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Original Article

Biochemical characterization of human peroxiredoxin 2, an antioxidative protein

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Human peroxiredoxin 2 (Prx2), which is abundant in erythrocytes, has been shown to play a key role in protecting erythrocytes against oxidative stress by scavenging reactive oxygen species as well as participating in cell signal transduction. Here, human *Prx2* gene was successfully cloned into *Escherichia coli* BL21 (DE3) for Prx2 expression. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis suggested that the recombinant protein was expressed mainly in a soluble form. The recombinant protein was purified by one-step Ni-nitrilotriacetic acid chelating affinity chromatography to a purity of up to 91.5%. The peroxidase activity of Prx2 to scavenge H_2O_2 was determined by a ferrithiocyanate assay. The ability of Prx2 to protect plasmid DNA was tested by using a mixed-function oxidation system, and results showed that Prx2 could prevent DNA from undergoing oxidative stress. Ultraviolet (UV)-induced cell apoptosis assay demonstrated that Prx2 is also able to protect NIH/3T3 cells from UV-induced damage, suggesting its possible applications in cosmetics and other areas.

Keywords   peroxiredoxin 2; ferrithiocyanate assay; cell apoptosis; plasmid DNA protection

Introduction

Under normal physiological conditions, cells are protected against oxidative stress by antioxidant enzymes, which mainly include superoxide dismutase (SOD), catalase, thioredoxin, and peroxiredoxins [1]. The imbalance between oxidative and reductive processes gives rise to oxidative stress, which is recognized as a risk factor in generating diabetes [2] and aging [3]. Thus, it is intriguing to investigate these antioxidant enzymes, which play key roles in scavenging superoxides.

Peroxiredoxin 2 (Prx2), the third most abundant protein in erythrocytes (after hemoglobin and carbonic anhydrase), is able to catalyze the reduction of hydrogen peroxide,

organic hydroperoxides, and peroxynitrite [4], thus protecting red blood cells (RBCs) against oxidative stress [5] as well as participating in cell proliferation, migration [6], and other signal transduction pathways.

Prx2 has been evaluated for its potential use in disease diagnosis and therapy. Proteomics research showed that Prx2 was a possible biomarker for the diagnosis of Alzheimer's disease [7], vascular tumors [8], and cervical cancer [9]. Prx2 is also a tumor survival factor as it can positively regulate Jun kinase-dependent DNA repair. Thus, the inhibition of Prx2 could sensitize tumor cells to chemotherapeutic agents [10]. It has also been found that inhibition of Prx2 sensitizes glioma cells to oxidative stress, indicating Prx2 may be a potential therapeutic target [11].

Generally, Prx2 can be extracted directly from erythrocytes [5] or expressed by using a baculovirus expression system [12]. In this study, to produce Prx2 more efficiently and further investigate its antioxidative and other properties, recombinant Prx2 was expressed in *Escherichia coli* BL21(DE3) and purified for antioxidation studies, including determination of its peroxidase activity and its ability to protect DNA, as well as its ability to protect cells from ultraviolet (UV)-induced apoptosis.

Materials and Methods

Strains, cells, and reagents

Plasmid containing the full-length human *Prx2* gene (BC003022.1) was purchased from Fulgene (Guangzhou, China). Prokaryotic expression vector pET-28 plasmid, supercoiled pUC18 plasmid, *E. coli* strain DH5α, and *E. coli* strain BL21(DE3) were purchased from TaKaRa (Dalian, China). Anti-His monoclonal antibody and annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit were purchased from Invitrogen (Carlsbad, USA). NIH/3T3 cells were stored in our own laboratory.

Expression of Prx2 protein in *E. coli*

Human *Prx2* gene was amplified by polymerase chain reaction (PCR) from the Prx2 cDNA sequence provided by

Fulgene using the following Prx2 primers with *EcoRI* and *XhoI* respectively as shown in bold: 5′-TTTA **GAATTC**ATGGCCTCCGGTAACGC-3′ (forward) and 5′-TTTC**CTCGAG**ATTGTGTTTGGAGAAATATTCC-3′ (reverse).

