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Effect of Malathion, Dimethoate and Chlorpyrifos on Radish Peroxidase

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ABSTRACT: This study focused on kietic characterization of Radish peroxidase and the effect of Malathion, Dimethoate and Chlorpyrifos on Radish peroxidase activity. H2O2- mediated oxidation of O-dianisidine by Radish Peroxidase was used to assess the enzymatic activity. The specific activity of Radish peroxidase was determined in presence of five different concentrations (0.2%, 0.4%, 0.6%, 0.8% and 1.0%) of three pesticides. Malathion and Dimethoate were found to inhibit Radish peroxidase activity whereas, chlorpyrifos showed no significant effect. The values of Km and Vmax in presence and absence of these pesticides were determined from Lineweaver-Burk plot by following the optimum pH and temperature conditions. Kinetic studies showed that the inhibition types were non-competitive for Radish peroxidase in presence of malathion and dimethoate.

KEYWORDS: Km, Vmax, Leneweaver-Burk plot, pesticides, pH.

I.INTRODUCTION

Peroxidase are a group enzyme which belongs to the class oxidoreductases that use hydrogen peroxide or alkyl peroxide as oxidant and catalyse the oxidation of various organic and inorganic electron donor substrates. Versatile biocatalysts peroxidase are a member of very large multigenic family having different physiological roles in different plants and animals. These peroxidases have an important impact on human society in context with various biotechnological and pharmaceutical applications, which stimulated vigorous research on this enzyme. Peroxidases are extracted mostly from plant cells and some animal organs and tissues [12] and by using simple chromogenic techniques their activities are measured. The research on peroxidase is carried out mostly on horseradish peroxidase until 1940s. The enzyme is typically investigated with respect to chemical modifications, organic solvent tolerance studies, reaction kinetics and kinetics in organic solvent, immunochemical and in biosensor fabrication. Use of horseradish peroxidase with a chromogenic donor has proven useful for assay system producing hydrogen peroxide as in the determination of glucose or galactose by their respective oxidase and in the determination of certain l-amino acids in conjugation with L-amino acid oxidase [14]. Though roots of horseradish serves as the major commercially available peroxidase, but the growing demand and wide application of peroxidase in different areas of biotechnology, biochemistry, food industry enhanced intense research on this enzyme in order to get new peroxidases from different plant sources are already employed for the remediation of commercial dyes. The most extensively studied and purified plant peroxidases are – oil palm [2], sweet potato tubers [9], melon [20], cauliflower and rubber tree. Peroxidases are also extracted and comparatively evaluated from turnip, tomato, radish, cucumber plants, beech and horseradish legume [5]. Peroxidases are isolated, purified, characterized from mango and quantitavely made available for industrial and medical use. The suitability of peroxidase for various applications is investigated through its kinetic characterization. Kinetic study is extensively done on the reaction of horseradish peroxidase with hydrogen peroxide [15, 6, 1]. A wide variety of compounds namely, metal cations and other inorganic species, organic compounds, pesticides, herbicides may act as peroxidase inhibitors. The crude enzyme preparation from bean leaves contains an inhibitor of peroxidase catalysed reactions [18]. A large number of inhibitor and stimulators of peroxidase are persisted in the environment. The present research work is aimed to investigate the kinetic characterization of Radish peroxidase along with the effect of different pesticides (Malathion, Dimethoate and Chlorpyrifos) on Radish peroxidase activity.

II.MATERIALS AND METHODS

For the enzyme kinetic studies Systronic UV-Visual spectrophotometer-117 with 1 cm. quarts cell was used. A water bath shaker was used to maintain a constant temperature for colour development. All the pH measurement and adjustments were done with digital pH meter.

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III.CHEMICALS

Chemicals used in the present study are of analytical grade obtained from commercial source. H_2O_2 (30%), BSA and o-dianisidine was obtained from E. Merk Ltd. (Mumbai, India). Double distilled water was used throughout the experiment. A (20 mM) 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 with deionized water.

A. Preparation of tissue extract for peroxidase activity:

Fresh root portion of *R. sativus* were collected from local agricultural field and stored at -20°C until used. Collected plant samples were washed with distilled water and 1 gm of the fresh tissue sample was weighed and homogenized in a blender using 10ml of 0.1M phosphate buffer of pH 7.0. The extract was passed through cheesecloth and centrifuged at 12000 rpm for 10 minutes at 4^oC and the supernatant was labelled as crude extract [16]. To inactivate any catalase present in the extract the extract was heated at 65 ºC for three minutes [19] cooled promptly by placing in ice bucket for 10 min. Different dilutions of the crude enzymes were examined for peroxidase activity assay.

