses increase sensitivity to infection. Therefore, although Th1 immune responses are key to protecting against most infections, the vaccines and passive immunization

rely on Th2 immune responses [86]. Under experimental conditions, Daifalla et al. (2011) [62] demonstrated the use of adjuvants, such as the Toll-like receptor 9 (TLR-9) agonist (CpG ODN) or the TLR-4 agonist (GLA-SE), to augment the immunogenicity of the recombinant *L. donovani* 2-Cys Prx (Prx4) in BALB/c mice. In addition, these immunizations led to increased immune responses to Th1 as high levels of IgG2a antibody titers were induced as well as of IgG1 antibody titers [62]. Thus, rHlPrx2 proteins with CpG ODN and/or GLA-SE could lead to an increased Th1 immune response and might be still considered as vaccine candidates against ticks because of their high immunogenicity.

In summary, I demonstrated that rHlPrx2 could induce high antibody titers of IgG1 related to a Th2 immune response. I also observed that rHlPrx2-immunized mouse sera could recognize native HlPrx2 protein in crude tick midgut proteins by Western blotting. Moreover, the effects of rHlPrx2 immunization in mice were studied using nymphal ticks, but the challenged ticks were not affected by rHlPrx2 immunization. Although the effects of rHlPrx2 immunization did not affect ticks in the present study, rHlPrx2 might still be considered a vaccine candidate against ticks because of its high immunogenicity and the possibility that the combination of Th1 immune response inducible adjuvants might improve my strategy of vaccine development against ticks.

Tables and Figures in CHAPTER 4

Table 4.1. Endotoxin levels of rHlPrx2

rHlPrx2 used for	Endotoxin levels (EU/30 μg of rHlPrx2)
Administration	0.010027 ± 0.000112
Booster	0.010216 ± 0.000156
PBS (Solvent for rHlPrx2 proteins)	0.000377 ± 0.000011

PBS, Phosphate Buffered Saline, which was used for a solvent of recombinant proteins; rHlPrx2, recombinant *Haemaphysalis longicornis* 2-Cys Peroxiredoxin; EU/30 μg of rHlPrx2. The endotoxin levels were calculated as one administration of rHlPrx2 to each mouse.

Table 4.2. Effects on nymphal ticks of rHlPrx2 immunization in mice

Antigen	Engorged rate (%)	Engorged body weight (mg)	Molting rate (%)	Survival rate (%)
PBS	96.7	4.5 ± 0.5	72.4	100
PBS + IFA	96.7	4.6 ± 0.4	82.8	95.8
rHlPrx2	92.9	4.8 ± 0.4	80.0	100
rHlPrx2 + IFA	95.7	4.8 ± 0.3	90.9	90

PBS, Phosphate Buffered Saline; IFA, Incomplete Freund's Adjuvant; rHlPrx2, recombinant *Haemaphysalis longicornis* 2-Cys peroxiredoxin; Engorged rate, the total number of the ticks that successfully engorged divided by the number of infested ticks; Molting rate, the total number of molted nymphal ticks to adult ticks divided by the number of successful engorged ticks; Survival rate, the survival rate after molting from nymphal to adult ticks.

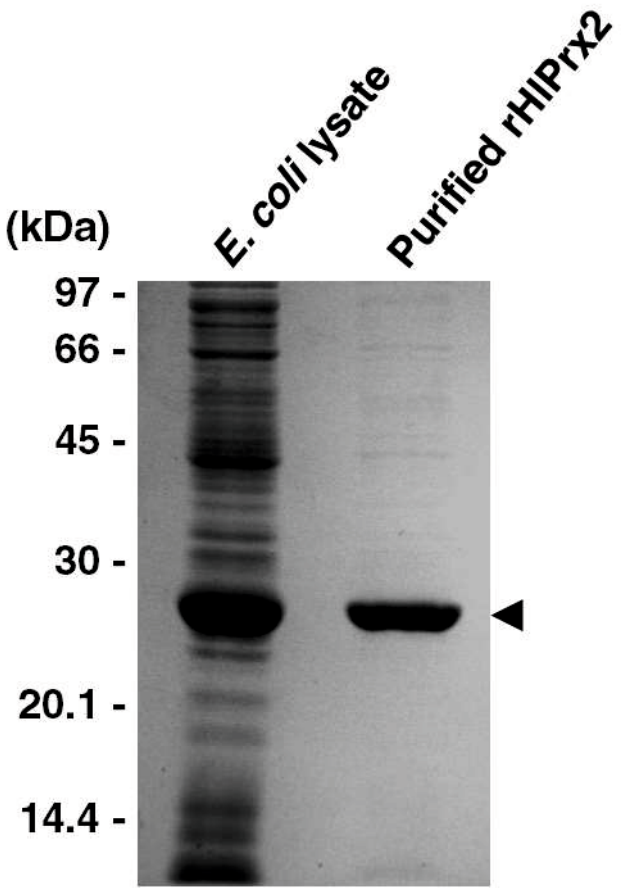

Fig. 4.1. Purification of recombinant *Haemaphysalis longicornis* 2-Cys peroxiredoxin (rHlPrx2). rHlPrx2 was expressed in *E. coli* and purified by fast protein liquid chromatography (FPLC). The *E. coli* lysate and purified rHlPrx2 were analyzed by SDS-PAGE and CBB staining. The molecular weight is shown on the left side of the image. The arrowhead indicates a band of rHlPrx2 protein.

