

EVALUATION OF PEROXIDASES FROM VARIOUS PLANT SOURCES

Ila Bania and Rita Mahanta

Department of Zoology, Cotton College,
Guwahati (Assam), INDIA

Abstract- Wide applications of peroxidase in different areas of clinical biochemistry, biotechnology, food industry etc. enhances the interest for further study on the enzyme. The distribution of peroxidase activity and the kinetic parameters of three plant species [*Nicotiana tabacum*, *Brassica oleraceae capitata var. albaL.* and *Raphanus sativus* (mooli in India, winter varieties)] were evaluated. To describe the rate of hydrogen peroxide decomposition for all the three plant peroxidases a first order kinetic expression with respect to both the substrate and the enzyme was used. Based on their linear regression analysis (Lineweaver-Burk plot) over the substrate concentration range of $0.05 \times 10^{-3}\text{M}$ to $0.5 \times 10^{-3}\text{M}$ the K_m and V_{max} of the three plant peroxidases (for H_2O_2 in presence of O-dianisidine) were: 0.250 mM and 9.09 uM min^{-1} (tobacco peroxidase), 0.370 mM and $11.11 \text{ uM min}^{-1}$ (cabbage peroxidase) and 0.277 mM and $10.02 \text{ uM min}^{-1}$ (radish peroxidase) respectively. Kinetic studies revealed that Tobacco peroxidase (source- *N. tabacum*) had lower apparent K_m values and it has more peroxidases than the other two plant peroxidases examined. The highest specific activity was recorded for cabbage peroxidases [in *B. oleraceae capitata var. albaL.* (1.6771 ± 0.12)]. Optimum pH and temperature for all the three plant peroxidases were also determined. *B. oleraceae capitata var. albaL.* had shown high thermal stability. These peroxidases may prove as a highly promising enzyme for practical application in biotechnological field.

Index Terms- Peroxidases, Cabbage, K_m , pH, Thermo stability

I. INTRODUCTION

Peroxidase (E.C. 1.11.1.7) are widely distributed in nature and are found in plants, micro-organisms and animals, where they catalyze the reduction of hydrogen peroxide (H_2O_2) to water, rendering it harmless. H_2O_2 is a common end product of oxidative metabolism, and being a strong oxidizing agent, could prove toxic if allowed to accumulate. Thus, peroxidases serve to rid plant cells of excess H_2O_2 under normal and stress conditions (Laloue *et al.*, 1997). Peroxidases are found mainly as haemoproteins and use hydrogen peroxide as the oxidizing substrate, although other more unusual peroxidases have been shown recently to contain either metal ions, such as selenium and vanadium, or a flavin prosthetic group. From the reviews of literature it was found that variety of electron donors were used, including aromatic amines, phenols and enediols like ascorbic

acid to detect peroxidase activity in plant extract. In this experiment a dye like o-dianisidine was used as electron donor to detect peroxidase because it gives stable colored oxidized product and shows high sensitivity for the reaction. On the basis of sequence similarity peroxidases were categorized into two super families: the mammalian peroxidase superfamily and the plant peroxidase superfamily (Dunford 1999, Welinder 1992). But di-heme cytochrome C peroxidase (Fulop *et al.*, 1995) or chloroperoxidase (Sundaramoorthy *et al.*, 1995) was excluded from these two superfamilies. They are typically named after either their sources, e.g. Horseradish peroxidase (HRP) and Soybean peroxidase (SBP) or their substrates, such as Lignine peroxidase (LiP) and cytochrome C peroxidase (CcP). Present study was done on three plant peroxidases namely- cabbage peroxidase, radish peroxidase and tobacco peroxidase (named after their sources).

