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Research Paper Biology

Effect of Malathion, Dimethoate and Chlorpyrifos on Cabbage Peroxidase (Brassica Oleracea) Activity.

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ABSTRACT

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

KEYWORDS : Cabbage peroxidase; inhibitors; pesticides; temperature; Linewaver-Burk plot.

Introduction:

Peroxidases are oxidoreductases having important impact on human society, in context with various biotechnological and biomedical applications, which stimulated intense research on this enzyme. They are well known for its wide applications in different fields such as in biosensors (Alonso-Lomilo et al., 2011), in wastewater treatment particularly in the removal of various organic and inorganic dyes, as effluents of textile industries (Maddhinni et al., 2006; Celebi et al., 2013). Purification and basic characterization of peroxidases from a wide number of plants were done in the recent years in search of a more efficient enzyme to meet the limitations and increasing demand of peroxidases (Krainer et al., 2014; Diao et al., 2014; Anbuselvi et al., 2013). Peroxidases can be easily extracted from most plant cells and some animal organ and tissue (Mahmoudi et al., 2008) and its activity can be easily measured by using simple chromogenic techniques. Hence they are also considered as a model enzyme for studying protein structure, enzyme reactions and enzyme functions, and also used for several practical applications (Hiraga et al., 2001). Peroxidases from plant sources, turnip, tomato, soybean, bitter gourd, white radish, and *Saccharum uvarum*, have been employed for the remediation of commercial dyes. Several studies are also reported on cabbage peroxidase. Spring cabbage peroxidase is suggested as a potential tool in biocatalysis and bioelectrocatalysis (Belcarz et al., 2008, Kong et al., 2003). Peroxidases are also used in food industry as an indicator of vegetable bleaching. Several naturally thermostable peroxidases are also described that include Soybean peroxidase (Mc Eldoon and Dordick, 1996), peroxidase from leaves of royal palm tree (Sakharov et al., 2001), spring cabbage peroxidase (Belcarz et al., 2008) etc.

Toxins and inhibitors persisted since organisms first began using chemical warfare to gain evolutionary advantage over their competitors and are affecting the environment. Pesticides such as Malathion, Dimethoate and Chlorpyrifos are well known pollutant of our environment. A wide variety of compounds namely, metal cations different organic and inorganic compounds may act as peroxidase inhibitors. L-cystine, dichromate ethylenethiourea, hydroxylamine, sulfide, vanadate, p-aminobenzoic acid and the divalent anions of Cd, Co, Fe, Mn, Ni and Pb have all been reported to inhibit HRP (Zollner, 1993). Malathion and Dimethoate 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 present study has been undertaken to determine the effect of malathion dimethoate and chlorpyrifos on cabbage peroxidase activity.

Materials and methods:

For all the spectroscopic studies an UV-visual spectrophotometer (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.

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.

Preparation of tissue extract for peroxidase activity:

Fresh leaf portion of *B. olaraceae* 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°C and the supernatant was labeled as crude extract (Nagaraja et al., 2009). To inactivate any catalase present in the extract the extract was heated at 65 °C for three minutes (Rehman *et al*. 1999) cooled promptly by placing in ice bucket for 10 min. Different dilutions of the crude enzymes were examined for peroxidase activity assay.

Peroxidase Activity Assay:

Assay of peroxidase activity was carried out according to the method of Malik and Sing (1980). 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 minute. 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.

Kinetic study:

Determination of the effect of pH and Temperature on Peroxidase Activity:

The pH optima of cabbage 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 (Koksal and Gulcin, 2008). 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 (Sakharov et al. 2002; Koksal and Gulcin 2008; Singh et al., 2010).

Influence of substrate concentrations:
Enzyme kinetic study was performed with ten samples, employing a range of substrate concentration (0.1 x 10^{-3}M to 1.0 x 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 i.e. pH 6.0 and 40°C for cabbage peroxidase. 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] (Lineweaver and Burk 1934) 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, Dimethoed, and Chlorpyrifos were assayed. The reaction rate was measured at various concentrations of substrate. The types of inhibitions were determined from Lineweaver-Burk plot.

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

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 ± standard deviation.

Result and discussion:
Cabbage peroxidase activity was assayed in presence and absence of different pesticides by following optimum pH and temperature conditions. The optimum pH for Cabbage peroxidase activity was found to be 6.0 and the optimum temperature was recorded at 40°C. Similar trend was reported in case of peroxidase isolated from Turnip where the optimum temperature for peroxidase activity was in the range of 40 to 50 °C (Duarte- Vazqueze et al., 2000). Most of the plant peroxidases reported to have optimum activity at high temperature. The tested plant peroxidase in this study showed maximum activity at higher temperature range which suggests the suitability of these enzymes for various biotechnological applications. This pH optimum of the peroxidase activity observed in the present investigation is closer to palm peroxidase (*Roystonea regia*) (Sakharow et al., 2002), clover peroxidase (Criquet et al., 2001) and *Prangos ferulacea* (Apiaceae) (Nadaroglu, 2009). The result of the present experiment can also be corroborated with the results of Civell et al. (1995) who reported optimum pH 6.0 to 6.5 for peroxidase activity of different vegetable and fruit sources. Mean specific activity of native Cabbage peroxidase was recorded as 131.89±7.55 U/mg whereas, in presence of malathion and dimethoate the activity of cabbage peroxidase was found to be decreased. On comparison of mean specific activity of Cabbage peroxidase in native condition with the mean activity of Cabbage peroxidase in presence of malathion and dimethoate it was found that the cabbage peroxidase activity decreases with the increasing concentrations of Malathion and Dimethoate. No considerable change in peroxidase activity in the tested plant peroxidase was observed in presence of Chlorpyrifos.