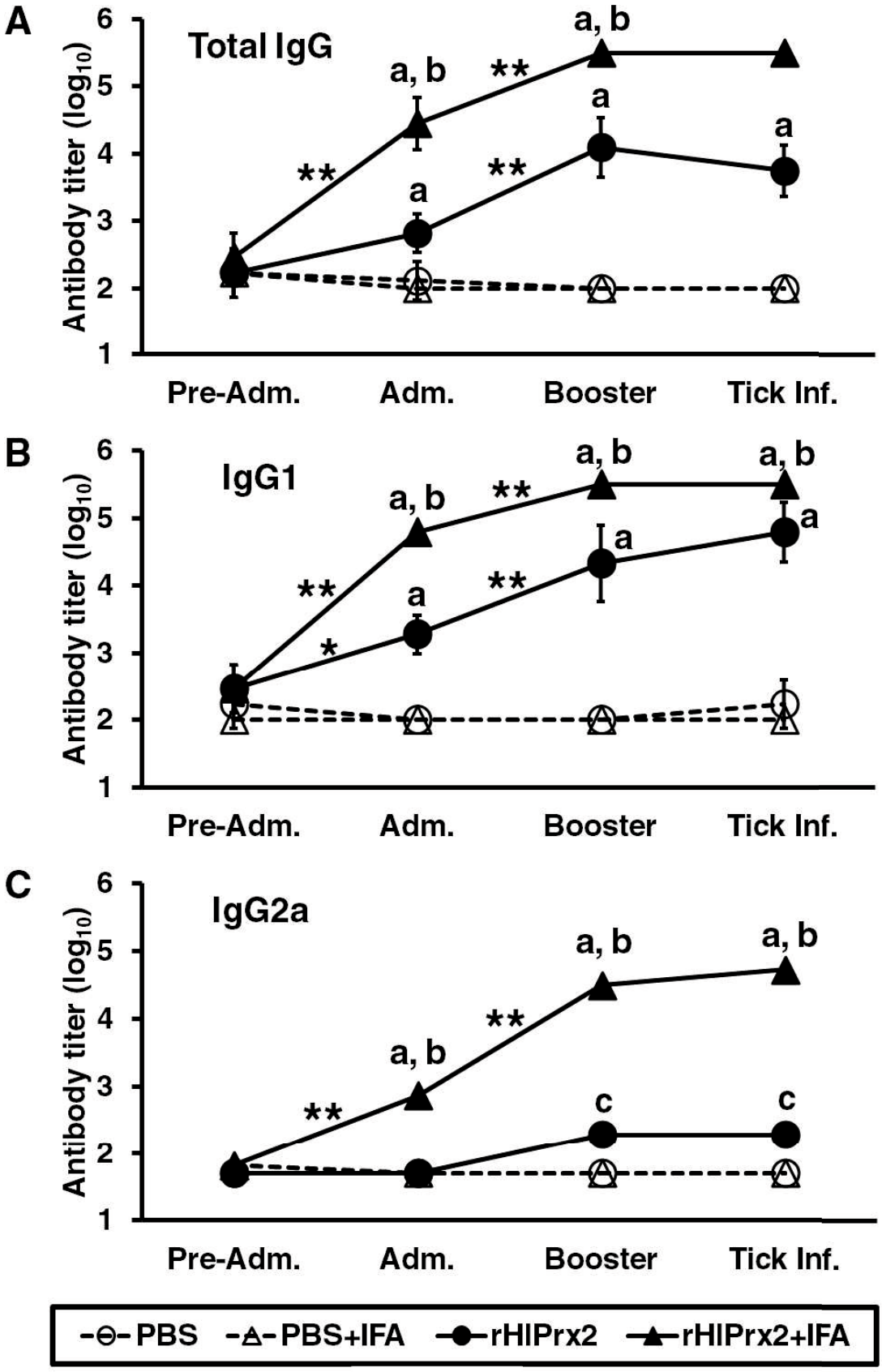
A
Total IgG
Antibody titer (log_{10})
6
5
4
3
2
1
a, b
a, b
**
**
**
a
a
a
Pre-Adm.
Adm.
Booster
Tick Inf.
B
IgG1
Antibody titer (log_{10})
a, b
a, b
a, b
**
**
**
*
a
a
a
Pre-Adm.
Adm.
Booster
Tick Inf.
C
IgG2a
Antibody titer (log_{10})
a, b
a, b
**
**
a, b
c
c
Pre-Adm.
Adm.
Booster
Tick Inf.
PBS
PBS+IFA
rHlPrx2
rHlPrx2+IFA

Fig. 4.2.

Fig. 4.2. The trend of the antibody titer in immunized mouse sera. Antibody titers were defined as the dilution rate (log_{10}) under $0.5 < OD_{450nm}$. Pre-Adm., Pre-Administration; Adm., 1st administration after one week; Booster, 2nd administration after one week; Tick Inf., after Tick infestation; [a]$P < 0.01$, significant differences vs. PBS and PBS+IFA groups; [b]$P < 0.01$, significant differences between rHlPrx2+IFA and rHlPrx2 groups; [c]$P < 0.05$, significant differences vs. PBS and PBS+IFA groups; [*]$P < 0.05$, significant differences between the states of immunization; [**]$P < 0.01$, significant differences between the states of immunization. PBS, Phosphate Buffered Saline; IFA, Incomplete Freund's Adjuvant; rHlPrx2, recombinant *Haemaphysalis longicornis* 2-Cys peroxiredoxin.

