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Isolation, Partial Purification and Characterization of Phospholipid Hydroperoxide Glutathione Peroxidase (Phgpx Enzyme from *Oryza Sativa* Seedlings

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Abstract

The recombinant Phospholipid hydroperoxide glutathione peroxidase isolated from *Oryza sativa* is a type of antioxidant enzyme. It is stable at pH 7-8 and temperature 27 and 62°C. The molecular weight of enzyme was found to be around 21-26 kDa by SDS PAGE. The peroxidation activity of PHGPx was inhibited by $HgCl_2$, EDTA, Potassium ferrocynate and was activated by $FeCl_2$ and $MnSO_4$ .These properties indicate that the possibilities for use of the PHGPx in the medical, clinical and cosmatic industry. This enzyme can be exploited commercially with some modification.

Keywords: PHGP, Enzyme activity, SDS Page, Antioxidant

Introduction

Glutathione peroxidase (E.C. 1.11.1.9) is an antioxidant enzyme family with peroxidase(oxidoreductase) activity whose main biological role is to protect the organism from oxidative damage.Glutathione peroxidases (GSHPx) are a group of selenium-dependent enzymes. Out of some are cytosolic GSHPxl, plasma GSHPx, phospholipid hydroperoxide PHGSHPx established the presence of glutathione peroxidase in mammalian erythrocyte. 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 [5,10]. The expression of the tomato PHGPx gene *(LePHGPx)* was observed to inhibit cell death induced by Bax and oxidative stress in yeast and plants [2]. These findings suggest that plant PHGPx might play a very important role in development and stress tolerance in plants. The present investigation is carried out in view to isolate, partial purify and characterize Phospholipid hydroperoxide glutathione peroxidase (PHGPx enzyme from *Oryza sativa* seedlings.

Materials and Methods

Isolation of Glutathione Peroxidase (PHGPx enzyme)

Phospholipid Hydroperoxide Glutathion Peroxidase (PHGPx) is an antioxidant enzyme and it's expression takes place in side (intracellular) the transformed cell present inside the LB broth. So recovery of Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) occurs after cell lysis by chemical method.

Purification of isolated of Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx)

Purification of enzyme Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) isolated from *Oryza sativa*. The Crude enzyme was precipitated by ammonium sulfate (ammonium sulfate precipitation method). The precipitated enzyme was passed through the process of dialysis by cellular membrane filter (treated with 3% sodium bi carbonate) for overnight and centrifuged the dialyzed enzyme at 6000-10000rpm for 10 minute then transferred the supernatant to a fresh tube and pure enzyme was separated by ion-exchange chromatography by DEAE cellulose.

Ammonium sulphate precipitation: The PHGPs enzymes were also purified by ammonium sulfate saturation. The protein fraction precipitated with 85 % ammonium sulfate. The suspension was centrifuged at 10,000rpm for 15 minutes. The precipitate was collected and dissolved in 25mM NaCl, 10MmTris base buffer. Followed by its dialysis for 1-2 days to make it more purified.

Dialysis: The precipitate obtained after ammonium sulphate precipitation was dissolved in 25mM NaCl; 10Mm Tris base buffer. Then it is put inside a semi permeable membrane bag and kept immersed in Tris base buffer for dialysis at 4°C for 1-2 days. After dialysis the enzyme was taken out and centrifuged at 8000rpm for 15minutes. The supernatant is collected as the pure enzyme after dialysis.

Characterization of pure enzyme Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx):

Protein estimation by Lowry's method: The protein was estimated by [8].

Enzyme assay: The enzyme activity of isolated Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) enzyme was measured by decreasing in absorbance at 340nm during detoxification of H_2O_2 (Hydrogenperoxide) and increase in relative activity and it was measured by continuous spectrophotometric rate determination and substrate concentration dependent assay.

Effect of temperature, pH, inhibiter and activator on enzyme activity: The enzymes was mixed with enzyme assay buffer and then

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incubated at different temperature for 30 minutes and absorbance was taken at 340 nm.

Estimation of molecular weight: The electrophoresis was conducted to determine the molecular weight of the visualized protein bands by comparing them with the molecular weight marker.

Zymography of Recombinant Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx): Zymography was done for visualization of protein bands.

Results and Discussion

Ammonium sulphate precipitation

Ammonium sulphate precipitation of recombinant Phospholipids Hydro peroxide Glutathione Peroxidase (PHGPs) enzyme was found at 85-90% saturation.

Spectrophotometric qualitative estimation of purified Recombinant enzyme (PHGPx)

Protein estimation by Lowry's method: The graph obtained from protein estimation was clearly showed that the concentration of enzyme PHGPs expressed by *E. coli* plasmid, P^{BR322}, and Pet EMBL21 was higher than standard (BSA) concentration respectively as shown in Figure 1.

