NOTE *Parasitology*

Cloning and Characterization of a 2-Cys Peroxiredoxin from *Babesia gibsoni*

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ABSTRACT. Peroxiredoxins (Prxs) are a family of antioxidant enzymes. Here, we cloned a 2-Cys Prx, *BgTPx-1*, from the canine *Babesia* parasite *B. gibsoni*. Sequence identity between BgTPx-1 and 2-Cys Prx of *B. bovis* was 81% at the amino acid level. Enzyme activity assay by using recombinant BgTPx-1 (rBgTPx-1) indicated that BgTPx-1 has antioxidant activity. Antiserum from a mouse immunized with rBgTPx-1 reacted with parasite lysates and detect a protein with a monomeric size of 22 kDa and also a 44 kDa protein, which might be an inefficiently reduced dimer. BgTPx-1 was expressed in the cytoplasm of *B. gibsoni* merozoites. These results suggest that the BgTPx-1 may play a role to control redox balance in the cytoplasm of *B. gibsoni*.

KEY WORDS: antioxidant activity, *Babesia gibsoni*, canine, peroxiredoxin.

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Babesia gibsoni is a tick-borne intraerythrocytic apicomplexan parasite that causes piroplasmosis in dogs [5, 10]. The disease is characterized by remittent fever, progressive anemia, marked splenomegaly and hepatomegaly and sometimes causes death [9]. *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe and Americas [18]. Generally, *B. gibsoni* infection is characterized by recurrent infections even after treatment with anti-babesia drugs. To establish a method for effective treatment of canine babesiosis, more detailed analysis of mechanisms essential for survival of *Babesia* parasites in the host is important. However, the biological properties and life cycle of this pathogenic parasite remain poorly understood.

Since aerobic parasites live in an oxygen-rich environment in their host bodies, the parasites are likely to be subjected to the toxic effects of reactive oxygen species (ROS) that could cause damage to membrane lipids, nucleic acid and proteins [22]. For those parasites, redox balance control is considered to be an important biological property. To protect biological molecules from the effect of ROS, aerobes have evolved efficient defense systems of enzymatic antioxidants [24]. The 4 major cellular antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (Gpx), catalase and peroxiredoxin (Prx). Prxs constitute a family of proteins structurally homologous to the antioxidant of yeast [6] and have been identified in all living organisms from bacteria to humans [7, 20, 29]. Prxs reduce and detoxify hydrogen peroxides through the action of the highly conserved redox-active cysteine [29]. The family is classified into 3 groups based on the number of active cysteine residues: 1-Cys, typical 2-Cys and atypical 2-Cys types [28, 29]. Since 2-Cys Prxs use electrons provided by the small protein thioredoxin, these enzymes are also called thioredoxin peroxidases (TPx) [15, 19]. In recent years, several Prxs of malaria parasites have been characterized, and the structural and functional properties of the enzymes have been determined as key factors for development of new drugs [3, 11, 13, 16, 21]. Recently, a Prx from the bovine *Babesia* parasite *B. bovis* (BbTPx-1) was identified, and its antioxidant activity was demonstrated [27]. However, Prx in *B. gibsoni* has not yet been characterized.

In this study, we found a predicted 2-Cys Prx gene, *BgTPx-1*, from an expressed sequence tag (EST) database of *B. gibsoni* [1]. The size of the open reading frame (ORF) of the *BgTPx-1* gene was 597 bp, and the gene coded for a protein comprised of 198 amino acid residues with a predicted molecular weight and theoretical isoelectric point of 21.95 kDa and 6.42, respectively (ExPASy Compute pl/Mw; http://web.expasy.org/). Amino acid sequence analysis using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/) showed that the protein had no signal peptide. Multiple sequence alignment of BgTPx-1 with 2-Cys Prxs from other apicomplexan parasites revealed that BgTPx-1 had 81% sequence similarity with *B. bovis* BbTPx-1 [27], 57% with *Toxoplasma gondii* TgPrx [25], 59% with *Cryptosporidium parvum* CpTPx [14] and 52% with *P. falciparum* PfTPx-1 [17] (Fig. 1A). The presence of two conserved cysteine