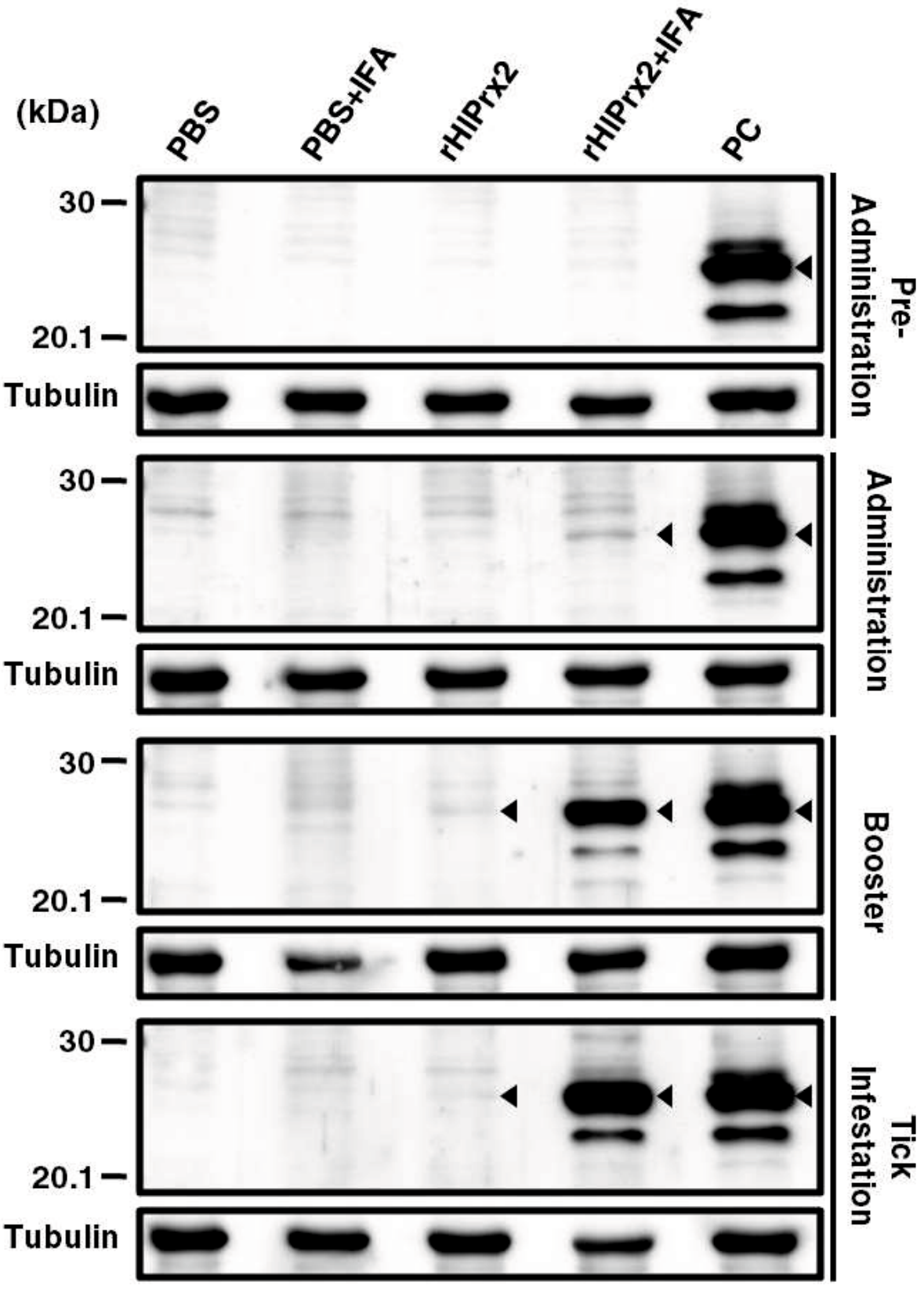
(kDa)
PBS
PBS+IFA
rHIPrx2
rHIPrx2+IFA
PC
30
20.1
Tubulin
Pre-Administration
Administration
Booster
Tick Infestation

Fig. 4.3.

Fig. 4.3. Detection of native HlPrx2 protein from partially fed ticks' midguts using rHlPrx2-immunized mouse sera. The left column indicates the molecular weight markers at 30 and 20.1 kDa. Tick tubulin protein served as the control. The right side indicates different stages of immunization. The serum from the different stages of immunization was from the same mouse. The anti-rHlPrx2 serum used for a positive control (PC) was from the previous study [13]. The arrowheads indicate the bands of native HlPrx2 protein having a molecular mass of approximately 23 kDa. PBS, Phosphate Buffered Saline; IFA, Incomplete Freund's Adjuvant; rHlPrx2, recombinant *Haemaphysalis longicornis* 2-Cys peroxiredoxin; PC, Positive control.

