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 PACE. The peroxidation activity of PHGPx was inhibited by HgCl\textsubscript{2}, EDTA, Potassium ferrocyanate and was activated by FeCl\textsubscript{3} and MnSO\textsubscript{4}. These properties indicate that the possibilities for use of the PHGPx in the medical, clinical and cosmetic 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 GSHPx, plasma GSHPx, phospholipid hydroperoxide PHGPx 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 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 PHGPx 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 amnonium sulphate precipitation was dissolved in 25mM NaCl; 10MmTris 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\textsubscript{2}O\textsubscript{2} (Hydrogen peroxide) and increase in relative activity and it was measured by continuous spectrophotometric rate determination and substrate concentration dependent assay.

Effect of temperature, pH, inhibitor 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 (PHGPx) 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 PHGPx expressed by E. coli plasmid, pBR322, 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 PHGPx higher in 50% fraction for Pet EMBL21 expressed enzyme, 50% fraction for PBR322 expressed enzyme and 25% fraction for E. coli plasmid expressed enzyme respectively as shown in Figure 2 and Table 1.

### 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

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**Table 1:** Absorbance of different fraction of enzyme.

<table>
<thead>
<tr>
<th>Phospholipids Hydro peroxide Glutathione Peroxidase (PHGPx) in different fraction of buffer</th>
<th>Absorbance at 280 nm</th>
<th>Absorbance at 280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pBR322</td>
<td>PEMBL21</td>
</tr>
<tr>
<td>Crude</td>
<td>2.371</td>
<td>2.211</td>
</tr>
<tr>
<td>0% fraction of PHGPx (first elution)</td>
<td>1.228</td>
<td>0.880</td>
</tr>
<tr>
<td>25% fraction of PHGPx</td>
<td>1.32</td>
<td>1.116</td>
</tr>
<tr>
<td>50% fraction of PHGPx</td>
<td>1.018</td>
<td>1.095</td>
</tr>
<tr>
<td>75% fraction of PHGPx</td>
<td>0.556</td>
<td>0.86</td>
</tr>
<tr>
<td>100% fraction of PHGPx</td>
<td>0.478</td>
<td>0.592</td>
</tr>
</tbody>
</table>

**Table 2:** Different RF value.

<table>
<thead>
<tr>
<th>Serial number</th>
<th>E.coli Plasmid</th>
<th>PBR322 VECTOR</th>
<th>Pet EMBL21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 spot RF value</td>
<td>0.18</td>
<td>0.18</td>
<td>0.304</td>
</tr>
<tr>
<td>2 spot RF value</td>
<td>0.64</td>
<td>0.64</td>
<td>0.54</td>
</tr>
<tr>
<td>3 spot RF value</td>
<td>0.79</td>
<td>0.91</td>
<td>0.79</td>
</tr>
<tr>
<td>4 spot RF value</td>
<td>0.975</td>
<td>0.96</td>
<td>0.975</td>
</tr>
</tbody>
</table>
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 PBR322 and Pet EMBL21 expressed intracellular enzyme activity: It is clearly showed that NaN₃, SDS and AgNO₃ showed minimum inhibition on enzyme activity and HgCl₂, EDTA and KFeCN completely effect the activity of PBR322 expressed intracellular enzyme and in case of Pet EMBL21 expressed intracellular enzyme NaN₃, SDS and AgNO₃ showed minimum inhibition on enzyme activity and HgCl₂, EDTA and KFeCN completely effect on enzyme activity. So it indicates that NaN₃, SDS and AgNO₃ are the good inhibitor (Figure 9-12).

It is clearly showed in Figure 10 that PBR322 expressed intracellular enzyme activity was greatly activated in the presence of FeCl₃, CaCl₂ and FeSO₄ and partially activated in the presence of MnCl₂, MnSO₄ and MgSO₄. So it indicates that that FeCl₃, CaCl₂ and FeSO₄ is the good activator.

In case of Pet EMBL21 expressed intracellular enzyme, enzyme activity was greatly activated in the presence of FeCl₃, CaCl₂ and FeSO₄ and partially activated in the presence of MnCl₂, MnSO₄ and MgSO₄. So it indicates that that FeCl₃, CaCl₂ and FeSO₄ is the good activator.
activity was greatly activated in the presence of FeCl₂, MnSO₄, and MnCl₂ and partially activated in the presence of CaCl₂, FeSO₄, and MgSO₄. So it indicates that that FeCl₂, MnSO₄ and MnCl₂ 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 standard marker respectively Figure 13.

Paper chromatography of recombinant PHGPs protein

The activity of OsPHGPs 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 H₂O₂ was measured. A linear decrease of $A_{340}$ (absorbance of NADPH) following the addition of H₂O₂ was observed, whereas no obvious declines were detected when GSH, H₂O₂ 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 H₂O₂ 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, HgCl₂ and potassium ferrocyanate acts as a good inhibitor for PHGPx while MnSO₄ and FeCl₂ 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 

\[ \text{H}_2\text{O}_2 \] was assayed with a fixed GSH concentration of 15mM. As illustrated in [2], the apparent \( K_m \) and \( V_{\text{max}} \) value for PHGPx activity were 55 × 10^{-3} M, 52 × 10^{-3} M and 0.068 mM/min/mg, 0.061 mM/min/mg, respectively.

The \( V_{\text{max}} \) 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_2O_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 PHGPxs 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 

\[ \text{H}_2\text{O}_2 \] using GSH as the electron donor. It is reasonable to believe that if the enzyme in plant cells.

\section*{References}