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Characterization of *Haemophilus influenzae* Peroxiredoxins

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Two open reading frames of *Haemophilus influenzae*, HI0572 and HI0751, showing homology to a yeast thioredoxin peroxidase II (TPx II) and an *E. coli* thiol peroxidase P_{20}, respectively, were cloned and expressed in *E. coli*, and then the proteins were subsequently purified and characterized. HI0751 protein showed the thioredoxin (Trx)-dependent peroxidase activity, whereas HI0572 protein showed glutathione-dependent peroxidase. The HI0572 is the first peroxiredoxin with glutathione peroxidase activity rather than thioredoxin peroxidase. Purified HI0572 and HI0751 proteins protected specifically the inactivation of glutamine synthetase by metal catalyzed oxidation (MCO) systems composed of Fe^{3+}, O_2 and mercaptans such as dithiothreitol, β-mercaptoethanol and glutathione (GSH). Unlike the HI0751 protein, the HI0572 protein was more effective in protecting glutamine synthetase from inactivation by the GSH/Fe^{3+}/O_2 system. It seems that these unique properties of the HI0572 protein are due to the structure containing a glutaredoxin domain at it's C-terminal in addition to a peroxiredoxin domain.

Keywords: Peroxiredoxin, *H. influenzae*, TPx, GPx, HI0572.

Introduction

Peroxiredoxin (Prx) is a family of peroxidases that exist from archaebacteria to humans with multiple isotypes (Chae *et al.*, 1994b; Rhee and Chae, 1994; Cha and Kim, 1996; Cha and Kim, 1998; Kang *et al.*, 1998b; Cha and Kim, 1999). All of the known Prx isotypes offer the protection of enzyme proteins against oxidative inactivation caused by a metal catalyzed oxidation (MCO) system that is composed of transition metal ion, oxygen, and thiol such as dithiothreitol (DTT) (Kim *et al.*, 1988; Rhee and Chae, 1994; Kang *et al.*, 1998a; Kang *et al.*, 1998b). However, they fail to support a protection of enzyme proteins against ascorbate/Fe^{3+}-containing MCO system (Chae *et al.*, 1993; Kim *et al.*, 1998). In a bacterial system, the existence of two distinct Prx isotypes, AhpC/TSA (Tartaglia *et al.*, 1990; Chae *et al.*, 1994b) and P_{20} (Cha *et al.*, 1995; Zhou *et al.*, 1997) have been identified and their employment of thioredoxin (Trx) as an immediate electron donor for the reduction of peroxide has also been confirmed. AhpC/TSA type Prx, the so called 2-Cys conserved Prx or thioredoxin peroxidase (TPx) has been found from organisms of all kingdoms (Chae *et al.*, 1994b). However, the P_{20} type Prx reported only from the bacterial system (Cha *et al.*, 1995; Zhou *et al.*, 1997). Eukaryotes, such as yeast (Jeong *et al.*, 1999) and human (Kang *et al.*, 1998;), contain another Prx isotype, a so called 1-Cys conserved Prx. In yeast, TPx-II that linked with Trx has been known as an 1-Cys conserved Prx (Jeong *et al.*, 1999). Two distinct 1-Cys conserved Prx isotypes have been reported in mammals. The physiological electron donor for one isotype is known as Trx (Seo *et al.*, 2000) and the other is unidentified (Kang *et al.*, 1998).

H. influenzae whole genome sequencing (Fleischmann *et al.*, 1995) revealed two distinct open reading frames of Prx isotypes, HI0751 and HI0572. These show homology to P_{20} of *E. coli* (Cha *et al.*, 1995; Zhou *et al.*, 1997) and partial homology to TPx-II of yeast (Jeong *et al.*, 1999) and mammalian PrxV (Seo *et al.*, 2000), respectively. Unlike other prokaryotic organisms, *H. influenzae* does not have AhpC/TSA type Prx, but has a larger and a novel Prx isotype instead. An open reading frame HI0572 from *H. influenzae* encodes a novel Prx isotype that consists of two functional domains which show homology to TPx-II of yeast and glutaredoxin-3 of *E. coli* (Aslund *et al.*, 1996).

We purified the gene product of two ORFs, HI0751 and HI0572, by using *E. coli* expression system and compared the peroxidase activity of two proteins. In this paper we report the HI0751 protein as a thioredoxin-dependent peroxidase and

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Abbreviations: Trx, thioredoxin; TR, thioredoxin reductase; DTT, dithiothreitol; TPx, thioredoxin peroxidase; PCR, polymerase chain reaction; MCO, metal catalyzed oxidation; GSH, glutathione; Prx, peroxiredoxin; *H. influenzae*, *Haemophilus influenzae*.

