Purification and Physicochemical Characterization of a Recombinant Phospholipid Hydroperoxide Glutathione Peroxidase from *Oryza sativa*

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Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an unique antioxidant enzyme that directly reduces lipid hydroperoxides in biomembranes. In the present work, the entire encoding region for *Oryza sativa* PHGPx was expressed in *Escherichia coli* M15, and the purified fusion protein showed a single band with 21.0 kD and p*I* = 8.5 on SDS- and IFE-PAGE, respectively. Judging from CD and fluorescence spectroscopy, this protein is considered to have a well-ordered structure with 12.2% α-helix, 30.7% β-sheet, 18.5% γ-turn, and 38.5% random coil. The optimum pH and temperature of the enzyme activity were pH 9.3 and 27°C. The enzyme exhibited the highest affinity and catalytical efficiency to phospholipid hydroperoxide employing GSH or Trx as electron donor. Moreover, the protein displayed higher GSH-dependent activity towards *t*-Butyl-OOH and H_2O_2. These results show that OsPHGPx is an enzyme with broad specificity for hydroperoxide substrates and yielded significant insight into the physicochemical properties and the dynamics of OsPHGPx.

Keywords: *Oryza sativa*, Phospholipid hydroperoxide glutathione peroxidase, Purification, Physicochemical characterization, Enzyme activity

Introduction

Since it was first identified as a peroxidation-inhibiting protein from pig liver in 1982 (Ursini *et al.*, 1982), phospholipid hydroperoxide glutathione peroxidase (PHGPx) has been extensively studied in mammals (Ursini *et al.*, 1999; Imai and

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Nakagawa, 2003). It has been characterized as a unique intracellular antioxidant enzyme that markedly reduces peroxidized phospholipids produced in cell membranes and is generally considered to be the main line of enzymatic defense against oxidative biomembrane damage in mammalian cells (Ursini *et al.*, 1999; Imai and Nakagawa, 2003). In contrast to the intensive studies on mammalian PHGPxs, only sporadic reports on PHGPx proteins in plants have been documented (Talia *et al.*, 1995; Yang and Liu, 2005). Nevertheless, cDNAs homologous to mammalian *PHGPx* have been identified from both dicot (Holland *et al.*, 1993; Sugimoto *et al.*, 1997; Sugimoto and Sakamoto, 1997; Mullineaux *et al.*, 1998) and monocot (Churin *et al.*, 1999; Li *et al.*, 2000) species. Furthermore, PHGPx mRNA levels have been shown to increase in plant tissues under stresses, such as salt (Holland *et al.*, 1993; Sreenivasulu *et al.*, 2004), heavy metals (Sugimoto and Sakamoto, 1997; Li *et al.*, 2000), oxidative damage (Sugimoto and Sakamoto, 1997; Li *et al.*, 2000), and infection by pathogens (Agrawal *et al.*, 2002). More recently, the radish PHGPx gene (*RsPHGPx*) introduced into a yeast PHGPx-deletion mutant was reported to significantly rescue the growth of recombinant cells exposed to linolenic acid, indicating a similar role to the yeast PHGPx3 gene (*ScPHGPx3*) in protection of membrane against lipid peroxidation (Yang *et al.*, 2005). Moreover, the expression of the tomato PHGPx gene (*LePHGPx*) was observed to inhibit cell death induced by Bax and oxidative stress in yeast and plants (Chen *et al.*, 2004). These findings suggest that plant PHGPx might play a very important role in development and stress tolerance in plants. However, our knowledge about biochemical and biophysical characterizations involved in plant PHGPx proteins is very limited.

It has been demonstrated that most deduced plant PHGPx proteins have a primary structure similar to those of mammalian PHGPx, except that the selenocysteine (SeCys) in the catalytic site is replaced by cysteine (Cys) in plant proteins (Maiorino *et al.*, 1990; Talia *et al.*, 1995; Yang and Liu, 2005). The SeCys residue is important for the catalytic activity of