Protein estimation of different fraction of purified intracellular enzyme: The graph obtained from protein estimation it was clearly showed that concentration of enzyme PHGPs higher in 50% fraction for Pet EMBL21 expressed enzyme, 50% fraction for PBR^{322} expressed enzyme and 25% fraction for *E. coli* plasmid expressed enzyme respectively as shown in Figure 2 and Table 1.

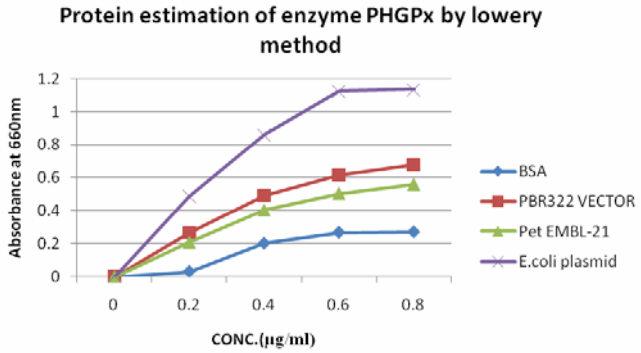

Figure 1: Protein Estimation of PHGPx enzyme by Lowry's method.

Phospholipids Hydro peroxide Glutathione Peroxidase (PHGPs)in different fraction of buffer	Absorbance at 280 nm	Absorbance at 280 nm	Absorbance at 280 nm at 280 nm
	P^{BR322}	PEMBL21	Natural *E.coli* plasmid
Crude	2.371	2.211	2.848
0% fraction of PHGPs (first elution)	1.228	0.880	1.732
25% fraction of PHGPs	1.32	1.116	1.983
50% fraction of PHGPs	1.018	1.095	1.357
75% fraction of PHGPs	0.556	0.86	0.853
100% fraction of PHGPs	0.478	0.592	0.603

Table 1: Absorbance of different fraction of enzyme.

Serial number	*E.coli* Plasmid	PBR^{322} VECTOR	Pet EMBL21
1 spot RF value	0.18	0.18	0.304
2 spot RF value	0.64	0.64	0.54
3 spot RF value	0.79	0.91	0.79
4 spot RF value	0.975	0.96	0.975

Table 2: Different RF value.

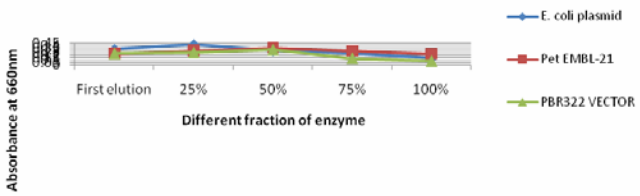

Figure 2: Protein Estimation of different fraction of PHGPs enzyme by Lowry's method.

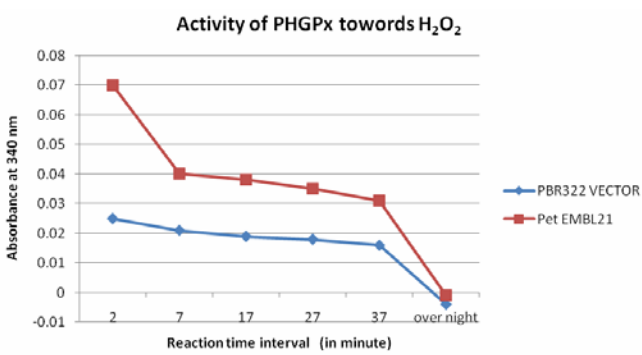

Figure 3: Activity of PHGPx towards H_2O_2 according to time.

Continuous spectrophotometric rate determination: Absorbance and activity of enzyme by Continuous spectrophotometry rate determination at different time interval as shown in Figure 3.

Characterization of enzyme Phospholipid Hydro peroxide Glutathione Peroxidase (PHGPx)

Effect of temperature on intracellular enzyme activity: It is clearly showed that all intracellular enzymes showed maximal activity at 27°C and 62°C, but at 37°C activity of enzyme was slightly decreased and as the temperature was gradually increases the activity decreases due to denaturation of the enzyme. It indicates that the enzyme was most active at an optimum temperature of 62°C (Figure 5).

Effect of temperature on extracellular enzyme activity: It is clearly showed that all intracellular enzymes showed maximal activity at 27°C and 62°C, but at 37°C activity of enzyme was slightly decreased and as the temperature was gradually increases the activity decreases due to denaturation of the enzyme. It indicates that the enzyme was most active at an optimum temperature of 27°C (Figure 6).