Peroxidases are versatile biocatalyst with an ever increasing number of applications (Colonn *et al.*, 1999, May 1999, Veitch and Smith 2001). Roots of horseradish serves at present as the major source of commercially available peroxidase, however, the researchers still investigate for new peroxidases of elevated stability and properties suitable for different biotechnological, biomedical and other application, eg. spring cabbage peroxidase was suggested as a potential tool in biocatalysis and bioelectrocatalysis (Anna *et al.*, 2007). Naturally thermostable peroxidase have been described and include Soybean peroxidase (Mc Eldoon and Dordick, 1996), peroxidase from leaves of royal palm tree (Sakharov *et al.*, 2001), spring cabbage peroxidase (Anna *et al.*, 2007). Chitinase and peroxidase which were isolated from soybean were considered to be involved in defense of plant against pathogens (Staehelin *et al.*, 1992). Being the most heat resistant enzyme, it is used in food industry as an index of blanching procedures (Reed, 1975). There is an empirical relationship between residual peroxidase activity and the development of flavors and odours in food (Burnette, 1977). Davis (1942) reported that the greatest peroxidase activity was in the inner seed coat in citrus fruits. With advances in spectral techniques and protein purification methods, Chance (1951) showed the existence of the peroxidase-hydrogen peroxide complexes, compound I and compound II. These are important intermediates formed between peroxidase and the oxidant in peroxidase-catalyzed reactions (Chance, 1952). Peroxidases have been identified on the root surface of many plant species (Mueller and Beckman, 1978; Smith and O'Brien, 1979; Zaar, 1979; Albert *et al.*, 1986). Peroxidases have got several biomedical applications too, eg. HRP is used in a large variety of

analytical and diagnostic systems which are very important in the clinical laboratory. There have been numerous reports on peroxidase activity in plants. Peroxidase activity has been shown in several species of plant [Padmarajaiah *et al.*, 2009]. Thus, there is a continual search for novel peroxidases for various applications. Although relevant reviews on peroxidase activity in plants are available broad effort for their characterization are very limited. The main objective of this study was to compare the peroxidase activity and kinetic parameters of three widely available angiospermic plant species [(*Nicotiana tabaccum*, *Brassica oleraceae capitata var. alba*L. and *Raphanus sativus* (mooli in India, winter varieties)]. As these enzymes are poorly studied compared to widely used, but less thermostable, horseradish peroxidase and thus relatively little is known about the kinetic properties of these plant peroxidases. So, this study seeks to find novel plant peroxidases these three different plant sources. We had compared their kinetic properties (K_m and V_{max}) and their specific enzyme activities. As these plants are abundant (easily cultivable) so with the advance in purification method they may prove as very cheap enzymatic source.

II. MATERIAL AND METHOD

An ultraviolet-visible spectrophotometer with 1 cm. quartz cell was used for all measurement. A water bath shaker was used to maintain a constant temperature for color development. All the pH measurement and adjustments were done with digital pH meter.

Reagents:

All chemical used in the assay were analytical grade obtained from commercial source. H_2O_2 (30%), BSA and o-dianisidine was purchased from E. Merk Ltd. (Mumbai, India). Double distilled water was used throughout the experiment. A (20mM) H_2O_2 stock solution was prepared daily and standardized by potassium permanganate method. Working standard solutions were prepared from the stock solution by dilution.

Plant Material Collection and Preparation of Extract:

As a source of peroxidase leaf portion (cabbage head) of *B. oleraceae* and *N. tabaccum* and root of *R. sativus* were collected from local agricultural field [Kamrup district, Assam (North East India)] and carried at 4°C to the laboratory and stored at -20°C until used. Samples (2g) were washed with distilled water and homogenized in a blender using 10ml of 0.1M phosphate buffer of pH 6.0. The extract was passed through cheesecloth, centrifuged at 12000g for 30 minutes and the supernatant was labeled as crude extract. The extract was heated at 65 °C for three minutes to inactivate any catalase present in extract (Rehman *et al.* 1999). Different dilutions of the crude enzyme were examined for peroxidase activity assay.

III. PROTEIN ESTIMATION

Total protein concentration was determined in triplicate by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Assay of Peroxidase (POX) Activity:

Assay of peroxidase was carried out according to the method of Malik and Sing with certain modifications. To 2ml of phosphate buffer (pH 6.0/ pH 7.0) 100ul of plant extract and 1ml of o-dianisidine solution were added. The reaction was initiated by adding 100ul of $0.2 \times 10^{-3}M$ H_2O_2 and the absorbance was read at (460 nm) every 30 second interval up to 5 minutes. The peroxidase activity was calculated using extinction co-efficient of o-dianisidine and the enzyme activity was expressed as unit per mg of protein.

