Fish & Shellfish Immunology 28 (2010) 795–801

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Molecular cloning, expression and antioxidant activity of a peroxiredoxin 2 homologue from *Lampetra japonica*

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ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form
7 January 2010
Accepted 28 January 2010
Available online 6 February 2010

Keywords:
Lampetra japonica
Peroxiredoxin
Sequence analysis
Oxidative damage
Antioxidant defense

ABSTRACT

Peroxiredoxin (Prx) is a cellular antioxidant protein family that plays important roles in oxidative stress and immune cytotoxicity. In this study, we cloned a homologue of the Prx2 from the buccal gland of *Lampetra japonica* (*L. japonica*). *L. japonica* Prx2 (Lj-Prx2) contained two highly conserved motifs and shared more than 70% identity with the homologs from other vertebrate species. Phylogenetic analysis revealed that Lj-Prx2 is closely related to other available teleost Prx2. The real-time PCR results demonstrated that the Prx2 gene was widely expressed in adult lamprey. In addition, the expression of Prx2 gene was particularly up-regulated in red blood cells (RBCs) after the experimental animals were challenged with lipopolysaccharide (LPS) *in vivo*. Lj-Prx2 gene was subcloned into the pET23b vector and expressed in *Escherichia coli* BL21 (DE3). The recombinant *L. japonica* Prx2 (rLj-Prx2) was purified by using His Bind affinity chromatography. Polyclonal antibody to rLj-Prx2 was generated in New Zealand Rabbit. Western blot analysis showed that the Lj-Prx2 is present in the buccal gland secretion, suggesting the secretory feature of it. The function assays revealed that rLj-Prx2 has the capability to reduce the H_2O_2 when dithiothreitol (DTT) is used as a reducing equivalent and to protect DNA from oxidative damage. These findings suggested that Lj-Prx2 probably plays an essential role in antioxidant defense in RBCs to keep lamprey alive.

1. Introduction

Peroxiredoxin (Prx), as a new class of antioxidants, contains a highly conserved Cys residue that undergoes a cycle of peroxide-dependent oxidation and thiol-dependent reduction during catalysis [1]. Prxs are present in all living organisms from prokaryotes to eukaryotes [2]. To date, there are three Prx isoforms in bacteria, five in yeast, ten in plants and six in mammals [2]. Mammalian Prxs can be divided into three classes, i.e., typical 2-Cys Prx (Prx1–4), atypical 2-Cys Prx (Prx5), and 1-Cys Prx (Prx6). They share the basic catalytic mechanism, i.e., an active-site cysteine (the peroxidatic cysteine, Cp-SH) is oxidized to a sulfenic acid (Cp-SOH) by the peroxide substrate [3]. The three classes differ from each other by the numbers and positions of conserved Cys residues [4]. In addition, the oxidized Cp-SOH of the typical 2-Cys Prx reacts rapidly with the resolving cysteine (C_R-SH) of the other subunit to form an intermolecular disulfide, which in turn is reduced by thioredoxin or other enzymes [4–6], whereas the atypical 2-Cys Prx forms an intra-molecular disulfide between the peroxidatic and resolving cysteine. As regards the 1-Cys Prx, Cp-SH is oxidized by peroxide to Cp-SOH which can be reduced by thiol but not form a disulfide [7,8]. Prxs possess various biological functions, including antioxidant activity, immune cytotoxicity, HIV-1 antiviral activity [9–11], and mediation of cell proliferation, differentiation and apoptosis [12–15].

Prx1 and Prx2, also known as natural killer cell enhancing factor (NKEF)-A and -B in human, are two members of the typical 2-Cys Prx. Both are found in the cytosol. Only the reduced form of NKEF-A can enhance NK cell-mediated cytotoxicity [16]. Prx2 is the third most abundant protein in the erythrocyte [17]. Previous study revealed that although human Prx1 and Prx2 are more than 90% homologous in their amino acid sequences, they are not duplicate proteins [18]. Human Prx1 contains a cysteine (Cys83) that is absent in Prx2. The mutation from Cys83 to Ser83 in Prx1 can result in dramatic changes in the structural and functional characteristics of Prx1 [18]. Prx1 is more efficient as a molecular chaperone, whereas Prx2 is always regarded as a peroxidase enzyme [18].

Abbreviations: BCA, bicinchoninic acid; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tags; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; *L. japonica*, *Lampetra japonica*; Lj-Prx2, *L. japonica* Prx2; LPS, lipopolysaccharide; MFO, mixed-function oxidase; NKEF, natural killer cell enhancing factor; ORF, open reading frame; Prx, peroxiredoxin; RBCs, red blood cells; rLj-Prx2, recombinant *L. japonica* Prx2; ROS, Reactive oxygen species; TCA, trichloroacetic acid.

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doi:10.1016/j.fsi.2010.01.018

Lampreys are considered to be the most scientifically accessible model of the remaining jawless vertebrates [19]. Researches in the evolution and development of immunological structures in lamprey may contribute to better understand how an adaptive immune system could have evolved [20]. Adult lampreys attach to the host fishes with a sucking mouth and feed on the blood and body fluids [21,22]. Some compounds in the buccal gland secretion, such as anaesthetic, anticoagulant and vasodilator, are indicated to facilitate parasitic lampreys to prey upon hosts [23–25].

Here, we report the molecular cloning and characterization of a Prx2 homologue from *Lampetra japonica* by analyzing the expressed sequence tags (ESTs) of the buccal gland cDNA library [26]. Our results suggest that the Prx2 gene is widely expressed in adult lampreys. Interestingly, the expression is found to increase in the RBCs of lamprey under LPS-stimulated condition. The rLj-Prx2 is a secretory protein and has the peroxidase activity. In addition, the potential effects of Prx2 on antioxidant defense and counteracting oxidative damage from the host are also discussed.

2. Materials and methods

2.1. Materials

Adult lampreys *L. japonica* (lengths: 36.4–58.4 cm, weights: 112–274.5 g) were obtained from the Tongjiang Valley of Songhua River, Heilongjiang Province, China, in December. The cDNA library construction and EST sequencing of the buccal gland had been previously reported elsewhere by our lab [26].