HI0572 protein, a novel Prx, as a glutathione dependent peroxidase. In addition we also discuss their possible physiological role for the respiration of *H. influenzae.*

Materials and Methods

Materials *Haemophilus influenzae* ATCC 51907 was obtained from Dr. Choi in Wonkwang University. Thioredoxin reductase (TR) and Trx were purified from *Saccharomyces cerevisiae.* Glutamine synthetase was purified from *E. coli pgln6* (Kim *et al.*, 1985)

Cloning and expression of *H. influenzae* HI0572 and HI0751 The DNA fragments having coding region of HI0572 and HI0751 were obtained by polymerase chain reaction (PCR) with genomic DNA of *H. influenzae* ATCC51907 as a template and the combination of the forward and reverse primers. The forward primers for HI0572 (5'-CAATAGGAGAAAAACATATG TCTAGTATGGAAGG-3') and for HI0751 (5'-AAACATATGAC AGTTAC ATTAGCAGG-3') contain a *Nde*I site. The start codon and the reversed primers for HI0572 (3'-CGTATTCGTTCATAAA TCGCTTAAGAATAAAC-5') and for HI0751 (3'-CGTAACCGT CACAATCGCATCTTAAGAAA-5') contain a *Eco*RI site and the stop codon. The PCR products were cloned into the pCRII vector (Invitrogen). The resulting pCRII constructs were digested with *Nde*I and *Eco*RI. The *Nde*I-*Eco*RI fragments containing the coding region were ligated pET-17b (Novgen). Strain BL21(DE3) pLysS (Novagen) was transformed for the recombinant pET-17b vector. HI0572 and HI0751 proteins were induced by adding 0.4 mM isopropyl-1-thio-β-D-galactopyranoside into mid-log cultures of the transformed *E. coli.*

Purification of recombinant *H. influenzae* Prx proteins expressed in *E. coli* Crude extracts of *E. coli* overexpressed *H. influenzae* Prx proteins, HI0572 or HI0751 protein, were applied to a DEAE-Sephacel anion exchange column (3 × 20 cm) that had been equilibrated with 25 mM Tris-HCl (pH 7.6). The proteins were eluted by a linear NaCl gradient from 0 M to 0.5 M. The activity of Prx was assayed by monitoring its ability to inhibit DTT/Fe^{3+}/O_2-mediated inactivation of glutamine synthetase and then collected and precipitated by addition of ammonium sulfate. Ammonium sulfate precipitate was dissolved in 20 mM HEPES-NaOH (pH 7.0) containing 1 M $(NH_4)_2SO_4$ and then applied to a Phenyl-Sepharose 4B column (1.2 × 15 cm). The proteins were eluted by decreasing gradient of ammonium sulfate from 1 M to 0 M. Fractions showing glutamine synthetase protection activity were dialyzed with 20 mM HEPES-NaOH (pH 7.0), were pooled, and then stored at -20°C until use.

Assay of TPx activity NADPH oxidation was monitored as the decrease in A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.2 mM NADPH, 10 μM yeast Trx, 0.3 μM yeast TR, 1 mM H_2O_2, and HI0572 protein or HI0751 protein. The reaction was started by addition of 50 μl of hydrogen peroxide solution, and the mixture was incubated at 30°C. One unit of peroxidase activity corresponds to the oxidation of 1 μmol of NADPH per minute (Chae *et al.*, 1994a).

Assay of glutathione-dependent peroxidase activity NADPH oxidation was monitored as the decrease in A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.2 mM NADPH, 10 μM GSH, 0.5 μM glutathione reductase, 1 mM H_2O_2, and HI0572 protein or HI0751 protein. The reaction was started by the addition of 50 μl of hydrogen peroxidase solution, and the mixture was incubated at 30°C. One unit of peroxidase activity corresponds to the oxidation of 1 μmol of NADPH per minute (Kim *et al.*, 1988).

Glutamine synthetase protection assay Glutamine synthetase inactivation was performed in a 25 μl reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 1 μg of glutamine synthetase, 10 mM DTT, 3 μM $FeCl_3$, and various concentrations of HI0572 protein or HI0751 protein. After 10 min at 37°C, the remaining glutamine synthetase activity was measured by the γ-glutamyltransferase method (Kim *et al.*, 1985; Kim *et al.*, 1988).

