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# PEROXIDASE ACTIVITY IN LEAVES OF PLANE TREE AS A MARKER OF AIR POLLUTION IN RASHT

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## ABSTRACT

Peroxidase (POD) induction is a general response of higher plants to the uptake of toxic amounts of heavy metals, low temperature, salt stress, wounding, pathogens, UV radiation and poisonous gases. Screening of plane trees (*Plantanus Orientalis*) located at a highly polluted urban area of Rasht demonstrated considerable peroxidase activity compared to the activity of enzyme in the leaf extracts of the same plant located in clean air area of the same city. Using the leaves of the tree as a source, the peroxidase content was isolated, partly purified and characterized. The specific activity of peroxidase was increased at various stages of purification process. However, in all cases it was found that the leaves of plane tree located at highly polluted urban area exhibited higher peroxidase activity (total and specific). It was suggested that variation in peroxidase activity could, therefore, be used as a marker to show the plant response to various environmental pollutions. The novel peroxidase extracted from polluted plane leaves showed characteristics similar to horseraddish peroxidase, a commercially availabe enzyme used extensively as antioxidant in food and cosmetic industry as well as in analytical techniques. Optimum pH and temperature of the novel peroxidase was measured and its substrate specificity obtained and compared to other peroxidases. It was, therefore, suggested that in addition to being a marker for air pollution, peroxidase extracted from highly polluted plants could also be used as an alternative to the commercially available peroxidase.

**Key Words :** Plane tree (*Plantanus Orientalis*), peroxidase, metal stress, air pollution

## 1. INTRODUCTION

Many industrial and natural environmental stresses can affect plants in a variety of ways. Emissions of motor vehicles, for example, are a very major source of heavy metals and toxic gases [Lagerwerff JV, Spetch AW.]. It is known that heavy metals originated from industrial and agricultural activities, can also be found in soils under natural conditions [Castellio FJ,]. Peroxidase (POD) induction is a general response of higher plants to the uptake of toxic amounts of heavy metals [Reddy GN, Prasad MNV.and, Shaw BP.], low temperature, salt stress, wounding, pathogens, UV radiation and poisonous gases [Ashraf MY et al. and Scalet M et al.]. Some trees such as: arborvitae, boxelder, douglas-fir, English oak, magnolia, read oak, white dogwood and white spruce are relatively tolerant to common air pollutants, while many trees including American elm, catalpa, jack pine, larch, ponderosa pine,

quaking aspen, Virginia pine, white pine and willow are relatively intolerant to common air pollutants.

Peroxidases (PODs, E.C. 1.11.1.7) are haemoproteins that are widely distributed in the plant kingdom [Lin Z et al.]. They can catalyze the oxidation of a wide variety of substances through a reaction with hydrogen peroxide [Sakuraka J et al. and Cilento G, Adam W]. Peroxoidases are considered to protect cell membrane against the active oxidants and enable plants to be resistant to stress factors [Edreva Asalcheva Ggeorgieva D.and , Everse J et al.].

The effect of heavy metals such as cadmium and lead on the peroxidase activity of *pinus pinea* [Baycu G et al.], water hyacinth [Meksongsee L et al.], and Pinto bean leaves [Peters J.L. et al.] and some other plants [Van Assche F and Clijters H.] have been studied. Many studies have shown that peroxidase increases due to increased levels of heavy metals both in soil and environment.

Plane tree is commonly grown on the sides of many roads and streets in order to provide pleasant atmosphere and image to the surrounding environment. There are few literatures available on the effects of environmental pollutants on the peroxidase activity in leaves of this tree.

## 2. MATERIALS AND METHODS

### 2.1 Material

Fresh and healthy leaves samples were selected from plane trees located at two environmentally different parts of the city.
Horseradish peroxidase, 1-phenyl-2,3-dimethyl-4-amino pyrazolan (AAP), folin-ciocateus, acrylamide, N,N-methylene-bis-acrylamide, hydrogen peroxide, 2-mercaptoethanol, ammonium persulphate, bromophenol blue, guaiacol, ascorbic acid were purchased from Merck Chemical Company, glycine and pyrogallol from BDH, tyrosine from Sigma and protein molecular weight marker from MBI.

### 2.2 Selection of leaf samples

The study was preformed during August (~ 26°C) when the leaves were fresh and undamaged by hot or cold weather. Fresh, young and healthy leaves of plane trees were picked and delivered to the laboratory within three to four hours. Rasht is a comparatively large city with congested population and many factories located near the city. The road traffic is extremely high in some parts of the city; however, there are some streets far from heavy traffic jams with nice clean and fresh air.

