

## Putative phospholipid hydroperoxide glutathione peroxidase from *Antrodia camphorata*

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### ARTICLE INFO

#### Article history:

Received 16 November 2007

Received in revised form 7 December 2008

Accepted 9 December 2008

#### Keywords:

*Antrodia camphorata*

Antioxidation

Phospholipid hydroperoxide glutathione peroxidase (PHGPx)

Taiwanofungus

### ABSTRACT

Glutathione Peroxidases (GPxs) play important roles in antioxidation. A cDNA (Ac-PHGPx, 764 bp) encoding a putative phospholipid hydroperoxide glutathione peroxidase (PHGPx) from *Antrodia camphorata* has been cloned. The deduced amino acid sequence is conserved among the reported GPxs. To characterize the Ac-PHGPx, the coding region was subcloned into pYEX-S1 and transformed into *Saccharomyces cerevisiae*. The recombinant 6His-tagged Ac-PHGPx was expressed and purified by Ni<sup>2+</sup>-nitrilotriacetic acid Sepharose. The purified enzyme showed a predominant band with molecular mass of ~18 kDa on 12% SDS-PAGE. The enzyme retained 50% activity at 60 °C for 8 min. The enzyme was most active at pH 9. The enzyme showed 42% activity after incubation with trypsin at 37 °C for 40 min. In addition, the ability of Ac-PHGPx to protect intact supercoiled plasmid DNA from ·OH induced nicking was demonstrated.

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### 1. Introduction

*Antrodia camphorata* (*A. camphorata*) is a unique Taiwanofungus species found only in Taiwan which traditionally has been used as a remedy for drug intoxication, abdominal pain, and liver cancer, among others. *A. camphorata* has been shown to exhibit antioxidative (Song & Yen, 2002), vasorelaxative (Wang et al., 2003), and anti-inflammatory (Shen et al., 2004) effects. Recently, we established EST (expressed sequence tag) from fruiting bodies of *A. camphorata* in order to search for value-added physiologically active components. We have cloned and characterized a thermostable superoxide dismutase (Liau, Wen, Shaw, & Lin, 2007), a 1-Cys peroxidoredoxin (Wen, Huang, Juang, & Lin, 2007), a catalase (Ken, Chen, Chang, & Lin, 2008) and a glutathione-dependent formaldehyde dehydrogenase (Huang, Ken, Wen, & Lin, 2009) based on the established EST (data not shown). We continue to search for antioxidant enzymes in *A. camphorata* which are believed to have potential health food applications. Here, we report the cloning of an antioxidant enzyme, phospholipid hydroperoxide glutathione peroxidase (Ac-PHGPx) cDNA, from *A. camphorata* on the basis of EST.

PHGPx is an antioxidant enzyme that can reduce peroxidized phospholipids produced in cell membranes. The enzyme is considered to be the main line of enzymatic defense against oxidative

membrane damage. Aerobic organisms use O<sub>2</sub> for oxidation of nutrients to acquire energy. Oxygen is reduced to H<sub>2</sub>O through acceptance of 4 electrons. During the reduction of O<sub>2</sub>, some reactive oxygen species (ROS) are formed, including ·OH, and H<sub>2</sub>O<sub>2</sub>. These ROS will attack cell components (protein, DNA, and lipid), and they sometimes cause lethal damage to cells. Among the ROS, ·OH and HOO· can remove the allylic H of unsaturated fatty acids to form an alkyl radical (L·) (Inoue, Matsuda, Sugiyama, Iza-wa, & Kimura, 1999). The L· is oxidized by O<sub>2</sub> to generate a peroxy radical (LOO·), and the LOO· reacts with LH to afford LOOH and L·. A radical reaction chain is then propagated. LOOH also belongs to the ROS, and the occurrence of the LOOHs in cell membranes may represent major oxidative damage to the cells. Organisms have enzymatic systems to defend against these ROS. The enzyme systems include superoxide dismutase which catalyzes O<sub>2</sub><sup>·-</sup> to O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> is then decomposed to H<sub>2</sub>O and O<sub>2</sub> by peroxidases such as glutathione peroxidase, or peroxidoredoxin. Ascorbate can also be used as a reductant for ascorbate peroxidase in plants.

There are five types of GPx in mammalian cells. The GPx1 is found in the cytosol and the matrix of mitochondria; GPx2 (GI-GPx) is found in the gastrointestinal; GPx3 is found extracellularly; Phospholipid hydroperoxide GPx calls GPx4 or PHGPx; and GPx5 is epididymis-specific secretory GPx (Jung et al., 2002). All animal GPx family members, except Cys-containing GPx3 and GPx5, possess selenocysteine (SeCys) at their active sites. GPx4 and GPx5 exist as a monomer, the other GPx isoforms form tetramers of identical subunit. The preferred GPx4 substrates are phospholipid

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hydroperoxides. During the catalytic mechanism of GPx, the Cys-SeH residue reacts with H<sub>2</sub>O<sub>2</sub> to produce cysteine selenenic acid (Cys-SeOH). GSH then binds and reduces Cys-SeOH to Cys-SeH (Jung et al., 2002).