SUMMARY AND CONCLUSION

Ticks lack heme synthesis and catabolism pathways because they are unable to produce σ-aminolevulinic acid, a heme precursor, even at genomic levels [46,47]. Therefore, they rely on heme from their host and heme stored in hemosomes of the midgut without digestion [48]. These facts suggest that ticks might have difficulty producing proteins that contain heme, such as catalase and peroxidase, which are both H_2O_2-scavenging enzymes [17]. Moreover, ticks must acquire nutrients from the host blood meal and metabolize these nutrients via catabolism and anabolism [49]. Plasmodium parasites also take in nourishment from host blood and are likely to utilize members of the Prx family as the principal enzymes for reducing peroxides, including H_2O_2, because they lack catalase and peroxidase [40]. Therefore, Prxs might be similarly essential for regulating the H_2O_2 concentration in ticks.

In Chapter 1, I identified a 2-Cys Prx gene from *H. longicornis* (HlPrx2) and prepared some kinds of recombinant proteins of HlPrx2 (rHlPrx2), including three mutants. I determined that Cys51 is necessary for the antioxidant activity of rHlPrx2 for removing H_2O_2, and both Cys51 and Cys172 are essential for the thioredoxin-dependent activity. Moreover, either Cys51 or Cys172 is critical for the dimerization of rHlPrx2

but not for oligomerization. Therefore, HlPrx2 may play an important role in the antioxidant activity, including chaperone activity, in ticks.

In Chapter 2, mRNA and protein expression profiles of HlPrx2 and the localization of this protein in tick tissues were investigated. Real-time PCR showed that *HlPrx2* gene expression in whole bodies and internal organs was significantly upregulated during blood-feeding. However, protein expression was constant throughout blood-feeding. Moreover, a knockdown experiment of *HlPrx2* was performed using RNAi to evaluate its function in ticks. The knockdown of the *HlPrx2* gene caused significant differences in body weight, egg weight and hatching rate in engorged ticks as compared to those of the control group. Finally, the detection of H_2O_2 after the double knockdown of *HlPrxs* in ticks showed that H_2O_2 concentration increased before and after blood-feeding. Therefore, HlPrx2 can be considered important for successful blood-feeding and reproduction through the regulation of H_2O_2 concentrations in ticks during blood-feeding.

In Chapter 3, I observed that paraquat could induce H_2O_2 in ISE6 cells as an oxidative stress inducer. In addition, I established the H_2O_2 detection method in ISE6 cells using an intracellular H_2O_2 probe (BES-H_2O_2-Ac). Moreover, the gene silencing of *IsPrxs* led to high levels of H_2O_2 in ISE6 cells under the microplate reader and the

fluorescence microscope. These results suggest that paraquat acts as an H_2O_2 inducer, and *Prx* genes are important for the regulation of the H_2O_2 concentration in ISE6 cells.

In Chapter 4, I demonstrated that rHlPrx2 could induce high antibody titers of IgG1 related to a Th2 immune response. I also observed that rHlPrx2-immunized mouse sera could recognize native HlPrx2 protein in crude tick midgut proteins by Western blotting. Moreover, the effects of rHlPrx2 immunization in mice were studied using nymphal ticks, but the challenged ticks were not affected by rHlPrx2 immunization. Although the effects of rHlPrx2 immunization did not affect ticks in the present study, rHlPrx2 might still be considered a vaccine candidate against ticks because of its high immunogenicity and the possibility that the combination of Th1 immune response inducible adjuvants might improve my strategy of vaccine development against ticks.

In conclusion, Prxs are multifunctional molecules related to antioxidants and immunity in organisms. In parasites such as nematodes, Prxs play a role in regulating the host immune response for ticks' survival in the host body. Prxs in the hard ticks are considered to be multifunctional molecules. Taken altogether, the results in this dissertation indicate that tick Prxs can be important for successful blood feeding and reproduction through the regulation of H_2O_2 concentrations in ticks during blood feeding. These evidences contribute to the search for furthers understanding of the tick's

oxidative stress coping mechanism during blood feeding and a candidate target for tick control.

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References

1. Sies, H. (1991). Oxidative stress: from basic research to clinical application. *Am. J. Med.*, ***91***, 31S-38S.

2. Halliwell, B., Gutteridge, J. M. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.*, ***246***, 501-514.

3. Robinson, M. W., Hutchinson, A. T., Dalton, J. P., Donnelly, S. (2010). Peroxiredoxin: a central player in immune modulation. *Parasite Immunol.*, ***32***, 305-313.

4. Rhee, S. G. (2006). Cell signaling. H_2O_2, a necessary evil for cell signaling. *Science*, ***312***, 1882-1883.

5. Hall, A., Nelson, K., Poole, L. B., Karplus, P. A. (2011). Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. *Antioxid. Redox. Signal.*, ***15***, 795-815.

6. Chae, H. Z., Kim, H. J., Kang, S. W., Rhee, S. G. (1999). Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res. Clin. Pract.*, ***45***, 101-112.

7. Lee, T.-H., Kim, S.-U., Yu, S.-L., Kim, S. H., Park, D. S., Moon, H.-B., Dho, S. H., Kwon, K.-S., Kwon, H. J., Han, Y.-H., Jeong, S., Kang, S. W., Shin, H.-S., Lee, K.-K., Rhee, S. G., Yu, D.-Y. (2003). Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. *Blood*, ***101***, 5033-5038.

8. Grandjean, O. (1983). Blood digestion in *Ornithodorus moubata* Murray sensu stricto Walton females (Ixodoidea: Argasidae) II. Modifications of midgut cells related to the digestive cycle and to the triggering action of mating. *Ann. Parasitol. Hum. Comp.*, ***58***, 493-514.

