Protein Expression and Purification 72 (2010) 1–7

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression of N-terminally truncated forms of rat peroxiredoxin-4 in insect cells

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ARTICLE INFO

Article history:
Received 10 November 2009
and in revised form 2 February 2010
Available online 6 February 2010

Keywords:
Peroxiredoxin
Peroxidase
Polymerization
Baculovirus
Insect cell
Signal peptide

ABSTRACT

Peroxiredoxins (Prxs), a family of thioredoxin-dependent peroxidases, are highly conserved in many organisms and function in detoxifying reactive oxygen species as well as other cellular processes. Six members of the Prx family are known in mammals, i.e., Prx-1 through -6. Among these proteins, only Prx-4 appears to contain a signal peptide that serves for localization in the endoplasmic reticulum, membrane translocation and secretion into the extracellular space, as demonstrated in a previous study using a baculovirus-insect cell system. The present study was conducted to determine whether the signal peptide-truncated mutant of rat Prx-4 is expressed as an enzymatically active form and is produced in large amounts. Two N-terminally truncated mutants were prepared by deletion of only the signal peptide and the larger region encompassing both the signal and the unique extension to Prx-4. These mutants were successfully produced within *Spodoptera frugiperda* 21 cells by infection with the recombinant baculoviruses, rather than by extracellular secretion. Both mutants were efficiently purified to homogeneity by two column chromatography steps. Biochemical characterization of the purified proteins showed that the truncated enzymes are enzymatically active and form an oligomeric structure, as reported for the mammalian Prx family. The findings also suggest that the unique extension plays a role in the regulation of non-covalent oligomerization. More than 4 mg of the purified proteins can be obtained from cells grown in monolayer cultures in twenty 75 cm^2 tissue culture flasks. The procedures described in this study permit recombinant Prx-4 to be prepared more efficiently and easily for purposes of crystallization and antibody preparation.

Introduction

The peroxiredoxin (Prx)[1] family catalyzes the reduction of peroxides in a thioredoxin (Trx)-dependent manner, and is thought to play roles in the detoxification of reactive oxygen species (ROS) [1–7]. It has been suggested that the enzymes of this protein family are associated with carcinogenesis [8–13], inflammatory states [13–16] and the pathogenesis of neural degenerative diseases [17–19].

Six members, Prx-1 through -6, have been reported in the mammalian Prx family [1,20], and, of those members, Prx-1 to -4 are known to be typical two Cys types, which are characterized by turnover between thiols and disulfide formation of redox-active cysteine residues of the two subunits [21]. During a catalytic cycle, the reaction of one cysteine thiol with a peroxide leads to the formation of cysteine sulfenic acid, which subsequently reacts with a second cysteine thiol, resulting in the formation of an intermolecular disulfide in the homodimeric protein unit. The resulting oxidized form is then reduced by a combination of Trx and Trx reductase in which NADPH serves as the reducing agent.

In earlier reports, Prx-4 was referred to as AOE372, an abbreviation of an antioxidant enzyme, by Jin et al., and was reported to be involved in a redox pathway that regulates NF-kB activation [22]. Consistent with the presence of an N-terminal signal peptide [22,23], it is known that Prx-4 is uniquely localized in the endoplasmic reticulum and can be secreted into the extracellular space. In addition, it is associated with the plasma membrane [23,24], while other Prx proteins appear to be generally localized within cells. Prx-4 also contains a unique N-terminal extension, which corresponds to the peptide region between the signal peptide and the homologous catalytic domain and, as a result, remains intact after proteolytic processing for secretion [24]. A wide variety of mammalian and bacterial Prxs have been structurally analysed, in attempts to develop a better understanding of the catalytic and regulatory mechanisms of the Prx family [25,26]. However, such a structural analysis has not yet been reported for mammalian Prx-4, even though recombinant rat Prx-4 has been successfully expressed in both bacterial and insect cells and then purified [22,24]. The hydro-

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[1] *Abbreviations used:* Prx, peroxiredoxin; Trx, thioredoxin; ROS, reactive oxygen species; DEAE, diethyaminoethyl; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; *t*-BuOOH, *t*-butyl hydroperoxide; t-PA, tissue plasminogen activator; GGT, γ-glutamyltranspeptidase.

1046-5928/$ - see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.pep.2010.02.001

phobic signal peptide of the protein might introduce problems in an analysis, and it is also possible that the capacity for cellular processing to secrete proteins is insufficient, thus leading to a decreased yield of the soluble form of Prx-4.

As suggested by the high structural homology, it is likely that the catalytic mechanism is essentially the same among typical two Cys type Prxs, including Prx-4 [25]. However, a unique region that is specific to each Prx isoform may confer or specify individual functions, for example, as indicated by the mitochondrial targeting of Prx-3 by its signal peptide [27,28]. Hence, a detailed investigation of the biological functions of the unique structural feature of Prx-4, by structural and functional analyses would be highly desirable, as has been carried out for other Prx family members. In this study, we report on a more convenient method for constructing the N-terminally truncated forms of rat Prx-4 in order to improve the expression and handling of the recombinant protein by avoiding the secretion pathway. When the truncated form was expressed using a baculovirus-insect cell system, the protein was intracellularly produced in a soluble and enzymatically active form. The recombinant protein was produced in sufficiently high yield to allow it to be readily produced in large amounts, which would facilitate further structural analyses.