IV. EFFECT OF PH AND TEMPERATURE ON ENZYME ACTIVITY

The effect of temperature on peroxidase activity was determined by incubating the reaction mixture at different temperatures (in hot water bath) including 10, 20, 30, 40, 50, 60, and 70°C for 5 minutes. The pH optima of the three peroxidases were determined using acetate buffer (pH 4.0-5.0) and potassium phosphate buffer (pH 6.0-8.0) in the range from 0.4-0.7. All the peroxidase activities were assayed under standard conditions (Sakharov *et al.* 2002; Koksall and Gulcin 2008 a, b; Singh *et al.* 2010) and percent relative activities were calculated. Percent relative activity is described in terms of the change of absorbance per minute or per second amount of sample added to the extract or to the mixture.

$$[100 \times \text{Sample (Treated) OD}]$$

$$\% \text{ relative activity} = \frac{\text{[100 X Sample (Treated) OD]}}{\text{[Control (Untreated) OD]}}$$

Thermo stability of these enzymes was determined by incubating them for 30 minutes, one day and ten days at specific temperatures (of 20, 30, 40, 50 and 60 °C).

V. KINETIC STUDIES

Enzyme kinetic studies were performed in ten samples, employing a range of substrate concentration ($0.05 \times 10^{-3}M$ to $0.5 \times 10^{-3}M$) with constant enzyme level in a final volume of 3 ml. All reactions were carried out at a fix (optimum) pH and temperature i.e. pH 6.0 and 50°C for cabbage peroxidase, pH 6.0 and 40°C for radish peroxidase and pH 7.0 and 40 °C for tobacco peroxidase respectively. Controls in which distilled water replaced the enzyme for each substrate concentration were run in parallel and marked as blank. All the reaction mixtures were monitored at a wavelength of 460 nm (showed highest absorbance). The apparent K_m and V_{max} were determined from the Lineweaver-Burk plot $1/V$ versus $1/S$ (Lineweaver and Burk 1934) by following the optimum pH and temperature conditions.

VI. STATISTICAL ANALYSIS

For all the experiments three plant samples were analyzed and all the assays were carried out ten times. The results were expressed as mean \pm standard deviation.

VII. RESULT AND DISCUSSION

One important factor in the preparation of peroxidase from the plant is the tissue to buffer (t/b) ratio. In this study (t/b) ratio from 1:2 to 1:151 g ml⁻¹ were examined and a suitable result was obtained at 1:10 g ml⁻¹ ratio. Common feature of all the three plant peroxidases were their optimum pH and temperature (in the range of 6.0 to 7.0 and 40-50°C respectively). The optimum pH of cabbage peroxidase and radish s found to be 6.0 but for the tobacco peroxidase the optimum pH was found to be 7.0 (fig: 1).

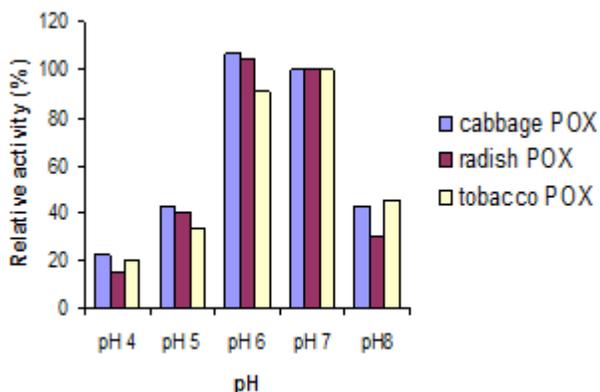


Fig 1: Effect of pH on peroxidase (POX) activities of the three plant peroxidases.

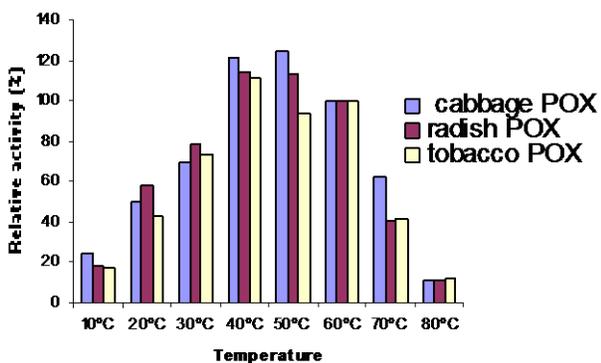


Fig 2: Effect of temperature on peroxidase (POX) activities of the three plant peroxidases.