### 2.3 Preparation of plane leaf extract

3 g leaves of plane trees approximately 5 m in height were homogenized in liquid nitrogen. A 10 ml solution of 0.10 M phosphate buffer (pH 7.0) containing 0.1mM EDTA (to prevent possible reaction of proteases), 1 mM mercaptoethanol (to inhibit peroxidase oxidation) and 0.1 M NaCl was then added. The resulting solution was centrifuged at 7500 rpm at 5°C for 40 minutes. The total protein content was

measured in the supernatant using Lawry protein assay. The activity of peroxidase was measured in the presence of 1-phenyl-2,3-dimethyl-4-amino pyrazolan and $H_2O_2$.

**2.4 Enzyme assay**

Peroxidase activity was determined spectrophotometrically using a method similar to [Sakharov I Yu, Bautista G.]. 50 μl of enzyme solution was added to 475 μl of 0.2 M phosphate buffer containing both substrates (2.5 mM 1-phenyl-2,3-dimethyl-4-amino pyrazolan and 1.7 mM hydrogen peroxide) and the absorbance change at 510 nm was measured at 25°C. One unit of activity (U) is the amount of peroxidase oxidating 1 μmole of substrate per minute. Specific activity is units of activity per mg of protein.

**2.5 Enzyme purification**

The leaves (350 g) from plane trees were milled and homogenized in 1 liter solution of 0.10 M phosphate buffer (pH 7.0) containing 0.1 mM EDTA and the homogenate was incubated for 1 h at ambient temperature. Tissue derbis was removed by filtration and centrifugation, and the extract fractionated by adding ammonium sulphate, $(NH_4)_2SO_4$ to 85% at 4°C and controlled pH. The sample was mixed for another 30 minutes followed by centrifugation at 5000 rpm at 5°C for 30 minutes. 2 ml of 0.2 M phosphate buffer was then added to the precipitate and mixed well. The mixture was centrifuged again under the same conditions for 10 minutes. 2 ml of 10 mM phosphate buffer was added to the resulting precipitate and the solution was dialyzed 3 times against 500 ml of 10 mM phosphate buffer (pH 7.0) using dialysis bags with pores less than 12000 daltons. The resulting peroxidase solution was applied to a DEAE-Sepharose column (1 × 50 cm) equilibrated with the same buffer. The elution was carried out with 10 mM tris-HCl buffer (pH 8.3) at a flow rate of 25 ml/h. Peroxidase passed through the column, but colored compounds of the leaf extract were absorbed. The fraction containing active peroxidase was stored at 5°C.

**2.6 Thermal stability**

Thermal stability of a $4 \times 10^5$ M of purified peroxidase was measured at temperatures of 10-90 °C. The enzyme was incubated at the desired temperature for 15 minutes and the change in its absorbance was recorded as discussed in section 2.4.

**2.7 Optimum pH**

2.5 mM 1-phenyl-2,3-dimethyl-4-amino pyrazolan was used as electron donor and 1.7 mM hydrogen peroxide as electron acceptor and using the following buffers different pH ranges were made: glycine/HCl (pH 3.0), sodium acetate (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0), tris/HCl (pH 8.0) and glycine/NaOH (pH 9.0).

**2.8 Analytical methods**

The purity and molecular weight of the extracted enzyme was determined by sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE). Electrophoresis was performed under denaturing conditions [Electrophoretic Theory Catalogue] and the gels were stained using Colloidal Brilliant Blue-G (B.B.G). The protein content

of each solution was measured by Lawry method using our purified peroxidase as standard.

**2.8 Substrate specificity of purified peroxidase**

The substrate specificity of plane tree peroxidase was examined using known peroxidase substrates. The change in substrate absorbance due to the peroxidase action was monitored. The wavelength used depends on the type and structure of the substrate, i.e. 290 nm for ascorbic acid, 470 nm for guaiocol and 1-phenyl-2,3-dimethyl-4-amino pyrazolan, 420 nm for pyrogalol and 260 nm for tyrosine.