B.Peroxidase Activity Assay:

Assay of peroxidase activity was carried out according to the method of Malik and Sing (1980) [13]. In a test tube, 3.5 ml of phosphate buffer (pH 7.0) was taken and 0.1 ml of O-dianisidine was added and mixed thoroughly. 0.2 ml of plant extract was added to the reaction mixture. The reaction was initiated by adding 0.2 ml of H_2O_2 and kept the tube for 5 minutes. A blank tube was prepared by adding all the above reagents except plant extract (enzyme extract). In place of plant extract 0.2 ml of phosphate buffer was added to the reaction mixture. The absorbance was read at 460 nm after 5 minutes against reagent blank. The peroxidase activity was calculated using extinction co-efficient of O-dianisidine and the enzyme activity was expressed as unit per mg of total protein.

C.Kinetic study:

Determination of the effect of pH and Temperature on Peroxidase Activity:
The pH optima of Radish peroxidase was determined by using 0.01 M acetate buffer, pH 3-4.5, 0.01 M phosphate buffer, pH 5-7.5, 0.01 M Tris/HCl buffer, pH 8-9 [7]. All indications were made with hydrogen peroxide and O-dianisidine. The effect of temperature on peroxidase activity was determined by incubating the reaction mixture prepared for enzyme assay at different temperatures (in hot water bath) including 20, 30, 40, 50, 60, 70, 80 and 90°C for 5 minutes. At a certain temperature, enzyme activity was determined by the addition of enzyme to the mixture as rapidly as possible. Peroxidase activity was assayed under standard conditions [21, 7, 22].

Influence of substrate concentrations:

Enzyme kinetic study was performed with ten samples, employing a range of substrate concentration (0.1×10^{-3}M to 1.0×10^{-3}M) with constant enzyme level in a final volume of 4 ml. All reactions were carried out at a fix (optimum) pH and temperature. Controls in which distilled water was taken as zero, was run in parallel and marked as blank. All the reaction mixtures were monitored at a wavelength of 460 nm (showed highest absorbance). The K_m and V_{max} were determined from the Lineweaver-Burk plot 1/V versus 1/[S] [10] by following the optimum pH and temperature conditions.

Effect of Pesticides on peroxidase activity:

To study the effect of different pesticides on the enzymatic activity of cabbage peroxidase, the concentrations of all compounds, i.e. H_2O_2, O-dianisidine and enzyme were kept constant and five (0.1%, 0.2%, 0.3% 0.4% and 1%) different concentrations of Malathion, Dimethoate, and Chlorpyrifos were assayed. The reaction rate was measured at various concentrations of substrate. The types of inhibitions were determined from Lineweaver-Burk plot.

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D.Protein Estimation:

Total protein concentration was determined in triplicate by the method of Lowry et al. (1951) [11] using bovine serum albumin as a standard.

E.Statistical Analysis:

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

IV.RESULT AND DISCUSSION

The mean values (Mean±SD) of specific activities of Radish peroxidase is found as 186.23±15.03 U/mg of total protein. Specific activity of Radish Peroxidase in presence of five different concentrations of malathion are presented in the table IV.1. Mean activity of Radish in native condition is compared with the mean activity of Radish peroxidase in presence of malathion.

Table:1. Presenting specific activity of Radish peroxidase (U/mg of total protein) in presence of five different concentrations of Malathion.

Sample	Enzyme Activity (U/mg of total protein)	
	MEAN, SEM, SD	
Radish Peroxidase (Without Malathion)	Mean	186.23
	±SEM	5.01
	±SD	15.03
Radish Peroxidase in presence of 0.2% Malathion	Mean	67.62
	±SEM	1.63
	±SD	3.26
Radish Peroxidase in presence of 0.4% Malathion	Mean	49.52
	±SEM	3.11
	±SD	6.95
Radish Peroxidase in presence of 0.6% Malathion	Mean	36.18
	±SEM	0.93
	±SD	2.08
Radish Peroxidase in presence of 0.8% Malathion	Mean	20.87
	±SEM	0.46
	±SD	1.03
Radish Peroxidase in presence of 1 % Malathion	Mean	18.58
	±SEM	0.338
	±SD	0.756

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Table: 2. Presenting specific activities of Radish peroxidase (U/mg of total protein) in presence of five different concentrations of Dimethoate.

Sample	Enzyme Activity (U/mg of total protein) MEAN, SEM, SD	
Radish peroxidase (Without Dimethoate)	Mean	186.23
	±SEM	5.01
	±SD	15.03
Radish Peroxidase in presence of 0.2% Dimethoate	Mean	113.19
	±SEM	0.553
	±SD	1.238
Radish Peroxidase in presence of 0.4% Dimethoate	Mean	78.62
	±SEM	0.269
	±SD	0.602
Radish Peroxidase in presence of 0.6 % Dimethoate	Mean	54.29
	±SEM	0.406
	±SD	0.908
Sample	Enzyme Activity (U/mg of total protein) MEAN, SEM, SD	
Radish Peroxidase in presence of 0.8% Dimethoate	Mean	39.95
	±SEM	0.612
Radish Peroxidase in presence of 1 % Dimethoate	±SD	1.370
	Mean	35.973
	±SEM	1.09
	±SD	2.45

Table: 3. Presenting specific activities of Radish Peroxidase (U/mg of total protein) in presence of five different concentrations of Chlorpyrifos.

