A peroxiredoxin cDNA from Taiwanofungus camphorata: role of Cys31 in dimerization

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Abstract

Peroxiredoxins (Prxs) play important roles in antioxidant defense and redox signaling pathways. A novel Prx isozyme cDNA (TcPrx2, 745 bp, EF552425) was cloned from *Taiwanofungus camphorata* and its recombinant protein was overexpressed. The purified protein was shown to exist predominantly as a dimer by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrolysis) in the absence of a reducing agent. The protein in its dimeric form showed no detectable Prx activity. However, the protein showed increased Prx activity with increasing DTT (dithiothreitol) concentration which correlates with dissociation of the dimer into monomer. The TcPrx2 contains two Cys residues. The Cys^{60} located in the conserved active site is the putative active peroxidatic Cys. The role of Cys^{31} was investigated by site-directed mutagenesis. The C31S mutant ($C^{31}\rightarrow S^{31}$) exists predominately as a monomer with noticeable Prx activity. The Prx activity of the mutant was higher than that of the corresponding amount of the wild-type protein nearly 2 fold at 12 μg/mL. The substrate preference of the mutant was H_2O_2 > cumene peroxide > t-butyl peroxide. The Michaelis constant (K_M) value for H_2O_2 of the mutant was 0.11 mM. The mutant enzyme's half-life of deactivation at 48 °C was 5 min, and its thermal inactivation rate constant K_d was 0.14 min^{-1}. The mutant enzyme was active under a broad pH range from 6 to 10. The results suggest a role of Cys^{31} in dimerization of the TcPrx2, a role which, at least in part, may be involved in determining the activity of Prx. The C^{31} residue does not function as a resolving Cys and therefore the TcPrx2 must follow the reaction mechanism of 1-Cys Prxs. This TcPrx2 represents a new, novel isoform of Prx

35 family.

36 **Keywords** *Taiwanofungus camphorata* · Peroxiredoxin · Peroxide · Three-dimension (3-D) structural

37 model

38

Introduction

Peroxiredoxins (Prxs) are a family of thiol-dependent peroxidases found in all known organisms [1]. The enzymes play important roles in antioxidant defense systems and cellular redox signaling pathways [2-4]. Prxs perform the protective antioxidant roles by reducing hydrogen peroxide and alkyl hydroperoxides to water and alcohol, respectively using thiols as reductants [5]. Prxs use the conserved redox active peroxidatic cysteine (C_P) to reduce peroxide substrates [6]. During peroxidase reaction, the C_P residue in the active site is first oxidized to sulfenic acid (C_P-SOH), and then the substrate hydrogen peroxide or alkyl hydroperoxides are reduced to water or the corresponding alcohol in resolving stage [7, 8]. Finally thiols reductants such as thioredoxin, glutaredoxin, glutathione, cyclophilins or ascorbate play as electron donors to regenerate the active form of Prxs [8]. Prxs are classified into six groups, A to F, based on sequence and structural homology as well as location of the conserved Cys residues [8]. Among them, the E-type Prx is found in bacteria and F-type in archaea, while A to D types are common in higher plants. The A-type Prx corresponds to typical 2-Cys peroxiredoxin (2-Cys Prx), contains a second conserved resolving Cys (C_R) residue at the C-terminal portion of the molecule. The B-type Prx corresponds to typical 1-Cys peroxiredoxin (1-Cys Prx) [9]. The C-type Prx corresponds to peroxiredoxin Q (PrxQ), and the D-type Prx to type II peroxiredoxin (PrxII), both are also termed atypical 2-Cys Prx [8, 10]. In typical 2-Cys Prxs, the C_P-SOH reacts with the C_R-SH residue located in the C-terminal portion of the second subunit of the enzyme homodimer to form an intermolecular disulfide [11]. In atypical 2-Cys Prxs, the C_P-SOH reacts with the C_R-SH residue within the same subunit forming intramolecular disulfide. The disulfide is then reduced by thioredoxin or glutathione [12] completing the catalytic cycle.