2.2. EST analysis

Based on the analysis of cDNA library and ESTs [26], we discovered a Prx2 homolog in *L. japonica* by using NCBI's Basic Local Alignment Search Tool (BLAST).

2.3. Cloning of Lj-Prx2 gene

Total RNA was isolated from *L. japonica* buccal gland by using Trizol (GIBCO BRL). The cDNA was obtained according to the manufacturer's instruction of High Fidelity PrimeScript™ RT-PCR Kit (TaKaRa). PCR amplification was performed using two primers as follows: forward: 5′-TCATGTCTGCTGGCAACGCT-3′, reverse: 5′-CAGGAGTTGACACATCTTGA-3′. The product was then purified and cloned into pMD19-T vector using DNA Ligation kit (TaKaRa). TaKaRa sequenced both strands of the plasmid DNA.

2.4. Amino acid sequence analysis and phylogenetic reconstruction

Additional 31 homologue protein sequences of Prx1 and Prx2 were obtained from ExPASy (Expert Protein Analysis System) at http://www.expasy.ch/tools/blast/. A multiple sequence alignment was obtained using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw/). The program MEGA 4 was used to construct a phylogenetic tree [27].

2.5. Real time quantitative PCR

Six lampreys were randomly divided into two groups, one was control group and the other was LPS-stimulated group. Lampreys in LPS-stimulated group were each injected by 0.2 mL (50 μg/mL) LPS at about 4 °C of water temperature. After 24 h, total RNA was extracted from buccal glands, hearts, livers, kidneys, gills, intestines, RBCs and white blood cells using Trizol (GIBCO BRL). The real time quantitative PCR was carried out with the TaKaRa SYBR® PrimeScript™ RT-PCR Kit according to the manufacturer's protocol. Reverse transcription was performed as described previously [28]. The PCR mix was composed of 12.5 μL SYBR *Premix Ex* Taq (2×), 1 μL of each primer (10 μM), 2 μL of DNA in a final volume of 25 μL. The amplification was carried out on TaKaRa PCR Thermal Cycler Dice Real Time System with the profile as follows: 95 °C for 10 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s. Specific primers for Lj-Prx2 gene were 5′-ACGAGATCCTCCGCTTGGT-3′ (forward) and 5′-CTGGCTTGATGGTGTCCTTG-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and primers for *L. japonica* GAPDH gene were 5′-AACCAACTGCCTGGCTCCT-3′ (forward) and 5′-GTCTTCTGCGTTGCC GTGT-3′ (reverse) [28]. Each sample was analyzed three times. Data were analyzed with the Thermal Cycler Dice Real Time System analysis software (TaKaRa).

2.6. Expression vector construction and in vitro expression of rLj-Prx2

The open reading frame (ORF) of Lj-Prx2, flanked by an *EcoR* I restriction site and a *Not* I restriction site, was amplified and subcloned into the pET23b vector with the His-tag. The recombinant plasmid was expressed in *Escherichia coli* BL21 (DE3) as described in our previous study [29] with minor modifications. The expression of the recombinant protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Subsequently, cells were centrifuged and the pellet was re-suspended in 20 mM Tris–HCl buffer containing 5 mM imidazole and 500 mM NaCl (pH7.9). The cell suspension was sonicated for 15 min on ice, centrifuged again at 5000 rpm for 15 min at 4 °C. The pellet was re-suspended in 1× Binding buffer (20 mM sodium phosphate, 500 mM NaCl, 45 mM imidazole, pH 7.4) with 8 M Urea, 90 mM DTT, and then incubated at room temperature for 1 h, and centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was collected and the histidine tagged fusion protein was purified by HIS TAG – Metal affinity column (Novagen). The concentration of rLj-Prx2 was measured using a Bicinchoninic Acid (BCA) Protein Assay kit (BEYOTIME).

2.7. Preparation of polyclonal antibody and western blot analysis

Polyclonal antibody preparation of rLj-Prx2 and western blot analysis that was used to prove Lj-Prx2 was secretory was performed as described before [29].

2.8. In vitro peroxidase activity

Each of the rLj-Prx2 samples with concentrations of 0, 20, 50 and 100 μg/mL was incubated with 50 mM Hepes–HCl (pH7.0) containing 5 mM DTT for 10 min at room temperature. The reaction was initiated by the addition of 6 μL 30% H_2O_2, and then incubated for 0, 2, 5 and 10 min, respectively. The reaction was stopped by adding 100 μL 100% (w/v) trichloroacetic acid (TCA). The remaining peroxide content was determined by measurement of the red-colored ferrithiocyanate complex after adding 10 mM Fe $(NH_4)_2(SO_4)_2$ and 2.5 M KSCN to the reaction mixture at room temperature. The absorbance was measured at 475 nm [30].

2.9. DNA cleavage assay

Supercoiled pUC18 plasmid DNA (TaKaRa) was used as substrate for detecting DNA damage by the thiol-dependent mixed-function oxidation (MFO) system [31]. The reaction mixture containing 3 μM $FeCl_3$, 10 mM DTT and 1 μg pUC18 plasmid DNA in 50 mM Hepes, pH7.0 was incubated with or without different concentrations of

