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Research Paper Biology

Cabbage Peroxidase activity in an in- vitro assay system supplemented with Indole-3-acetic acid for hydrogen peroxide scavenging.

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ABSTRACT

Peroxidases are promising enzyme having attractive biocatalytic properties and wide applications in the field biotechnology and biomedicine. The effect of Indole-3-Acetic Acid on the oxidation of o-dianisidine catalyzed by a cabbage peroxidase-H_2O_2 system was studied following the optimum pH and temperature conditions. Indole-3-Acetic Acid was found to be a potent inhibitor on the peroxidase catalyzed reaction under given condition. The feasible inhibition types of peroxidase oxidation of o-dianisidine in the presence of inhibitor along with the values of K_m and V_{max} were determined from Lineweaver-Burk plot. K_m for native cabbage peroxidase (without indole-3-acetic acid) was 0.34 mM and V_{max} was 10.2 µM/Min. Kinetic studies showed that the inhibition type was uncompetitive with considerable decrease in K_m (0.266 mM) and increased Vmax (7.69 µM/Min).

KEYWORDS : Peroxidase, Lineweaver- Burk Plot, pH, Temperature, K_m, V_{max}.

Introduction:

Peroxidases (E.C. 1.11.1.7) are ubiquitous oxidoreductases that catalyses the redox reaction between H_2O_2 as an electron acceptor and different kinds of organic and inorganic substrates, which make peroxidase usefull in a number of biotechnological applications. The family of plant peroxidase comprises of yeast cytochrome C peroxidase, plant ascorbate peroxidase, fungal peroxidase and classical plant secretory peroxidase. Among the various peroxidases found in plant, animal and microorganisms class III plant peroxidase are widely studied. They are members of a large multigenic family, found in tonoplast and plasmalemma, inner and outer side of the cellular wall both in soluble as well as, ionically bound forms (Chen et. al., 2002; Passardi et al., 2005), integument cotyledons and embryo axis (Scialabba et. al., 2002) of plants. Studied on peroxidases have suggested that in plants they have been implicated in various physiological processes which includes lignification (Whetten et. al., 1998; Quiroga et. al., 2000) antioxidant protection during stress (Huseynova et al., 2013), phenol oxidation (Bratkovskaja et al.,2004), suberization (Espelie et. al., 1986), salt stress tolerance (M'barek, 2007; Martinez et al., 2014) protection of tissue from damage and infection by microorganisms (Sat, 2008; Bashan, 1987) senescence (Abeles et. al., 1997), Protection of Plants from Ultraviolet Radiation Stress (Jansen et. al., 2001), as biomarker of metallic stress and as Biochemical Markers for Biocontrol Efficacy (Jouili et. al., 2011; Seleim et. al., 2014) removal of carcinogenic aromatic amines from industrial aqueous effluents (Klivanov et al., 1981) and the oxidative degradation of the major endogenous auxin, IAA (Krylov and Dunford, 1996; Normanly, 1997; Gazaryan et al., 1998). Indole-3-Acetic Acid (IAA) is a naturally occurring auxin and the role of IAA in plant growth and development is an important one. Ambatkar and Mukundan (2014) studied the interaction of peroxidases and indole-3-acetic acid in *Armoracia rusticana* and suggest that the peroxidase content of in vitro horseradish plantlets can be improved significantly using a low dose of IAA which can be helpful for growing Horseradish in tropical countries. Peroxidases from plant sources, turnip, tomato, soybean, bitter gourd, white radish, and Saccharum uvarum, have been employed for various biotechnological applications and for the remediation of commercial dyes. In the recent years cabbage peroxidase is also getting attention in peroxidase research for its various important biochemical properties and applications (Fortea, et al. 2012; Belcarz, et al., 2008). In this study the activity of cabbage peroxidase in presence of Indole-3-acetic acid was investigated in vitro.

Experimental:

An UV-visual spectrophotometer (Systronic UV-Visual spectrophotometer-117) with 1 cm. quarts cell was used for all the spectroscopic studies. 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.