Taiwanofungus camphorata is a medicinal mushroom found only in the forests of Taiwan which has traditionally been used in the treatment of liver cancer, drug intoxication, among others [13]. *T. camphorata* was named *Antrodia cinnamomea* in 1995 [14] and renamed as *Antrodia camphorata* in

1997 [15]. A phylogenetic analysis based on sequence data of ribosomal RNA genes of large ribosomal subunit indicated that *T. camphorata* is distantly related to other species in *Antrodia*. The fungus was subsequently classified in the new genus *Taiwanofungus* [16]. *T. camphorata* has been shown to exhibit anticancer properties, anti-inflammatory effects, anti-hepatitis B virus replication, anti-oxidant activities, hepatoprotective activity, neuroprotective effect, and antihypertensive effect [17, 18]. Majority of the research that aim at finding bioactive compounds in *T. camphorata* have been focused on the extracts of fruit body [19]. It is strongly believed that regular consumption of *T. camphorata* in the form of extract or mushroom powder will preserve human vitality and promote longevity. The rarity of *T. camphorata* fruit body has limited its use in scientific research, health food and medical applications. One approach to overcome such limitation is to use recombinant DNA technology. Recently, we established ESTs (expressed sequence tags) from the fruiting bodies of *T. camphorata* in order to search physiologically active components, including antioxidant enzymes. We have cloned and characterized several antioxidant enzymes including a 1-Cys peroxiredoxin [20], a 2-Cys peroxiredoxin [21], a superoxide dismutase [22], a catalase [23], a phospholipid hydroperoxide glutathione peroxidase [24], and a 2-Cys peroxiredoxin isozyme [25] based on the established ESTs from *T. camphorata.* This motivated us further to search more active components from *T. camphorata* for potential health food and medical applications.

Here, we report the cloning of a novel peroxiredoxin isozyme from *T. camphorata*, named TcPrx2. The TcPrx2 contains two Cys residues, one is a putative conserved peroxidatic Cys^{60}, whereas the other Cys^{31} locates closer to the N-terminal end unlike most known C_R-SH residue locates in the C-terminal portion of a 2-Cys peroxiredoxin. This nonperoxidatic Cys^{31} is conserved only in yeast and fungal Prxs [26]. In order to understand the role of Cys^{31}, we constructed a C31S mutant. Both the coding regions of the TcPrx2 cDNA and C31S mutant were introduced into an *E. coli* C41(DE3) expression system. The enzymes were purified and characterized. The Prx activity of the C31S is capable of reducing hydrogen peroxide and alkyl hydroperoxides suggesting a potential

application of the enzyme in food industry as a food antioxidant [27, 28].

Materials and methods

Materials

Fruiting bodies of *T. camphorata* grew in the hay of *Cinnamomum kanehirai* were obtained from Asian company, Taiwan (http://www.asian-bio.com/).

Identification of TcPrx2 cDNA

We have established an expressed sequence tag database from fruiting bodies of *T. camphorata* and sequenced all clones with insert size greater than 0.4 kb (data not shown). A Prx2 cDNA clone was identified by comparing the inferred amino acid sequence with homologous sequences in the nonredundant database (NRDB) at the National Center for Biotechnology Information, National Institutes of Health (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis revealed that the Prx2 cDNA covered an open reading frame of a putative peroxiredoxin isozyme (TcPrx2, 745 bp, EMBL no. EF552425).

Bioinformatics analysis of TcPrx2

Several homologous protein sequences retrieved by the BLASTP program were aligned using ClustalW2 program. The secondary structure of the TcPrx2 protein was predicted by SWISS-MODEL program and represented as α helices and β strands. A 3-D structural model of TcPrx2 was created by SWISS-MODEL [29] (http://swissmodel.expasy.org/SWISS-MODEL.html) based on the known crystal structure and solution nuclear magnetic resonance (NMR) of PtPrx (*Populus tremula x Populus tremuloides*, PDB code 1tp9) [30]. The model superimposed with this

PtPrx via the SPDBV_4 program was shown by using protein solid ribbons.