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                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                                      5         15         25         35         45         55         65
Human Prx1                       MSSG------ --NAKIGHPA PNFKATAVMP DGQFKDISLS DYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
Rat Prx1                         MSSG------ --NAKIGHPA PSFKATAVMP DGQFKDISLS DYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
Bovin Prx1                       MSSG------ --NAKIGHRA PQFKATAVMP DGQFKDISLA DYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
Mouse Prx1                       MSSG------ --NAKIGYPA PNFKATAVMP DGQFKDISLS EYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
Little brown bat Prx1            MSSG------ --NAKIGHPA PNFKATAVMP DGQFKDISLS DYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
Tammer wallaby Prx1              MSAG------ --NAKIGFPA PNFRATAVMP DRQFKDISLS DYKGKYVVLF FYPLDFTFVC PTEIIAFSDR
Chick Prx1                       MSSG------ --KAFIGKPA PDFTATAVMP DGQFKDIKLS DYRGKYVVFF FYPLDFTFVC PTEIIAYSDR
Chinese hamster Prx1             MSSG------ --NAKIGYPA PNFKATAVMP DGQFRDICLS EYRGKYVVFF FYPLDFTFVC PTEIIAFSDR
Japanese gecko Prx1              MSSG------ --NAYIGKLA PDFQATAVMP DGQFKEIKLS DYKGKYVVLF FYPLDFTFVC PTEIIAFSDR
Western clawed frog Prx1         MSSG------ --NAKIGHPA PDFTAKAVMP DGQFKDLKVS DYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
African clawed frog Prx1         MSVG------ --NAKIGQPA PDFTAQAVMP DGQFKNLKIS DYKGKYVVFF FYPLDFTFVC PTEIIAFSDR
Common carp NKEF-A               MAAG------ --KAHIGKPA PDFTAKAVMP DGQFKDLSLS EYKGKYVVLF FYPLDFTFVC PTEIIAFSDA
Channel catfish NKEF-A           MSAG------ --KAQIGKPA PDFTAKAVMP DGQFKDLKLS DYRGKYVVFF FYPLDFTFVC PTEIIAFSDA
Northern pike Prx1               MASG------ --KARIGHPA PHFKATAVMP DGQFKGISIS DYKGKYVVFF FYPLDFTFVC PTEIIAFSDA
Rainbow trout NKEF-A             MAAG------ --KARIGHLA PGFTAKAVMP DGQFKDISMS DYRGKYVVFF FYPLDFTFVC PTEIIAFSDA
Green puffer NKEF-A              MAAG------ --KAQIGKLA PDFTAKAVMP DGQFHDLKLS DYRGKYVVFF FYPLDFTFVC PTEIIAFSDA
Japanese flounder NKEF-A         MAAG------ --SAQIGKPA PDFTAKAVMP DGQFKDLTLS NYRGKYVVFF FYPLDFTFVC PTEIIAFSDA
Sablefish Prx1                   MAAG------ --KAQIGKLA PDFTAKAVMT DGQFNDLKLS DYRGKYVVFF FYPLDFTFVC PTEIIAFSDA
Human Prx2                       MASG------ --NARIGKPA PDFKATAVV- DGAFKEVKLS DYKGKYVVLF FYPLDFTFVC PTEIIAFSNR
Cynomolgus monkey Prx2           MASG------ --NARIGKPA PDFKATAVV- DGAFKEVKLS DYKGKYVVLF FYPLDFTFVC PTEIIAFSNR
Rat Prx2                         MASG------ --NAHIGKPA PDFTGTAVV- DGAFKEIKLS DYRGKYVVLF FYPLDFTFVC PTEIIAFSDH
Mouse Prx2                       MASG------ --NAQIGKSA PDFTATAVV- DGAFKEIKLS DYRGKYVVLF FYPLDFTFVC PTEIIAFSDH
Sheep Prx2                       MACG------ --KAHLGKPA PEFQATAVV- DGAFKEVKLS DYKGKYVVLF FYPLDFTFVC PTEIVAFSDR
Bovin Prx2                       MACVC----- --KAHVGKPA PEFQATAVV- DGAFKEVKLS DYKGKYVVLF FYPLDFTFVC PTEIVAFSDR
Chinese hamster Prx2             MASG------ --NAHIGKPA PDFTATAVV- DGAFKEVKLS DYRGKYVVLF FYPLDFTFVC PTEIIAFSNH
Common carp NKEF-B               MSSG------ --NAKIGQPA PQFKATAVV- DGQFKDVQLS EYRGKYVVLF FYPLDFTFVC PTEIIAFSER
Zebrafish Prx2                   MSAG------ --NAKIGQPA PQFKATAVV- DGQFKDIQLS DYRGKYVVLF FYPLDFTFVC PTEIIAFSER
Ayu NKEF-B                       MSSG------ --KAKIGQAA PQFKATAVV- DGQFKDIQLS DYKGKYVVFF FYPLDFTFVC PTEIVAFSDR
Green puffer NKEF-B              MSSG------ --SAKIGQPA PDFTAKAVV- DGQFKDLRLS DYRGKYVVFF FYPLDFTFVC PTEIVAFSDR
Western clawed frog Prx2         MSCPVGPGVR AVKTHIGQPA PAFKATAVV- NGEFKDIQLS DYLGKYVVLF FYPLDFTFVC PTEIIAFSDH
African clawed frog Prx2         MACPV----R AVKTHIGKPS PAFQATALV- NGEFKEIQLS DYLGKYVVLF FYPLDFTFVC PTEIIAFSNH
Lampetra japonica Prx2           MSAG------ --NASIGKPA PDFSGVAVV- NGAFKDIKLS NYKGKYVVLF FYPLDFTFVC PTEIIAFNDR
Clustal Consensus                *:           .: :*  : * * . *::  :  *. : :: :* *****:* ********** ****:*:.:

                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                                     75         85         95        105        115        125        135
Human Prx1                       AEEFKKLNCQ VIGASVDSHF CHLAWVNTPK KQGGLGPMNI PLVSDPKRTI AQDYGVLKAD EGISFRGLFI
Rat Prx1                         AEEFKKLNCQ VIGASVDSHF CHLAWINTPK KQGGLGPMNI PLVSDPKRTI AQDYGVLKAD EGISFRGLFI
Bovin Prx1                       AEEFKKLNCQ VIGASVDSHF CHLAWINTPK KQGGLGPMNI PLISDPKRTI AQDYGVLKAD EGISFRGLFI
Mouse Prx1                       ADEFKKLNCQ VIGASVDSHF CHLAWINTPK KQGGLGPMNI PLISDPKRTI AQDYGVLKAD EGISFRGLFI
Little brown bat Prx1            AEEFKKINCQ VIGASVDSHF CHLAWINTPK KQGGLGPMNI PLVSDPKRTI AQDYGVLKAD EGISFRGLFI
Tammer wallaby Prx1              AEEFKKLNCQ VIGASVDSHF CHLAWVNTVK KNGGLGAVNI PLMSDPKRTI AQDYGILKED EGISFRGLFI
Chick Prx1                       ADEFKKINCE IIGASVDSHF CHLAWINTPK KQGGLGTMKI PLVSDTKRVI AKDYGVLKED EGIAYRGLFI
Chinese hamster Prx1             AEEFKKLNCQ VIGASVDSHF CHLAWINTPK KQGGLGPMNI PLVSDPKRTI AQDYGVLKAD EGISFRGLFI
Japanese gecko Prx1              SEEFRKINCE VIGASVDSHF CHLAWINTPK KQGGLGSMHI PLVSDTKRVI AKDYGILKED EGISYRGLFI
Western clawed frog Prx1         VEEFKKLNCE VIGASGDSHF CHLAWISQPR KEGGLGKMNI PLVSDVQHTI AKDYGVFEEK EGVSFRGLFI
African clawed frog Prx1         VEEFKKINCE VIGASGDSHY CHLAWINQPR KEGGLGQMKI PLVADVQHTI AKDYGVFKED DGVSFRGLFI
Common carp NKEF-A               VEEFRKINCE VIGASVDSHF CHLAWINTPR KQGGLGHMNV PLVADSLRSI SQDYGVLKED EGIAYRGLFI
Channel catfish NKEF-A           AEEFKKINCE VIGASVDSHF CHLAWINTPR KQGGLGHMKV PLVADTKRSI SQDYGVLKED EGIAYRGLFI
Northern pike Prx1               AEEFRKIGCE VIGASVDSHF CHLAWTNTPR KQGGLGAMKI PLVADTLRSI STDYGVLKED EGIAYRGLFI
Rainbow trout NKEF-A             AEEFRKIGCE VIGASVDSHF CHLAWTNTPR KHGGLGAMKI PLVADTLRSI STDYGVLKED EGIAYRGLFI
Green puffer NKEF-A              AEDFRKIGCE VIAASVDSHF SHFAWTNTPR KQGGLGTMKI PLVSDTRHTI STDYGVLKED EGIAYRGLFI
Japanese flounder NKEF-A         AEQFRKIGCE VIAASVDSHF SHFAWTNTPR KQGGLGTMKI PLVSDTRRTI STDYGVLKED EGIAYRGLFI
Sablefish Prx1                   ADDFRKIGCE VIGASVDSHF SHFAWINTPR KQGGLGPMKI PLVSDTRRTI STDYGVLKED EGIAYRGLFI
Human Prx2                       AEDFRKLGCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTRRL SEDYGVLKTD EGIAYRGLFI
Cynomolgus monkey Prx2           AEDFRKLGCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTRRL SEDYGVLKTD EGIAYRGLFI
Rat Prx2                         AEDFRKLGCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTKSL SQNYGVLKND EGIAYRGLFI
Mouse Prx2                       AEDFRKLGCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTKSL SQNYGVLKND EGIAYRGLFI
Sheep Prx2                       AEEFHKLNCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTRKL SSDYGVLKED EGVAYRGLFV
Bovin Prx2                       AAEFHKLNCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTRKL SSDYGVLKED EGIAYRGLFV
Chinese hamster Prx2             AEDFRKLGCE VLGVSVDSQF THLAWINTPR KEGGLGPLNI PLLADVTRSL SENYGVLKTD EGIAYRGLFI
Common carp NKEF-B               AAEFRKIGCE VIAASTDSHF SHLAWINTPR KQGGLGSMNI PLVADLTQSI SRDYGVLKED EGLAYRGLFV
Zebrafish Prx2                   AAEFRKIGVE LIAASTDSHF SHLAWINTPR KQGGLGSMNI PLVADLTQSI SRDYGVLKED EGIAYRGLFV
Ayu NKEF-B                       AADFRKIGCE VIAASTDSHF SHLAWINTPR KQGGLGPMNI PLVADLTQSI SRDYGVLKEE DGIAYRGLFV
Green puffer NKEF-B              VQDFRSINCE VIGCSIDSHF THLAWINTPR KEGGLGEMKI PLVADLTKSI SKDYGVLKED DGIAYRGLFV
Western clawed frog Prx2         AGDFSKINCQ LIAVSVDSQF THLAWTNVPR KEGGLGPINI PLVSDLTHSI AKDYGVLKEE DGVAYRGLFI
African clawed frog Prx2         AEDFKKINCQ LIAVSVDSQF THLAWTKVPR KEGGLGPVNI PLVSDLTHSI AKDYGVLKEE DGVAYRGLFI
Lampetra japonica Prx2           LPEFTKLDCA VIAASTDSQF SHLAWVNTPR KQGGLGHMEI PLLADRTCNV AKNYGVLKED EGIAYRGLFI
Clustal Consensus                  :* .:.   ::. * **::  *:** .  : *.**** :.: **::*    : : :**::: . :*:::****:

                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|..
                                    145        155        165        175        185        195        205
Human Prx1                       IDDKGILRQI TVNDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK EYFSKQK
Rat Prx1                         IDDKGILRQI TINDLPVGRS VDEILRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVNKSK EYFSKQK
Bovin Prx1                       IDDKGILRQI TINDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK EYFSKQK
Mouse Prx1                       IDDKGILRQI TINDLPVGRS VDEIIRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVNKSK EYFSKQK
Little brown bat Prx1            IDDKGILRQI TVNDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK EYFSKQK
Tammer wallaby Prx1              IDDKGILRQI TINDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGLEPXSD TIKPDVQGSK EYFSKQE
Chick Prx1                       IDEKGILRQI TINDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK EYFSKQK
Chinese hamster Prx1             IDDKGILRQI TINDLPVGRS VDEILRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK EYFSKQK
Japanese gecko Prx1              IDDKGTLRQI TINDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGWQPGSD TIKPDVQKSK EYFSKHK
Western clawed frog Prx1         IDEKGILRQI TINDLPVGRS VDETLRLVQA FQFTDKYGEV CPAGWQPGSD TIKPDVKKSK EYFNKQK
African clawed frog Prx1         IDEKGILRQI TINDLPVGRS VDETLRLVQA FQFTDKYGEV CPAGWQPGSD TIKPDVQKSK EYFSKQK
Common carp NKEF-A               IDDKGILRQI TINDLPVGRS IDETLRLVQA FQFTDKHGEV CPAGWKPGKD TIKPDVQQSK DYFSKQH
Channel catfish NKEF-A           IDDKGILRQI TINDLPVGRS IDETLRLVQA FQFTDKHGEV CPAGWKPGKD TIKPDVQKSK DFFSKQN
Northern pike Prx1               IDDKGILRQI TINDLPVGRS VDETIRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK DFFSKQK
Rainbow trout NKEF-A             IDDKGVLRQI TINDLPVGRS VDETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK DFFSKQQ
Green puffer NKEF-A              IDPKGILRQI TINDLPVGRS VEETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK EFFSKH-
Japanese flounder NKEF-A         IDDKGILRQI TINDLPVGRS VEETLRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK DFFSKQ-
Sablefish Prx1                   IDDKGVLRQI TINDLPVGRS VEETMRLVQA FQFTDKHGEV CPAGWKPGSD TIKPDVQKSK DFFSKQ-
Human Prx2                       IDGKGVLRQI TVNDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWKPGSD TIKPNVDDSK EYFSKHN
Cynomolgus monkey Prx2           IDGKGVLRQI TVNDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWKPGSD TIKPNVDDSK EYFSKHN
Rat Prx2                         IDAKGVLRQI TVNDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWKPGSD TIKPNVDDSK EYFSKHN
Mouse Prx2                       IDAKGVLRQI TVNDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWKPGSD TIKPNVDDSK EYFSKHN
Sheep Prx2                       IDGKGVLRQV TVNDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWTPGSD TIKPNVDDSK EYFSKHN
Bovin Prx2                       IDGKGVLRQV TINDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWTPGSD TIKPNVDDSK EYFSKHN
Chinese hamster Prx2             IDAKGILRQI TVNDLPVGRS VDEALRLVQA FQYTDEHGEV CPAGWKPGSD TIKPNVDDSK EYFSKHN
Common carp NKEF-B               IDDKGILRQI TINDLPVGRS VDETLRLVQA FQHTDKYGEV CPAGWKPGSD TIVPDVQKSK EFFSKQ-
Zebrafish Prx2                   IDDKGILRQI TINDLPVGRS VDETLRLVQA FQHTDKYGEV CPAGWKPGSD TIVPDVQKSK EFFSKQ-
Ayu NKEF-B                       IDDKGILRQI TINDLPVGRS VDETLRLVQA FQHTDKYGEV CPAGWKPGSD TIVPDVQKSK EFFSKQ-
Green puffer NKEF-B              IDDKGVLRQI TVNDLPVGRS VDETLRLVQA FQFTDKYGEV CPAGWKPGSD TIVPDVEKSK TFFSKQN
Western clawed frog Prx2         IDGKGNLRQI TINDLPVGRS VEETLRLVQA FQYTDQHGEV CPAGWKPGSS TIKPNVKDSK EFFSKEY
African clawed frog Prx2         IDGKGILRQI TINDLPVGRS VDETLRLVQA FQYTDVHGEV CPAGWKPGSS IIKPNVKDSK EFFSKEY
Lampetra japonica Prx2           IDGKGILRQI TINDLPVGRD VDEILRLVQA FKFTDEHGEV CPAGWKPGKD TIKPDVQKSK EFFAKQ-
Clustal Consensus                ** ** ***: *:*******. ::* :***** *:.** :*** ****  * ..  * *:*. **  :* *.
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rLj-Prx2 at 37 °C for 2 h, and then applied to 1% agarose gel electrophoresis to examine DNA cleavage.

2.10. Data analysis

Data were shown as the mean ± SD. The results were analyzed using *t*-test, and values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cloning of Lj-Prx2 gene

A single EST homologous to Prx2 was found among the extensive EST sequences from the cDNA library of *L. japonica* buccal gland [26]. The ORF of Prx2 is 594 bp in length that ends at the termination codon TGA. It encodes a protein of 197 amino acids with a predicted molecular mass of 21.7 kDa (ProtParam program of ExPASy, http://www.expasy.ch/tools/protparam.html). The nucleotide sequence of Lj-Prx2 gene has been submitted to GenBank database with the accession number of GQ357639.

3.2. Amino acid sequence alignment and phylogenetic tree

Comparison of Lj-Prx2 with other Prxs in other species reveals common structural features. The Lj-Prx2 possesses the main feature of the typical 2-Cys Prx given that it contains two highly conserved cysteine residues: peroxidatic cysteine and resolving cysteine, which are found at positions 51 and 172, respectively. They are surrounded by two motifs FFYPLDFTFVCPTEI and GEVCPA, respectively (Fig. 1).

Lj-Prx2 shows high sequence similarities (>70%) with Prx1 and Prx2 in other species (Table 1). There are two clades in the phylogenetic tree. One is composed of all Prx1 (NKEF-A) sequences and the other comprises all Prx2 (NKEF-B) sequences. Lj-Prx2 is found to group with previously reported teleost Prx2 (Fig. 2).

3.3. Expression analysis of Prx2 mRNA in L. japonica

To determine the expression of Lj-Prx2 gene in various tissues, we performed real time quantitative PCR assay with GAPDH as an internal control. As shown in Fig. 3, Lj-Prx2 gene was ubiquitously expressed in a variety of tissues including buccal glands, hearts, livers, kidneys, gills, intestines, RBCs and white blood cells. The strongest expression was detected in RBCs and weakest in white blood cells. Obviously, the level of Lj-Prx2 gene expression in RBCs remained at a high level in LPS-stimulated group. Nearly 2-fold increase in mRNA level relative to control was observed.

3.4. In vitro expression and purification of recombinant protein

The Lj-Prx2 gene cloned into pET23b was expressed as a His-tagged fusion protein in *E. coli* BL21 (DE3) with the treatment of IPTG. As shown in Fig. 4a, the recombinant protein has a molecular mass of approximately 23 kDa. The concentration of the purified rLj-Prx2 (Fig. 4b) was about 0.5 mg/mL.

Table 1
The Prx2 sequence identity of *L. japonica* compared with homologs of other 31 species.