Results

Expression and purification of HI0572 and HI0751 proteins The genes encoding the HI0572 and HI0751 proteins were amplified using standard PCR techniques and then subcloned and expressed in *E. coli.* The purification of the recombinant proteins is shown in Fig. 1. The elution profiles of both proteins were almost the same in DEAE-Sephacel and Phenyl-Sepharose chromatographic separations (Fig. 1). The molecular weights of the purified HI0572 and HI0751 proteins were 27 kDa and 20 kDa on SDS-PAGE, respectively. This is the reported size of the ORF's (Fig. 2).

Peroxidase properties of HI0572 and HI0751 proteins All examined 2-Cys Prx enzymes use reduced Trx as an

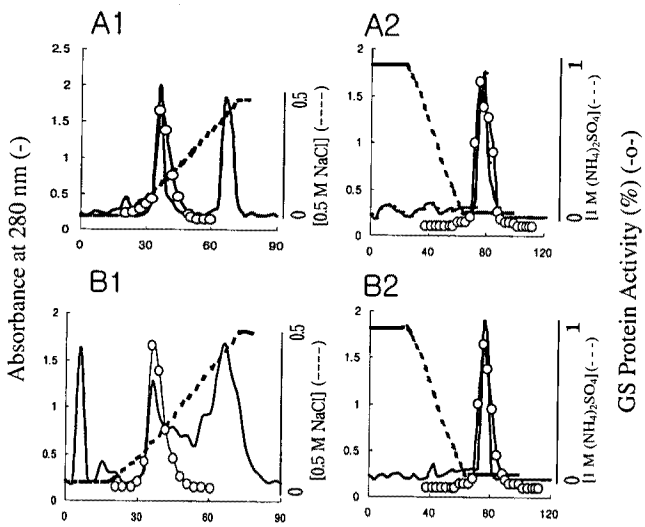


Fig. 1. Purification of *H. influenzae* peroxiredoxins overexpressed in *E. coli.* Crude extracts of overexpressed HI0572 or HI0751 protein was subjected to chromatography on DEAE-Sephacel (A1 and B1) and Phenyl-Sepharose 4B (A2 and B2) columns as described in *Materials and Methods.*

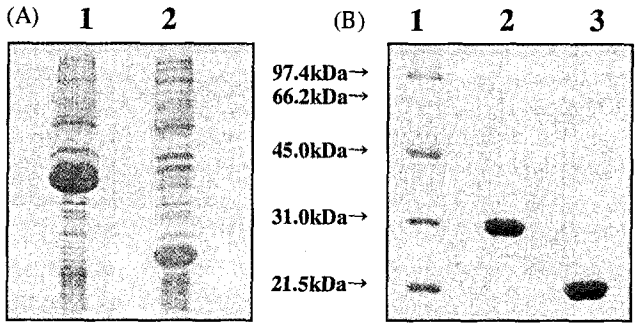


Fig. 2. SDS-PAGE analysis *H. influenzae* peroxiredoxins. (A) Crude extracts of *E. coli* overexpressed with of *H. influenzae* peroxiredoxins, HI0572 (lane 1) and HI0751 (lane 2). (B) Purified peroxiredoxins, HI0572 protein (lane 2) and HI0751 (lane 3) with molecular weight standard marker (lane 1).

immediate electron donor for the reduction of peroxides. We investigated whether or not the reducing equivalents required for peroxidase activity of *H. influenzae* Prx proteins could be provided by the Trx. The rate of peroxide degradation was measured by monitoring the decrease in A_{340} that is attributable to the oxidation of NADPH. The oxidation of NADPH was observed in the presence of all three protein components (HI0751, TR, and Trx) with hydrogen peroxide (Fig. 3A). The thioredoxin-dependent peroxidase activity of the HI0751 protein was 3.0 μmole/min/mg.protein for H_2O_2 as the peroxide substrate. The peroxidase activities of HI0751 protein for alkylhydroperoxide substrates, such as cumene hydroperoxide and t-butyl hydroperoxide, were almost same as for the activity for H_2O_2 substrate (Fig. 4). The HI0572 protein, however, didn't show the thioredoxin-dependent peroxidase activity. We investigated whether or not GSH can provide reducing equivalents for the reduction of H_2O_2 by monitoring NADPH oxidation with a reaction mixture containing H_2O_2, glutathione, GSH reductase, NADPH, and the *Haemophilus* Prx proteins. No NADPH oxidation was observed for the HI0751 protein; whereas, the HI0572 protein showed glutathione-dependent peroxidase activity (Fig. 3B). The specific activities of glutathione-dependent peroxidase of the HI0572 protein for hydrogen peroxide, *t*-butyl hydroperoxide, and cumene hydroperoxide were 9.3, 0.95, and 5.9 μmole/min/mg. protein, respectively.