PHGPx, as replacement of SeCys by Cys greatly reduces the activity of the enzyme (Maiorino *et al.*, 1990). The enzymatic activity of the first found plant PHGPx, citrus PHGPx, was reported to be 500-fold weaker than that of pig heart PHGPx, and similar to that of engineered pig heart PHGPx, in which the SeCys was replaced by a Cys residue through point mutagenesis (Eshdat *et al.*, 1997). However, replacement of the Cys residue by a SeCys residue in citrus PHGPx only displayed a 4-fold enhancement of peroxidase activity as compared with the Cys-containing analog (Hazebrouck *et al.*, 2000). In general, the overall enzymatic activity of plant PHGPx with phospholipid hydroperoxide as a substrate and reduced glutathione (GSH) as the electron donor is three orders of magnitude lower than that of animal proteins. Such results suggest the great complexity of the relationships between PHGPxs' structure and their activity and raise the question of whether plants contain PHGPx with higher activity equal to that of their counterpart in animals. Therefore, more studies are necessary to analyze the physicochemical and enzymatic characterization of PHGPx proteins in plants.

We previously isolated a cDNA for PHGPx from *Oryza sativa* (*OsPHGPx*) and characterized its expression patterns (Li *et al.*, 2000). Furthermore, a systematic investigation has shown that the *OsPHGPx* gene was induced by a variety of defense/stress-related stimuli and modulated by phosphatase of the kinase-signaling cascade (Agrawal *et al.*, 2002). These results suggest its potential role in rice stress response. In the present work, we focused our attention on an important aspect of the physicochemical and enzymatic properties of the OsPHGPx protein. For this study, the *Escherichia coli* strain M15 and pQE30 vector were used to produce recombinant OsPHGPx as a histidine-tagged protein. The product was soluble and could be purified to homogeneity with high yield. Herein, we report the physiochemical characterization and enzymatic properties of the recombinant protein. These results will greatly help us in the future to study the physiological function of this important enzyme in plant cells.

Materials and methods

Bacterial strains, plasmids, reagent, and chemicals. The cDNA of *OsPHGPx* was previously cloned in our laboratory (Li *et al.*, 2000). Host cell *E. coli* M15 strain and expression vector pQE30 were purchased from Qiagen, Germany. All chemicals were of analytical grade and purchased from Sigma Chemicals, USA, unless stated otherwise. Restriction endonucleases, *Bam*HI and *Hin*dIII, T4 DNA ligase, and pGEM-T Easy were from Promega, USA. The plasmid extraction kit, acrylamide, bisacrylamide, and SDS were also from Promega. Oligonucleotides were synthesized by Shenggong, China. Yeast thioredoxin (Trx) and Trx reductase (TR) were kindly gifted by Dr. Sang Won Kang and purified from the *Escherichia coli* transformants as reported (Kim *et al.*, 2005).

Construction of expression plasmid. The cloned cDNA of *OsPHGPx* encoding a protein with 169 amino acids was directly

used as a template for PCR amplification. DNA encoding OsPHGPx with a six-histidine tag and a subsequent enteropeptidase recognition site (Asp-Asp-Asp-Asp-Lys) at its N-terminus and was produced using primers 5'-GTGGATCCGATGACGATGACAAA ATGGGGGCGGCGGAAT-3' and 5'-CCCAAGCTTCTAATCCTC GAGCGCC-3'. The PCR products were cloned into pGEM-T Easy vector. Then, a fragment was excised with *Bam*HI and *Hin*dIII and inserted into pQE30 to yield pQE-PHGPx plasmid. The identity of the DNA construct was confirmed by sequencing.

Expression and purification of recombinant OsPHGPx. *E. coli* M15 cells transformed with pQE-PHGPx were cultured at 37°C in 200 mL of Luria-Bertani (LB) medium supplemented with 100 μg/ mL ampicillin and 25 μg/mL kanamycin to OD_{600nm} = 0.6-0.8. Protein expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 12 h of induction at 25°C, bacterial cells were harvested by centrifugation, resuspended in 6 mL of lysis buffer (50 mM sodium phosphate, 500 mM NaCl, pH 7.8), and disrupted by sonication in an ice bath. After centrifugation (12,000 × g, 40 min, 4°C), the supernatant was loaded to 2 mL of a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) pre-equilibrated with the lysis buffer and extensively washed with washing buffer (50 mM sodium phosphate, pH 7.8, 1 M NaCl and 20 mM imidazole). The bound histidine-tagged protein was eluted with elution buffer (50 mM sodium phosphate, pH 7.8, 250 mM imidazole). The protein solution was further loaded on a Mono S column (Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer (SPB) of pH 7.8 and was eluted with a linear gradient of 0-1.0 M NaCl in the same buffer. The protein solution was concentrated using a Centricon 10 filter from Amicon (Millipore, Bedford), and then was loaded on a Superdex^{TM} 75 HR 10/30 column (Amersham Biosciences) and eluted with 20 mM SPB, 150 mM NaCl of pH 7.8. The peak protein fractions were then combined and measured with a BCA^{TM} Protein Assay Kit (Pierce) using bovine serum albumin as a standard.