Species	Gene	Uniport No	Sequence identity (%)
Common carp	NKEF-B	A8CV51	77
Zebrafish	Prx2	Q6DGJ6	77
Rat	Prx2	P35704	76
Mouse	Prx2	Q61171	75
Chinese hamster	Prx2	Q8K3U7	75
Cynomolgus monkey	Prx2	Q2PFZ3	75
Human	Prx2	P32119	75
Ayu	NKEF-B	C1KUR3	75
Bovine	Prx2	Q9BGI3	74
Green puffer	NKEF-B	Q4S7T1	72
Western clawed frog	Prx2	Q6P8F2	72
Sheep	Prx2	C8BKC5	72
African clawed frog	Prx2	Q6ING3	71
Channel catfish	NKEF-A	Q643S2	77
Common carp	NKEF-A	O93241	76
Chicken	Prx1	P0CB50	76
Japanese gecko	Prx1	Q6DV14	76
Human	Prx1	Q06830	75
Mouse	Prx1	B1AXW7	75
Rat	Prx1	Q63716	75
Green puffer	NKEF-A	Q4ZJF5	75
Japanese flounder	NKEF-A	Q4ZH88	75
Chinese hamster	Prx1	Q9JKY1	75
Bovine	Prx1	Q5E947	74
Little brown bat	Prx1	Q6B4U9	74
Northern pike	Prx1	C1BWR2	74
Sablefish	Prx1	C3KHC7	73
Rainbow trout	NKEF-A	Q9I886	73
Tammar wallaby	Prx1	B4XEM9	73
Western clawed frog	Prx1	Q5XH88	72
African clawed frog	Prx1	Q6GQB3	72

3.5. ELISA and western blot analysis

ELISA showed that the titer of the antibody against rLj-Prx2 was about 1:12,800. As from the western blotting analyses (Fig. 4c), we observed that the polyclonal antibody could recognize both rLj-Prx2 and natural Prx2 protein in the buccal gland secretion, indicating that the Prx2 from buccal gland of *L. japonica* is secretory.

3.6. Characterization of recombinant protein

The peroxidase activity of rLj-Prx2 was measured by the decrease of H_2O_2 in the reaction mixture with or without DTT (Fig. 5). As expected, when DTT was present in the reaction mixture, the rate of H_2O_2 degradation was gradually increased as the concentration of recombinant protein increased. It was higher than the control group without rLj-Prx2 (Fig. 5a). In comparison, the rLj-Prx2 had almost no effect on the degradation of H_2O_2 without DTT (Fig. 5b). These results show that the peroxidase activity of rLj-Prx2 is thiol-dependent.

To evaluate the ability of rLj-Prx2 in protecting DNA from oxidative damage, a thiol-dependent MFO system was chosen (Fig. 6). In the MFO system, the supercoiled plasmid DNA was converted into nicked form (lane 2). The rLj-Prx2 was found to prevent supercoiled DNA from degrading in a dose-dependent

Fig. 1. Multiple sequence alignment of Lj-Prx2 with other species Prx1 and Prx2. The ExPASy accession number used are as follows: common carp NKEF-B, A8CV51; rat Prx2, P35704; mouse Prx2, Q61171; Chinese hamster Prx2, Q8K3U7; cynomolgus monkey Prx2, Q2PFZ3; human Prx2, P32119; ayu NKEF-B, C1KUR3; bovine Prx2, Q9BGI3; green puffer NKEF-B, Q4S7T1; zebrafish Prx2, Q6DGJ6; western clawed frog Prx2, Q6P8F2; sheep Prx2, C8BKC5; African clawed frog Prx2, Q6ING3; channel catfish NKEF-A, Q643S2; common carp NKEF-A, O93241; chicken Prx1, P0CB50; Japanese gecko Prx1, Q6DV14; human Prx1, Q06830; mouse Prx1, B1AXW7; rat Prx1, Q63716; green puffer NKEF-A, Q4ZJF5; Japanese flounder NKEF-A, Q4ZH88; Chinese hamster Prx1, Q9JKY1; bovine Prx1, Q5E947; little brown bat Prx1, Q6B4U9; northern pike Prx1, C1BWR2; Sablefish Prx1, C3KHC7; rainbow trout NKEF-A, Q9I886; tammar wallaby Prx1, B4XEM9; western clawed frog Prx1, Q5XH88; African clawed frog Prx1, Q6GQB3. Invariant residues are shown in asterisks (*). Strong and weak homologous residues are indicated in colons (:) and dots (.), respectively. Two signature motifs FFYPLDFTFVCPTEI and GEVCPA are covered with vertical bars.

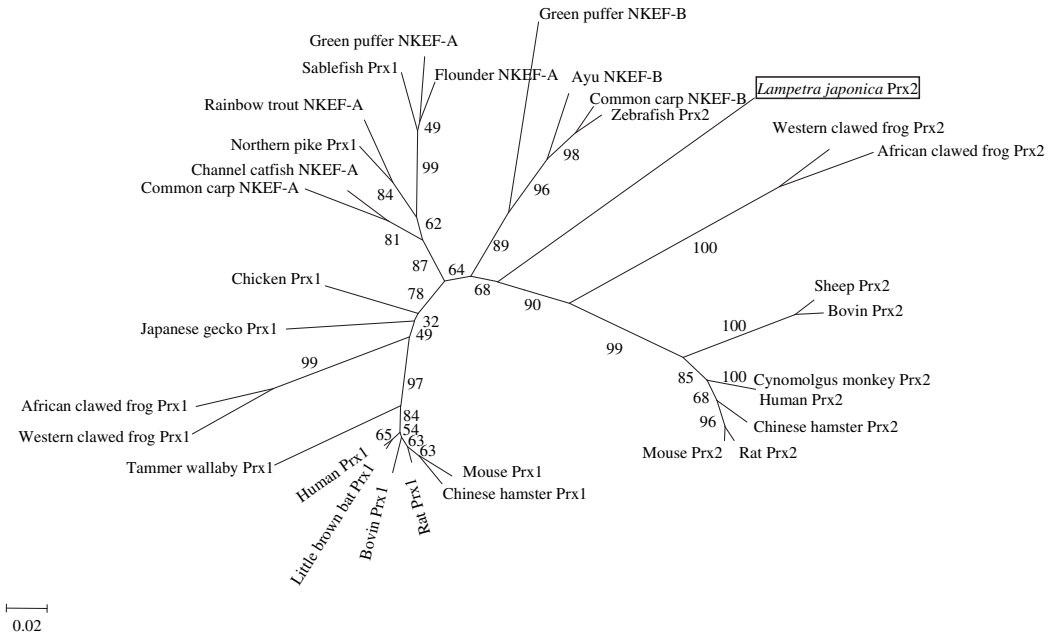

Fig. 2. Phylogenetic tree constructed using the neighbor-joining method. GenBank accession numbers for the sequences used are the same as those listed in Fig. 1 legend.

manner (lane 3–9), suggesting that rLj-Prx2 can act as an antioxidant protein.