The PCR product was digested with *Eco*RI and *Xho*I and then ligated into expression vector pET-28. Positive clones were picked up and grown overnight at 37°C in Luria-Bertani (LB) medium supplemented with 50 µg/ml kanamycin. After being cultured overnight, the *E. coli* cells were inoculated into fresh LB medium containing 4% kanamycin (*v/v*). When A_{600} reached 0.6, different concentrations (0.4, 0.8, 1.2, and 1.6 mM) of isopropyl-β-D-thiogalactopyranoside (IPTG) were added to induce protein expression. To obtain soluble Prx2 protein rather than inclusion body, different induction temperatures (18, 25, and 37°C) were tested. After 5 h of induction, the cells were harvested and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis.

Purification of Prx2 protein

Recombinant *E. coli* cells were harvested by centrifugation at 3800 *g* for 10 min. After removing the supernatant, cells were resuspended in buffer A (20 mM phosphate buffer supplemented with 500 mM NaCl) and disrupted by sonication. After centrifugation at 4°C, 18,000 *g* for 30 min, the supernatant was passed through a Hitrap column (Bellefonte, USA) pre-equilibrated with buffer A. Then, the target protein was eluted with buffer B (20 mM phosphate buffer supplemented with 500 mM NaCl and 500 mM imidazole). Fractions with different concentrations of imidazole were collected and then desalted by application to a desalting column. The eluted protein was analyzed by SDS-PAGE.

Western blot analysis

After SDS-PAGE, proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane (Bio-Rad, Richmond, USA) at a constant voltage of 100 V for 40 min. The membrane was then blocked with 5% bovine serum albumin (BSA) for 2 h and incubated with anti-His tag monoclonal antibody (1 : 2000) at ambient temperature for 3 h. Protein bands were visualized by using a chemiluminescent substrate kit (Pierce, Rockford, USA) following the manufacturer's instructions.

Ferrithiocyanate assay

The antioxidant activity of recombinant human Prx2 was determined as described previously [13]. Briefly, 5 µl of Prx2 protein (about 1.5 µg) was incubated with 85 µl reaction buffer (1 mM dithiothreitol/0.03 × phosphate buffered saline/0.5% glycerol, pH 7.4) at ambient temperature for 2 min. The reaction was initiated by adding 10 µl of H_2O_2 at 37°C. After 10 min, 40 µl of 26% (*w/v*) trichloroacetic acid was added to terminate the reaction. Then 40 µl of $Fe(NH_4)_2(SO_4)_2$ and 20 µl of potassium thiocyanate (KSCN) were added to form a red-colored ferrithiocyanate complex with H_2O_2. The red-colored complex was quantified by measuring the absorbance at 475 nm.

DNA cleavage assay

Supercoiled pUC18 plasmid was used for a DNA cleavage assay as described previously [14] with some modifications. Briefly, different concentrations of Prx2 were incubated with reaction mixture (50 mM Tris-HCl containing 40 µM $FeCl_3$, 10 mM DTT, and 1 µg of pUC18 plasmid DNA at pH 7.0) at 37°C for 1 h, and then the plasmid DNA was analyzed on ethidium bromide stained agarose gel.

Flow cytometry assay for UV-induced reactive oxygen species production and cell apoptosis

NIH/3T3 cells were treated with UV in the absence or presence of purified Prx2 (∼100 µg/ml). The UV-treated group was placed vertically 50 cm below the UV light. Prx2 was added 30 min before UV irradiation and the depth of the liquid above the cells was kept to ∼1 mm. After 30 min of irradiation, UV-treated cells were cultured for another 3 h before being harvested. Cells were incubated with a fluorescence probe DCFH-DA (2′,7′-dichlorofluorescin diacetate; Molecular Probes Inc., Eugene, USA) in the dark for 30 min at 37°C, and reactive oxygen species (ROS) production was detected by flow cytometry using FACS Calibur (BD Biosciences, Mountain View, USA). Alternatively, cells were stained with FITC-conjugated annexin V and propidium iodide (PI) using an Alexa Fluor 488 annexin V/ dead cell apoptosis kit (Invitrogen) before flow cytometric analysis for apoptosis assay.