GPx is one of the most important antioxidant enzymes. The possibility that the *A. camphorata* GPx could increase human longevity remains to be investigated. In this report, *A. camphorata* PHGPx cDNA was cloned and expressed in a yeast expression system. The active GPx enzyme has been purified and its properties studied.

## 2. Materials and methods

### 2.1. *Antrodia camphorata*

Fruiting bodies of *A. camphorata* which grown in the hay of *C. kanehirai* were obtained from Nanto, central part of Taiwan.

### 2.2. Total RNA preparation and cDNA synthesis

Fresh fruiting bodies (wet weight 5 g) were frozen in liquid nitrogen and ground to powder in a ceramic mortar. Poly(A)<sup>+</sup> RNA was prepared using Straight A's mRNA Isolation System (Novagen, Madison, WI, USA). The mRNA (22 µg) was obtained. Three µg of the mRNA was used for cDNA synthesis using a ZAP-cDNA kit from Stratagene (La Jolla, CA).

### 2.3. Isolation of Ac-PHGPx cDNA

We have previously established an EST database from fruiting bodies of *A. camphorata* and sequenced all clones with insert size greater than 0.4 kb (kilobase pairs, data not shown). The identity of an Ac-PHGPx cDNA clone was assigned by comparing the inferred amino acid sequence in databases using the basic local alignment search tool (BLAST, <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

### 2.4. Subcloning of Ac-PHGPx cDNA

The coding region of the Ac-PHGPx cDNA was amplified using two gene-specific primers. The 5' upstream primer contains EcoRI recognition site (5' GGA ATT CGA TGT CCT CTT TCT ACG GCC TG 3') and the 3' downstream primer contains a 6His-tag and EcoRI recognition site (5' GGA ATT CTA GTG GTG GTG GTG GTG CAA 3'). Using 0.1 µg of *A. camphorata* cDNA as the template, and 10 pmole of each 5' upstream and 3' downstream primer, a 0.5 kb fragment was amplified by PCR. The fragment was ligated into pCR2.1 and transformed into *E. coli* TOP10. Plasmid DNA was isolated from the clone and digested with EcoRI. The digestion product was separated on a 0.8% (w/v) agarose gel. The 0.5 kb insert DNA was gel purified and subcloned into the EcoRI site of pYEX-S1 expression vector (Clontech, Mountain View, CA). The recombinant DNA was introduced into *Saccharomyces cerevisiae* (trp-ura-) and the transformed yeast cells were selected by YNBDT [0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, and 2% (w/v) glucose] agar plates containing 20 µg Trp/ml. The presence of Ac-PHGPx cDNA in selected transformants was verified by PCR using gene specific flanking primers. The recombinant Ac-PHGPx protein was overexpressed in yeast in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] or YWA-ANDT medium [0.17% (w/v) yeast nitrogen base without amino acid and ammonium sulfate, 0.5% (w/v) asparagine, and 2% (w/v) glucose] containing 20 µg Trp/ml. Functional recombinant Ac-PHGPx protein was identified by activity staining as described below.

### 2.5. Expression and purification of the recombinant Ac-PHGPx

The transformed yeast containing the Ac-PHGPx gene was grown at 30 °C in 250 ml of YPD medium. The culture was incubated for 5 days at shaking at 180 rpm, and then the cells were harvested by centrifugation. Cells were suspended in 4 ml of PBS containing 1% (v/v) glycerol and 1 g glass beads. The content was vortexed for 10 min, frozen at -70 °C for 10 min, thawed, and centrifuged at 10,000 g for 5 min. The supernatant was saved and the pellet was re-extracted. The extraction procedure was repeated three times, and the supernatants were combined. The final crude extract (12 ml) was loaded on a Ni-nitrilotriacetic acid Sepharose superflow (Qiagen, Duesseldorf, Germany) column (with 3 ml bed volume) as described before (Ken, Hsiung, Huang, Juang, & Lin, 2005). The purified enzyme (18 ml) was dialyzed against 200 ml 0.33× PBS containing 5% (v/v) glycerol at 4 °C for 4 h. Fresh 0.33× PBS containing 5% (v/v) glycerol was changed once during dialysis. The dialyzed sample was used directly for analysis or stored at -20 °C until use.

### 2.6. Analysis of the purified Ac-PHGPx

To confirm the identity of the purified recombinant Ac-PHGPx protein, the protein (0.25 µg/µl) was sent to Yao-Hong Biotechnology Company (Taiwan) for peptide-mass fingerprinting using a Micromass Q-TOF ESI/MS/MS. The protein (100 µg) was digested with trypsin and fragments analyzed by Q-TOF ESI/MS/MS (Micromass, Manchester, England).