9. Mori, H., Galay, R. L., Maeda, H., Matsuo, T., Umemiya-Shirafuji, R., Mochizuki, M., Fujisaki, K., Tanaka, T. (2014). Host-derived transferrin is maintained and transferred from midgut to ovary in *Haemaphysalis longicornis* ticks. *Ticks Tick Borne Dis.*, ***5***, 121-126.

10. Galay, R. L., Umemiya-Shirafuji, R., Bacolod, E. T., Maeda, H., Kusakisako, K., Koyama, J., Tsuji, N., Mochizuki, M., Fujisaki, K., Tanaka, T. (2014). Two kinds of ferritin protect ixodid ticks from iron overload and consequent oxidative stress. *PLoS ONE*, ***9***, e90661.

11. Citelli, M., Lara, F. A., da Silva Vaz, I., Oliveira, P. L. (2007). Oxidative stress impairs heme detoxification in the midgut of the cattle tick, Rhipicephalus (Boophilus) microplus. *Mol. Biochem. Parasitol.*, ***151***, 81-88.

12. Kumar, D., Budachetri, K., Meyers, V. C., Karim, S. (2016). Assessment of tick antioxidant responses to exogenous oxidative stressors and insight into the role of catalase in the reproductive fitness of the Gulf Coast tick, *Amblyomma maculatum*. *Insect Mol. Biol.*, ***25***, 283-294.

13. Kusakisako, K., Galay, R. L., Umemiya-Shirafuji, R., Hernandez, E. P., Maeda, H., Talactac, M. R., Tsuji, N., Mochizuki, M., Fujisaki, K., Tanaka, T. (2016). 2-Cys peroxiredoxin is required in successful blood-feeding, reproduction, and antioxidant response in the hard tick *Haemaphysalis longicornis*. *Parasit. Vectors*, ***9***, 457.

14. Kusakisako, K., Masatani, T., Miyata, T., Galay, R. L., Maeda, H., Talactac, M. R., Tsuji, N., Mochizuki, M., Fujisaki, K., Tanaka, T. (2016). Functional analysis of recombinant 2-Cys peroxiredoxin from the hard tick *Haemaphysalis longicornis*. *Insect Mol. Biol.*, ***25***, 16-23.

15. Tsuji, N., Kamio, T., Isobe, T., Fujisaki, K. (2001). Molecular characterization of a peroxiredoxin from the hard tick *Haemaphysalis longicornis*. *Insect Mol. Biol.*, ***10***, 121-129.

16. Wood, Z. A., Schröder, E., Robin Harris, J., Poole, L. B. (2003). Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.*, ***28***, 32-40.

17. Mitozo, P. A., de Souza, L. F., Loch-Neckel, G., Flesch, S., Maris, A. F., Figueiredo, C. P., Dos Santos, A. R. S., Farina, M., Dafre, A. L. (2011). A study of the relative importance of the peroxiredoxin-, catalase-, and glutathione-dependent systems in neural peroxide metabolism. *Free Radic. Biol. Med.*, ***51***, 69-77.

18. Hofmann, B., Hecht, H.-J., Flohé, L. (2002). Peroxiredoxins. *Biol. Chem.*, ***383***, 347-364.

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

20. Seo, M. S., Kang, S. W., Kim, K., Baines, I. C., Lee, T. H., Rhee, S. G. (2000). Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate. *J. Biol. Chem.*, ***275***, 20346-20354.

21. Choi, H. J., Kang, S. W., Yang, C. H., Rhee, S. G., Ryu, S. E. (1998). Crystal structure of a novel human peroxidase enzyme at 2.0 A resolution. *Nat. Struct. Biol.*, ***5***, 400-406.

22. Boldbaatar, D., Umemiya-Shirafuji, R., Liao, M., Tanaka, T., Xuan, X., Fujisaki, K. (2010). Multiple vitellogenins from the *Haemaphysalis longicornis* tick are crucial for ovarian development. *J. Insect Physiol.*, ***56***, 1587-1598.

23. Kelley, L. A., Sternberg, M. J. E. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.*, ***4***, 363-371.

24. Maeda, H., Kurisu, K., Miyata, T., Kusakisako, K., Galay, R. L., Rio, T. M., Mochizuki, M., Fujisaki, K., Tanaka, T. (2015). Identification of the *Babesia*-responsive leucine-rich repeat domain-containing protein from the hard tick *Haemaphysalis longicornis*. *Parasitol. Res.* ***114***, 1793-1802.

25. Sauri, H., Butterfield, L., Kim, A., Shau, H. (1995). Antioxidant function of recombinant human natural killer enhancing factor. *Biochem. Biophys. Res. Commun.*, ***208***, 964-969.

26. Masatani, T., Asada, M., Ichikawa-Seki, M., Usui, M., Terkawi, M. A., Hayashi, K., Kawazu, S.-I., Xuan, X. (2014). Cloning and characterization of a 2-Cys peroxiredoxin from *Babesia gibsoni*. *J. Vet. Med. Sci.*, ***76***, 139-143.

27. Low, F. M., Hampton, M. B., Peskin, A. V., Winterbourn, C. C. (2007). Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte. *Blood*, ***109***, 2611-2617.