SDS-PAGE and IEF analyses. The molecular mass of the purified protein was estimated by SDS–PAGE (12.5 % polyacrylamide gel) according to the method of Laemmli (1970). An electrophoresis calibration kit (Amersham Pharmacia Biotech) was used for the estimation of the molecular mass as standard proteins. Isoelectric focusing (IEF) was performed at room temperature using a previously described method (Liu *et al.*, 2002). Calibration standards were between pH 4.55 and 8.65. Both gels were stained with 0.1% Coomassie Brilliant Blue R-250.

Fluorescence and CD spectroscopy. Fluorescence measurements were carried out on a Hitachi 850 spectrofluorimeter (Hitachi) at room temperature (25°C). The protein concentration was 4.5 μM and the spectra were obtained in 20 mM sodium phosphate at pH 7.8 in 150 mM NaCl. The excitation wavelength was 280 nm and the intensity of fluorescence emission was measured between 250 and 450 nm.

Far-UV CD spectra of purified OsPHGPx were obtained on a Jasco J-715 spectropolarimeter (Jasco Corporation) equipped with a Peltiereffect device for temperature control. Spectra were recorded using a cuvette with a 0.2 cm path length from 250 to 190 nm at room temperature. The scan speed was set to 50 nm/min with a 1-s

response time, 0.2-nm data pitch and 1.0-nm bandwidth, and the protein concentration was 4.5 μM. For all spectra, an average of six scans was obtained and the background spectra of the buffer were subtracted.

Determination of enzyme activity. The glutathione-dependent glutathione peroxidase activity of recombinant OsPHGPx towards phosphatidylcholine hydroperoxide [1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine, PLPC-OOH], *tert*-butyl hydroperoxide (*t*-Butyl-OOH) and H_2O_2 was measured by spectrophotometrically monitoring the decrease in the A_{340} resulting from simultaneous oxidation of NADPH in the presence of glutathione (GSH)/glutathione reductase (GR) or thioredoxin (Trx)/ thioredoxin reductase (TrxR). The phosphatidylcholine hydroperoxide (PLPC-OOH) substrate was prepared by the hydroperoxidation of 1-palmitoyl-2-linoleoyl-L-3-phosphatidylcholine (PLPC, Sigma) dispersion in deoxycholate using soybean lipoxygenase (Sigma) exactly as described by Maiorino *et al.* (Maiorino *et al.*, 1990). PLPC-OOH was purified by passage through a PepRPC HR5/5 column (Amersham Pharmacia Biotech, Sweden) with methanol elution (Bao *et al.*, 1995). The standard reaction buffer consisted of 100 mM Tris-HCl, pH 8.0, 1 mM NaN_3, 5 mM EDTA, 0.12% Triton X-100, 0.3 mM NADPH, and an electron donor, either GSH (15 mM glutathione and 3 units/mL glutathione reductase) or Trx (10 μM Trx and 1 μM TrxR). For the standard assay (1.0 mL), the above mixture plus enzyme (10 μg of OsPHGPx protein/ml) was preincubated for 5 min at a constant temperature, and the catalytic reaction was started by the addition of hydroperoxide solution. Rates of NADPH consumption were corrected for (a) hydroperoxide-independent NADPH-oxidizing activity by omitting the hydroperoxide substrate, (b) GSH- or Trx-independent NADPH-oxidizing activity by omitting GSH or Trx, and (c) nonenzymatic NADPH oxidizing activity by omitting the OsPHGPx protein. A millimolar absorption coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADPH was used. One unit of activity was defined as the amount of NADPH/min oxidized by 1 mg protein (extract) at 25°C.