### 2.7. Protein concentration measurement

Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

### 2.8. Ac-PHGPx activity assay (ferrithiocyanate assay)

The recombinant Ac-PHGPx (1.0 µg protein) was incubated in 45–47 µl 0.33× PBS containing 1 mM DTT and 5% (v/v) glycerol for 2 min at room temperature. The reaction was initiated by addition of 3–5 µl 1 mM H<sub>2</sub>O<sub>2</sub>. At 0 and 10 min reaction times, 50 µl aliquot of the reaction mixture were taken and 20 µl of 26% (w/v) trichloroacetic acid were added to stop the reaction. The peroxidase activity was determined by following the disappearance of the peroxide substrate (the total peroxide, at the beginning of the reaction minus the remaining amount after 10 min incubation). The remaining peroxide content was determined as a red-colored ferrithiocyanate complex formed by addition of 20 µl 10 mM Fe(II)(NH<sub>4</sub>)(SO<sub>4</sub>)<sub>2</sub> and 10 µl 2.5 M KSCN to the 70 µl reaction mixture which was quantified by absorbance measurement at 475 nm (Thurman, Ley, & Scholz, 1972).

### 2.9. Enzyme characterization

The enzyme sample was tested for activity under various conditions. Aliquots of the Ac-PHGPx sample were treated as follows:

1. Thermal effect. The enzyme sample was heated to 60 °C for 2, 4, 8 or 16 min.
2. pH effect. The enzyme sample was adjusted to the desired pH by adding a half volume of buffer with different pHs: 0.2 M citrate buffer (pH 2.2, or 5.4), 0.2 M Tris-HCl buffer (pH 7.8, or 9.0) or 0.2 M glycine-NaOH buffer (pH 10.4, or 11.2). Each sample was incubated at 37 °C for 1 h.

3. SDS effect. SDS, a protein denaturing reagent, was added to the enzyme sample to the levels of 0.5, 1, or 2% (w/v) and incubated at 37 °C for 30 min.
4. Imidazole effect. During protein purification, the Ac-PHGPx enzyme was eluted with imidazole. Therefore, the effect of imidazole on protein activity was examined. Imidazole was added to the enzyme samples at levels of 0.2, 0.4 or 0.8 M and incubated at 37 °C for 1 h.
5. Proteolytic susceptibility. The enzyme was incubated with one-tenth its weight of trypsin or chymotrypsin at pH 8.0, 37 °C for a period of 10, 20 or 40 min. In the chymotrypsin digestion, CaCl<sub>2</sub> was added to 5 mM. Aliquots of each sample were removed at various time intervals for analysis. After each treatment, 2/3 of each sample was electrophoresed on a 12% native gel to determine any changes in protein content and mobility. The other 1/3 of each sample was used for Ac-PHGPx activity assay.

### 2.10. Thiol mixed-function oxidation (MFO) assay

Ac-PHGPx-dependent inactivation of DNA cleavage was evaluated by thiol mixed-function oxidation (MFO) assay (Kawazu et al., 2000). The assay generates ROS by autoxidation of thiol in the presence of iron. Reaction mixture (15 µl) containing 40 µM FeCl<sub>3</sub>, 10 mM dithiothreitol (DTT), 25 mM HEPES (pH 7.0) and 0.75 µg of pUC19 plasmid DNA was incubated with or without the Ac-PHGPx protein (0.28 or 0.56 µg) at 25 °C for 0.5 or 1 h. After incubation, nicking of the supercoiled plasmids by the MFO was evaluated on 1% (w/v) agarose gels stained with ethidium bromide.

## 3. Results

### 3.1. Cloning and characterization of a cDNA encoding Ac-PHGPx

Approximately 20,000 *A. camphorata* cDNA clones were sequenced. Nucleotide sequences and the inferred amino acid sequences of these clones were compared to the sequences in various nucleic acid and protein data banks using appropriate BLAST. A putative Ac-PHGPx cDNA clone was identified by sequence homology to the published PHGPxs and GPxs (Inoue et al., 1999; Avery & Avery, 2001; Jung et al., 2002). The Ac-PHGPx cDNA (764 bp; EMBL accession no. DQ021913) encodes a protein of 159 amino acid residues with calculated molecular mass of 18 kDa. Fig. 1 shows the amino acid sequence alignment of the putative Ac-PHGPx with GPx from several sources. This Ac-PHGPx shared 48–63% identity with PHCC-TPx (Chinese cabbage; AF411209), and yeast PHGPx3 (*S. cerevisiae*; Y1R037W), shared 32–49% identity with At PHGPx3 (*Arabidopsis thaliana*; CAA61965), Hs PHGPx (*Homo sapiens*; AAC32261), and Hs cGPx (*Homo sapiens*; CAB37833). Thus, the alignment results suggest that the deduced protein more closely resembled PHGPx than GPx.