Sample	Enzyme Activity (U/mg of total protein) MEAN, SEM, SD	
Radish peroxidase (Without chlorpyrifos)	Mean	186.23
	±SEM	5.01
	±SD	15.03
Radish Peroxidase in presence of 0.2% Chlorpyrifos	Mean	184.87
	±SEM	2.43
	±SD	5.69
Radish Peroxidase in presence of 0.4% Chlorpyrifos	Mean	186.98
	±SEM	4.12
	±SD	7.86
Radish Peroxidase in presence of 0.6 % Chlorpyrifos	Mean	188.81
	±SEM	2.76
	±SD	6.17
Radish Peroxidase in presence of 0.8% Chlorpyrifos	Mean	185.35
	±SEM	0.774
	±SD	2.54
Radish Peroxidase in presence of 1 % Chlorpyrifos	Mean	189.34
	±SEM	1.67
	±SD	3.61

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In presence of 0.2%, 0.4%, 0.6%, 0.8% and 1.0% malathion the specific activities of Radish peroxidase are recorded as 67.62±3.26, 49.52±6.95, 36.18±2.08, 20.87±1.03 and 18.58±0.76 U/mg of total protein respectively against 186.23±15.03 U/mg of total protein specific activity of Radish peroxidase in native condition. Specific activities of Radish peroxidase are found to be decreased with the increasing malathion concentration.

The mean values of specific activity of native Radish peroxidase (Table. 2) is found as 186.23±15.03 U/mg of total protein. In presence of 0.2%, 0.4%, 0.6%, 0.8% and 1.0% dimethoate the specific activities of Radish peroxidase are recorded as 113.19±1.23, 78.62±0.60, 54.29±0.908, 39.95±1.37 and 35.97±2.45 U/mg of total protein respectively. Specific activities of Radish peroxidase are found to be decreased with the increasing dimethoate concentration.

No considerable change in peroxidase activity in Radish peroxidase is observed in presence of pesticide chlorpyrifos.

Peroxidase kinetics: The optimum pH and temperature for Radish peroxidase is found as 7.0 and 40 ^{o}C respectively. The finding is similar with the reported result in which peroxidase isolated from Turnip showed the same pH optima at pH 7.0 [4].

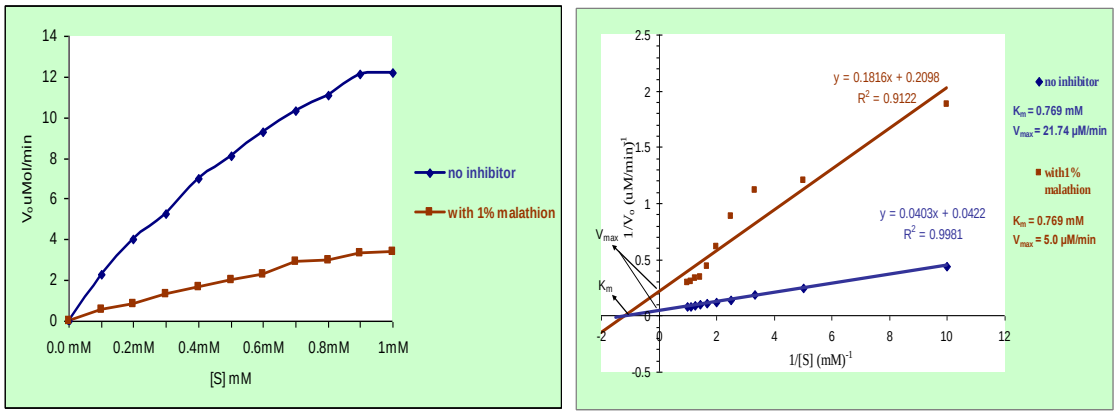

Figure.1: Michaelis Menten and Leneweaver Burk plot of Radish peroxidase activity on hydrogen peroxide in the presence and absence of 1% Malathion at pH 7, 40 ^{o}C.