Subcloning of TcPrx2 cDNA into an expression vector

The coding region of the TcPrx2 cDNA was amplified using gene specific flanking primers. The 5' upstream primer contains *Eco*RI recognition site (5' GAATTCG ATG GCC CCT TCC ATC AAA 3') and the 3' downstream primer contains *Xho*I recognition site (5' CTCGAG GAG GTG AGC CAA AAC GGC 3'). The restriction enzyme sites were indicated as underline. Using 0.2 μg of TcPrx2 cDNA as a template, and 10 pmole of each 5' upstream and 3' downstream primers, a 0.5 kb fragment was amplified by PCR. The fragment was ligated into pCR®4-TOPO® (Invitrogen, Grand Island, NY) and transformed into *E. coli*. The recombinant plasmid was isolated and digested with *Eco*RI and *Xho*I. The digestion products were separated on a 1% agarose gel. The 0.5 kb insert DNA was gel purified and subcloned into *Eco*RI and *Xho*I site of pET-20b(+) expression vector (Novagen, Darmstadt, Germany). The recombinant DNA was then transformed into *E. coli* C41(DE3). The recombinant protein was overexpressed in *E. coli* and its function identified by activity assay as described below.

Site-directed mutagenesis (C31→S31)

The recombinant TcPrx2 DNA prepared above was used as a template for site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The C31S mutant was created by replacing the TGT codon with TCT. The sequence of the mutant DNA was verified by nucleotide sequencing.

Expression and purification of the recombinant wild-type TcPrx2 and its C31S mutant

The transformed *E. coli* C41(DE3) containing the recombinant TcPrx2 DNA or its C31S mutant was

grown at 37 °C in 20 mL of Luria-Bertani containing 50 μg/mL ampicillin until A_{600} reached 0.8. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated at 80 rpm for an additional 16 h at 37 °C. The cells were harvested and soluble proteins were extracted in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) with glass beads as described before [31]. Both recombinant proteins were purified by Ni-NTA affinity chromatography (elution buffer: 30% PBS containing 100 mM imidazole) according to the manufacture's instruction (Qiagen). The purified proteins were checked by a 10% SDS-PAGE. Each purified protein was dialyzed against 200 mL of 30% PBS containing 2% glycerol and 1 mM DTT for 4 h at 4 °C (3 changes). The dialyzed sample was either used directly for analysis or stored at –65 °C until use. Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

Molecular mass analysis via Electrospray Ionization Quadrupole-Time-of-Flight (ESI Q-TOF)

The purified TcPrx2 (0.5 mg/mL) was prepared in 0.3% PBS containing 0.05 mM imidazole and 0.05% glycerol. The sample (5 μL) was used for molecular mass determination using an ESI Q-TOF mass spectrometer (Micromass, Manchester, England).

Prx activity assay

The recombinant TcPrx2 or its C31S mutant (1.0 μg) was incubated in 47-48 μL buffer (30% PBS/2% glycerol/1 mM DTT) for 10 min at room temperature. The reaction was initiated by addition of 2-3 μL of 1 mM H_2O_2 (or t-butyl peroxide, or cumene peroxide). At the reaction intervals, 20 μL of 26% trichloroacetic acid was added to the 50 μL reaction mixture to stop the reaction. The

peroxidase activity was determined by following the disappearance of the peroxide substrate (the total peroxide, 2-3 nmol at the beginning of the reaction minus the remaining amount at the end of 10 min). The remaining peroxide content was determined as a red-colored ferrithiocyanate complex formed by addition 20 μL of 10 mM $Fe(II)(NH_4)(SO_4)_2$ and 10 μL of 2.5 M KSCN to the 70 μL of reaction mixture. The color intensity was quantified by absorbance measurement at 475 nm [32].