### 3.2. Expression and purification of the recombinant Ac-PHGPx

The coding region of Ac-PHGPx (0.5 kb) was amplified by PCR and subcloned into an expression vector, pYEX-S1 as described in the Section 2. Positive clones were verified by DNA sequence analysis. The recombinant Ac-PHGPx protein was expressed, and the proteins were analyzed using a 12% SDS–PAGE in the presence of a reducing agent without boiling (Fig. 2). The recombinant Ac-PHGPx was expressed as a 6His-tag fusion protein and was purified by affinity chromatography with nickel chelating Sepharose. A band with molecular mass of ~18 kDa (expected size of the Ac-PHGPx monomer) was detected in Ni-NTA eluted fractions by SDS–PAGE (Fig. 2, lanes 4–8). This is consistent with the literature

results that most PHGPxs exist predominantly as a monomer, while the other GPx isoforms form tetramers of identical subunit. The Ni-NTA eluted fractions containing pure protein were pooled and characterized further. The yield of the purified 6His-tag Ac-PHGPx was 80 µg from 200 ml of culture. The purified Ac-PHGPx was digested with trypsin and peptide-mass fingerprinting performed using Q-TOF ESI/MS/MS. The masses of six peptides analyzed showed a complete match with the putative Ac-PHGPx sequence, confirming the identity of the protein. Functional Ac-PHGPx was detected by activity assay as describe below.

### 3.3. Antioxidant activity of the recombinant Ac-PHGPx

The ability of the Ac-PHGPx to remove H<sub>2</sub>O<sub>2</sub> in the presence or absence of a thiol reagent was evaluated using the ferrithiocyanate system. The data shown in Fig. 3 were analyzed by ANOVA and Scheffe's test. This Ac-PHGPx showed ability to remove H<sub>2</sub>O<sub>2</sub> without supplementing thiols (Fig. 3). There was a significance decrease in the level of H<sub>2</sub>O<sub>2</sub> with increasing Ac-PHGPx concentration from 10 to 40 µg in the absence of thiol (–) ( $p < 0.01$ ). The activity was promoted in the presence of 2 mM DTT. The H<sub>2</sub>O<sub>2</sub> level was significantly lower in the presence of DTT than that in the absence of thiol (–) at Ac-PHGPx 20 and 40 µg. Addition of GSH did not increase the activity above that seen in the absence of thiol. The result indicates that DTT is capable of reducing the oxidized form of Ac-PHGPx to regenerate its activity.

### 3.4. Characterization of the purified Ac-PHGPx

To examine the heat stability of the purified Ac-PHGPx, the protein was heat-treated as described in the Materials and Methods section. The heat-treated samples were then analyzed by 12% native gel or assayed for its activity. As shown in Fig. 4A, heat treatment may have altered the protein conformation and causing band smearing. Fig. 4B showed that approximately 50% of the Ac-PHGPx activity was lost at 60 °C after 8 min. The Ac-PHGPx protein was most active at pH 9.0 as shown in Fig. 5A, suggesting that tyrosine residue(s) may be involved in the active site of the enzyme. The enzyme retained 30% activity in the presence of 2% SDS (Fig. 5B), and 40% activity in the presence of 0.2 M imidazole (Fig. 5C). The enzyme showed 40% activity after 20 min of incubation at 37 °C with one-tenth its weight of chymotrypsin (data not shown), and 50% activity after 40 min of incubation at 37 °C with trypsin (Fig. 5D).

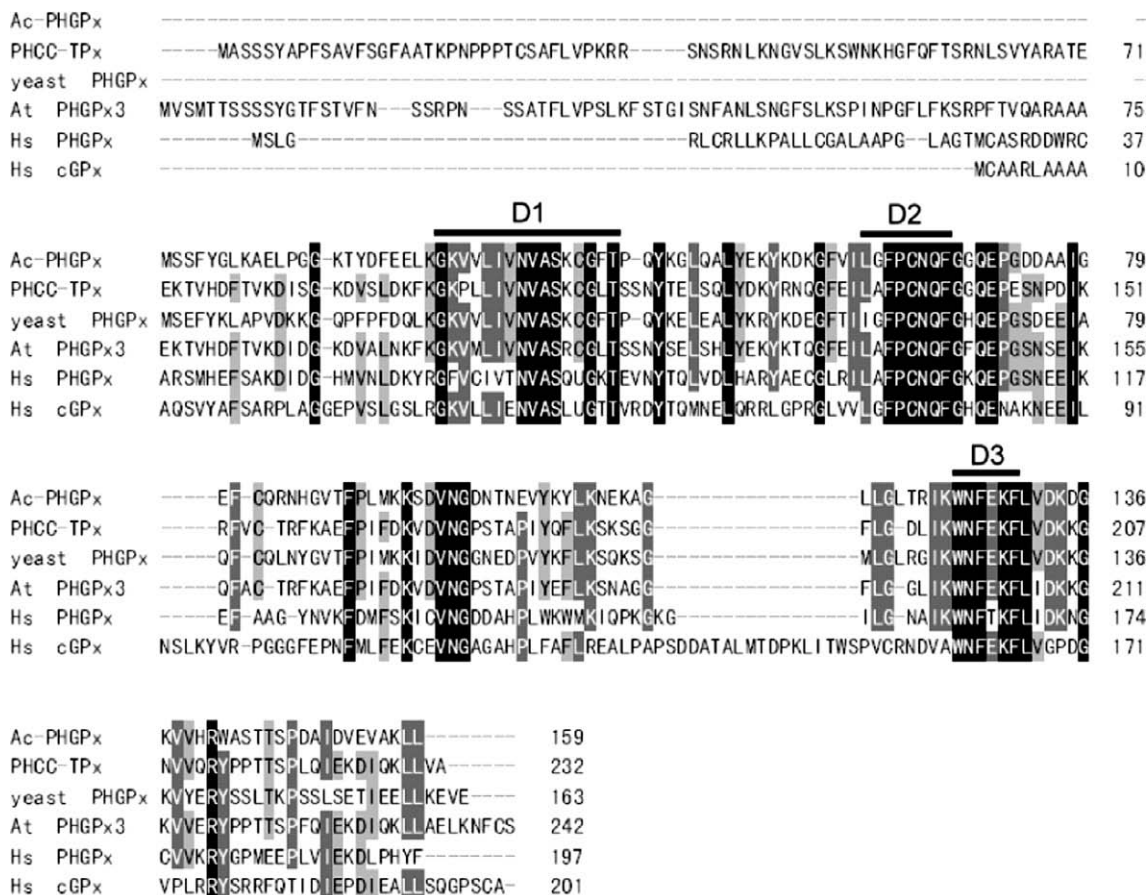
Using ExpASY Peptide Cutter tool, we predicted potential cleavage sites in the Ac-PHGPx protein cleaved by trypsin and chymotrypsin. The Ac-PHGPx protein contains 21 potential trypsin cleavage sites and 17 potential chymotrypsin-high specificity (C-term to [FYW], not before P) cleavage sites. However, the enzyme appeared to be susceptible to chymotrypsin but a little resistant to digestion by trypsin even at a high enzyme/substrate (w/w) ratio of 1/10 (Fig. 5D).