## 3.RESULTS AND DISCUSSIONS

Screening of plane trees planted in two environmentally different area of Rasht showed higher peroxidase activity in the leaves of polluted trees. Table 1 shows changes in specific activity of the enzyme extracted from leaves of plane tree collected in a highly polluted area during various stages of purification. Using the step by step purification processes in this study, plane tree peroxidase was specifically isolated with a purification fold of 39. A similar procedure was used for purification of peroxidase from leaves of the same tree located in an exceptionally air clean area. In this case also and the success in purification was the same. Table 2 compares total protein and peroxidase activity in leaves of plane trees grown in two environmentally different roads measured directly after homogenation.

The results also showed that the decrease in peroxidase activity is proportional to the increase in air pollution especially in terms of heavy metal concentration (mostly lead and cadmium) in the leaf extracts (the data not shown here, to be published later). Sakharov et al [Sakharov I Yu et al.] have shown that peroxidase activity in leaf extracts of royal palm tree does not depend on the age and height of the plant or on the time of the year. We, therefore, assumed that peroxidase measured in August did not change considerably during other seasons. A comparison between total and specific activity of peroxidase in leaf extracts of polluted and not polluted plant shows that peroxidase assay may be used as a quick test to show the level of pollution in urban area. The total peroxidase activity is about 1.5 times in plants living in polluted area when compared to the leaves of the same plant living in an environmentally clean area.

Table 2. Purification of peroxidase from plane tree leaves grown in a highly air polluted area.

| Procedure | Volume (ml) | Protein (mg) | Specific activity (U/mg protein) | Total activity (U) | Yield (%) | Purification fold |
|---|---|---|---|---|---|---|
| Homogenate | 5.32 | 4.8 | 73.3 | 351.6 | 100 | 1 |
| $(NH_4)_2SO_4$ | 1.35 | 0.312 | 586.4 | 183 | 52 | 8 |
| DEAE-sepharose | 0.081 | 0.072 | 2053 | 156 | 44 | 28 |
| Sephacryl S100 | 0.012 | 0.192 | 2871 | 55.1 | 15.5 | 39 |

Table 2. Peroxidase difference in homogenates of leaf extracts form plane trees located at different environmental conditions.

| Tree location | Homogenate volume (ml) | Total protein (mg) | Specific activity (U/mg protein) | Total activity (U) |
|---|---|---|---|---|
| Light traffic road | 5.70 | 4.36 | 61.9 | 270 |
| Highly busy road | 5.320 | 4.80 | 73.3 | 351.6 |

Extraction of peroxidase from leaves of plane tree was carried out using 0.10 M phosphate buffer (pH 7.0) containing 0.1mM EDTA. Exclusion of EDTA from the extracting buffer did not change the yield at the extraction step, suggesting that most of peroxidase in plane tree leaves is not bound to the cell walls but is present in a soluble form. This was also assumed for peroxidase from royal palm tree leaves [Sakharov I Yu et al.].

Addition of $(NH_4)_2SO_4$ in the second step of purification increased the specific activity of the enzyme to about 8-fold and removed some colored substances and wax from the extract, as the solution was almost clear and lighter in color after this stage. The next step in the presence of DEAE-sepharose, followed by elution of the chromatographic column with elution with 10 mM tris-HCl buffer caused peroxidase passage through the column, while colored compounds in the plane leaf extract were absorbed. The resulting solution was, therefore, clear and colorless. The final step of purification was a gel filtration process on Sephacryl S100. The purified plane tree peroxidase showed a specific activity of 2871 U/mg of protein in the case of highly polluted leaves. Lawry protein assay was performed to measure the protein concentration using purified enzyme instead of bovine serum albumin as a standard. A yield of 15.5% after 39 fold purification is a fairly high yield compared to the values obtained for other peroxidases [Sakharov I Yu et al.], suggesting that tree leaf wastes from highly polluted area are good sources for peroxidase extraction and its possible use in industrial applications.

The UV-Visible spectrum of the novel peroxidase extracted from leaves of highly polluted plane trees showed the typical Soret maximum at 403 nm observed for all plant peroxidases [Smulevich G] The purity index (RZ value) was 2.76 calculated from the product of the absorbance at 403 and 280 nm as follows

$$RZ = A_{403}/A_{280} = 2.76$$

The molecular weight of plane tree peroxidase was 55.6 kDa estimated from its single band in SDS-electrophoresis. This molecular weight value is lower than that reported for peroxidase extracted from leaves of African oil palm tree (57 kDa) [Sakharov I Yu et al.], but higher than peroxidase from royal palm tree [Electrophoretic Theory Catalogue] and peroxidases from other sources [Gazaryan I.G.]. The difference in molecular weight of various peroxidases is probably due to different degree of glycosylation of the enzyme.