Antioxidant activity of HI0572 and HI0751 proteins All reported Prx enzymes showed the inhibition activity of enzyme inactivation caused by the thiol-containing MCO systems. The antioxidant activity of the HI0572 and HI0751 proteins were examined. Both proteins also protect glutamine synthetase against inactivation by the DTT/Fe^{3+}/O_2 MCO system but not by the ascorbate. DTT, electron donor for MCO, system and for Prx enzyme, can be replaced by other thiol compounds. Both proteins also protect glutamine synthetase against inactivation by the other thiol-containing MCO systems (Fe^{3+}, O_2 and GSH or β-mercaptoethanol).

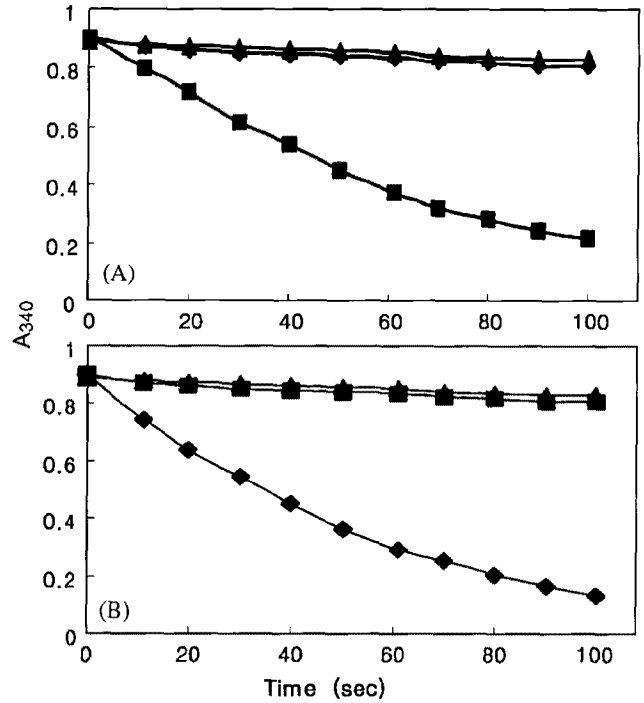


Fig. 3. Peroxidase activity of *H. influenzae* peroxiredoxins. (A) Thioredoxin-dependent peroxidase activity of HI0572 (◆) and HI0751 (■) proteins. NADPH oxidation was monitored as the decrease in A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.18 μM TR, 8 μM Trx1, 0.2 mM NADPH, 1 mM H_2O_2 and 0.2 μM purified HI0572 or HI0751 protein. (B) glutathione-dependent peroxidase activity of HI0572 (◆) and HI0751 (■) proteins. NADPH oxidation was monitored as the decrease in A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.15 μM GR, 0.5 mM GSH, 0.2 mM NADPH, 1 mM H_2O_2 and 0.2 μM purified HI0572 or HI0751 protein. For control (▲) assay mixture was not added with HI0572 or HI0751 protein.

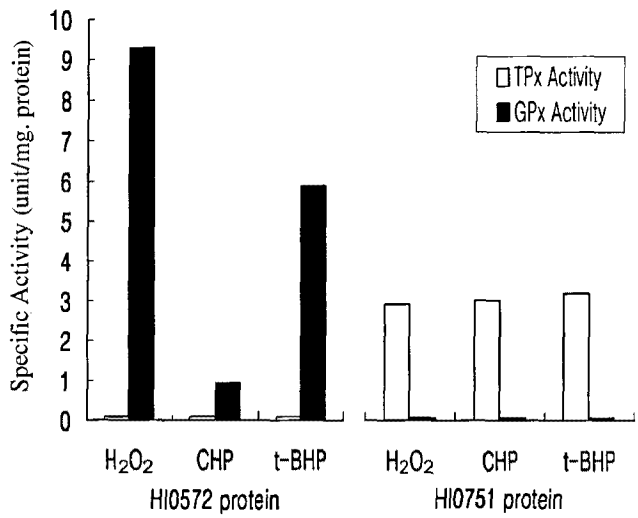


Fig. 4. Peroxidase activity of HI0572 and HI0751 protein for peroxide substrates. The activity was measured as described in Fig. 3.