To test the peroxidase activity of the Ac-PHGPx, we studied the ability of Ac-PHGPx to protect supercoiled plasmid from hydroxyl radical induced nicking using the MFO system. In the absence of Ac-PHGPx protein, the supercoiled plasmid became nicked as evident by a shift in gel mobility of the plasmid, presumably by the hydroxyl radicals produced in the system (Fig. 6 lane 2). In the presence of 0.28 or 0.56 µg of Ac-PHGPx, a fraction of supercoiled plasmid was protected from nicking after one hour incubation, demonstrating antioxidant activity of Ac-PHGPx.

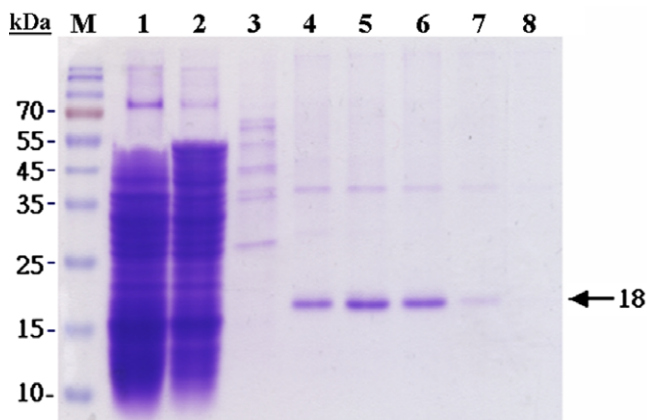
## 4. Discussion

Isolation and characterization of PHGPx proteins has been reported from various organisms. Sequence alignment showed that





**Fig. 1.** Optimal alignment of the amino acid sequences of GPxs from various sources. Ac-PHGPx (this study), PHCC-TPx (Chinese cabbage, AF411209), yeast PHGPx3 (YIR037W), At PHGPx3 (*Arabidopsis thaliana*, CAA61965), Hs PHGPx (*Homo sapiens*, AAC32261), Hs cGPx (*Homo sapiens* cytosolic GPx, CAB37833). Identical amino acids in all sequences are shaded black, 5 identical amino acids in all sequences are shaded deep gray, and 4 identical amino acids in all sequences are shaded light gray.

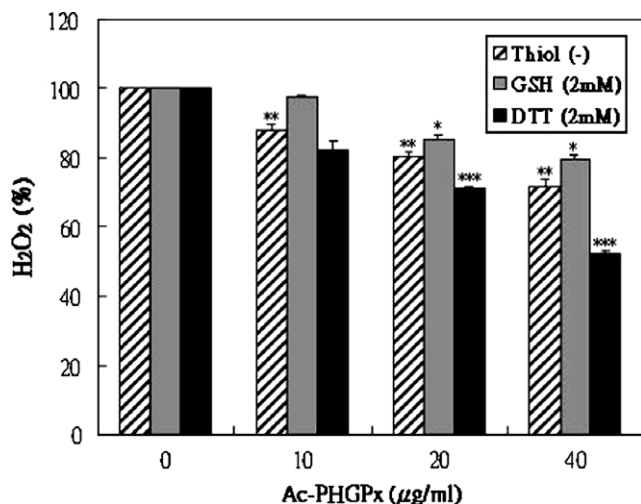


**Fig. 2.** Coomassie blue-stained SDS-polyacrylamide gel showing the purification of a 6His-tag Ac-PHGPx expressed in *S. cerevisiae*. Fifteen micro liters (without boiling) of each fraction was loaded into each lane of the 12% SDS-PAGE. Lane 1, crude extract from yeast expressing Ac-PHGPx; 2, flow-through proteins from the Ni-NTA column; 3, wash; lanes 4–8, purified Ac-PHGPx eluted from Ni-NTA column fractions 1–5. Molecular masses (in kDa) of standards are shown at left. Arrow denotes monomer.