28. Kim, J.-A., Park, S., Kim, K., Rhee, S. G., Kang, S. W. (2005). Activity assay of mammalian 2-cys peroxiredoxins using yeast thioredoxin reductase system. *Anal. Biochem.*, ***338***, 216-223.

29. Miyata, T., Harakuni, T., Tsuboi, T., Sattabongkot, J., Ikehara, A., Tachibana, M., Torii, M., Matsuzaki, G., Arakawa, T. (2011). Tricomponent immunopotentiating system as a novel molecular design strategy for malaria vaccine development. *Infect. Immun.*, ***79***, 4260-4275.

30. Moon, J. C., Hah, Y.-S., Kim, W. Y., Jung, B. G., Jang, H. H., Lee, J. R., Kim, S. Y., Lee, Y. M., Jeon, M. G., Kim, C. W., Cho, M. J., Lee, S. Y. (2005). Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H_2O_2-induced cell death. *J. Biol. Chem.*, ***280***, 28775-28784.

31. Matte, A., Bertoldi, M., Mohandas, N., An, X., Bugatti, A., Brunati, A. M., Rusnati, M., Tibaldi, E., Siciliano, A., Turrini, F., Perrotta, S., De Franceschi, L.

(2013). Membrane association of peroxiredoxin-2 in red cells is mediated by the N-terminal cytoplasmic domain of band 3. *Free Radic. Biol. Med.*, ***55***, 27-35.

32. Schröder, E., Littlechild, J. A., Lebedev, A. A., Errington, N., Vagin, A. A., Isupov, M. N. (2000). Crystal structure of decameric 2-Cys peroxiredoxin from human erythrocytes at 1.7 A resolution. *Structure*, ***8***, 605-615.

33. Hall, A., Karplus, P. A., Poole, L. B. (2009). Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. *FEBS J.*, ***276***, 2469-2477.

34. Kim, K., Kim, I. H., Lee, K. Y., Rhee, S. G., Stadtman, E. R. (1988). The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O_2 mixed-function oxidation system. *J. Biol. Chem.*, ***263***, 4704-4711.

35. Koshkin, A., Knudsen, G. M., Ortiz De Montellano, P. R. (2004). Intermolecular interactions in the AhpC/AhpD antioxidant defense system of *Mycobacterium tuberculosis*. *Arch. Biochem. Biophys.*, ***427***, 41-47.

36. Liao, S.-J., Yang, C.-Y., Chin, K.-H., Wang, A. H.-J., Chou, S.-H. (2009). Insights into the alkyl peroxide reduction pathway of *Xanthomonas campestris* bacterioferritin comigratory protein from the trapped intermediate-ligand complex structures. *J. Mol. Biol.*, ***390***, 951-966.

37. Wang, X., Wang, L., Wang, X., Sun, F., Wang, C. (2012). Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4. *Biochem. J.*, ***441***, 113-118.

38. König, J., Galliardt, H., Jütte, P., Schäper, S., Dittmann, L., Dietz, K.-J. (2013). The conformational bases for the two functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone. *J. Exp. Bot.*, ***64***, 3483-3497.

39. Emsley, P., Lohkamp, B., Scott, W. G., Cowtan, K. (2010). Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.*, ***66***, 486-501.

40. Kawazu, S., Komaki-Yasuda, K., Oku, H., Kano, S. (2008). Peroxiredoxins in malaria parasites: parasitologic aspects. *Parasitol. Int.*, ***57***, 1-7.

41. Dalton, J. P., Robinson, M. W., Mulcahy, G., O'Neill, S. M., Donnelly, S. (2013). Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. *Vet. Parasitol.*, ***195***, 272-285.

42. Fujisaki, K. (1978). Development of acquired resistance precipitating antibody in rabbits experimentally infested with females of *Haemaphysalis longicornis* (Ixodoidea: Ixodidae). *Natl. Inst. Anim. Health Q (Tokyo)*, ***18***, 27-38.

43. Umemiya-Shirafuji, R., Tanaka, T., Boldbaatar, D., Tanaka, T., Fujisaki, K. (2012). Akt is an essential player in regulating cell/organ growth at the adult stage

in the hard tick *Haemaphysalis longicornis*. *Insect Biochem. Mol. Biol.*, ***42***, 164-173.

44. Galay, R. L., Takechi, R., Umemiya-Shirafuji, R., Talactac, M. R., Maeda, H., Kusakisako, K., Mochizuki, M., Fujisaki, K., Tanaka, T. (2016). Impaired cellular immune response to injected bacteria after knockdown of ferritin genes in the hard tick *Haemaphysalis longicornis*. *Parasitol. Int.*, ***65***, 251-257.

45. Oba, Y., Yoshida, M., Shintani, T., Furuhashi, M., Inouye, S. (2012). Firefly luciferase genes from the subfamilies Psilocladinae and Ototretinae (Lampyridae, Coleoptera). *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, ***161***, 110-116.

46. Braz, G. R., Coelho, H. S., Masuda, H., Oliveira, P. L. (1999). A missing metabolic pathway in the cattle tick *Boophilus microplus*. *Curr. Biol.*, ***9***, 703-706.

47. Perner, J., Sobotka, R., Sima, R., Konvickova, J., Sojka, D., Oliveira, P. L. de, Hajdusek, O., Kopacek, P. (2016). Acquisition of exogenous haem is essential for tick reproduction. *elife,* ***5***, e12318.