Determination of temperature and pH optimum for recombinant OsPHGPx. The temperature optimum of OsPHGPx was studied by assaying the enzyme activity at different temperatures (0-50°C). To determine the pH optimum of OsPHGPx, the enzyme activity was measured at final purified protein concentrations of 4.5 μM in 50 mM buffer of various pH values from 6 to 13 (pH 6.0-7.0, potassium phosphate; pH 8.0-9.0, Tris-HCl buffer; pH 10.0-13.0, Gly-NaOH).

Kinetic analysis of recombinant OsPHGPx. To investigate the substrate preference of OsPHGPx, enzyme activity towards various concentrations of H_2O_2, *t*-Butyl-OOH and PLPC-OOH at the optimum temperature and pH was measured as described above. Enzyme activities were plotted against hydroperoxide concentrations and used to calculate the apparent kinetic V_{max} and K_m for OsPHGPx with the Michaelis-Menten equation ($v = V_{max}[S]/K_m + [S]$) by SigmaPlot software. Data represent the mean of three independent experiments.
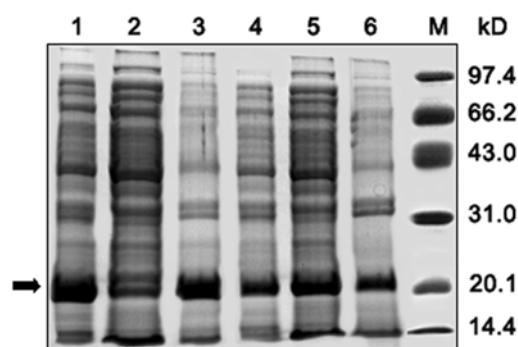

Fig. 1. SDS-PAGE analysis of the recombinant OsPHGPx protein produced in *E. coli* M15 cells. Each extract (approximately 10 μg of protein) was analyzed by 12.5% SDS-PAGE. Lanes 1-6 represent the total proteins (lanes 1 and 4), the soluble proteins (lanes 2 and 5) and the insoluble proteins (lanes 3 and 6) of pQE30-PHGPx-transformed cells induced with 1 mM IPTG at 37°C for 6 h (lanes 1-3) or with 0.1 mM of IPTG at 25°C for 6 h (lanes 4-6). The arrow indicates the migration position of the recombinant OsPHGPx. Positions and sizes of molecular mass protein markers are shown on lane M in kilodaltons (kD).

Results and discussion

Expression of recombinant OsPHGPx. In order to express recombinant OsPHGPx in *E. coli*, the mature protein coding region was generated by PCR and was inserted in frame into pQE30 to yield expression vector pQE-PHGPx containing a six-histidine tag at its N-terminus. With this fusion tag, the expressed OsPHGPx fusion protein could be purified by Ni^{2+} affinity chromatography. Between His-tag and OsPHGPx, an enteropeptidase recognition site was also introduced to release the intact OsPHGPx. After induction with IPTG, *E. coli* M15 cells transformed with pQE-PHGPx produced a fusion protein of approximately 21.0 kDa as shown in Fig. 1. The size of the fusion protein matched its theoretical molecular weight well. To obtain the maximum soluble fusion protein, expression conditions were optimized. The soluble His-tagged OsPHGPx was greatly produced at 25°C, 0.1 mM IPTG for 12 h induction (Fig. 1, lane 5), but expressed at low levels at 37°C, 1.0 mM IPTG (Fig. 1, lane 2). This was most likely due to growth at 37°C and higher IPTG concentration causing much protein to accumulate as inclusion bodies (Fig. 1, lane 3), whereas incubation at 25°C and lower IPTG concentration decreased the rate of protein synthesis and resulted in more protein solubility (Fig. 1, lane 5).