Enzyme characterization

The C31S mutant enzyme was characterized under various conditions as described below. Aliquots of the C31S mutant samples (5 μL, 0.2 μg/μL, 10 μM) were treated as follows: (1) *Buffer effect.* Enzyme activity was tested after C31S mutant was purified and dialyzed against 30% PBS, pH 7, containing 2% glycerol or 10 mM Tris-HCl, pH 7, containing 5 mM NaCl and 2% glycerol. (2) *pH effect*. Each 5 μL sample (0.2 μg/μL, 10 μM) was adjusted to desired pH by adding a volume of buffer with different pHs: 0.2 M citrate buffer (pH 2.5, or 4), 0.2 M Tris-HCl buffer (pH 6, 7 or 8) or 0.2 M glycine-NaOH buffer (pH 10, or 11). Each 10 μL sample (0.1 μg/ μL, 5 μM) was incubated at 37 °C for 1 h. (3) *Thermal effect.* Each 10 μL enzyme sample (0.1 μg/μL, 5 μM) was heated at 48 °C for 2, 4, 8 or 16 min. Each treated sample was tested for Prx activity using H_2O_2 as substrate and/or analyzed by SDS-PAGE.

Kinetic studies

The kinetic properties of the C31S mutant (1.0 μg, 1 μM) was determined by varying the concentrations of H_2O_2 (0.02 to 0.16 mM) with fixed amount of 1 mM DTT in 30% PBS/2% glycerol. The change in absorbance at 475 nm was recorded between 0 and 2 min. The K_M, V_{max} and k_{cat} were calculated from the Lineweaver-Burk plots.

Results

Cloning and characterization of a cDNAencoding TcPrx2

A putative TcPrx2 cDNA clone was identified on the basis of the consensus pattern and sequence homology to the published Prxs in the NCBI data bank. The coding region of TcPrx cDNA was 513 bp that encodes a protein of 171 amino acid residues with a calculated molecular mass of 18.2 kDa (EMBL accession no. EF552425). Fig. 1 shows the optimal alignment of the amino acid sequences of TcPrx2 with 6 selected Prx sequences. This TcPrx2 shares 47% identity with LkPrx (*Lipomyces kononenkoae*, Q01116), 46% identity with MfPrx (*Malassezia furfur*, P56578), 40% identity with AtTPX1 (*Arabidopsis thaliana*, NP_176773), 38% identity with PtPrx **(***Populus tremula x P. tremuloides*, AAL90751), 36% identity with VpPrx (*Vibrio parahaemolyticus* RIMD 2210633*,* BAC62636) and 35% identity with HsPrx5 (*Homo sapiens,* CAB62210) using ClustalW2 multiple sequence alignment program. The secondary structure was predicted by SWISS-MODEL program and represented as α helices and β strands (Fig. 1A). Four key Prx catalytic residues totally conserved in all known Prxs active site [3] are the C_P at C^{60} surrounded by P^{53}, T^{57}, and R^{138}. This Prx belongs to the D-type as it contains all eleven conserved residues reported in the D-type prxs [26]. These conserved residues correspond to K^{45}, P^{53}, F^{56}, T^{57}, C^{60}, H^{61}, H^{64}, W^{97}, D^{112}, R^{138} and E^{154} in the TcPrx2 protein. The second Cys residue, C^{31} is located closer to N-terminus unlike other known resolving Cys (C_R) residue which is located at the C-terminal portion of the protein. The C^{31} is only conserved in yeast and fungal Prxs (Fig. 1A) [26]. This C^{31} residue does not function as a resolving Cys, instead it is responsible for dimerization and inactivation of the TcPrx2. Therefore, it is concluded that the TcPrx2 follows the reaction mechanism of 1-Cys Prxs as only one Cys residue, the C_P at the C^{60} appears to involve in the peroxidase activity.

194 A 3-D structural model of TcPrx2 was created based on the known crystal structure and solution
195 NMR structures of PtPrx (*P. tremula x P. tremuloides*, PDB code 1tp9). The model superimposed
196 with PtPrx (green) via the SPDBV_4 program was shown using protein solid ribbons (Fig. 1B).