the Ac-PHGPx exhibits higher sequence homology with PHGPxs than GPxs. The most distinctive structural motifs of GPx proteins are in the D1–D3 domains. These domains contain highly conserved amino acids found in GPxs, including a Cys residue (position

36) at a position normally occupied by SeCys (named U) in domain D1, a Gln residue (position 70) near to domain D2, and a Trp (position 125) in domain D3. These residues are believed to be important for the formation of the catalytic triad of GPx proteins (Jung et al., 2002). Most animal GPx family members, GPx3 and GPx5 contain a Cys residue at D1 (correspond to position 36), GPx1, GPx2 and GPx4 (PHGPx) possess selenocysteine (SeCys equals to U) at the same position, but all PHGPxs listed in Fig. 1 except Hs PHGPx contain a Cys instead of U, this indicates Ac-PHGPx more closely resembles PHGPx from Chinese cabbage, yeast, and *Arabidopsis thaliana* but is different from human PHGPx.

Jung et al. (2002) reported that the recombinant PHCC-TPx protein utilized Trx but not GSH as an electron donor to efficiently reduce H<sub>2</sub>O<sub>2</sub> and organic hydroperoxides. Site-directed mutagenesis of the individual conserved Cys residues of the PHCC-TPx (Cys107, Cys136, and Cys155 correspond to Cys36, Cys64, and Cys82 of the Ac-PHGPx) showed that all the three Cys residues are important in H<sub>2</sub>O<sub>2</sub> reduction. Cys107 is the primary attacking site by peroxide, and oxidized Cys107 then reacts with Cys155-SH to make an intramolecular disulphide bond. It is believed that these three Cys residues play important roles in antioxidant activity of the Ac-PHGPx. It is possible that Cys82 of the Ac-PHGPx forms an intramolecular disulphide bond with either Cys36 or Cys64, which may explain why the Ac-PHGPx existed predominantly as monomeric form in PAGE (Fig. 2). A minor band of 35–40 kDa can be seen in Fig. 2. This band may represent a dimeric form of Ac-PHGPx, although PHGPx has not been reported to exist as a dimer from any other sources.

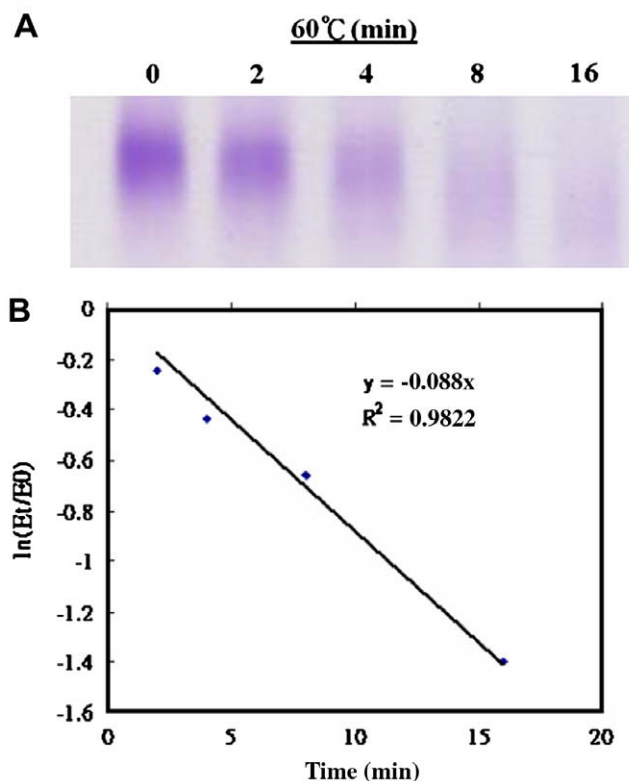


**Fig. 3.** Peroxidase activity of Ac-PHGPx. An effect of 2 mM DTT or 2 mM GSH on the activity of Ac-PHGPx to remove  $H_2O_2$  was examined in a 50  $\mu$ l reaction volume. Various concentrations of the Ac-PHGPx protein were incubated with  $H_2O_2$  (60  $\mu$ M) in the presence or absence of indicated thiols for 10 min. The remaining  $H_2O_2$  in the reaction mixture was measured using the ferrithiocyanate system. The results were expressed as the percentage of  $H_2O_2$  recorded with the Ac-PHGPx relative to that recorded without the Ac-PHGPx. Data are means  $\pm$  S.D. of three similar experiments. A significant decrease in the level of  $H_2O_2$  with increasing Ac-PHGPx concentration from 10 to 40  $\mu$ g/ml was observed in the absence of Thiol (-) ( $p < 0.01$ ). The  $H_2O_2$  level was significantly lower in the presence of DTT than in the absence of Thiol (-) at Ac-PHGPx 20 and 40  $\mu$ g/ml ( $p < 0.01$ ). In the presence of 2 mM GSH, significant difference was only observed at 20 and 40  $\mu$ g/ml GPx ( $p < 0.01$ ).