Table-1: Specific activity of cabbage peroxidase in presence of five different concentrations of Malathion, Dimethoate and Chlorpyrifos.

Sl no.	Pesticide concentrations	Specific activity of cabbage peroxidase (U/mg)			
		MEAN, SEM, SD	In presence of Malathion	In presence of Dimethoate	In presence of Chlorpyrifos
1	Native Cabbage peroxidase (no pesticide)	Mean 131.89			
		± SEM 2.51			
		±SD 7.55			
2	0.2%	Mean	78.45	60.89	131.11
		±SEM	0.594	1.24	2.87
		±SD	1.33	2.77	5.66
3	0.4%	Mean	56.03	51.41	133.87
		±SEM	0.414	0.960	3.56
		±SD	0.925	2.146	7.12
4	0.6%	Mean	40.64	41.50	137.21
		±SEM	0.313	0.827	3.84
		±SD	0.7	1.849	7.11
5	0.8%	Mean	28.82	28.140	137.99
		±SEM	0.276	0.845	3.54
		±SD	0.61	1.889	8.91
6	1.0%	Mean	13.24	12.62	133.53
		±SEM	0.436	0.57	0.892
		±SD	0.975	1.288	2.56

The kinetic constants for Cabbage peroxidase were determined for the leaf portion of cabbage following standard pH and temperature condition. Gradual and sustained increase in peroxidase activity was found with increased substrate concentrations from 0.1 to 1.0 mM which can be interpreted with the Michaelis-Mententen curve (figure-1).

The Lineweaver-Burk plot for the same set of data on Cabbage peroxidase indicates that the K_m and V_{max} for cabbage peroxidase were 0.833 mM and 17.24 µM/ min respectively (figure-1). The R value from the trend line of Lineweaver-burk plot was found to be 0.915, which exhibits high degree of correlation between the two kinetic determinants.

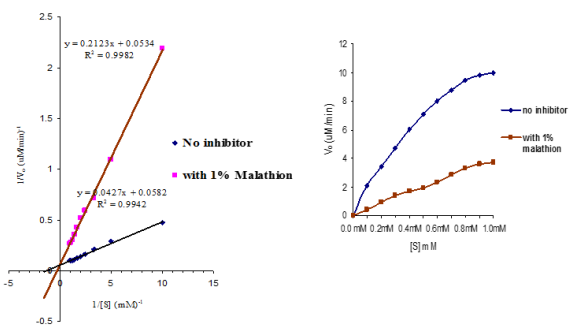

Figure-1: Lineweaver-Burk plot of Cabbage peroxidase activity on hydrogen peroxide in the presence and absence of 1% Malathion. The unit of X and Y axes are $(mM)^{-1}$ and $(\mu M/min)^{-1}$. Inset: Michaelis Menten plot for Cabbage peroxidase activity.

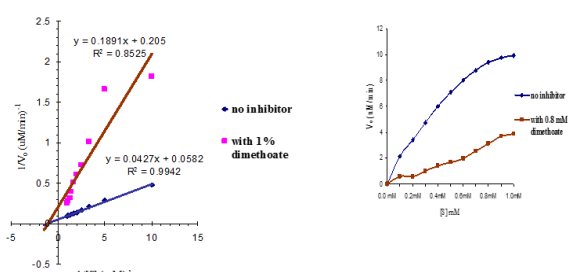

Figure-2: Lineweaver-Burk plot of Cabbage peroxidase activity on hydrogen peroxide in the presence and absence of 1% Dimethoate. The unit of X and Y axes are $(mM)^{-1}$ and $(\mu M/min)^{-1}$. Inset: Michaelis-Menten plot for Cabbage peroxidase activity.

The study on cabbage peroxidase activity in presence of 1% malathion showed that Malathion is a competitive inhibitor of o-dianisidine oxidation by Cabbage peroxidase, shown by the extrapolated lines for $1/V_o$ versus 1/[S] which intersect each other on the Y-axis in the Lineweaver-Burk plot (Figure-1). From the Lineweaver-Burk plot enzyme's V_{max} in presence of malathion is observed as 17.24 µM/min and its K_m is 7.69 mM which shows that the K_m was highly increased

from the controlled cabbage peroxidase (0.833 mM) while the V_{max} was unchanged.

Effect of five different concentrations (0.2%, 0.4%, 0.6% 0.8% and 1.0%) of dimethoate on Cabbage peroxidase was studied in the present investigation and it was found that the maximum rate decreases with increasing inhibitor concentration, confirming its inhibition effect. The Lineweaver-Burk plot in presence and absence of dimethoate was linear with a common intercept in the abscissa indicating noncompetitive inhibition for cabbage peroxidase against 1 % dimethoate (Figure-2). The V_{max} for cabbage peroxidase decreased from 17.24 µM/min for the controlled to 5.0 µM/min in presence of the dimethoate while K_m remained unaffected (0.833 mM).

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 and Herbicides 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 Malathione and Dimethoate are strong inhibitors of Cabbage peroxidase activity.

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