4. Discussion

Reactive oxygen species (ROS), which derived from the interaction of metabolism with oxygen, include not only the radicals hydroxyl, peroxyl, nitric oxide and superoxide but also the non-radicals hypochlorous acid, singlet oxygen, peroxynitrite, ozone and H_2O_2 [32]. In living cells, the major source of endogenous ROS are hydrogen peroxide and superoxide anion, which lead to a continuous threat to cells and cause many diseases due to their oxidative damage to DNA, protein and lipids [33,34]. During the long-term evolution, a series of antioxidant defense systems have been formed so as to maintain intracellular redox homeostasis [34]. Prx is one of the most important antioxidants which protect organism against oxidative damage. So far researches have focused mainly on Prxs in jawed vertebrates [35–39], little is known about the existence of Prx in lamprey which is one of the most ancient jawless vertebrates. In the present study, we obtained Prx2 gene from *L. japonica* and this is the first report about Prx gene in lamprey. The expression pattern of Lj-Prx2 gene was examined. The antioxidant activity of the recombinant protein was identified.

To further analyze the relationship between Lj-Prx2 and other vertebrate Prx1 and Prx2, a phylogenetic tree was constructed. Although the deduced amino acid sequence of Lj-Prx2 shares 77% identity with channel catfish NKEF-A (Table 1), it does not group with the Prx1 subfamily (Fig. 2). As seen from the sequence comparison and phylogenetic tree, our study demonstrated that Lj-Prx2 should be classified into the Prx2 subfamily.

Comparing with other cell types, RBCs are more exposed to oxidative stress because of high cellular concentration of heme iron and oxygen, which can generate H_2O_2 and lipid peroxide [40]. RBC membrane may be damaged under conditions of excessive oxidative stress [40]. Thus, there may be a potential antioxidant defense system against peroxides in RBCs to keep them integrated. As shown in Fig. 3, the highest expression level of Prx2 in RBCs indicated that Lj-Prx2 can act as a very effective antioxidant to remove oxidative stress in RBCs to protect lamprey from damage by ROS and therefore ensure its own survival. In addition, the observation that the expression of Prx2 in RBCs under normal condition was almost 2-fold lower than the level after LPS stimulation indicated that Prx2 may be an inflammatory stress inducible gene associated with anti-bacterial defense. Furthermore, stable expression of Lj-Prx2 was found in other tissues tested (Fig. 3). Liver and intestine take charge of detoxification, digestion and absorption, while gill is responsible for respiration and osmoregulation. Given that all of these organs are continuously exposed to the attack of ROS, Lj-Prx2 in liver, intestine and gill may therefore also act as the antioxidant. Because the cardiovascular system is prone to be injured by oxidants, Lj-Prx2 may also play a protective role in cardiac oxidative stress.

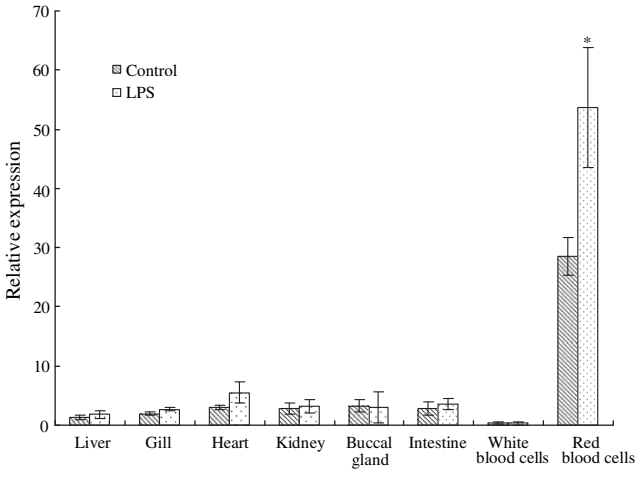

Fig. 3. Expression of Lj-Prx2 after LPS stimulation. Relative expression of Prx2 in *L. japonica* was detected using real time RT-PCR. The group without LPS stimulation was used as control. Values shown are the mean (±SD) of three experiments. The strongest expression was found in RBCs. The significant difference ($P < 0.05$) of Prx2 expression between the LPS-stimulated group and the control group were indicated with asterisks.

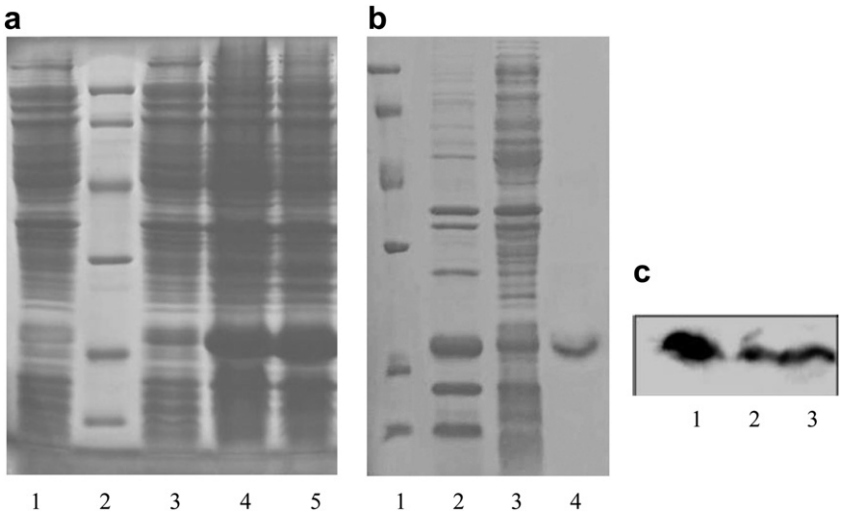

Fig. 4. Expression of rLj-Prx2 and western blot analysis. (a) SDS-PAGE analysis of recombinant protein expressed in *E. coli* BL21. 1, induced expression of BL21/pET23b; 2, low molecular weight protein marker. From the top down, the molecular weight of each band is 97.2, 66.4, 44.3, 29.0, 20.1 and 14.3 kDa, respectively; 3, non-induced expression of BL21/pET23b-Prx2; 4 and 5, induced expression of BL21/pET23b-Prx2. (b) Purification of recombinant protein. 1, low molecular weight protein marker. 2, the precipitate before purification; 3, non-induced expression of BL21/pET23b-Prx2; 4, the purified recombinant protein; (c) Western blot analysis. 1, the hybridization between buccal gland secretion (1:10 dilution with PBS) and polyclonal antibody; 2, the hybridization between buccal gland secretion (1:20 dilution with PBS) and polyclonal antibody; 3, the hybridization between rLj-Prx2 and polyclonal antibody.