The expressed protein in yeast cells was extracted with PBS containing 1% (v/v) glycerol by vortexing with glass beads. The extraction procedure recovered only soluble proteins. We did not check to see if the insoluble material contained any of the expressed target protein. The recombinant Ac-PHGPx contained a 6His-tag which is different from endogenous PHGPx enzymes. Using the one step affinity column to purify Ac-PHGPx, we excluded endogenous PHGPx enzymes. The identity of the Ac-PHGPx was further verified by peptide-mass fingerprinting. The yield of purified Ac-PHGPx protein was 40  $\mu$ g/g wet cells (2 g wet cells were obtained from 200 ml cultures).

The Ac-PHGPx enzyme showed some ability to remove hydrogen peroxide in a concentration-dependent manner. As shown in Fig. 3, there was a significance decrease in the level of  $H_2O_2$  with increasing GPx concentration from 10 to 40  $\mu$ g in the absence of thiol (-) or in the presence of 2 mM DTT ( $p < 0.01$ ). The  $H_2O_2$  level was significantly lower in the presence of DTT than in the absence of thiol (-) at GPx 20 and 40  $\mu$ g. In the presence of 2 mM GSH, significant difference was seen only at 20 and 40  $\mu$ g GPx ( $p < 0.01$ ). In the present study, we showed that the Ac-PHGPx can use DTT, an artificial reductant, *in vitro*. Although our results showed that GSH is not an effective reductant *in vitro*, its ability to serve as an electron donor *in vivo* for reduction of peroxides remains to be investigated. Manevich et al. (2002) reported that depletion of GSH diminished the activity of human PrxVI in intact cells, indicating that GSH can serve as a physiological electron donor. In addition, GSH can serve as an electron donor *in vitro* for PrxVI (1-Cys Prx) when  $\pi$ GST is present (Manevich, Feinstein, & Fisher, 2004).

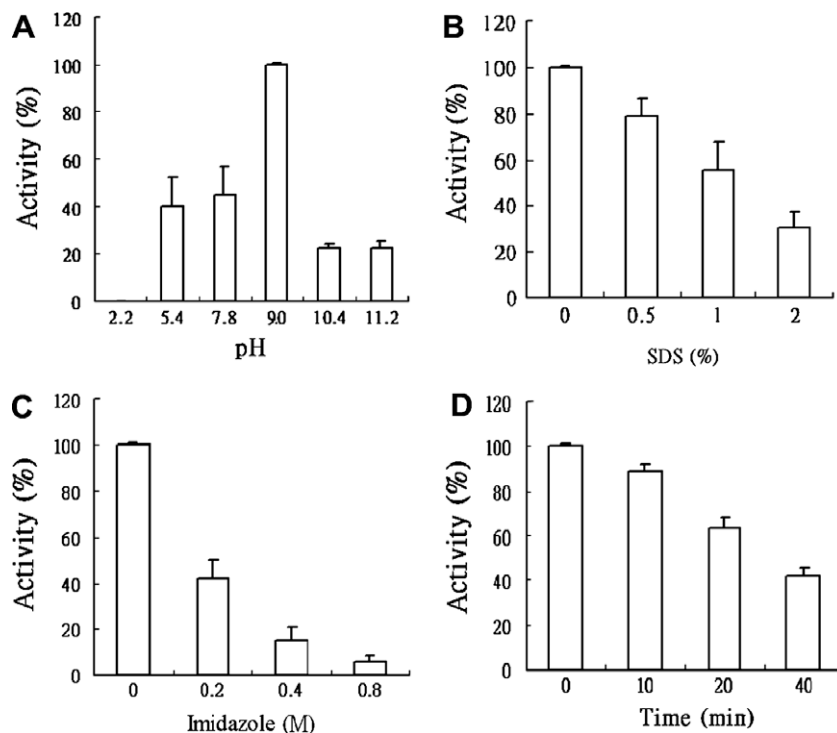
The enzyme was characterized as to its heat stability, the influence of SDS, imidazole or protease sensitivity. Heat stability and imidazole effects were tested because the information is useful for developing enzyme purification protocols. Protease tests may be useful in understanding the effect of the digestive enzymes on the Ac-PHGPx. The *A. camphorata*, rich in antioxidants, has been used as folk medicine and as health food for a long time. We point