48. Lara, F. A., Lins, U., Paiva-Silva, G., Almeida, I. C., Braga, C. M., Miguens, F. C., Oliveira, P. L., Dansa-Petretski, M. (2003). A new intracellular pathway of haem detoxification in the midgut of the cattle tick *Boophilus microplus*: aggregation inside a specialized organelle, the hemosome. *J. Exp. Biol.*, ***206***, 1707-1715.

49. Tsuji, N., Miyoshi, T., Battsetseg, B., Matsuo, T., Xuan, X., Fujisaki, K. (2008). A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. *PLoS Pathog.*, ***4***, e1000062.

50. Kitaoka, S. (1961). Physiological and ecological studies on some ticks. VII. Parthenogeneticand bisexual races of *Haemaphysalis bispinosa* in Japan and experimental crossing between them. *Natl. Inst. Anim. Health Q. (Tokyo)*, ***1***, 142-149.

51. Avarre, J.-C., Lubzens, E., Babin, P. J. (2007). Apolipocrustacein, formerly vitellogenin, is the major egg yolk precursor protein in decapod crustaceans and is homologous to insect apolipophorin II/I and vertebrate apolipoprotein B. *BMC Evol. Biol.*, ***7***, 3.

52. Seehuus, S.-C., Norberg, K., Gimsa, U., Krekling, T., Amdam, G. V. (2006). Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc. Natl. Acad. Sci. U S A*, ***103***, 962-967.

53. Sekiya, M., Mulcahy, G., Irwin, J. A., Stack, C. M., Donnelly, S. M., Xu, W., Collins, P., Dalton, J. P. (2006). Biochemical characterisation of the recombinant peroxiredoxin (FhePrx) of the liver fluke, *Fasciola hepatica*. *FEBS Lett.*, ***580***, 5016-5022.

54. Rudenko, N., Golovchenko, M., Edwards, M. J., Grubhoffer, L. (2005). Differential expression of *Ixodes ricinus* tick genes induced by blood feeding or *Borrelia burgdorferi* infection. *J. Med. Entomol.*, ***42***, 36-41.

55. Narasimhan, S., Sukumaran, B., Bozdogan, U., Thomas, V., Liang, X., DePonte, K., Marcantonio, N., Koski, R. A., Anderson, J. F., Kantor, F., Fikrig, E. (2007). A tick antioxidant facilitates the Lyme disease agent's successful migration from the mammalian host to the arthropod vector. *Cell Host Microbe.*, ***2***, 7-18.

56. Peterson, T. M. L., Luckhart, S. (2006). A mosquito 2-Cys peroxiredoxin protects against nitrosative and oxidative stresses associated with malaria parasite infection. *Free Radic. Biol. Med.*, ***40***, 1067-1082.

57. Zhang, L., Lu, Z. (2015). Expression, purification and characterization of an atypical 2-Cys peroxiredoxin from the silkworm, *Bombyx mori*. *Insect Mol. Biol.*, ***24***, 203-212.

58. Hu, Z., Lee, K. S., Choo, Y. M., Yoon, H. J., Lee, S. M., Lee, J. H., Kim, D. H., Sohn, H. D., Jin, B. R. (2010). Molecular cloning and characterization of 1-Cys and 2-Cys peroxiredoxins from the bumblebee *Bombus ignitus*. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, ***155***, 272-280.

59. Veal, E. A., Day, A. M., Morgan, B. A. (2007). Hydrogen peroxide sensing and signaling. *Mol. Cell*, ***26***, 1-14.

60. Donnelly, S., Stack, C. M., O'Neill, S. M., Sayed, A. A., Williams, D. L., Dalton, J. P. (2008). Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. *FASEB J.*, ***22***, 4022-4032.

61. Mendes, R. E., Pérez-Ecija, R. A., Zafra, R., Buffoni, L., Martínez-Moreno, A., Dalton, J. P., Mulcahy, G., Pérez, J. (2010). Evaluation of hepatic changes and local and systemic immune responses in goats immunized with recombinant Peroxiredoxin (Prx) and challenged with *Fasciola hepatica*. *Vaccine*, ***28***, 2832-2840.

62. Daifalla, N. S., Bayih, A. G., Gedamu, L. (2011). Immunogenicity of *Leishmania donovani* iron superoxide dismutase B1 and peroxidoxin 4 in BALB/c mice: the contribution of Toll-like receptor agonists as adjuvant. *Exp. Parasitol.*, ***129***, 292-298.

63. Raina, O. K., Nagar, G., Varghese, A., Prajitha, G., Alex, A., Maharana, B. R., Joshi, P. (2011). Lack of protective efficacy in buffaloes vaccinated with *Fasciola*

gigantica leucine aminopeptidase and peroxiredoxin recombinant proteins. *Acta Trop.*, ***118***, 217-222.

64. Munderloh, U. G., Liu, Y., Wang, M., Chen, C., Kurtti, T. J. (1994). Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. *J. Parasitol.*, ***80***, 533-543.

65. Yoshii, K., Goto, A., Kawakami, K., Kariwa, H., Takashima, I. (2008). Construction and application of chimeric virus-like particles of tick-borne encephalitis virus and mosquito-borne Japanese encephalitis virus. *J. Gen. Virol.*, ***89***, 200-211.