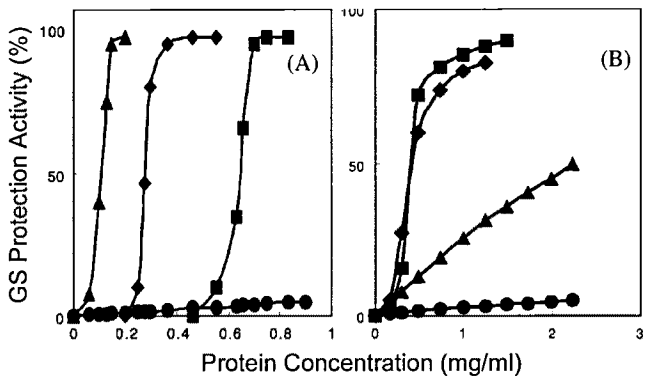


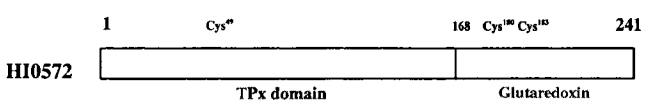
Fig 5. Glutamine synthetase protection activity of *H. influenzae* peroxiredoxins. Protection of glutamine synthetase by HI0572 (A) or HI0751 (B) protein against DTT, GSH, β-mercaptoethanol or ascorbate/Fe^{3+} system. Data were expressed as a percentage of the remaining activity glutamine synthetase in the absence of MCO system. Oxidation system was contained DTT (◆), GSH (▲), β-mercaptoethanol (■), or ascorbate (●).

The antioxidant potencies of HI0572 and HI0751 proteins against thiol containing MCO systems were evaluated. IC_{50} (inhibition concentration with 50%) of HI0572 and HI0751 proteins against the DTT/Fe^{3+}/O_2 MCO system were 11.2 μg/ml and 18 μg/ml, respectively (Fig. 5). The HI0751 protein was much less effective in the MCO system containing GSH and all of the reported Prx (Fig. 5B). The HI0572 protein, however, showed a higher potency against GSH MCO system (Fig. 4A). IC_{50} of the HI0572 protein against GSH MCO system was 4.8 μg/ml.

Discussion

The *H. influenzae* HI0751 protein shows sequence homology to P_{20} protein, thiol peroxidase, which is a Prx type that is only found in the bacterial system. The HI0751 protein is 63.1% identical to *E. coli* P_{20}. The protein prevented oxidative damage of enzyme caused by mercaptan-containing MCO systems and showed thioredoxin-dependent peroxidase activity. Those properties of HI0751 protein were very similar to that of *E. coli* P_{20} (Cha *et al.*, 1995; Zhou *et al.*, 1997) and other 2-Cys Prx such as TPx type I (Chae *et al.*, 1993) and II (Jeong *et al.*, 1999) of yeast *Saccharomyces cerevisiae*.

The *H. influenzae* HI0572 protein was composed of two domains which show homology to TPx II of *Saccharomyces cerevisiae* and glutaredoxin of *E. coli*. The N-terminal part of the HI0572 protein (1-168) is 29.8 % identical to the yeast TPx II and the C-terminal part of the protein (169-241) is 42.5 % to *E. coli* glutaredoxin 3. The HI0572 protein is the first Prx having other domain with a known function. The HI0572 Prx



did not appear thioredoxin-dependent peroxidase activity. The protein showed glutathione-dependent peroxidase activity, which is a unique property. Up to now, there was no reported Prx showing glutathione-dependent peroxidase activity. Moreover, the HI0572 protein was more effective in inhibiting glutamine synthetase inactivation in the GSH/Fe^{3+}/O_2 system, unlike HI0751 or other known Prx. The HI0572 protein has a putative C-terminal glutaredoxin-like sequence. Thus, this sequence might be involved in the peroxidase activity of the protein. This report suggests that HI0572 is the first Prx to be identified that uses GSH as an immediate hydrogen donor, and its glutaredoxin-moiety acts a role for reduction of peroxide.

Members of the genus *Haemophilus* are obligate parasites, which constitute a part of the normal flora of the respiratory tract of humans and many animal species (Edwin *et al.*, 1994). The type species *H. influenzae* is responsible for a variety of disease in humans, ranging from chronic respiratory infection to meningitis. These HI0572 and HI0751 proteins will be important role for cell survival against detrimental environment. To know the pathogenic bacteria defense mechanism related to antioxidant proteins, will be important to understand the human pathology.

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