Purification of recombinant OsPHGPx. The OsPHGPx fusion protein was purified from the supernatants of the cell lysates using a 2 mL Ni-NTA column. Elution with sodium phosphate buffer containing 250 mM imidazole yielded over 6 mg of OsPHGPx fusion protein per liter of culture at >98% purity, and an intense band corresponding to the molecular
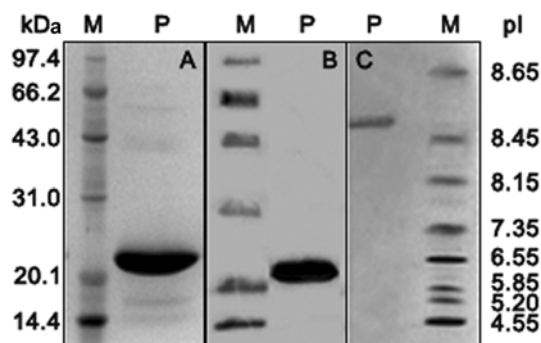
Fig. 2. Analysis by SDS-PAGE and IFE-PAGE of the purified recombinant OsPHGPx. A-B, the recombinant OsPHGPx purified by affinity chromatography (A) or gel-filtration chromatography (B) on 12.5% SDS-PAGE; C, the recombinant OsPHGPx on IEF-PAGE using a 7.5% polyacrylamide gel. M indicates protein marker, P represents the purified recombinant OsPHGPx protein. Positions and sizes of molecular mass protein markers are shown on the left side of the panel in kilodaltons (kDa), while positions and values of pI protein markers are shown on the right side of the panel.

weight of the expected protein was shown at 21.0 kDa for OsPHGPx fusion protein on SDS-PAGE gel (Fig. 2A). Further purification of recombinant OsPHGPx on a Mono S column using a salt gradient formed one major peak (data not shown). The concentrated protein solution corresponding to this major peak was then loaded on a Superdex 75 column

and eluted with SPB. Such preparation of recombinant protein was >99% pure as determined by densitometric analysis of SDS-PAGE electrophoresis (Fig. 2B). The yield of the purified recombinant protein was ~5 mg/L of culture. Isoelectric focusing electrophoresis (IFE) of the protein exhibited a single band of pI = 8.5 (Fig. 2C), similar to its theoretical pI of 8.865 predicted by the program: Compute pI/Mw tool at http://cn.expasy.org. These data collectively indicate that the protein purity is suitable for analysis of the physicochemical properties of this protein.

Spectroscopic properties of recombinant OsPHGPx. A UV scan of the purified recombinant OsPHGPx showed a maximum UV peak at 278 nm (Fig. 3A) on a UV spectrophotometer (Hitachi UV-7500, Japan). This is the typical UV spectroscopic characteristic of most proteins resulting from Trp and Tyr in the primary sequence of OsPHGPx. The circular dichroism of OsPHGPx in SPB was recorded in the far UV range to assess the secondary structure content of OsPHGPx (Fig. 3B). The CD spectrum of recombinant OsPHGPx showed a broad negative band with a minimum at 207 nm. The Secondary Structure Estimation Program using the method of Yang et al. (1986) calculated the α-helix, β-sheet, γ-turn and random coil contents of the protein to be about 12.2, 30.7, and 18.5 and 38.5%, respectively. Based on the CD spectrum, the protein would contain a significant portion of unstructured region. The fluorescence emission spectrum of OsPHGPx (which contains 13 phenylalanine, 6 tyrosine, and 1 tryptophan residues),
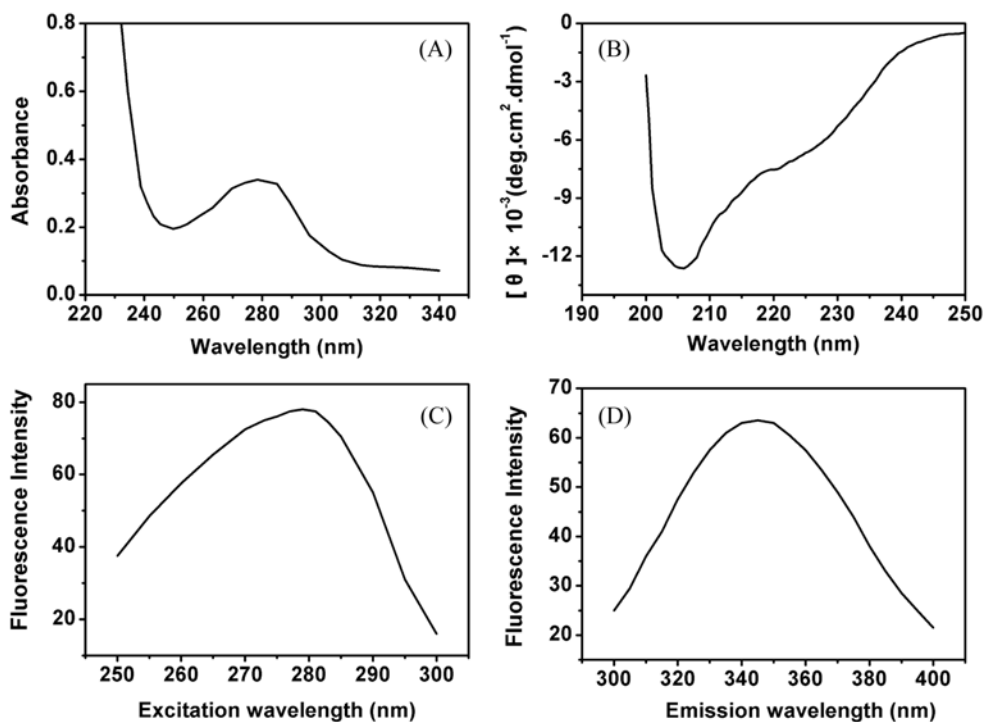