197 Expression and purification of the recombinant TcPrx2 and its C31S mutant

198 The coding region of TcPrx2 was amplified by PCR and subcloned into an expression vector,
199 pET-20b(+) as described in the Materials and methods. Positive clones were verified by DNA
200 sequence analysis. The C31S mutant was created via QuikChange Site-Directed Mutagenesis. Both
201 the wild-type recombinant TcPrx2 and its C31S mutant were expressed and the proteins analyzed by
202 a 10% non-reducing SDS-PAGE without boiling (Fig. 2). The recombinant TcPrx2 was expressed as
203 a His_6-tagged fusion protein and was purified by affinity chromatography with nickel chelating
204 Sepharose. A major and a minor band with molecular mass of approximately 41 kDa (expected size
205 of TcPrx2 dimer) were seen in Ni-NTA eluted fractions by a SDS-PAGE (Fig. 2A, lanes 3-8). The
206 presence of the two bands may due to different disulfide bond patterns in the dimers that lead to
207 different conformations. The dimers may be linked by two disulfide bonds between Cys^{31}-Cys^{60} and
208 Cys^{60}-Cys^{31}, or between Cys^{31}-Cys^{31} and Cys^{60}-Cys^{60}. Alternatively, the dimers may be linked by
209 only one disulfide bond between Cys^{31}-Cys^{60}, or Cys^{31}-Cys^{31}, or Cys^{60}-Cys^{60} [11]. The purified C31S
210 mutant showed multiple bands with approximate molecular mass of 20 kDa, expected size of
211 monomers (Fig. 2B, lanes 3-8). These multiple monomeric bands presumably with different
212 conformations may be due to formation of intramolecule epoxidation between the –OH of Ser^{31}
213 and –SH of Cys^{60}. The epoxidation conformation should be more compact, therefore migrated faster
214 in the SDS-PAGE. In Fig. 2B (lanes 3-6), near 41 kDa, 3 minor dimeric bands of different
215 conformations were visible. These dimers might be linked by a disulfide bond between Cys^{60}-Cys^{60}
216 or epoxidation formed between –OH of Ser^{31} and –SH of Cys^{60} or –OH of $Ser^{31}$and –OH of Ser^{31}.

The Ni-NTA eluted protein fractions were pooled, dialyzed, and characterized further. Analysis of the TcPrx2 by ESI Q-TOF confirms the presence of one major protein band with molecular mass of 40,620 Da. This indicates that recombinant wild-type TcPrx2 is predominantly dimeric in nature and recombinant C31S mutant exists predominantly as monomer. The yield of the purified His_6-tagged TcPrx2 was 650 µg from 20 mL of culture. The yield of C31S mutant was 400 µg from 20 mL of culture.

Reductive dissociation of TcPrx2 dimer and enzyme activity depends on reducing agent (DTT)

To examine the effect of DTT on reductive dissociation of TcPrx2 dimers and enzyme activity, aliquots of the TcPrx2 were incubated with increasing concentrations of DTT (0-50 mM) for 10 min at room temperature. The samples were analyzed by SDS-PAGE and Prx activity. As shown in Fig. 3A, progressive dissociation of the TcPrx2 dimer was observed with increasing DTT. In the absence of DTT, the TcPrx2 was in dimeric form (Fig. 3, lane 1). As the DTT concentration increased from 2 to 50 mM, the dimeric band decreased gradually with a concomitant increase in the monomeric form. The results indicate that formation of intermolecular disulfide bond(s) is responsible for TcPrx2 dimerization. Prx activity of the DTT-treated samples was determined by the enzyme's ability to eliminate t-butyl peroxide (Fig. 3B). The increase in Prx activity was proportional to the increase in DTT concentration from 2 to 50 mM (Fig. 3B) which correlated with the increase in levels of monomer. In other words, the enzyme is active in its monomic form. However, the activity was unaffected by GSH up to 50 mM (Fig. 3B). The data shown in Fig. 3B and Fig. 4B were subjected to analysis of variance (ANOVA) and Scheffe's test [ref n1].

Enzymatic activity of TcPrx and C31S mutant

The recombinant TcPrx2 activity was tested in the absence or in the presence of 50 mM DTT using

239 H_2O_2 or t-butyl peroxide or cumene as the substrate (Fig. 4A). Our results showed a linear
240 relationship between elimination of peroxide substrate (H_2O_2, t-butyl peroxide, or cumene) with
241 increasing TcPrx2 levels from 2-20 µg/mL in the presence of 50 mM DTT (Fig. 4A, closed symbols).
242 The enzyme worked well with all three substrates, but slightly better with H_2O_2 in the range of 4-10
243 µg/mL of TcPrx2. In the absence of DTT, no activity was detected with t-butyl peroxide or cumene
244 as substrate, and very little activity (20-30%) was detected with H_2O_2 as substrate at high levels of
245 TcPrx2 from 12-20 µg/mL (Fig. 4A, open symbols).