Chemicals:

All chemical used in the present study were of 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 (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.

Plant Material Collection and Preparation of Extract:

As a source of peroxidase fresh leaf of *Brassica oleraceae* was collected from local agricultural field [Morigaon district, Assam (North East India)] and carried at 4°C to the laboratory and stored at -20°C until used. Collected plant samples were washed with distilled water and 1 gm of the fresh tissue sample was weighted 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).. 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.

Assay of Peroxidase (POX) Activity:

Assay of peroxidase was carried out according to the method of Malik and Sing , 1980, with certain modifications. To 3.5 ml of phosphate buffer (pH 6.0) 0.2ml of plant extract and 0.1ml of o-dianisidine solution were added. The reaction was initiated by adding 0.2 ml of 0.2 x 10^{-3}M H_2O_2 and the absorbance was read at (460 nm) every 30 second interval up to 3 minutes. The peroxidase activity was measured by a change in absorbance at 460 nm, due to the oxidation of o-dianisidine in the presence of hydrogen peroxide. The enzyme activity was expressed as unit per mg of protein.

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. At a certain temperature, enzyme activity was determined by the addition of enzyme to the mixture as rapidly as possible. The process was carried out in a circulatory water bath in a temperature range between 30 to 80 °C. The pH optima of peroxidase was determined using acetate buffer (pH 3.0-5.0, 0.01M), potassium phosphate buffer (pH 6.0-8.0, 0.01M) and Tris/HCl buffer (pH 8-9, 0.01M). All the peroxidase activities were assayed under standard conditions (Sakharov et al., 2002; Koksal, 2011; Singh et al., 2010).

Kinetic Studies

Enzyme kinetic studies were performed in 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 condition. 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). Tobacco peroxidase kinetics was represented through Michaelis Mentan plot and 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.

Determination of the effect of indole-3-acetic acid:

To study the effect of indole-3-acetic acid 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 different concentrations of indole-3-acetic acid (20 µM, 40 µM, 60 µM, 80 µM, 100 µM) were assayed. The reaction rate was measured at various concentration of H_2O_2 in the presence of indole-3-acetic acid.

The type of inhibition was determined from Lineweaver-Burk plot 1/V versus 1/S (Lineweaver and Burk 1934).

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 showed optimum activity at pH 6 and a significant decrease in activity was seen with increasing and decreasing pH. The effect of temperature on cabbage peroxidase activity was assayed by heating the reaction mixture to the appropriate temperature in hot water bath before introduction of the enzyme. The observed activity was found to be highest at 50°C. The optimum absorption spectra for the cabbage peroxidase catalyzed reaction was recorded at 460 nm and further analysis were done in that wavelength of spectrophotometer.

Effect of indole-3-acetic acid:

The effect of indole-3-acetic acid on the oxidation of o-dianisidine catalyzed by a tobacco peroxidase-H_2O_2 system was studied following` the optimum pH (pH 6.0) and temperature (50°C) conditions. The tested indole-3-acetic acid of five different concentrations (20 µM, 40 µM, 60 µM, 80 µM, 100 µM) showed variable responses against the peroxidase catalyzed reaction (Table-1). The native cabbage peroxidase (without amino acids) showed a specific activity of 131.89±7.55 U/mg. The enzyme activity showed considerable decrease with increasing concentration of indole-3-acetic acid.

