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Recombinant Human Peroxiredoxin VI: Preparation and Protective Properties *in vitro*

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Abstract—cDNA of human peroxiredoxin VI, one of the recently discovered novel antioxidant proteins, was expressed in *Escherichia coli* cells. The expression product was obtained in water-soluble form and purified by a two-step chromatographic procedure using DEAE-Sepharose and Sephacryl S-200. According to CD data, the polypeptide chain of the recombinant human peroxiredoxin VI contains ~40% α-helical region and 30% β-structure, which is the same as for native rat peroxiredoxin VI. The protective properties of the recombinant protein determined as its ability to prevent the inactivation of glutamine synthetase from *E. coli* in a model oxidation system were comparable with the protective properties of native rat peroxiredoxin VI.

Key words: reactive oxygen species, antioxidant, peroxiredoxin VI

Single electron reduction of molecular oxygen in the process of cell respiration results in the formation of reactive oxygen species, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$), which are extremely toxic to cells [1, 2]. Aerobic organisms acquired protective mechanisms to render harmless of H_2O_2 and oxygen radicals by their reduction or dismutation in antioxidant enzyme-catalyzed reactions. These enzymes include superoxide dismutase, catalase, and glutathione peroxidase [3] and, additionally, peroxiredoxins discovered over the last decade [4].

Peroxiredoxins are a novel protein family consisting of over 100 members that have been found in all living organisms from bacteria to human beings and belonging to the thiol peroxidases [5]. Six isotypes (peroxiredoxins I-VI) have been revealed in mammals, differing in amino acid sequence, mechanism, and localization in the cell and organism [5]. Molecules of all peroxiredoxins contain a highly conservative region with a cysteine residue, representing the enzyme active site [4, 5]. Using a model system generating H_2O_2 *in vitro* it was demonstrated that peroxiredoxins prevent the inactivation of glutamine synthetase in the presence of Fe^{3+}, O_2, and dithiothreitol (DTT), which is an electron donor for all peroxiredoxins [6]. The property of preventing the inactivation of glutamine synthetase is due to peroxidase activity of these H_2O_2-reducing proteins. Peroxiredoxins I-V utilize thioredoxin [5] as an electron donor to reduce H_2O_2, whereas peroxiredoxin VI uses glutathione [7] or cyclophilin A [5].

To date, peroxiredoxin has been identified in many mammalian organs and tissues, including eye, olfactory epithelium, liver, skin, and brain [8]. Determination of the primary structure of a 28 kD secretory protein isolated earlier from rat olfactory epithelium and revealing of the homologies with other proteins [9-11] identified rat peroxiredoxin VI [12]. It was also shown that rat peroxiredoxin VI concentration is increased in organs directly exposed to the environment, such as trachea, bronchial epithelium, lungs, skin epidermis, and olfactory epithelium [13]. It was assumed that rat peroxiredoxin VI is the main contributor into neutralization of reactive oxygen species in these tissues *in vivo* and is involved in the reparation of epithelium tissues [12, 13], and hence may be employed as a novel drug, promoting the regeneration of damaged tissues and organs.

The aim of the present work was to obtain the functionally active human peroxiredoxin VI by cloning and expression its cDNA in *E. coli* cells as well as the investigation of its protective properties *in vitro*.

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MATERIALS AND METHODS

NaOH, HCl, H_3BO_3, CH_3COOH, trichloroacetic acid (TCA), NaCl, CH_3COONa, Na_2HPO_4, $Na_2S_2O_8$, SDS, KCl, KH_2PO_4, $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_3$, KH_2AsO_4, $(NH_4)_2SO_4$, Tris, Hepes, EDTA, 2-mercaptoethanol, DTT, acrylamide, N,N′-methylene-bis-acrylamide, Temed, ethanol, methanol, glycerol, agarose, Tween-20, hydroxylamine, ampicillin, ADP, Gln, BSA, bromophenol blue, ethidium bromide, and brilliant blue R were from Sigma (USA); tryptone, yeast extract, and bactoagar were from Difco (USA); Taq-polymerase; restriction endonucleases, phage T4 DNA-ligase, calf intestinal alkaline phosphatase, and Wizard Plus Minipreps DNA Purification System for plasmid DNA isolation were from Promega (USA); UltraClean DNA Purification Kit for agarose gels was from Mo Bio Laboratories (USA); Sequenase 7-deaza-dGTP DNA Sequencing Kit was from Amersham (USA); marker proteins: lysozyme (14.4. kD), β-lactoglobulin (18.4 kD), *Bsp*981 restrictase (25.0 kD), lactate dehydrogenase (35.0 kD), ovalbumin (45.0 kD), BSA (66.2 kD), β-galactosidase (116.0 kD), and isopropyl β-D-thiogalactopyranoside (IPTG) were from MBI Fermentas (Lithuania); DEAE-Sepharose and Sephacryl S-200 were from Pharmacia (Sweden); BA 85-SB membrane was from Schleicher and Schull (Germany).