246 Prx activity of the C31S mutant also showed an increase as the amount of the protein increased
247 from 4-12 µg/mL in the presence of 1 mM DTT and H_2O_2 (Fig. 4B, white bar). The Prx activity of
248 the mutant was higher than that of the corresponding amount of the wild-type protein under the same
249 assay conditions; nearly 2 fold at 12 µg/mL (Fig. 4B). The substrate preference of the C31S mutant
250 was H_2O_2 > cumene > t-butyl peroxide (Fig. 4B).

251 The effect of DTT on Prx activity of the wild-type TcPrx2 and C31S mutant was compared (Fig. 5).
252 As the concentration of DTT increased, enzyme activity of TcPrx2 increased and peaked at 40 mM
253 (Fig. 3B and Fig. 5). The activity of C31S was unaffected by DTT. The results suggest that C31 is
254 responsible for dimerization of the TcPrx2.

255 The wild-type TcPrx2 has a long shelf life; it maintained high enzymatic activity in presence of 50
256 mM DTT after storage at -20 °C for 2 years. In contrast, C31S was inactive under the same storage
257 conditions (data not showed). The results suggest that the enzyme was well protected and preserved
258 as an inactive dimer.

259 Characterization and kinetic properties of the purified C31S mutant

260 As shown in Fig. 6A-C, the C31S enzyme had higher activity in PBS than in Tris-HCl buffer at the
261 same pH 7. The enzyme was active in a broad pH range with optimal pH 6-8. The half-life of

deactivation at 48 °C was 5 min, and its thermal inactivation rate constant K_d was 0.14 min^{-1}.

As shown in Fig. 6D, the Lineweaver-Burk plot of the velocity (1/V) against 1/[H_2O_2] gave the K_M = 0.11 mM, V_{max} = 0.04 mM/min, and k_{cat} = 41.0 min^{-1}. The enzyme appears to have low efficiency in contrast to most Prxs which have fast catalytic rates with H_2O_2 on the order of $1x10^5$ to $1x10^7$ $M^{-1}S^{-1}$ [3, 4].

Discussion

This study reported the first cloning and expression of a novel redox enzyme, TcPrx2, from *T. camphorata*. The enzyme contains two Cys residues Cys^{31} and Cys^{60}; Cys^{60} is the conserved peroxidatic cysteine at the active site. The nonperoxidatic Cys^{31} located closer to the N-terminal end is conserved only in yeast and fungal Prxs [26]. Our results suggested that the Cys^{31} is responsible for dimerization and inactivation of TcPrx2 (Fig. 2, 4). The enzyme is active in its monomeric form: in the presence of enough DTT that dissociated the dimers or in the C31S mutant form that prevents the formation of dimer (Figs. 3-5). The C^{31} residue does not function as a resolving Cys and therefore the TcPrx2 must follow the reaction mechanism of 1-Cys Prxs. Diverse isoforms of Prx, characterized by different catalytic mechanisms and associated with various thiol-containing agents, are known to exist [3, 4]. This TcPrx2 represents a new, novel isoform of Prx family. Although the physical role of the TcPrx2 in *T. camphorata* is unclear, its activity is likely to be regulated by the level of yet to be determined thiol-containing agent(s). Further investigations are needed to establish the functions of the TcPrx2. The antioxidant activity of the enzyme suggests a potential use in food industry as food antioxidative agent in preventing lipid peroxidation [27, 28].