The optimum temperature for cabbage peroxidase was found to be 50 °C, though its activity remains almost constant in the temperature range of 40 °C to 50 °C (Fig: 2). It showed remarkable decreases in its activity at the temperature range of 70-80 °C. Cabbage peroxidase had also shown highest thermostability on incubation at different temperature (20 °C to 60 °C) for thirty minutes, one day and ten days.

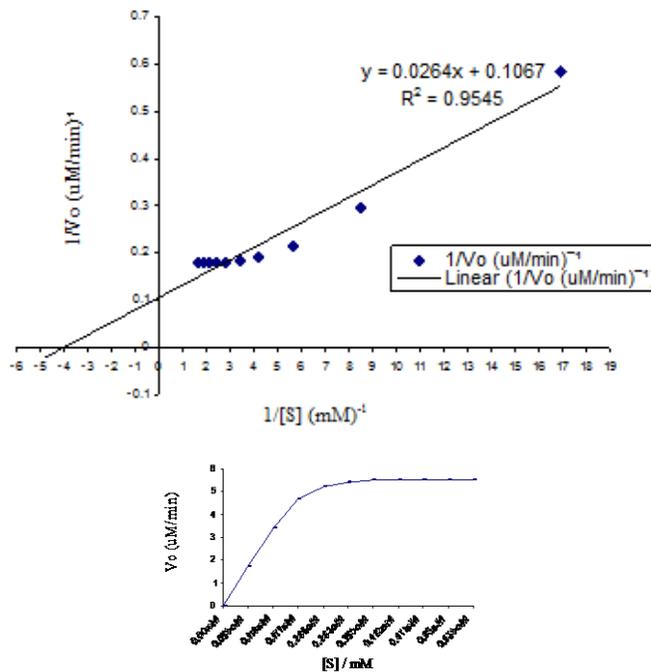


Fig: 3. Lineweaver-Burk Plot for Tobacco peroxidase (*N. tabacum*) where [S] represents substrate concentration of H₂O₂ (0.05 x 10⁻³M to 0.5 x 10⁻³M) and V_o represents initial reaction velocity of tobacco peroxidase. Inset: Michaelis-Menten plot for Tobacco peroxidase.

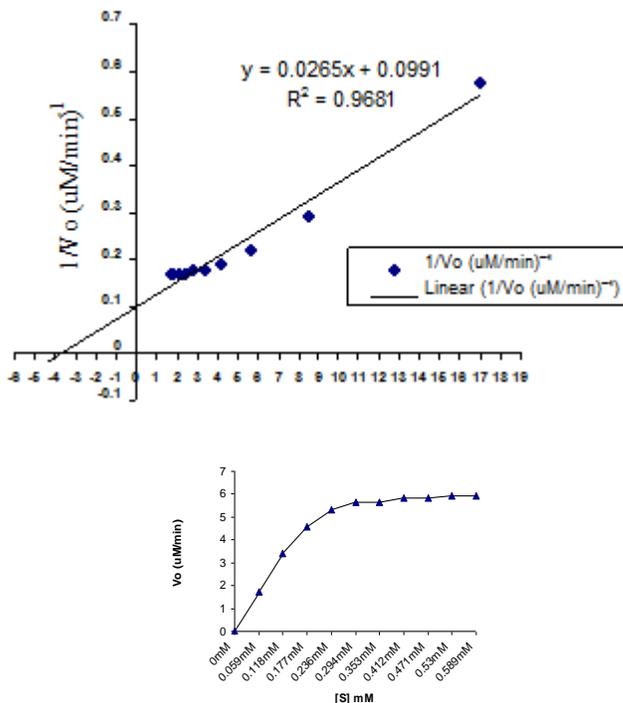


Fig: 4. Lineweaver-Burk Plot for Radish peroxidase (*R. sativus*) where [S] represents substrate concentration of H₂O₂ (0.05 x 10⁻³M to 0.5 x 10⁻³M) and V_o represents initial reaction velocity of Radish peroxidase. Inset: Michaelis-Menten plot for Radish Peroxidase.