Effect of pH on intracellular enzyme activity: It is clearly showed

that all intracellular enzymes showed maximal activity at range of pH 5-8 respectively, but as the pH was gradually increased above pH 8 and decreases below pH 5, the activity decreases due to denaturation of the enzyme. It indicates that the enzyme was most active at an optimum pH of 8 as shown in Figure 7.

Effect of pH on extracellular enzyme activity: It is clearly showed that all extracellular enzymes showed maximal activity at range of pH 5-8 respectively, but as the pH was gradually increased above pH 8 and decreases below pH 5, the activity decreases due to denaturation of the enzyme. It indicates that the enzyme was most active at an optimum pH of 8 Figure 8).

Effect of inhibitor and activator on P^{BR322} and Pet EMBL21 expressed intracellular enzyme activity: It is clearly showed that NaN_3, SDS and $AgNo_3$ showed minimum inhibition on enzyme activity and $HgCl_2$, EDTA and KFeCN completely effect the activity of P^{BR322} expressed intracellular enzyme and in case of Pet EMBL21 expressed intracellular enzyme NaN_3, SDS and $AgNO_3$ showed minimum inhibition on enzyme activity and $HgCl_2$, EDTA and KFeCN completely effect on enzyme activity. So it indicates that NaN_3, SDS and $AgNo_3$ are the good inhibitor (Figure 9-12)

It is clearly showed in Figure 10 that P^{BR322} expressed intracellular enzyme activity was greatly activated in the presence of $FeCl_2$, $Cacl_2$ and $FeSO_4$ and partially activated in the presence of $MnCl_2$, $MnSO_4$ and $MgSO_4$. So it indicates that that $FeCl_2$, $Cacl_2$ and $FeSO_4$ is the good activator.

In case of Pet EMBL21 expressed intracellular enzyme, enzyme

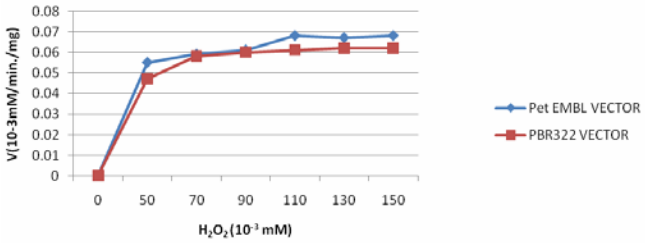

Figure 4: Effect of concentration of H_2O_2 on enzyme PHGPx activity.

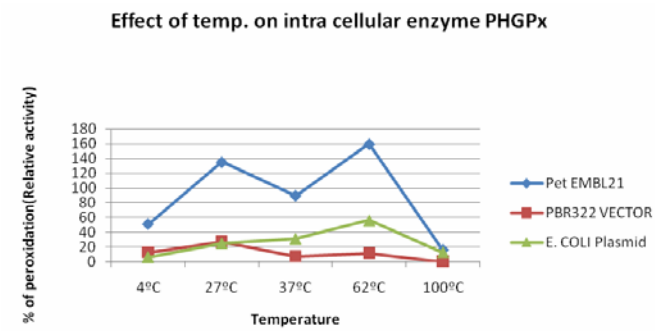

Figure 5: Effect of different temperature on intracellular PHGPx enzyme.

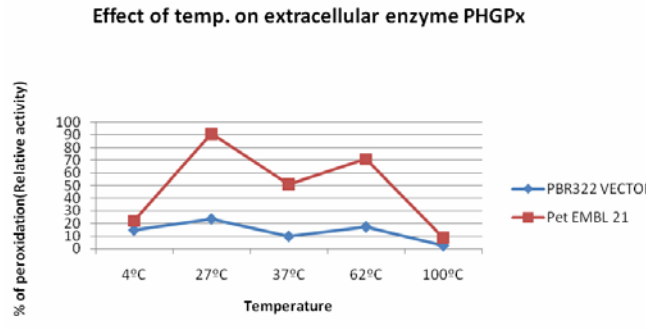

Figure 6: Effect of different temperature on extra cellular PHGPx enzyme.