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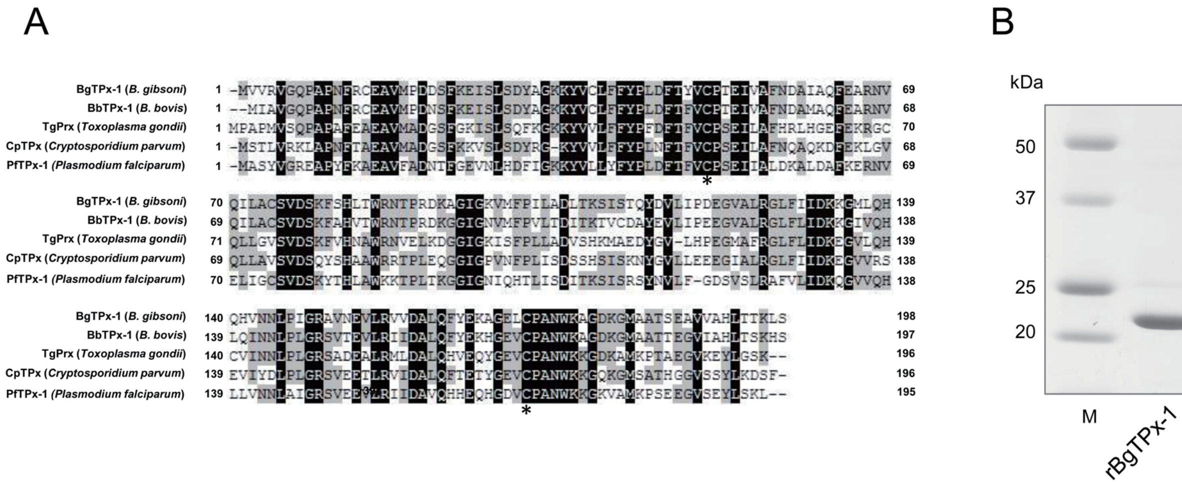

Fig. 1. Amino acid sequence alignment and SDS-PAGE analysis of BgTPx-1 protein. (A) Multiple sequence alignment of *B. gibsoni* BgTPx-1 protein (deduced sequence) with the sequences of other 2-Cys Prxs of apicomplexan parasites. Sequences are from *B. bovis* (BbTPx-1; XP_001610019), *T. gondii* (TgPrx; AAG25678), *C. parvum* (CpTPx; ACV31867) and *P. falciparum* (BAA97121). Black boxes with white letters show identical residues, and gray boxes with black letters show chemically similar residues. The dashes indicate gaps introduced between the sequences. Two conserved cysteine residues that correspond to Cys47 and Cys170 of the yeast Prx [5] are marked with asterisks. (B) Expression of BgTPx-1 protein by using the *E. coli.* expression system and SDS-PAGE analysis. A recombinant plasmid containing the sequence of *BgTPx-1* in pGEX-6P1 was transformed in *E.coli* strain BL21 (DE3), and the transformed colony was cultured in 1 l of LB broth with ampicillin sodium (100 μg/m*l*) at 37°C. When the optical density at 600 *n*m reached 0.6, expression of the recombinant fusion protein was induced by adding 1 mM isopropyl thio-β-D-galactoside (IPTG) and incubating for another 5 hr at 24°C. The bacterial cultures were lysed with PBS containing 100 μg/m*l* lysozyme and 1.5% Triton X-100 with sonication. The supernatant was subjected to protein purification using Glutathione-Sepharose 4B beads and PreScission protease. An SDS-PAGE image of rBgTPx-1 protein is shown. M, protein marker.

residues in BgTPx-1 (Cys50 and Cys171), corresponding to Cys47 and Cys170 of the yeast Prx [6], suggested that it is a typical 2-Cys type Prx. The full sequence of the *BgTPx-1* gene was deposited in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) under accession number AB829722.

To demonstrate the enzymatic activity of this BgTPx-1, we produced a recombinant BgTPx-1 protein (rBgTPx-1) in *Escherichia coli*. Total RNA of *B. gibsoni* was prepared from dog erythrocytes infected with *B. gibsoni* Oita strain maintained *in vitro* as previously described [26] by using TRI reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.). The dogs were housed, fed and given clean drinking water in accordance with the stipulated rules for the care and use of research animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan (approval number: 24–117). Parasite cDNA was synthesized from the total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). The *BgTPx-1* gene ORF was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primer sets: forward primer (5′- CCC GAA TTC GTA GTT CGC GTA GGA CAG CCT GC −3′) and reverse primer (5′- CCC CTC GAG TTA AGA GAG TTT AGT GGT GAG GTG G −3′) (underlined sequences containing the *Eco*RI site and *Xho*I site, respectively). The PCR product was digested with *Eco*RI and *Xho*I and then ligated to the pGEX-6P1 vector containing an ORF encoding a glutathione *S*-transferase (GST)-fusion protein (GE Healthcare, Piscataway, NJ, U.S.A.). rBgTPx-1 was expressed as a GST-fusion protein in *E. coli* and purified using Glutathione-Sepharose 4B beads (GE Healthcare) as previously described [12]. Then, GST was cleaved by PreScission protease (GE Healthcare) according to the manufacturer's instructions. The purified recombinant protein had an apparent molecular weight after treatment with PreScission protease of approximately 22 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie brilliant blue staining (Fig. 1B).