Results

Expression and purification of recombinant Prx2

The pET28-Prx2 plasmid was successfully constructed and transformed into *E. coli* BL21(DE3) for recombinant expression. Prx2 was mainly expressed as soluble protein at the different induction temperatures. As shown in **Fig. 1(A)**, the yield of Prx2 at 25°C is higher than that at 18 or 37°C. There was no significant difference in Prx2 yields when different concentrations of IPTG (0.4– 1.6 mM) were used (data not shown). After induction with 0.8 mM IPTG at 25°C for 12 h, recombinant Prx2 accounted for <60% of the total cellular protein, which was determined by using BandScan software.

The protein was purified using a Ni-affinity column from a total crude extract. Fractions collected at different concentrations of imidazole were analyzed by SDS-PAGE, and the
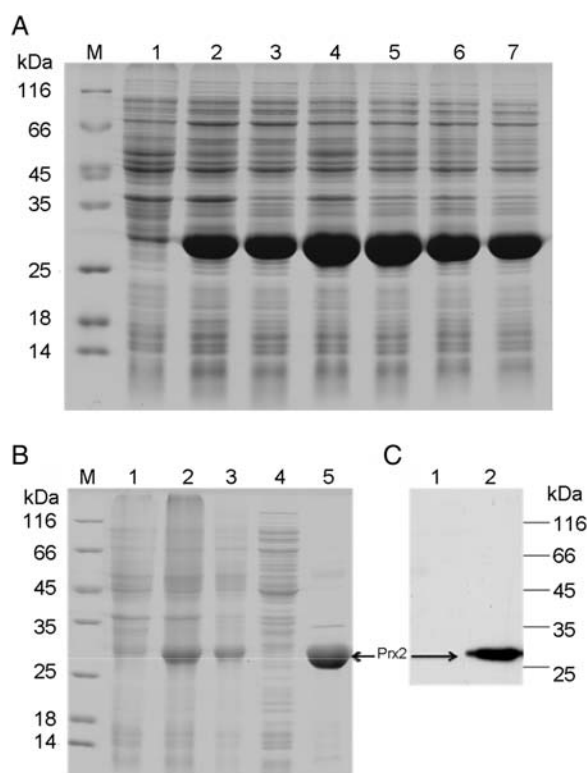
Figure 1 Expression, purification, and western blot analysis of recombinant Prx2 (A) Optimization of Prx2 expression at different temperatures. M, protein molecular weight marker; lane 1, uninduced sample; lane 2, total protein of induced sample at 18°C; lane 3, supernatant of induced sample at 18°C; lane 4, total protein of induced sample at 25°C; lane 5, supernatant of induced sample at 25°C; lane 6, total protein of induced sample at 37°C; and lane 7, supernatant of induced sample at 37°C. (B) Expression and purification of recombinant Prx2. Lane 1, uninduced sample; lane 2, total protein of induced sample; lane 3, supernatant of induced sample; lane 4, flow through solution; and lane 5, eluted with 500 mM imidazole. (C) Western blot analysis of recombinant Prx2. Lane 1, uninduced sample as control; and lane 2, purified recombinant Prx2.

target protein was found mainly in fractions collected at 500 mM imidazole [**Fig. 1(B)**]. The final concentration and purity of Prx2 were 1.3 mg/ml and 91.5%, determined by Protein assay kit and Bandscan software, respectively.

Western blot analysis showed that the purified protein was recognized by the His-tag monoclonal antibody [**Fig. 1(C)**], suggesting that recombinant Prx2 protein was obtained after induction and purification.