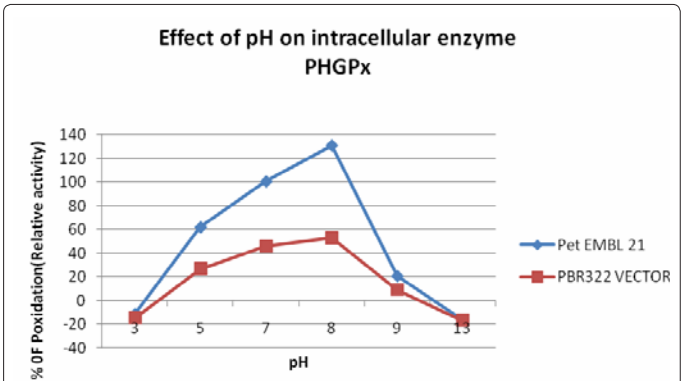

Figure 7: Effect of different pH on intracellular PHGPx enzyme.

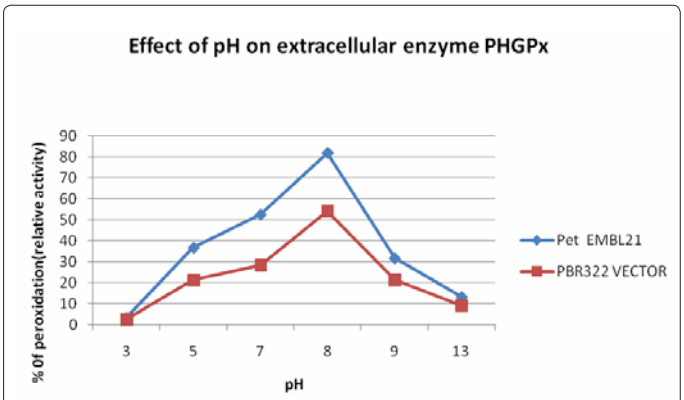

Figure 8: Effect of different pH on extra cellular PHGPx enzyme.

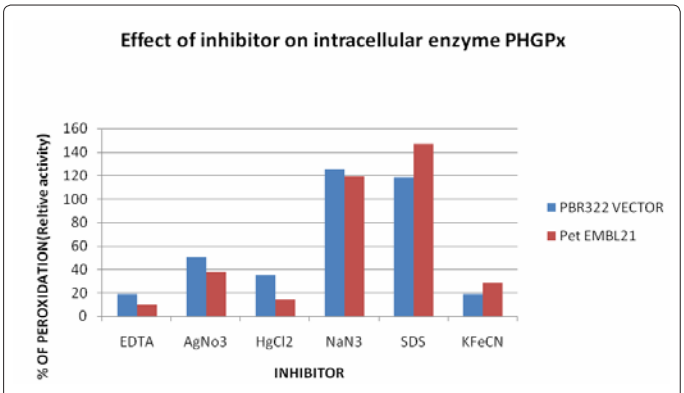

Figure 9: Effect of inhibitor on intra cellular PHGPx enzyme.

activity was greatly activated in the presence of $FeCl_2$, $MnSO_4$ and $MnCl_2$ and partially activated in the presence of $Cacl_2$, $FeSO_4$ and $MgSO_4$. So it indicates that that $FeCl_2$, $MnSO_4$ and $MnCl_2$ is the good activator.

Estimation of molecular weight of recombinant PHGPs protein by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein bands was obtained on SDS PAGE was compared to the standard marker (BSA -66.2 kDa, ova albumin- 45kDa and lysozyme-14 kDa).The molecular weight of PBR32,Pet EMBL21 and *E.coli* plasmid expressed protein was about 21 – 25kDa compared with standrd marker respectively Figure 13.

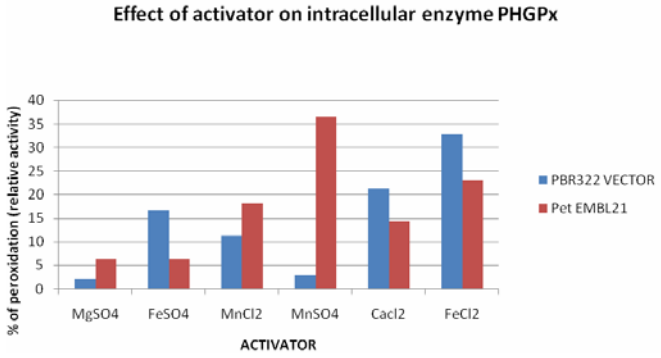

Figure 10: Effect of activator on intra cellular PHGPs enzyme.

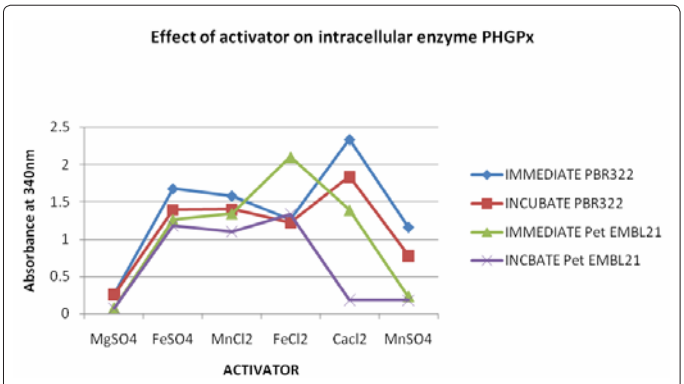

Figure 11: Effect of activator on intra cellular PHGPs enzyme.