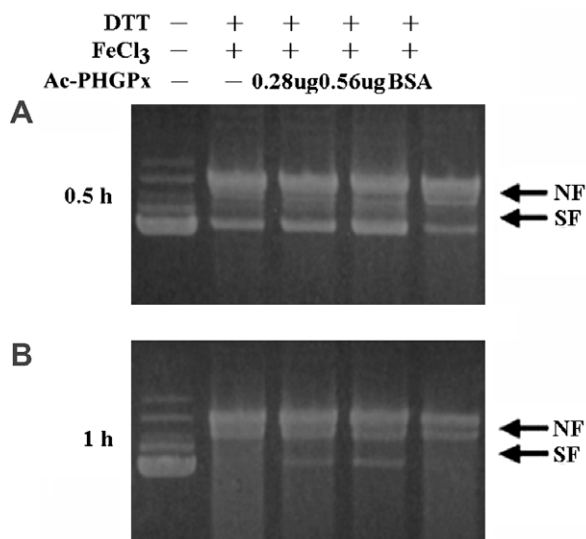
**Fig. 4.** Effect of temperature on the purified Ac-PHGPx. The enzyme sample was heated at 60  $^{\circ}$ C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8 or 16 min and analyzed by 12% native-PAGE or assayed for enzyme activity. (A) Staining for protein (2.2  $\mu$ g protein/lane) after separation on a 12% native-PAGE. (B) Plot of thermal inactivation kinetics. Ac-PHGPx activity assay (1.1  $\mu$ g protein/ time interval) was done using the ferrithiocyanate assay.  $E_0$  and  $E_t$  represented original activity and residual activity after being heated for different time intervals, respectively. Data are means of three experiments.

out to the possibility that the Ac-PHGPx may be one of the active ingredients in the *A. camphorata*. Further investigation of the enzyme's suitability as health food is required. Our results showed that the enzyme may escape digestion by the digestive enzymes. It would be interesting to see if the enzyme can enter cells by pinocytosis and if it could function in the blood stream. It has been reported that Mn-SOD, an antioxidant, is an anti-inflammatory agent and its half-life in serum was 5–6 h. In contrast, the half-life of Cu/Zn-SOD was only 6–10 min, thus Mn-SOD may be superior to Cu/Zn-SOD for the treatment of chronic diseases (Gorecki et al., 1991).

The Ac-PHGPx protein was most active at pH 9.0 as shown in Fig. 5A, which is consistent with that of its rice counterpart (Wang, Wang, Duan, & Liu 2007). The pH optima are determined by the ionization constants ( $pK_a$ ) of the catalytic residues. The  $pK_a$  values of Cys and Tyr are approximately 8.55 and 9.84, respectively (Thurkill, Grimsley, Scholtz, & Pace 2006). These values are relatively close to the enzyme's optimal pH. The results suggest that Cys and Tyr residues may be involved in the active site of the enzyme. As discussed before, it has been well established that Cys residues are in the active site of several reported glutathione peroxidases, although ionization of the Cys residues are not involved. Near the putative active Cys residue (position 36 in the Ac-PHGPx), there is a totally conserved Tyr residue at position 42 and highly conserved Tyr residues at positions 49 and 52. The conservation implies the importance of these residues and their contribution in the function of Ac-PHGPx can now be investigated by site-directed mutagenesis. It is interesting to note that tyrosine serves as an



**Fig. 5.** Effect of pH, SDS, imidazole and trypsin on the purified Ac-PHGPx. (A) The enzyme samples were incubated with different pH buffer at 37 °C for 1 h and then assayed for Ac-PHGPx activity (1 µg protein/pH point). (B) The enzyme samples were incubated with various concentration of SDS at 37 °C for 30 min and then checked for activity (1 µg protein/SDS level). (C) The enzyme samples were incubated with various concentration of imidazole at 37 °C for 1 h and then checked for Ac-PHGPx activity (1 µg protein/imidazole level). (D) The enzyme samples were incubated with trypsin at 37 °C for various times and then checked for Ac-PHGPx activity (1 µg protein/time interval). Data are means ± S.D. of three similar experiments ( $n = 3$ ) under each condition.



**Fig. 6.** Functional activity of recombinant Ac-PHGPx. The reaction was performed in a mixture consisting of 40 µM FeCl<sub>3</sub>, 10 mM DTT, 25 mM HEPES (pH 7.0) and 0.75 µg of pUC19 plasmid DNA. The reaction was stopped after incubation for 0.5 h (A), 1 h (B) and the plasmid DNA was analyzed on ethidium bromide stained agarose gel. Lane 1, only DNA; 2, DNA + Fe<sup>3+</sup> + DTT; 3, DNA + Fe<sup>3+</sup> + DTT + 0.28 µg Ac-PHGPx; 4, DNA + Fe<sup>3+</sup> + DTT + 0.56 µg Ac-PHGPx; 5, Fe<sup>3+</sup> + DTT + 0.56 µg BSA. SF, supercoiled form; NF, nicked form.

active site residue in glutathione S-transferase (Zheng & Ornstein, 1997).

The most significant finding in this paper is that the recombinant Ac-PHGPx can enzymatically detoxify ·OH. Using a MFO system to generate ·OH *in vitro*, we have demonstrated the ability of

Ac-PHGPx to protect intact supercoiled plasmid DNA from ·OH induced nicking. Further investigation is required to evaluate the possible applications of Ac-PHGPx including removal of peroxide (especially hydroxyl radicals) in wounded tissue to promote healing. The enzyme expressed in yeast should be superior for many applications to enzyme produced in *E. coli* as it will be free of endotoxin such as lipopolysaccharide during purification.

#### Acknowledgement

This work was supported partially by the National Science Council of the Republic of China under Grant NSC 97-2313-B-019-001-MY3 to C.-T. L.

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