Fig. 3. Spectroscopic characterization of the purified recombinant OsPHGPx. A, ultraviolet absorption spectrum of 4.5 μM purified protein at 25°C in aqueous solution; B, Far-UV CD spectrum of 4.5 μM purified protein at 25°C in sodium phosphate buffer, pH 7.8; C-D, Excitation (C) and Emission fluorescence spectrum (D) of 4.5 μM purified protein at 25°C in sodium phosphate buffer, pH 7.8.
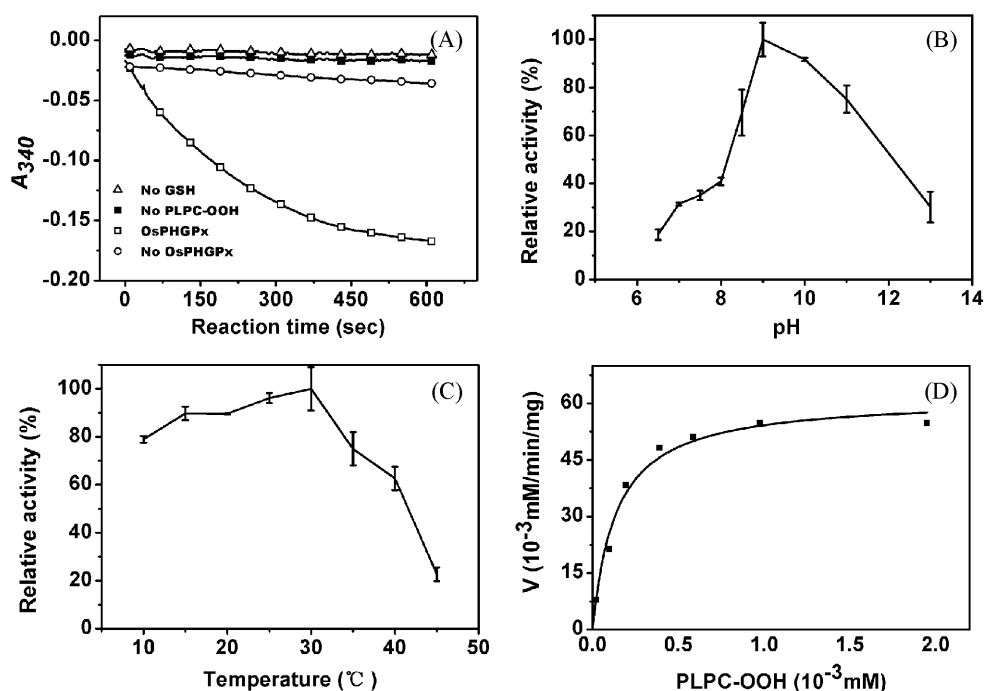
Fig. 4. Enzymatic kinetics analyses of the purified recombinant OsPHGPx. A, The glutathione peroxidase activity of the OsPHGPx towards PLPC-OOH in the presence of glutathione/glutathione reductase, which involved following NADPH oxidation by measuring changes in absorbance at 340 nm; B, Effects of pH on the OsPHGPx activity; C, Effects of temperature on the OsPHGPx activity; D, Effect of the concentration of PLPC-OOH on the RsPHGPx activity.

upon excitation at 282 nm (Fig. 3C), had a maximum fluorescence wavelength of 342 nm (Fig. 3D), indicating that the tryptophan residues may be in a hydrophobic environment in the native protein.