Peroxiredase activity and plasmid DNA protective ability of purified Prx2

The peroxidase activity of Prx2 was checked by detecting the decrease of H_2O_2 in the reaction mixture. As shown in **Fig. 2**, significant decrease of A_{475} was measured when the recombinant Prx2 was added indicating that H_2O_2 could be scavenged by Prx2. The relative activity of Prx2 catalyzing H_2O_2 was calculated as $\sim 1.86 \times 10^{-2}$ ΔA_{475}/min/μg·ml^{-1} Prx2, which was $\sim$30% higher than that of Prx2 from *Lampetra japonica* (1.25×10^{-2} ΔA_{475}/min/μg·ml^{-1} Prx2) [14].
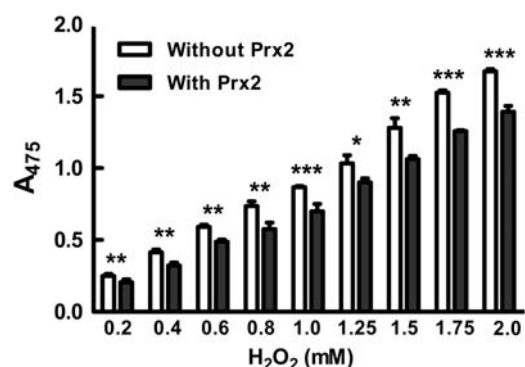

Figure 2 Peroxidase activity of recombinant Prx2 Various concentrations of H_2O_2 were incubated in the reaction buffer with or without recombinant Prx2 protein for 10 min, then the remaining H_2O_2 in the reaction mixture was measured. A_{475} of the reaction mixture with Prx2 was compared with A_{475} without Prx2. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.
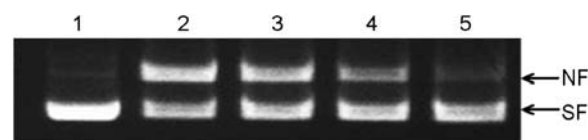

Figure 3 Plasmid protection activity of recombinant Prx2 Supercoiled pUC18 plasmid was incubated with different concentrations of Prx2 at 37 °C for 1 h. Plasmid DNA was analyzed on ethidium bromide stained agarose gel. Lane 1, plasmid DNA only; lane 2, DNA + Fe^{3+}; lane 3, DNA + Fe^{3+} + 1 μg of Prx2; lane 4, DNA + Fe^{3+} + 3 μg of Prx2; and lane 5, DNA + Fe^{3+} + 5 μg of Prx2. SF, supercoiled form; NF, nicked form.

A thiol-dependent mixed-function oxidation system [14] was used to test the ability of Prx2 to protect plasmid DNA from oxidative cleavage by ROS. As a result of this damage, the supercoiled form of plasmid DNA is converted into the nicked form, as evidenced by a shift in the gel (**Fig. 3**). The conversion of the plasmid form was prevented by adding Prx2 to the reaction system, and this process was also shown to be dose-dependent.

Protection of NIH/3T3 cells from UV-induced damage by Prx2

We further investigated whether Prx2 treatment inhibited the UV-mediated apoptosis. As shown in **Fig. 4(A)**, increased apoptotic cell population was observed in UV-exposed NIH/3T3 cells compared with the controls, and the increase was inhibited by the pretreatment with Prx2. The quantitative analysis from three independent experiments showed that the percentage of apoptotic cells (UV group) was 46.41% ± 3.55% [**Fig. 4(B)**]. However, in the presence of Prx2, the percentage of apoptotic cells was 14.28% ± 1.28% (Prx2 group), which was significantly lower than that of the UV group ($P = 0.0135$) [**Fig. 4(B)**]. No significant difference was detected between the UV group and the UV-treated group with BSA (**Fig. 4**). This demonstrates that Prx2 can protect mammalian cells from UV-induced damage and indicates its potential value.