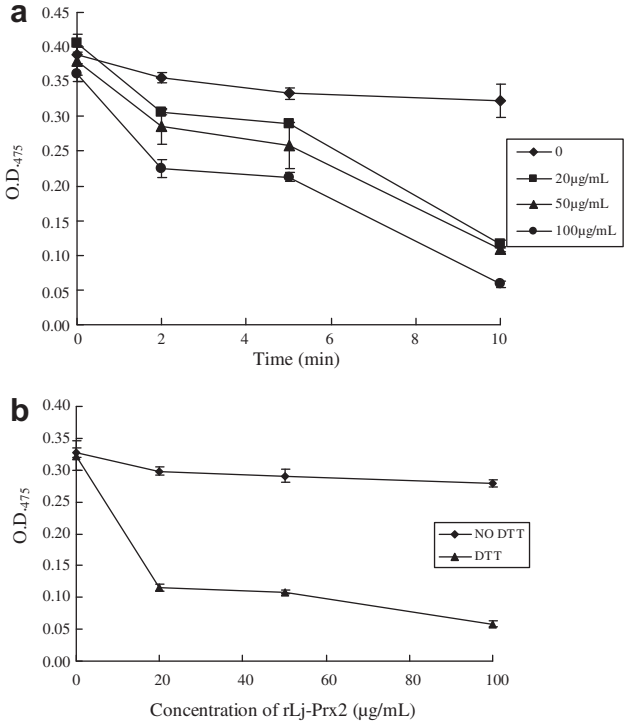

Fig. 5. H_2O_2 eliminable activity of rLj-Prx2. (a) Peroxidase activity of rLj-Prx2 in different concentrations and incubation times. Abscissa indicates incubation time. Solid diamond, square, triangle and circle represent different concentration of recombinant protein (0, 20, 50, 100 μg/mL) added in the reaction mixture. Values shown are the mean (±SD) of three experiments. Along with the increase of incubation time and protein concentration, the peroxidase activity of rLj-Prx2 in reaction mixture is obviously higher than controls. (b) The effect of DTT on recombinant protein activity. The reaction mixture with or without DTT is indicated by solid diamond and triangle, respectively. The incubation time is 10 min. Along with the increase of protein concentration, the peroxidase activity of rLj-Prx2 in reaction mixture with DTT is significant higher than controls.

Although, like other homologues from teleosts and mammals, the signal peptide of Lj-Prx2 was absent (SignalP 3.0 Server. http://www.cbs.dtu.dk/services/SignalP/), our result proved that it is a secretory protein. A recent study shows that human Prx1 is secreted through a non-classical ER/Golgi-independent secretory pathway, and the process of secretion is induced by TGF-β1 [41]. In view of this, it is likely that Lj-Prx2 might also be secreted in a similar manner. Expression of Prx2 in buccal gland secretion may be related to the parasitic habit of lamprey. Earlier study has shown that the buccal gland secretion of lamprey acts as an anticoagulant [23]. Our study revealed that it may also have an antioxidant effect. Due to the fact that during a long-term parasitical state, lamprey is likely to endure ROS generated by metabolic processes and immune defenses from host, we predict that the lamprey buccal gland can secrete an antioxidant to suppress the oxidative assault from host and make lamprey alive. In addition, our results demonstrated that the rLj-Prx2 can scavenge H_2O_2 and protect DNA, leading us to propose that the recombinant Prx2 may also act as a potential drug for ROS-associated diseases.

In conclusion, we have cloned a homologue of the typical 2-Cys Prx2 from the buccal gland of *L. japonica*. Lj-Prx2, as a secretory protein, has the ability to remove H_2O_2 and protect DNA from oxidative injury. The strong expression of Prx2 in RBCs indicates that it may participate in antioxidant defense which is important to maintain the lifespan of lamprey.

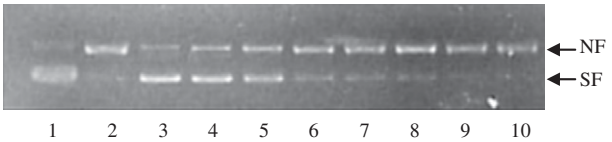

Fig. 6. DNA protection of rLj-Prx2 in MFO system. 1. DNA only; 2. Fe^{3+} + DTT; 3. Fe^{3+} + DTT + 16 μg rLj-Prx2; 4. Fe^{3+} + DTT + 12 μg rLj-Prx2; 5. Fe^{3+} + DTT + 10 μg rLj-Prx2; 6. Fe^{3+} + DTT + 8 μg rLj-Prx2; 7. Fe^{3+} + DTT + 4 μg rLj-Prx2; 8. Fe^{3+} + DTT + 2 μg rLj-Prx2; 9. Fe^{3+} + DTT + 1 μg rLj-Prx2; 10. Fe^{3+} + DTT + BSA. SF, supercoiled form; NF, nicked form.

Acknowledgment

This work was joint supported by the National High Technology Research and Development Program of China (No. 2007AA09Z428), the National Natural Science Foundation of China (No. 30671083) and the National Basic Research Program of China (No. 2007CB815802), and Program for Innovative Research Team in University of Liaoning Province (No. 2007T089 and 2008T103). We gratefully acknowledge Bo Yu and Shuang Feng, Life Sciences of Liaoning Normal University for their help in this study.

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