Catalytic and kinetic properties of recombinant OsPHGPx. The activity of OsPHGPx towards several hydroperoxides was determined in the presence of glutathione/glutathione reductase or Trx/TrxR, which involved following NADPH oxidation by measuring changes in absorbance at 340 nm. First, GSH-dependent enzymatic activity of recombinant OsPHGPx towards PLPC-OOH was measured. A linear decrease of A_{340} (absorbance of NADPH) following the addition of PLPC-OOH was observed, whereas no obvious declines were detected when GSH, PLPC-OOH, or OsPHGPx was omitted (Fig. 4A), indicating that the peroxidase activity of OsPHGPx was remarkably GSH-dependent. When the effects of pH and temperature on GSH-dependent OsPHGPx activity towards PLPC-OOH were evaluated, as shown in Fig. 4B and 4C, the pH optimum was in the range of pH 9.0-10.0 with a maximum of activity at approximately pH 9.3, and the dependence of enzyme activity *versus* temperature from 10 to 45°C yielded an obvious temperature optimum at approximately 27°C, with activity demonstrated in a wide temperature range of 10-35°C. These results show the temperature- and pH-dependence of GSH-dependent OsPHGPx enzymatic activity. The optimum pH range for catalysis of OsPHGPx was

consistent with that of its well-known mammalian counterpart (Flohé and Günzler, 1984), while its optimum temperature coincides with that required for plant growth.

Considering the broad specificity for hydroperoxide substrates of PHGPx (Maiorino et al., 1990), the hydroperoxide substrate preference for recombinant OsPHGPx was investigated. Peroxidase activity towards different concentrations of PLPC-OOH, t-Butyl-OOH, and H_2O_2 were assayed with a fixed GSH concentration of 15 mM. As illustrated in Fig. 4D, the plot of GSH-dependent OsPHGPx activity versus PLPC-OOH concentration was consistent with normal Michaelis-Menten kinetics. From this plot, the apparent K_m and V_{max} value for PLPC-OOH was calculated to be 0.14 ± 0.01 μM and 0.0641 ± 0.0034 μM/min per mg of protein (Table 1). The GSH-dependent activity of recombinant OsPHGPx towards H_2O_2 and t-Butyl-OOH was then examined using the same method and conditions. As shown in Table 1, apparent K_m and V_{max} values of the protein for H_2O_2 and t-Butyl-OOH were 798.81 ± 17.57 and 4344.12 ± 34.31 μM, and 258.15 ± 5.461 and 200.02 ± 1.982 μM/min/mg, respectively. In Trx-dependent peroxidase assays, OsPHGPx only exhibited activity towards PLPC-OOH with K_m and V_{max} value as 36.18 ± 4.12 and 0.131 ± 0.0065, respectively and no activity towards others substrates. So, in GSH- and Trx-dependent peroxidase assays, among the three hydroperoxide substrates, OsPHGPx exhibited the highest affinity to PLPC-OOH, and a weaker affinity towards H_2O_2 and t-Butyl-OOH, as indicated by their respective

Table 1. Glutathione-dependent peroxidase activities of OsPHGPx towards different substrates

Substrates	App. V_{max} (µmol/min/mg)	App. K_m (µM)	V_{max}/K_m
PLPC-OOH	0.0641 ± 0.0034	0.14 ± 0.01	0.44
H_2O_2	258.15 ± 5.461	798.81 ± 17.57	0.32
t-Butyl-OOH	200.023 ± 1.982	4344.12 ± 34.31	0.046

Glutathione peroxidase assays were performed as described in Materials and methods with a fixed concentration of GSH (15 mM) using seven or eight different concentrations of peroxide. Apparent maximum velocities (App. V_{max}), apparent maximum Michaelis constant (App. K_m) values (± SEM) and V_{max}/K_m ratios are shown as the average of three independent experiments.