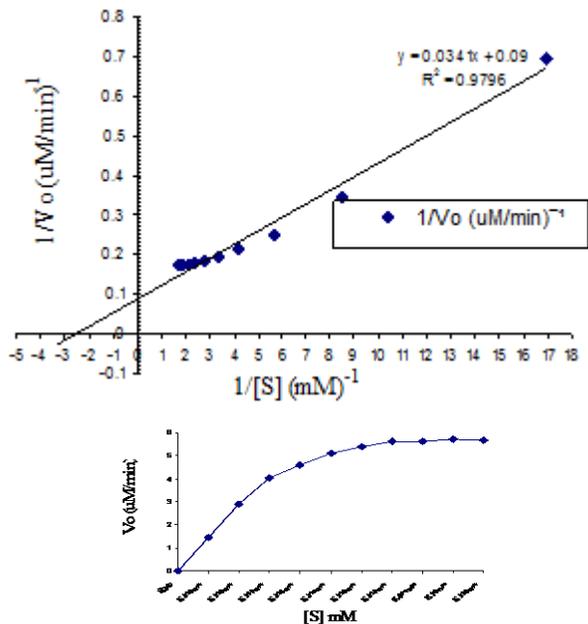


Fig 5: Lineweaver-Burk Plot for Cabbage peroxidase (*Brassica oleraceae capitata var. albaL.*) where [S] represents substrate concentration of H₂O₂ (0.05 x 10⁻³M to 0.5 x 10⁻³M) and V_o represents initial reaction velocity of Cabbage peroxidase. Inset: Michaelis-Menten plot for Cabbage peroxidase.

Table I: Determination of kinetic constant and peroxidase (POX) activity in plant extract.

| Source | K _m (mM) | V _{max} (uM min ⁻¹) | Peroxidase activity (Unit/ mg) |
|--|---------------------|--|--------------------------------|
| <i>Nicotiana tabaccum</i> | 0.250 | 9.095 | 0.9862±0.016 |
| <i>Brassica oleraceae capitata var. albaL.</i> | 0.370 | 11.111 | 1.6771±0.12 |
| <i>Raphanus sativus</i> (mooli in India, winter varieties) | 0.277 | 10.012 | 0.8761±0.017 |

All the three peroxidases showed first order reaction kinetics for H₂O₂ with o-dianisidine. The *k_m* and *V_{max}* values were calculated for peroxidase reaction with the substrate using Lineweaver-Burk plot transformation of Michaelis-Menten equation. Respective values of their *K_m* and *V_{max}* evaluated from double reciprocal plot of substrate concentration versus enzyme activity were: 0.250 mM and 9.09 uM min⁻¹ (for tobacco peroxidase, Fig: 3), 0.370 mM and 11.11 uM min⁻¹ (for cabbage peroxidase, Fig: 5) and 0.277 mM and 10.02 uM min⁻¹ (for radish peroxidase Fig: 4) respectively. High R² values (very close to 1) were observed for Lineweaver-Burk plots of all the three

plant peroxidases, proving the greater fit of the data to the line. From the Lineweaver Burk plot it was found that both *N. tabaccum* and *R. sativus* shares almost similar kinetic properties. *N. tabaccum* had lower apparent *K_m* value (Table I). *N. tabaccum* had also showed the lowest specific activity values (Table I), whereas *B. oleraceae capitata var. albaL.* showed the highest specific activity and very high thermostability. Because of these significant characteristics *B. oleraceae capitata var. albaL.* may prove as a potential peroxidase source for biotechnological application. So, with the advance in purification method these plant peroxidases may prove as potential enzymatic source.

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Assam, INDIA, Pin code: 781014

Email- ila.bania@gmail.com

Second Author – Dr. Rita Mahanta,
Associate Professor, Department of Zoology, Cotton College,
Guwahati (Assam), INDIA, Pin code: 781001
Email- ritamahanta@yahoo.co.in

Correspondence Author— Ila Bania, Research Fellow (Ph.D.),
Division of Biochemistry, Department of Zoology, Cotton
College (Gauhati University) Assam, INDIA, Pin code: 781014

AUTHORS

First Author – Ila Bania, Research Fellow (Ph.D.), Division
of Biochemistry, Department of Zoology, cotton College,
(Gauhati University),