66. Marengo, B., Nitti, M., Furfaro, A. L., Colla, R., Ciucis, C. D., Marinari, U. M., Pronzato, M. A., Traverso, N., Domenicotti, C. (2016). Redox homeostasis and cellular antioxidant systems: crucial players in cancer growth and therapy. *Oxid. Med. Cell Longev.* ***2016***, 6235641.

67. Winterbourn, C. C. (2008). Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.*, ***4***, 278-286.

68. Adimora, N. J., Jones, D. P., Kemp, M. L. (2010). A model of redox kinetics implicates the thiol proteome in cellular hydrogen peroxide responses. *Antioxid. Redox Signal.*, ***13***, 731-743.

69. Perkins, A., Nelson, K. J., Parsonage, D., Poole, L. B., Karplus, P. A. (2015). Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. *Trends Biochem. Sci.*, ***40***, 435-445.

70. Budachetri, K., Kumar, D., Karim, S. (2017). Catalase is a determinant of the colonization and transovarial transmission of *Rickettsia parkeri* in the Gulf Coast tick *Amblyomma maculatum*. *Insect Mol. Biol.*, ***26***, 414-419.

71. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, ***65***, 55-63.

72. Rhee, S. G., Chae, H. Z., Kim, K. (2005). Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic. Biol. Med.*, ***38***, 1543-1552.

73. Yamada, S., Ding, Y., Sasaguri, Y. (2012). Peroxiredoxin 4: critical roles in inflammatory diseases. *J. UOEH*, ***34***, 27-39.

74. Ding, Y., Yamada, S., Wang, K.-Y., Shimajiri, S., Guo, X., Tanimoto, A., Murata, Y., Kitajima, S., Watanabe, T., Izumi, H., Kohno, K., Sasaguri, Y. (2010). Overexpression of peroxiredoxin 4 protects against high-dose

streptozotocin-induced diabetes by suppressing oxidative stress and cytokines in transgenic mice. *Antioxid. Redox Signal.*, ***13***, 1477-1490.

75. Anisuzzaman, Islam, M. K., Alim, M. A., Miyoshi, T., Hatta, T., Yamaji, K., Matsumoto, Y., Fujisaki, K., Tsuji, N. (2012). Longistatin is an unconventional serine protease and induces protective immunity against tick infestation. *Mol. Biochem. Parasitol.*, ***182***, 45-53.

76. Brito, L. A., Singh, M. (2011). Acceptable levels of endotoxin in vaccine formulations during preclinical research. *J. Pharm. Sci.*, ***100***, 34-37.

77. Gretes, M. C., Poole, L. B., Karplus, P. A. (2012). Peroxiredoxins in parasites. *Antioxid. Redox Signal.*, ***17***, 608-633.

78. Yanase, N., Toyota, H., Hata, K., Yagyu, S., Seki, T., Harada, M., Kato, Y., Mizuguchi, J. (2014). OVA-bound nanoparticles induce OVA-specific IgG1, IgG2a, and IgG2b responses with low IgE synthesis. *Vaccine*, ***32***, 5918-5924.

79. Wood, Z. A., Poole, L. B., Hantgan, R. R., Karplus, P. A. (2002). Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins. *Biochemistry*, ***41***, 5493-5504.

80. Satoh, T., Takeuchi, O., Vandenbon, A., Yasuda, K., Tanaka, Y., Kumagai, Y., Miyake, T., Matsushita, K., Okazaki, T., Saitoh, T., Honma, K., Matsuyama, T.,

Yui, K., Tsujimura, T., Standley, D. M., Nakanishi, K., Nakai, K., Akira, S. (2010). The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat. Immunol.*, ***11***, 936-944.

81. Abuodeh, R. O., Shubitz, L. F., Siegel, E., Snyder, S., Peng, T., Orsborn, K. I., Brummer, E., Stevens, D. A., Galgiani, J. N. (1999). Resistance to *Coccidioides immitis* in mice after immunization with recombinant protein or a DNA vaccine of a proline-rich antigen. *Infect. Immun.*, ***67***, 2935-2940.

82. Miller, M. A., Skeen, M. J., Ziegler, H. K. (1995). Nonviable bacterial antigens administered with IL-12 generate antigen-specific T cell responses and protective immunity against *Listeria monocytogenes*. *J. Immunol.*, ***155***, 4817-4828.

83. Mountford, A. P., Anderson, S., Wilson, R. A. (1996). Induction of Th1 cell-mediated protective immunity to *Schistosoma mansoni* by co-administration of larval antigens and IL-12 as an adjuvant. *J. Immunol.*, ***156***, 4739-4745.

84. Li, X., Sambhara, S., Li, C. X., Ewasyshyn, M., Parrington, M., Caterini, J., James, O., Cates, G., Du, R. P., Klein, M. (1998). Protection against respiratory syncytial virus infection by DNA immunization. *J. Exp. Med.* ***188***, 681-688.

85. Cárdenas-Freytag, L., Cheng, E., Mayeux, P., Domer, J. E., Clements, J. D. (1999). Effectiveness of a vaccine composed of heat-killed *Candida albicans* and

a novel mucosal adjuvant, LT(R192G), against systemic candidiasis. *Infect. Immun.*, ***67***, 826-833.

86. Spellberg, B., Edwards, J. E. (2001). Type 1/Type 2 immunity in infectious diseases. *Clin. Infect. Dis.*, ***32***, 76-102.