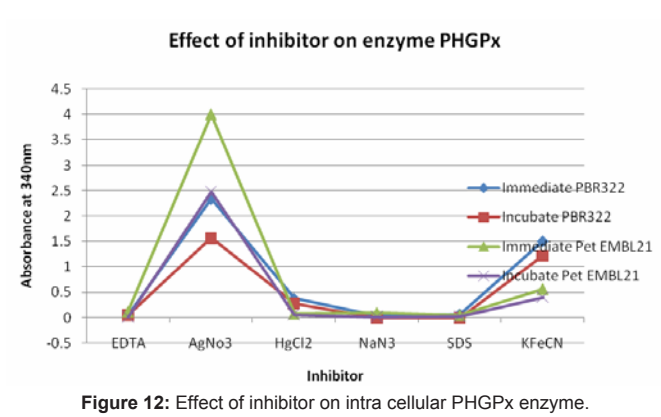

Figure 12: Effect of inhibitor on intra cellular PHGPx enzyme.

Figure 13: Protein band on polyacrylamide gel.

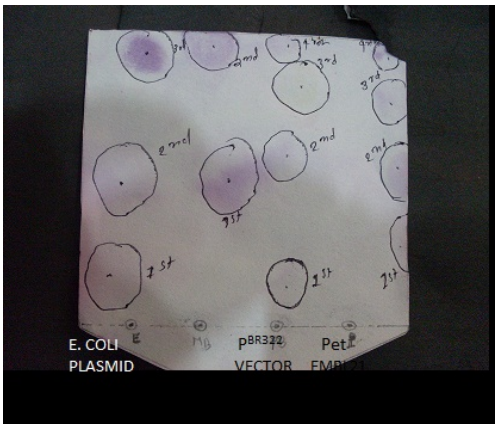

Figure 14: Paper chromatograph.

Paper chromatography of recombinant PHGPs protein

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 H_2O_2 was measured. A linear decrease of A_m (absorbance of NADPH) following the addition of H_2O_2 was observed, whereas no obvious declines were detected when GSH, H_2O_2 or OsPHGPx was omitted, indicating that the peroxidase activity of OsPHGPx was remarkably GSH-dependent Figure 14. When the effects of pH and temperature on GSH-dependent OsPHGPx activity towards H_2O_2 were evaluated, according to [12], the pH optimum was in the range of pH 9.0-10. With a maximum of activity at approximately pH 9.3, but in contrast to our study the optimum pH was observed in the range of pH 7.0-8.0 with a maximum of activity at approximately pH 8. 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, in contrast to present study enzyme stable in the range 27-62°C with maximum activity approximately 27°C that is same result was observed. In the present study indicated that EDTA, $HgCl_2$ and potassium ferrocynate acts as a good inhibitor for PHGPx while $MnSO_4$ and $FeCl_2$ acts as a good activator. 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 [3], while its optimum temperature coincides with that required for plant growth.

Considering the broad specificity for hydroperoxide substrates of PHGPx [9], the hydroperoxide substrate preference for recombinant OsPHGPx was investigated. Peroxidase activity towards different concentrations of H_2O_2 was assayed with a fixed GSH concentration of 15mM. As illustrated in, the plot of GSH-dependent OsPHGPx activity versus H_2O_2 concentration was consistent with normal Michaelis-Menten kinetics. From this plot, the apparent K_m and V_{max} value in case of P^{BR322} expressed enzyme and Pet EMBL21 expressed enzyme for H_2O_2 was 55×10^{-3} mM, 52×10^{-3} mM and 0.068 mM/min/mg, 0.061 mM/min./mg respectively.

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 [2,4]. The results also demonstrated that OsPHGPx was active on H_20_2 indicating a similar broadness of substrate specificity to mammalian PHGPx [9].

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 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 [9,11]. Interestingly, OsPHGPx showed higher catalytic efficiency and V_{max} for H_2O_2, but it displayed a lower affinity to H_2O_2. 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 [6]. 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 [9,11], we suppose that phospholipid hydroperoxides might be the main natural substrates of OsPHGPx.

Conclusion

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 H_2O_2. 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 [7,1]. 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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