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362

363 **Legends**

364 **Fig. 1** Alignment of the amino acid sequences of TcPrx2 with Prxs from other sources and a 3-D
365 structural model. (**A**) Sequence alignment: TcPrx2 was identified from this study, LkPrx isolated
366 from *Lipomyces kononenkoae*, MfPrx from *Malassezia furfur*, AtTPX1 from *Arabidopsis thaliana*,
367 PtPrx from *Populus tremula x Populus tremuloides*, VpPrx from *Vibrio parahaemolyticus* RIMD
368 2210633 and HsPrx5 from *Homo sapiens*. Identical amino acids in all sequences are shaded black,
369 conservative replacements are shaded gray. Protein secondary structure was predicted by
370 SWISS-MODEL program and represented as α helices and β strands. (**B**) A 3-D structural model of
371 TcPrx2. The 3-D structural model of the TcPrx2 was created based on the known crystal structure
372 and solution NMR of PtPrx (*Populus tremula x Populus tremuloides*, PDB code 1tp9) via
373 SWISS-MODEL program and was superimposed to obtain structure alignment via SPDBV_4
374 program. Superimposition of TcPrx2 (green) and PtPrx (white) was shown using protein solid
375 ribbons. * denotes Cys^{31} and Cys^{60}.

376 **Fig. 2** Expression and purification of recombinant TcPrx2 (**A**) and its C31S mutant (**B**) in *E. coli.*
377 Fifteen μL (loading buffer without β-mercaptoethanol and without boiling) of each fraction was
378 loaded into separated lanes of a 10% SDS-PAGE followed by Coomassie Brilliant Blue R-250
379 staining. Lane 1, crude extract from *E. coli* expressing TcPrx2 or mutant; 2, flow-through proteins
380 from the Ni-NTA column; 3-8, enzymes eluted from the Ni-NTA column. Molecular masses (in kDa)
381 of standards are shown at left. The target protein bands were indicated as d (dimer) and m
382 (monomer).

383 **Fig. 3** Reductive dissociation of TcPrx2 dimer and enzyme activity depends on reducing agent (DTT).

(**A**) Progressive reduction of TcPrx2 as the DTT concentration increased: the amounts of dimers decreased with a concomitant increase of monomers. (**B**) Reaction velocities of TcPrx2 against elimination of t-BOOH were proportional to the increase in concentration of DTT from 2 to 50 mM. The rates were unaffected by GSH.

Fig. 4 (**A**) TcPrx2 catalyzed reduction of peroxides (H_2O_2, t-butyl peroxide, cumene) is proportional to the amount of TcPrx2 in the presence of 50 mM DTT. In the absence of DTT, no activity was observed against t-butyl peroxide or cumene; little activity was observed against H_2O_2 only at high TcPrx2 concentration (12-20 μg/mL). Prx activity was measured as described in the Materials and methods. (**B**) TcPrx2 or C31S catalyzed reduction of peroxides (H_2O_2, t-butyl peroxide, cumene) is proportional to the amount of TcPrx2 in the presence of 1 mM DTT. C31S mutant has higher catalysis efficiency to H_2O_2 than to t-butyl peroxide and cumene. Data are means of three experiments. The data shown in B were analyzed by ANOVA and Scheffe's test. Star indicates significant difference. t-butyl peroxide (t-BOOH), cumene peroxide ($C_6H_5C(CH_3)_2OOH$).

Fig. 5 Comparison of Prx activity of the wild-type TcPrx2 and C31S mutant in the presence of 5 to 50 mM DTT. Prx activity was measured as described in the Materials and methods. C31S mutant exhibited Prx activity independent of DTT.

Fig. 6 Effect of buffer, pH, and temperature on the purified C31S mutant and its kinetic property using H_2O_2 as the substrate. (**A**) The enzyme samples were assayed in 30% PBS containing 2% glycerol or 10 mM Tris-HCl containing 5 mM NaCl and 2% glycerol. (**B**) The enzyme samples were incubated with different pH buffers at 37 ^oC for 1 h and then assayed for Prx activity. (**C**) The enzyme samples were heated at 48 ^oC for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8 or 16 min and assayed for Prx activity. Thermal inactivation of the activity was plotted. E_0 and E_t represent original activity and residual activity after being heated, respectively. (**D**) Double-reciprocal plot of varying H_2O_2 versus the enzyme activity. The initial rate of the enzymatic reaction was measured at 1 mM DTT with the H_2O_2 concentration varied from 0.02 to 0.16 mM. The

409 K_M, V_{max} and k_{cat} were calculated from the Lineweaver-Burk plots. Data are means of three
410 experiments.