Bacterial strains. *E. coli* DH5α strain (Life Technologies, USA) was used for production of plasmid DNA and transformation during the cloning procedure. *E. coli* BL-21 (DE-3) strain (Stratagene, USA), containing T7 RNA polymerase gene under an inducible *lac*-promotor was used for the expression of human peroxiredoxin VI cDNA cloned in the vector controlled by the T7 phage promotor [14].

Cloning of human peroxiredoxin VI cDNA. Operations with recombinant DNA were performed according to conventional techniques [15]. A fragment of human peroxiredoxin VI cDNA for cloning designed with non-altered open reading frame in the expressing vector were obtained by a PCR technique using oligonucleotides with point mutations to form the corresponding restriction sites as primers. 5′-ATCACCGTCCATATGCCCG-GAGG-3′ (the *Nde*I restriction site is underlined) and 5′-CCAGAATTCTTAAGGCTGGGGTGTG-3′ (the *Eco*RI restriction site is underlined) were used as forward and backward primers, respectively. A plasmid containing human peroxiredoxin VI cDNA sequence was used as a template for PCR (HA0683, GenBank™ accession number D14662, kindly provided by S. I. Fenstein, USA). The reaction mixture contained ~1 ng of plasmid DNA, 20 pmol of each primer, 5 μl of 10× buffer (Promega, USA), 200 μM each dNTP, 5 units of Taq-polymerase (Promega) in a final volume of 50 μl. Reaction was started from the preliminary denaturation (94°C, 5 min), after that 30 PRC cycles were run under the following conditions: denaturation (94°C, 30 sec), annealing with primers (60°C, 30 sec), elongation (72°C, 45 sec) with subsequent incubation for 5 min at 72°C. After the treatment with corresponding restriction endonucleases the human peroxiredoxin VI cDNA was cloned into pET23-a(+) plasmid from Novagen (USA) at *Nde*I-*Eco*RI sites.

Expression of human peroxiredoxin VI cDNA. *E. coli* BL-21 (DE-3) strain containing T7 RNA polymerase gene under the control of inducible *lac*-promotor was used as a host strain for the expression of human peroxiredoxin VI cDNA [14]. Transformation of *E. coli* competent BL-21 (DE-3) cells was performed using conventional techniques [15]. For the production of recombinant protein the cells were grown at 37°C until the absorbance of the cultivation medium at 600 nm reached the value of 0.6 optical density units. After that the expression of a recombinant protein was triggered by addition of *lac*-promotor inducer (IPTG) up to the final concentration of 0.4 mM and the sample was incubated for an additional 4-5 h.

Electrophoretic analysis of recombinant protein. The cells were precipitated by centrifugation at 10,000*g* for 5 min at room temperature. The precipitate from 1 ml of cell culture was resuspended in 100 μl of buffer containing 0.045 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 1% SDS, 0.01% bromophenol blue, and 0.05 M DTT, then heated at 100°C for 5 min and applied to the gel (10-20 μl).

SDS-PAGE was conducted in a 12.5% polyacrylamide gel according to the Laemmli method [16]. The gel was then treated with a solution containing 0.3% of brilliant blue R dye, 25% methanol, and 10% CH_3COOH and rinsed with 25% methanol–10% CH_3COOH.