Prx2 treatment decreased ROS production in NIH/3T3 cells induced by UV exposure. ROS is reported to be critical for UV-mediated apoptosis [15,16]. Prx2 can protect NIH3T3 cells from UV treatment. We further determined whether Prx2 mediates the decrease of intracellular ROS production upon UV treatment, DCFH-DA was used to determine the ROS production in NIH/3T3 cells after exposure to UV. As shown in **Fig. 5(A)**, an increase in the DCF fluorescence was seen in UV-treated NIH/3T3 cells compared with the untreated cells, indicating the enhanced ROS production after UV treatment. When cells pretreated with purified Prx2 protein for 30 min and then exposed to UV, the ROS production decreased to the levels similar with the control group (untreated group). The quantitative analysis from three independent experiments showed that Prx2 treatment can reduce ROS production

significantly compared with UV treatment group ($P = 0.0127$) [**Fig. 5(B)**]. BSA was used as a control protein without oxygen-reduction activity, and there was no significant difference in DCF fluorescence between UV-treated cells and BSA-pretreated cells (**Fig. 5**). Our results suggested that purified Prx2 protein can protect UV-mediated NIH/3T3 damage by the impairment of ROS production.

Discussion

To our knowledge, Prx2 has either been extracted directly from erythrocytes or expressed by using a baculovirus expression system. Anion-exchange chromatography has been applied with size-exclusion chromatography to purify Prx2 from human plasma-free erythrocytes [5]. However,
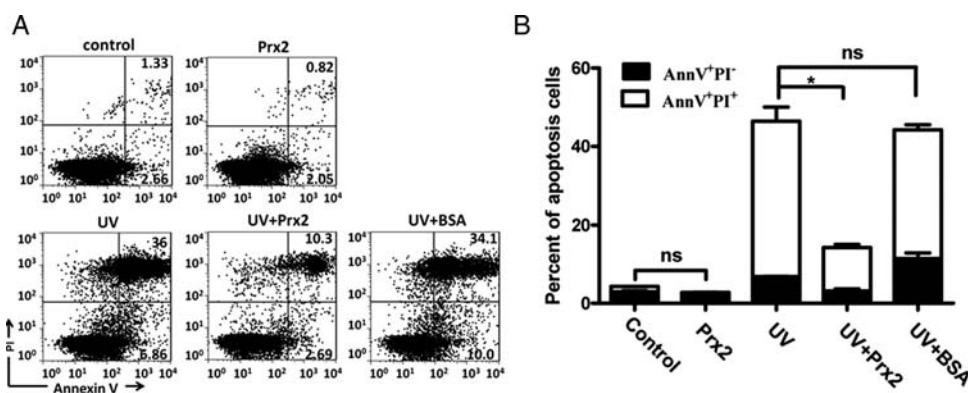

Figure 4 Prx2 protection of NIH/3T3 cells from UV-induced cell apoptosis NIH/3T3 cells were treated with UV in the absence or presence of purified Prx2 (~100 µg/ml) or BSA (100 µg/ml). Untreated cells were used as controls. Cells were harvested and stained with FITC-conjugated annexin V and PI and then applied to flow cytometry. (A) The dot plot results from one representative experiment. The value in the lower or upper right quadrant of the dot plots represents the percentage of early apoptotic cells and late apoptotic cells, respectively. (B) The complied data are from three independent experiments. Data are shown as the mean ± SEM, and analyzed by Student's t-test. *$P<0.05$.
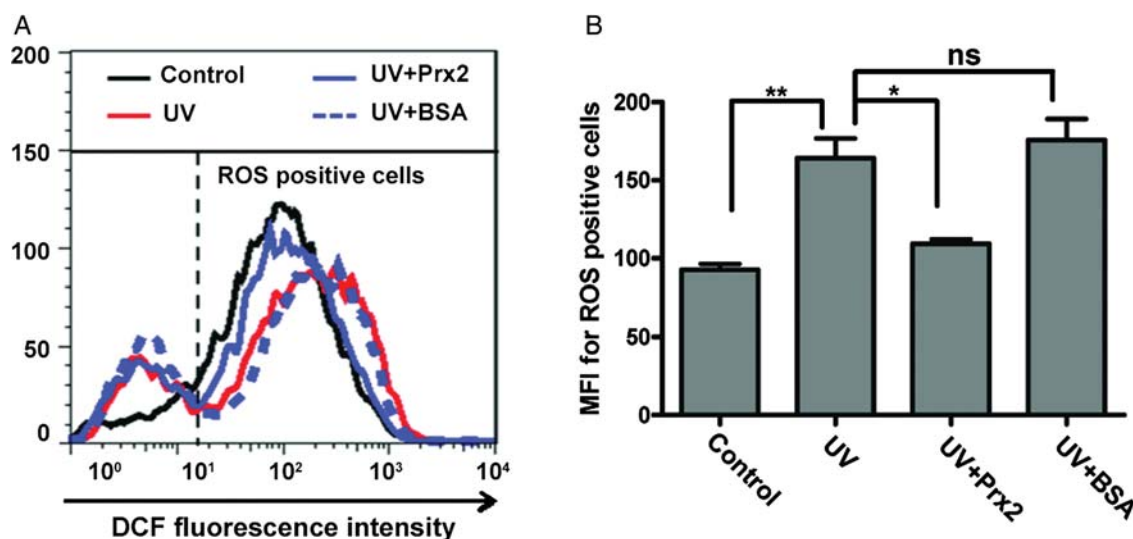