Materials and methods

Materials

Restriction endonucleases and DNA modifying enzymes were purchased from Takara or New England Biolabs. Oligonucleotide primers were synthesized by Hokkaido System Science. Other common chemicals were obtained from Wako pure chemicals or Nacalai tesque.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to Kunkel [29], as described previously [30] for truncation of the N-terminal regions of the rat Prx-4 sequence to produce cytosolic soluble forms of the protein. The 5′ Xho I – Eco RI 0.3 kb fragment of rat Prx-4 cDNA [23] was subcloned into the pBluescript SK+(Stratagene). The uracil-substituted single stranded template was prepared from *Escherichia coli* CJ236, which had been transformed by the plasmid. The uracil-template was used with a synthetic oligonucleotide primer to create an initiation codon and to remove the N-terminal portion. The oligonucleotide primers used were 5′-CTG TTC CTG TTA GGA TCC ATG GCT TTG CAG GGC TTG-3′ for the M1 mutant and 5′-GTT TCG GTC GCA GGA TCC ATG GTG CAC CTA AGC AAA GCC-3′ for M2 mutant (see the Results and discussion). The resulting mutations were verified by dideoxy sequencing, as were the entire sequences that had been subjected to mutagenesis.

Construction of the transfer plasmid

An internal Eco RI – EcoRI 0.1 kb and a 3′ Eco RI – Bam HI 0.5 kb fragment of rat Prx-4 cDNA were successively inserted into the plasmid containing each of the mutated fragments as described above, to prepare a full-length cDNA for the N-terminally truncated protein. The Bam HI DNA fragment, encoding an open reading frame of the truncated protein, was excised from the plasmid then ligated to the Bam HI site of a transfer vector, pVL1393 vector (PharMingen) in the correct orientation. The resulting transfer plasmids for the M1 and M2 mutants were used in transfection experiments after purification using a QIAGEN plasmid mini kit.

Cell culture and general manipulation of viruses

Spodoptera frugiperda (Sf) 21 cells were maintained at 27 °C in Grace's insect media (GIBCO) supplemented with 10% fetal bovine serum, 3.33 g/l yeastolate, 3.33 g/l lactalbumin hydrolysate and 100 mg/l kanamycine. Recombinant viruses were manipulated as described previously [31].

Preparation of recombinant viruses

The purified transfer plasmid (1 μg) was co-transfected into 5×10^5 Sf21 cells with 10 ng of BaculoGold DNA (PharMingen), which was used as the *Autographa californica* nuclear polyhedrosis viral genome. The transfection experiments were carried out using the Lipofectin (GIBCO) method [32], as described previously [33,34]. Media containing the recombinant viruses generated by homologous recombination were collected 6 days after transfection. The recombinant viruses were further amplified to more than 5×10^7 plaque forming units/ml prior to use.

Expression of rat Prx-4 proteins in insect cells

2×10^8 Sf21 cells, which were cultured in twenty 75 cm^2 flasks, were infected with the recombinant viruses at a multiplicity of infection in excess of 8, and were harvested about 70 h post-infection for purification of the expressed protein.

Purification of the recombinant rat Prx-4

The harvested cells were rinsed with PBS and then centrifuged at 3000 rpm for 5 min at room temperature. The precipitated cells were homogenized in 15 ml of 20 mM Tris–HCl, 1 mM EDTA, 5 mM DTT (pH 8.0) with a Dounce homogenizer. The homogenate was centrifuged at 15,000 rpm for 30 min, and the resulting supernatant was treated with a 0.04% (v/v) aqueous polyethyleneimine solution to remove contaminated polynucleotides. After centrifugation, the clear supernatant was dialyzed against 3 L of 20 mM Tris–HCl, 1 mM EDTA, 0.5 mM DTT (pH 8.0) at 4 °C overnight with one change of buffer. The dialyzed sample was loaded on a DE52 column (bed volume, 10 ml) that had been pre-equilibrated with the same buffer as the dialysis buffer. After washing the column with the buffer, the Prx-4 absorbed to the column was eluted by an NaCl gradient from 0 to 0.5 M. The eluted Prx-4, the peak of which was detected by SDS–PAGE, was recovered and further purified on a Sephacryl S-200 gel filtration column (bed volume, 160 ml; 1.5 × 95 cm) using 20 mM Tris–HCl, 120 mM NaCl, 1 mM EDTA, 0.5 mM DTT (pH 7.5) as the solvent. The flow rate was 0.4–0.5 ml/min.

Electrophoresis and immunoblot analysis

Proteins were subjected to SDS–PAGE analysis on 12% gels, according to Laemmli [35], and the protein bands were visualized by staining with Coomassie brilliant blue R-250. The separated proteins were transferred onto nitrocellulose membrane (PROTRAN, Whatman), and blocked with 1% bovine serum albumin in phosphate-buffered saline. The membrane was incubated with anti-rat Prx-4 [24] rabbit IgG. A horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as the second antibody. 4-Chloro-1-naphthol and H_2O_2 were used to visualize the immunoreactive bands.

Assay for enzyme activity

The peroxidase activity of Prx-4 was measured by coupling with a Trx/Trx reductase system, essentially as described previously