Isolation and purification of recombinant human peroxiredoxin VI. For preparative production of the recombinant protein the cultivation medium volume was increased to 250-1000 ml. The cells were centrifuged at 4,000*g* for 20 min at 4°C. The cell precipitate was resuspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) at 4°C, adding 50 μl of buffer for 1 ml of the cell culture. The resulting suspension was frozen, then thawed and sonicated on an ice bath (5 × 1 min, pause 1 min) at 200-300 W power using an ultrasonic disintegrator (UP 50 H Ultrasonic Processor, Dr. Hielscher, Germany). The suspension was centrifuged at 10,000*g* for 10 min at 4°C. All further procedures were performed at the same temperature (4°C). The total protein was precipitated in saturated $(NH_4)_2SO_4$ solution and dialyzed against 12 mM Tris-HCl buffer, pH 7.8, containing 1 mM $MgCl_2$ and 1 mM DTT. The dialyzed lysate was applied to DEAE-Sepharose chromatography in a NaCl gradient as described earlier for the native rat peroxiredoxin VI [9, 17]. The fractions were analyzed by SDS-PAGE. The fractions containing recombinant peroxiredoxin were subjected to further purification by gel filtration using Sephacryl S-200 as described previously [9, 17].

Western blot. The proteins were separated by SDS-PAGE and then transferred onto BA 85-SB nitrocellulose membrane. Electroblotting was performed using a 2051 Midget Multiblot (LKB, Sweden) in buffer containing 20 mM $NaHCO_3$, pH 8.5, and 10% methanol for 50 min at 55 mA. After this process was finished the membrane was rinsed with water and incubated in TBS buffer (50 mM Tris-HCl, pH 7.0-8.0, 150 mM NaCl) containing 3% of BSA, 20% glycerol, and 0.5% Tween 20 to prevent nonspecific adsorption of primary antibodies. After that the membrane was rinsed three times with TBS buffer containing 0.5% Tween 20. Polyclonal rabbit antibodies against native rat peroxiredoxin VI produced as described earlier [9] were used as primary antibodies. Incubation with primary antibodies was performed for 1 h at 37°C. Further experiments were carried out according to the standard technique [18].

CD spectroscopy. Measurements were performed under non-denaturing conditions (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) using a J-500A instrument (Jasco, Japan). For the calculations the molecular weight of one amino acid residue was assumed to be equal to 115 daltons. The secondary structure content was calculated using previously suggested techniques [19].

Determination of protective properties of human peroxiredoxin VI *in vitro*. Glutamine synthetase was isolated from *E. coli* DH5α strain as described earlier [20] and then inactivated in the presence of Fe^{3+}, O_2, and DTT, a model oxidation system that generates H_2O_2 [6, 21]. The inactivation reaction was conducted in a reaction mixture (60 μl) containing 5 μg of glutamine synthetase, 50 mM Hepes-NaOH, pH 7.4, 3 mM DTT, and 3 μM $FeCl_3$ in the presence of different peroxiredoxin concentrations for 10 min at 37°C. Residual activity of *E. coli* glutamine synthetase was determined as described previously [6]. Protective properties of peroxiredoxin toward *E. coli* glutamine synthetase were defined as the ratio between the enzyme residual activity after inactivation in the presence of different peroxiredoxin concentrations and activity of the native glutamine synthetase.

RESULTS AND DISCUSSION

Full size human peroxiredoxin VI cDNA was cloned into pET23-a(+) vector at the *Nde*I and *Eco*RI restriction sites (see "Materials and Methods"). pET23-a(PrxVI hum) plasmid, containing the open reading frame under the control of the T7-inducible promotor and including initiating ATG codon, human peroxiredoxin VI cDNA, and stop codon TAA was selected for further experiments (Fig. 1). The resulting plasmid was used for *E. coli* BL-21(DE-3) cell transformation.

The recombinant protein with the expected molecular weight was found in the water-soluble protein fraction obtained after the ultrasonic lysis of the cells. Figure 2