Next, the antioxidant activity of rBgTPx-1 was evaluated by mixed-function oxidation (MFO) assay (Fig. 2) [23]. A reaction mixture containing 40 mM $FeCl_3$, 10 mM dithiothreitol (DTT), 20 mM EDTA and 25 mM HEPES (pH 7.0) was pre-incubated with or without the rBgTPx-1 protein (10–400 μg/m*l*) at 37°C for 1 hr. After the pre-incubation period, 0.5 μg of pRSET-B plasmid DNA (Invitrogen, Carlsbad, CA, U.S.A.) was added, and the reaction mixture was incubated for another 1.5, 2 and 3 hr. Nicking of the supercoiled plasmids by MFO was evaluated on 1% agarose gel stained with ethidium bromide. In the absence of or in the presence of a low concentration (10–50 μg/m*l*) of rBgTPx-1, $FeCl_3$ and DTT produced hydroxyl radicals giving nicks in the supercoiled plasmid DNA, and thus, reaction time-dependently changing the migration pattern of the DNA in the gel due to differences in migration between supercoiled DNA and linearized DNA (Fig. 2, lanes 4–7). However, the presence of rBgTPx-1 in the reaction mixtures at concentra-

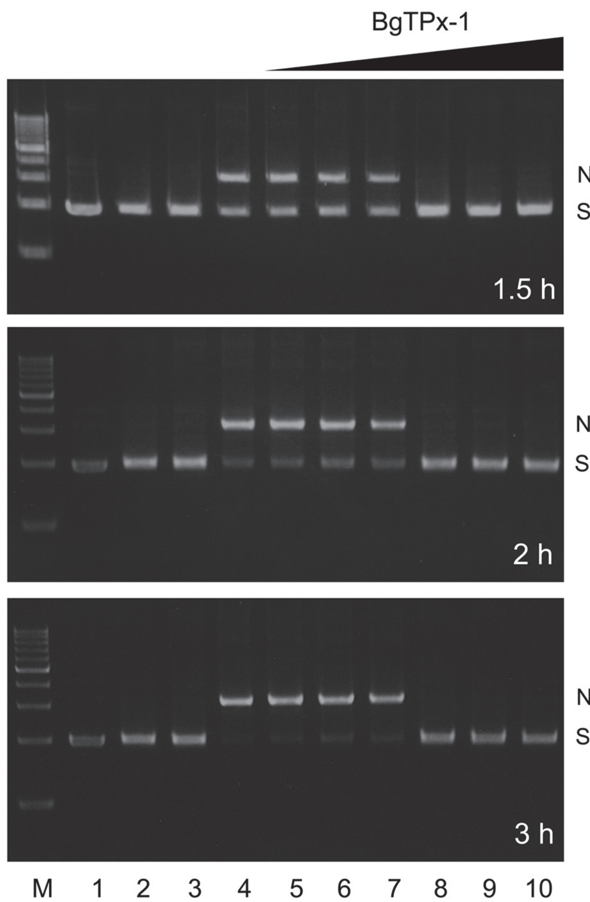

Fig. 2. Antioxidant activity of rBgTPx-1. After incubation for 1.5, 2 and 3 hr, nicking of the supercoiled plasmids by MFO was evaluated on 1% agarose gel stained with ethidium bromide. The nicked form (NF) and supercoiled form (SF) of the plasmids are indicated on the right. M, 100-bp DNA ladder marker. lane 1, pRSET DNA; lane 2, pRSET DNA and DTT; lane 3, pRSET DNA and $FeCl_3$; lane 4, pRSET DNA, $FeCl_3$ and DTT; lanes 5–10, pRSET DNA, $FeCl_3$, DTT, and 10, 25, 50, 100, 200 and 400 μg/m*l* of rBgTPx-1 protein, respectively.

tions of 100–400 μg/m*l* prevented nicking of the supercoiled plasmid DNA (Fig. 2, lanes 8–10). This result indicated that BgTPx-1 has antioxidant activity with threshold concentration in our experimental condition.