Figure 5 ROS production in NIH/3T3 cells after exposure to UV with or without Prx2 treatment NIH/3T3 cells were pretreated with or without purified Prx2 protein or BSA before exposure to UV irradiation. Untreated cells were used as controls. ROS production was measured by flow cytometry using fluorescent probe DCHF-DA. (A) The histogram results are from one representative experiment. (B) The complied data are from three independent experiments. Data are shown as the mean ± SEM, and analyzed by Student's t-test. *$P<0.05$ and **$P<0.01$.

erythrocytes may not be readily available, especially when a large quantity of Prx2 is required. A baculovirus expression system has also been tried as a way to produce recombinant Prx2, but the procedure is complicated and highly purified Prx2 is difficult to achieve [12]. Here, we used one-step Ni-nitrilotriacetic acid chelating affinity chromatography to purify recombinant Prx2 economically and efficiently. As a result, 1.33 mg/ml of Prx2 was obtained on average and the purity reached 91.5%.

Prx2 is able to act as an antioxidative enzyme without the participation of a cofactor such as a coenzyme or metal ion, which indicates that its characteristics are different from those of catalase and SOD. This is partly because the structural basis for its peroxidase activity is the thiol-specific Cys-51 in the active site ($2 \ R'\text{-SH} + \text{ROOH} = R'\text{-S-S-}R' + H_2O + \text{ROH}$) [17]. It has been demonstrated that a mutation in Cys-51 results in the loss of its catalytic activity [18]. Arginine-127 was also found to play a role in providing a positive charge to lower the pKa of Cys-51, thus enhancing its reactivity with hydrogen peroxide [19].

The peroxidase activity of Prx2 was checked by detecting the reduction of H_2O_2 when the recombinant Prx2 was added to the ferrithiocyanate system. However, the ability of Prx2 to catalyze H_2O_2 reduction was considered to be less efficient compared with catalase and cytoplasmic glutathione peroxidase [20]. Taking its abundance into account, Prx2 is sensitive to changes in H_2O_2 concentration under normal physiological conditions, which is consistent with its function of protecting RBCs from over-oxidative stress. Meanwhile, by adjustment of the H_2O_2 concentration, Prx2 could become available to participate in a group of intracellular signaling cascades.

Under acute UV light irradiation, ROS, and free radicals are overproduced and cause an inflammatory response or even cell apoptosis [21]. Our results showed that UV treatment induced NIH/3T3 cells to produce ROS. And Prx2 pretreatment could decrease ROS production and protect NIH/3T3 cells from UV-induced damage to a certain extent. The ability of Prx2 to protect cells from UV-induced apoptosis was considered to be relevant to its role in scavenging peroxide. However, the mechanism underlying its protective function remains to be unraveled. Most of the erythrocyte Prx2 is cytosolic under native conditions, but an estimated 0.05% is bound to the membrane as well [22,23], which affords the possibility that Prx2 could perform its scavenger role through membrane receptors without entering the cells. The antioxidation studies of Prx2 provide the basis for investigating its potential use in cosmetics, the food industry, and other areas.

Funding

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