apparent K_m values (Table 1). Obviously, PLPC-OOH was the optimum hydroperoxide substrate under *in vitro* conditions, suggesting a high probability that phospholipid hydroperoxides may be the primary physiological substrates. On the other hand, the protein displayed a higher catalytic efficiency towards PLPC-OOH and H_2O_2 and a weaker catalytic efficiency towards t-Butyl-OOH, as indicated by their respective V_{max}/K_m values (Table 1). It should be noted that although the V_{max} value for PLPC-OOH was still lower than the specific activity of mammalian PHGPx (Maiorino *et al.*, 1990), the V_{max} value of OsPHGPx was at least 50% higher than those of all other known plant homologues, including citrus, tomato, and sunflower PHGPx (Chen *et al.*, 2004; Herbette *et al.*, 2002). The results also demonstrated that OsPHGPx was active on H_2O_2, t-Butyl-OOH, and PLPC-OOH, indicating a similar broadness of substrate specificity to mammalian PHGPx (Maiorino *et al.*, 1990). However this is in contrast to other reported plant PHGPxs, which displayed no activity on H_2O_2 at all when glutathione was employed as a reducing substrate (Herbette *et al.*, 2002).

It is also noteworthy that the overall enzymatic activity of plant PHGPxs recorded so far with phospholipid hydroperoxide as a substrate is generally three orders of magnitude lower than those of the mammalian proteins. This low activity has made it difficult to explore the potential physiological role of PHGPx in higher plants and also raises the question of whether higher plants indeed do not maintain their PHGPx with higher activity equal to that of their mammalian counterpart. In this report, the presented results showed an unusual finding: recombinant OsPHGPx exhibits enzymatic activity against not only phospholipid hydroperoxide, but also H_2O_2 and t-Butyl-OOH using GSH as the electron donor. It is reasonable to believe that if the cysteine at the catalytic site of OsPHGPx is replaced by a selenocysteine residue, OsPHGPx's catalytic activity may be enhanced to a higher level. Our kinetic characterization displayed obvious similarity in substrate preference between plant OsPHGPx and its mammalian counterpart: both of them could use H_2O_2, organic hydroperoxide, and phospholipid hydroperoxide as substrates, but preferred phospholipid hydroperoxide (Maiorino *et al.*, 1990; Ursini *et al.*, 1995). Interestingly, OsPHGPx showed higher catalytic efficiency and V_{max} for H_2O_2, but it displayed a lower affinity to H_2O_2 (Table 1). This probably means that under normal physiological conditions OsPHGPx is predominantly concerned

with reduction of phospholipid hydroperoxides rather than competing with other H_2O_2-detoxifying peroxidases for H_2O_2 reduction, such as ascorbate peroxidase, which has higher affinity to H_2O_2 (Kvaratskhelia *et al.*, 1997). On the other hand, OsPHGPx may also participate in the detoxification of H_2O_2 when endogenous H_2O_2 accumulates to a very high concentration. By analogy with mammalian PHGPx whose main *in vivo* oxidative substrate has proven to be phospholipid hydroperoxides (Maiorino *et al.*, 1990; Ursini *et al.*, 1995), we suppose that phospholipid hydroperoxides might be the main natural substrates of OsPHGPx.

In conclusion, a plant phospholipid hydroperoxide peroxidase, OsPHGPx, was produced in *E. coli* and purified to near homogeneity. The purified recombinant protein showed GSH-dependent peroxidase activity towards PLPC-OOH, H_2O_2 and t-Butyl-OOH and Trx-dependent peroxidase activity twards PLPC-OOH. In addition, the enzyme (OsPHGPx) was also found to have a glutathione-dependent hydroperoxide peroxidase activity towards H_2O_2. The results clearly demonstrated that OsPHGPx is an enzyme with broad specificity for hydroperoxide substrates. Previous works have demonstrated that the OsPHGPx gene is expressed at a relatively high level in rice flag leaves and markedly induced by a variety of oxidative stress and defense-related stimuli, strongly suggesting that the product of the gene plays a key role in defense against oxidative damage in rice (Li *et al.*, 2000; Agrawal *et al.*, 2002). Taken together, this evidence *in vivo* and *in vitro* has provided significant insight into the induction expression of the gene, the biochemical and biophysical properties of the protein, and the dynamics of the OsPHGPx enzyme, and will greatly help us in the future to uncover the exact physiological function of this important enzyme in plant cells.

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