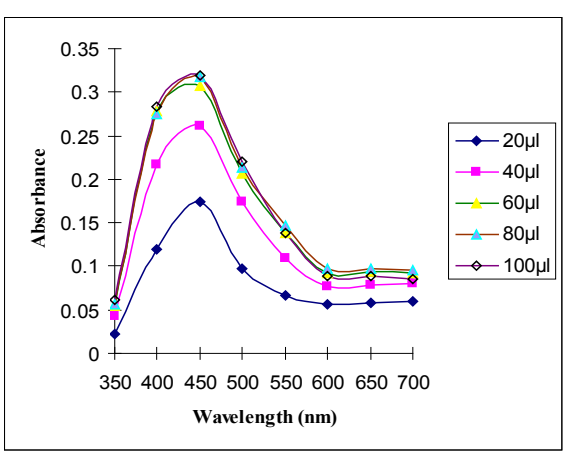

Fig1: Absorption spectrum of different concentrations of Cabbage Peroxidase at 3 min after addition of 1mM H_2O_2, 1mM O-dianisidine and phosphate buffer of pH 6.0.

Kinetic Studies:

The values of K_m and V_{max} for tobacco peroxidase were determined for the substrate hydrogen peroxide (Fig.2). The enzyme activities were measured at ten different concentrations of substrate and constant concentration of o-dianisidine. The K_m and V_{max} for native tobacco peroxidase (controlled) was found to be 0.34 mM and 10.2µM/Min respectively.

Table-1: Effect of different concentrations of indole-3-acetic acid on Cabbage peroxidase.

Sample	Enzyme Activity (U/mg)	
	MEAN, SEM, SD	
Native Cabbage POX (Without indole-3-acetic acid)	Mean=	131.89
	SEM= ±	2.51
	SD= ±	7.55
20 µM indole-3-acetic acid / Cabbage Peroxidase	Mean=	105.29
	SEM= ±	1.77
	SD= ±	2.22
40 µM indole-3-acetic acid / Cabbage Peroxidase	Mean=	99.75
	SEM= ±	0.54
	SD= ±	2.10
60 µM indole-3-acetic acid / Cabbage Peroxidase	Mean=	94.21
	SEM= ±	1.01
	SD= ±	2.78
80 µM indole-3-acetic acid / Cabbage Peroxidase	Mean=	79.43
	SEM= ±	0.028
	SD= ±	.676
100 µM indole-3-acetic acid / Cabbage Peroxidase	Mean=	72.04
	SEM= ±	1.47
	SD= ±	3.11

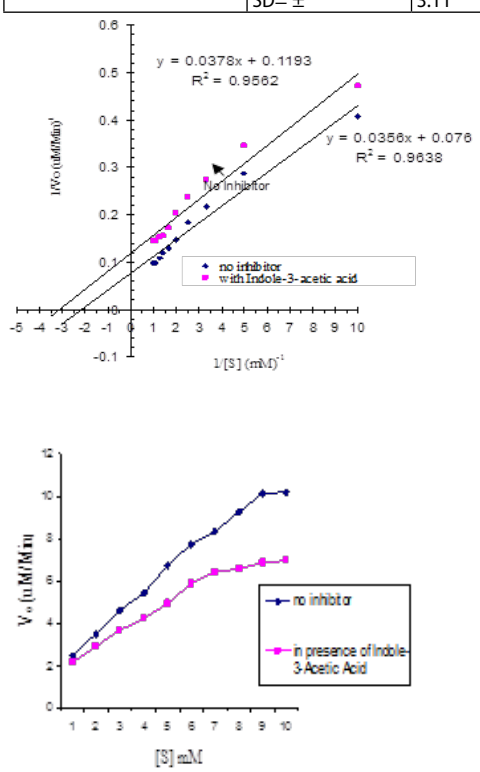

Fig-2: Lineweaver-Burk plot of Cabbage peroxidase activity on hydrogen peroxide in the presence and absence of indole-3-acetic acid.

The unit of Y and X axes are $(mM)^{-1}$ and $(\mu M/min)^{-1}$. The inset curve is Michaelis-Menten plot.

From Lineweaver-Burk plot considerable decrease in K_m (0.266 mM) was recorded in presence of indole-3-acetic acid whereas the V_{max} increases (7.69 µM/Min). Kinetic studies showed that the inhibition type was uncompetitive for cabbage peroxidase. The study shows the kinetic behaviour of cabbage peroxidase in vitro in the presence and absence of indole-3-acetic acid.

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