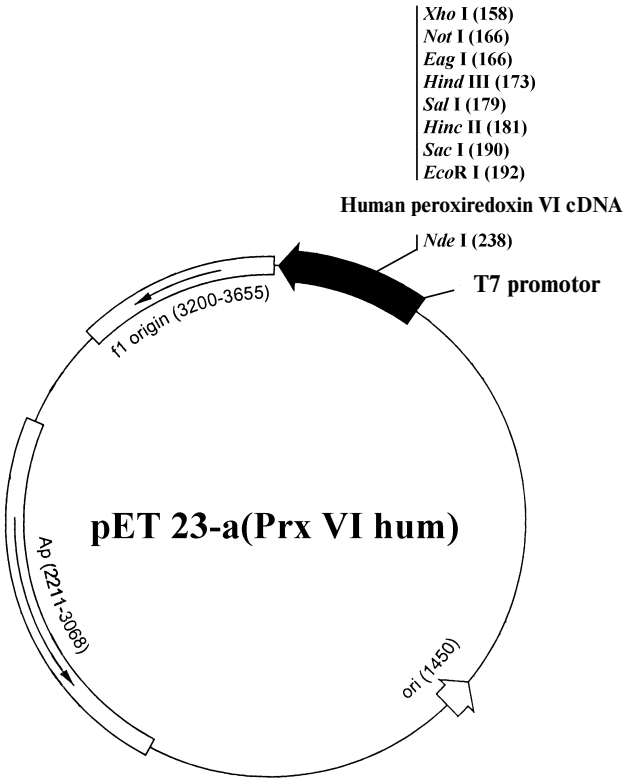

Fig. 1. pET23-a(PrxVI hum) expressing vector. Location of human peroxiredoxin VI cDNA and restriction sites in polylinker is shown at the top, right.

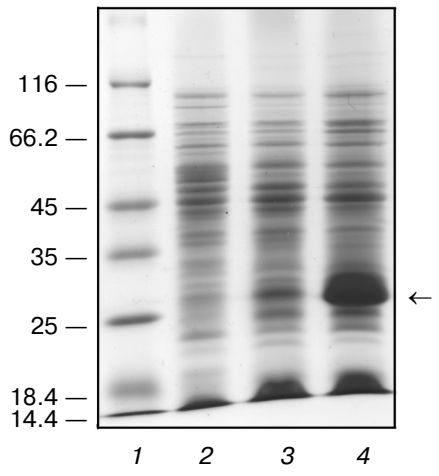

Fig. 2. Analysis of human peroxiredoxin VI cDNA expression. 12.5% SDS-PAGE of the total cell protein from *E. coli* BL-21(DE-3) cells containing the initial pET23-a(+) (*2*); pET23-a(PrxVI hum) before (*3*) and 4 h after (*4*) IPTG induction. *1*) Marker proteins, the molecular weight (kD) is shown on the left. The arrow indicates the position of the recombinant protein.

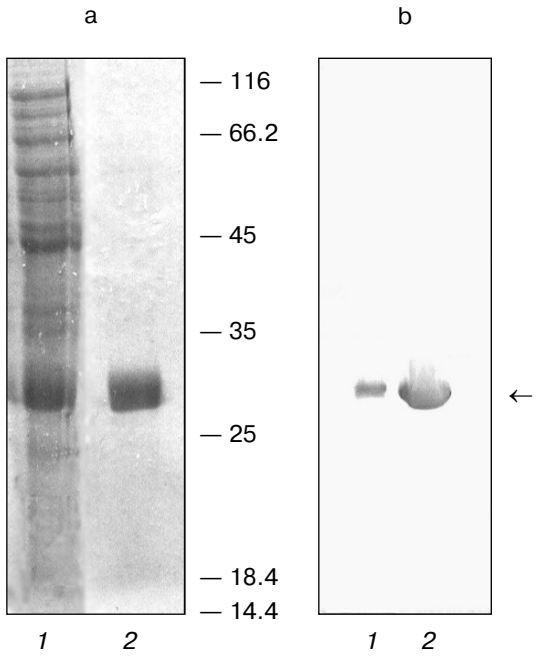

Fig. 3. Electrophoregram (a) and western blot (b) of the total cell protein from *E. coli* BL-21(DE-3) cells containing pET23-a(PrxVI hum) plasmid 4 h after the IPTG induction (*1*) and recombinant protein preparation after purification by DEAE-Sepharose and Sephacryl S-200 chromatography (*2*). Figures in the center show the molecular weight (kD) of the protein markers. The arrow indicates the position of the recombinant protein.

shows the electrophoretic image of the water-soluble protein from cell lysate before and after IPTG induction. Comparison of lanes *3* and *4* shows that intensive accumulation of protein with molecular weight of 27 kD occurred after the IPTG expression induction (Fig. 2). The protein yield was ~60 mg for 1 liter of cell culture.

Identification of recombinant human peroxiredoxin VI was carried out by Western blot analysis using rabbit polyclonal antibodies towards native rat peroxiredoxin VI (Fig. 3b). Since the degree of amino acid homology between rat and human peroxiredoxins VI is 92%, the data indicate that the protein with molecular weight of 27 kD whose concentration in *E. coli* cells dramatically increases after the IPTG induction is human peroxiredoxin VI.

For further investigation of recombinant protein properties it was obtained in preparative amounts and purified as described in "Materials and Methods". The yield of electrophoretically pure protein was ~30 mg for 1 liter of cell culture.