Since the optimal conditions for catalysis by different peroxidases are not identical the optimum pH and temperature for plane tree peroxidase was obtained prior to measuring the substrate specificity (Figures 3 and 4 respectively). Using some known peroxidase substrates, i.e. ascorbic acid, guaiocol, 1-phenyl-2,3-dimethyl-4-amino pyrazolan (4-AAP) and tyrosine the substrate specificity of our peroxidase was examined under optimum pH and temperature (Table III). It was shown that for all substrates the pH optima occurred between 6.0-6.2. We found that the plane tree peroxidase is relatively unstable to changes in pH. The enzyme stability remained almost unchanged at pH 6.0-7.0 as shown in Figure 1. Measuring thermal stability of plane peroxidase, we showed that a wide range of temperature is optimal of the activity of this peroxidase (20-40°C). Increasing temperature beyond 40°C caused a steady decrease in the % activity (Figure 2) up to 60°C when plane tree peroxidase totally was unreactive. This kind of thermal stability has also been observed for peroxidase from leaves of *lipomoea palmetto* [Srinivas N.D et al.] and soybean peroxidase [R Sariri et al.].

Table 3. The activity of plane tree peroxidase on various substrates compared to horseradish peroxidase.

| Substrate | Activity of peroxidase (µmol/min/mg) | |
|---|---|---|
| | Plane tree | Horseradish |
| 4-AAP | 157 | 248 |
| Guaiacol | 103 | 184 |
| Ascorbic acid | 95 | 172 |
| Pyrogallol | 73 | 97 |
| Tyrosine | 3 | 49 |

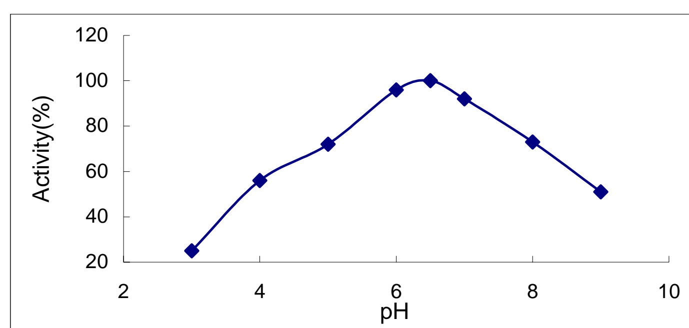


Figure 1. Dependence of plane tree peroxidase activity on pH.

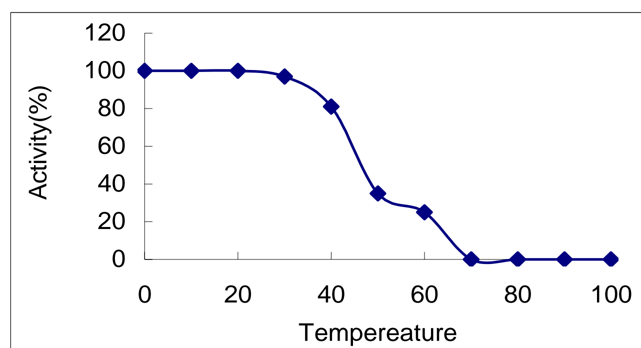


Figure 2. The effect of temperature of the activity of plane tree peroxidase.

## 4. CONCLUSIONS

1. Peroxidase activity in the leaves of polluted plane trees is higher than the activity of the enzyme extracted from the same tree located in a clean air area.
2. Decrease in peroxidase activity is proportional to the increase in air pollution.
3. The optimum pH of plane tree peroxidase is 60-6.2. Thermal stability of plane peroxidase is constant at 20-40°C and increasing temperature, slowly decreases the activity. The enzyme is totally inactive at 60°C.
4. The yield of purified peroxidase was15.5% after 39 folds purification. The relatively high yield suggests that leaf wastes from plane tree located at highly polluted environment are good sources of the enzyme.
5. The change in peroxidase activity in leaves of plants present in urban area could be used for monitoring the degree of air pollution.
6. The highest activity of plane tree peroxidase is exhibited when 1-phenyl-2,3-dimethyl-4-amino pyrazolan (4-AAP) and guaicol are used as its substrates.

## 5. ACKNOWLEDGEMENTS

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