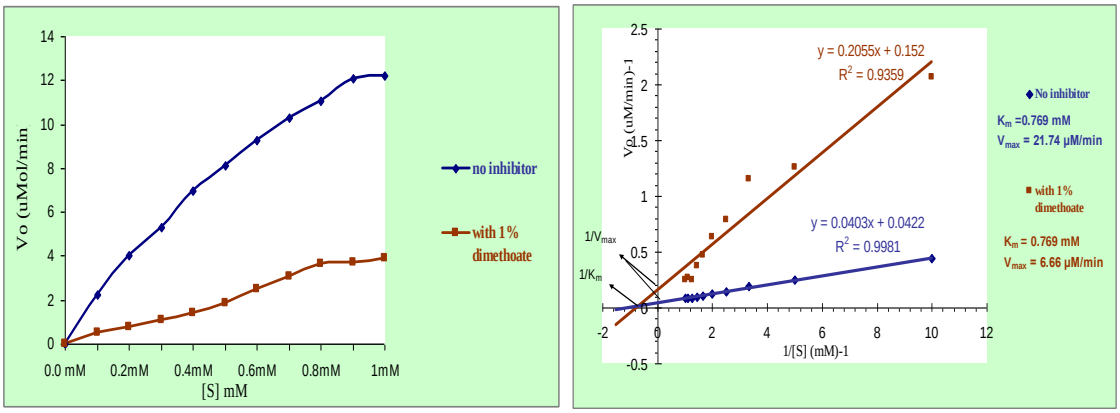

Figure. 2: Michaelis Menten and Lineweaver-Burk plot of Radish peroxidase activity on hydrogen peroxide in the presence and absence of 1% Dimethoate at pH 7, 40 ^{o}C.

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Kinetic study on Radish peroxidase shows that there is gradual and sustained increase in peroxidase activity with increase in substrate concentration upto 1.0 mM as presented in table.1, which is also interpreted with the Michaelis-Mententen curve (Figure- 1). The values of K_m and V_{max} are determined from the Lineweaver-Burk plot obtained with the same set of data on Radish peroxidase under same concentration gradients of the substrate H_2O_2 which are found to be 0.769 mM and 21.74 µM/min respectively (Figure-1). It is evident from the trend line of Lineweaver-Burk plot with a degree of co-relation with a value of R=0.999 between substrate concentration and velocity of Radish peroxidase indicates presence of very high dependency between the two kinetic determinants in the form of substrate concentrations and velocity. The observation of mean specific activity with a value of 186.23±15.03 in radish peroxidase tallies well with the observed relatively low K_m of 0.769 mM resulting in the observed activity due to the matched affinity.

Lineweaver-Burk plots used to analyze inhibition kinetics (Figure-1) show that the extrapolated lines for $1/V_o$ versus 1/[S] intersect each other on the X-axis, indicating that Malathion is a non competitive type of inhibitor binds to Radish peroxidase somewhere other than the active site. This changes the three dimensional structure of Radish peroxidase so that its active site can still bind H_2O_2 as substrate with the usual affinity, but no longer in optimal arrangement to stabilize the transition state and catalyze the reaction. Malathion as a non competitive inhibitor lowers the V_{max} thus Radish peroxidase simply cannot catalyze the reaction with the same efficiency as the native Radish peroxidase. In this condition, noncompetitive inhibition cannot be overcome by raising the H_2O_2 concentration. In presence of Malathion as noncompetitive inhibitor of Radish peroxidase, the value of K_m is nearly unchanged (0.769 mM), while V_{max} is decreased from 21.74 µM/min to 5.0 µM/min. The findings suggest that malathion inhibits Radish peroxidase activity by acting directly on the Radish peroxidase rather than on the substrate.

Pesticide Dimethoate showed inhibitory effect on Radish peroxidase. Analysing the results presented in figure. 2, it is observed that Lineweaver-Burk plots for Radish peroxidase in presence and absence of 1% dimethoate shows non-competitive inhibition. The values of kinetic parameter V_{max} for Radish peroxidase in presence of dimethoate is found as 6.66 µM/min which is much lower than the V_{max} of native Radish peroxidase and K_m in presence of the inhibitor is found as 0.769 suggesting non-competitive type of inhibition.

V.CONCLUSION

Pesticides are widely used in several applications and are of great concern in present time due to its toxic effects and widespread occurrence in the environment. The distribution, absorption, metabolism, toxicity and excretion of pesticides have been examined extensively in recent years but unfortunately past research has rarely focused on the its effect on peroxidases. In the present study, it is found that Malathion and Dimethoate are strong inhibitors of Radish peroxidase activity with the substrate hydrogen peroxide. The type of inhibition shown by different inhibitors is also studied which is found to non-competitive. Although this study could not completely represent in vivo information, it could still provide us some reference to realize the mechanism of inhibitions. The results indicates that Radish peoxidase have shown tolerance over a wide range of pH, temperature. Therefore, this peroxidase can be a potent source for bioanalytical or biotechnological applications, such as enzymatic reagents for clinical diagnosis, food analysis, biotransformation and degradation of various chemicals.

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