To analyze the expression of native BgTPx-1 in *B. gibsoni* merozoites, we produced antiserum against rBgTPx-1 in a mouse. One hundred micrograms of rBgTPx-1 was subcutaneously injected into an 8-week-old female ICR mouse (Clea Japan, Tokyo, Japan) with Gerbu adjuvant 10 (GERBU biotechnik GmbH, Heidelberg, Germany). On days 14, 28 and 42, the same antigens were subcutaneously injected with Gerbu adjuvant 10. The mouse serum was collected 10 days after the last immunization following the stipulated rules for the care and use of research animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (approval number: 24–118). By using the antiserum, we performed Western blotting (Fig. 3A). The antiserum against rBgTPx-1 bound to a protein of the expected monomeric size of 22 kDa as well as a larger protein with an apparent molecular weight of about 44 kDa, which might be an inefficiently reduced dimer of BgTPx-1, in extracts of *B. gibsoni*-infected erythrocytes. As shown in Fig. 3B, BgTPx-1 was observed around the nucleus of the parasite. This cytoplasmic expression pattern was also shown in typical 2-Cys peroxiredoxins of *P. falciparum* (PfTPx-1) [31], *P. vivax* (PvTPx-1) [11] and *B. bovis* (BbTPx-1) [27]. Recently, our group showed that BbTpx-1 gene disruption does not affect *in vitro* intraerythrocytic growth of B. bovis [2], as previously reported for the gene disrupted in P. berghei [30], indicating that the TPx-1 gene is not essential for the erythrocytic stage of *Babesia* parasites. In fact, *Babesia* parasites have other antioxidant proteins, such as catalase and Gpx [4, 8]. Thus, elucidation of the interaction between BgTPx-1 and other antioxidant *B. gibsoni* proteins is needed.

Taken together, in this study, we have characterized a functional, typical 2-Cys Prx antioxidant, BgTPx-1, from *B. gibsoni*. Since BgTPx-1 has antioxidant activity, we assume that BgTPx-1 plays a role in the reduction of ROS.

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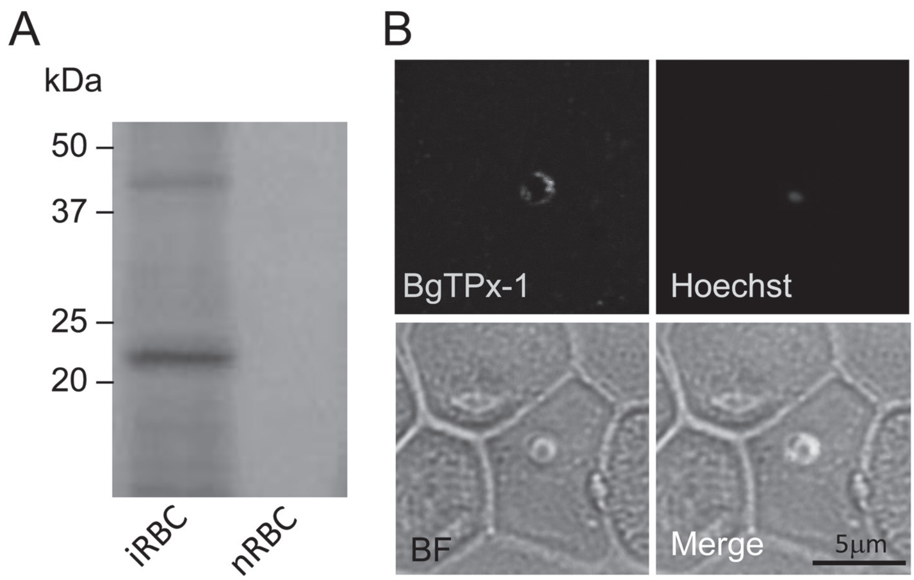

Fig. 3. Localization of native BgTPx-1 in *B. gibsoni* merozoites. (A) Western blot analysis of native BgTPx-1 using mouse anti-rBgTPx-1 serum. We lysed 10^9 of *B. gibsoni*-infected canine erythrocytes (iRBC, parasitemia of 5%) or the same amount of normal erythrocytes (nRBC, negative control) as reported previously [32]. Samples were dissolved in 2 × SDS-PAGE sample buffer and heated at 96°C for 5 min. Then, 10 *μl* of lysates containing 5×10^6 RBC were separated by SDS-PAGE. The positions of molecular mass standards are indicated on the left. (B) Indirect immunofluorescence microscopy to determine cellular localization of BgTPx-1 in the parasite cells. For nuclear staining, Hoechst 33342 (Lonza) was used. BF: bright field.

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