The purified protein is a water-soluble protein and appears on SDS-PAGE as a polypeptide with molecular weight of ~27 kD (Fig. 3a). The calculated molecular weight of human peroxiredoxin VI is 25,034 daltons. The difference between the calculated molecular weight and that determined from the experiment may be associated with abnormal recombinant peroxiredoxin behavior under SDS-PAGE. It was shown previously that the molecular weight of the native rat peroxiredoxin VI according to the electrophoretic data also exceeding its calculated molecular weight [11]. This suggests the similarity of physicochemical properties of these proteins.

To display enzymatic activity the protein should form the native secondary and tertiary structure. For confirmation of native conformation of the obtained recombinant protein, CD spectra for native rat peroxiredoxin VI and recombinant human peroxiredoxin VI were recorded. Figure 4 presents the CD spectra for the two proteins in the far UV region using non-denaturing conditions. The CD spectra indicate the presence of characteristic sec-

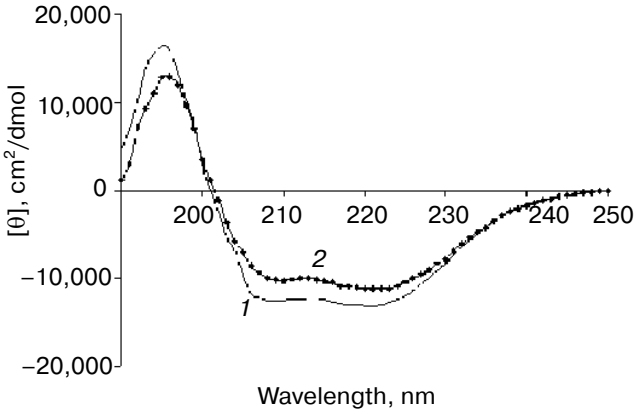

Fig. 4. CD spectra of native rat peroxiredoxin VI (*1*) and recombinant human peroxiredoxin VI (*2*) in the far UV region under non-denaturing conditions.

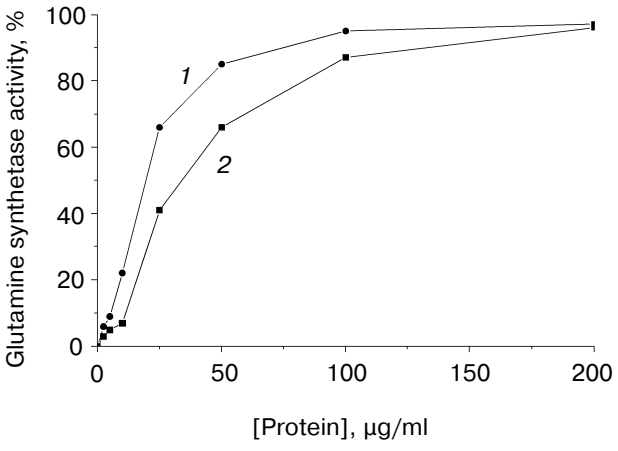

Fig. 5. Protective properties of native rat peroxiredoxin VI (*1*) and recombinant human peroxiredoxin VI (*2*) toward the inactivation of *E. coli* glutamine synthetase in a model oxidation system *in vitro*.

ondary structure in both polypeptides. The expansion of the experimental spectra in terms of α-helical, β-structural, and random components was carried out by the method of Provencher [19]. Since native and recombinant peroxiredoxins contain approximately the same number of α-helices (38 ± 2%) and β-structures (31 ± 3%), the conformational state of these proteins can be considered as identical, i.e., the recombinant protein is obviously in the native conformation.

It was shown earlier that the native rat peroxiredoxin VI is able to prevent the inactivation of *E. coli* glutamine synthetase in a model oxidation system [12, 13]. The protective properties of the recombinant protein were investigated according to the same technique. The native rat peroxiredoxin VI was used as a positive control in this experiment. A considerable protective effect of recombinant human peroxiredoxin VI was found at the protein concentration of 25 μg/ml (40% of glutamine synthetase activity was preserved), whereas at the concentration of 100 μg/ml the glutamine synthetase activity was fully preserved (Fig. 5), this being comparable with the results obtained with the native rat peroxiredoxin VI (Fig. 5).

Thus, we have developed a method for preparation and purification of recombinant human peroxiredoxin VI possessing its characteristic protective properties *in vitro*. At the present time we are investigating the protective properties of the protein *in vivo*.

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