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RESEARCH ARTICLE

KINETIC CHARACTERIZATION AND ACTIVITY ASSAY OF TOBACCO PEROXIDASE IN PRESENCE OF DIFFERENT AMINO ACIDS

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ABSTRACT

The effect of a number of amino acids on the catalytic activity of tobacco peroxidase was studied in an in vitro assay system following the optimum pH and temperature conditions. The tested amino acids of two different concentrations (0.2 mM amd 0.8 mM) showed variable responses against the Tobacco peroxidase catalyzed reaction. Amino acids like D-alanine, L-proline, DL-valin, DL-Tryptophan were found to stimulate the activity of peroxidase catalyzed reaction under given condition while certain other amino acids like L-lysine, Arginine, Histidine, Glutamic acid etc. showed no significant effect. Moderate inhibition was observed in case of aspartic acid at relatively higher concentration (0.8mM). However, a very low concentration of L-cysteine (0.2mM) acts as a strong inhibitor of Tobacco Peroxidase activity. Kinetic studies showed that the inhibition type was mixed idnhibition for cystein and noncompetitive inhibition for aspartic acid. The feasible inhibition types of peroxidase oxidation of o-dianisidine in the presence of each inhibitor along with the values of K_m and V_{max} were determined from Lineweaver-Burk plot.

INTRODUCTION

Peroxidases (EC.1.11.1.7) are biocatalyst and haemprotein enzyme that catalyses the one or two electron oxidation of various organic and inorganic substances in the presence of hydrogen peroxide. These haem containing peroxidases are classified under two broad superfamilies, one isolated from plants, fungi and bacteria (plant peroxidase superfamily) and another from mammals (animal peroxidase superfamily). On the basis of sequence similarity plant peroxidase superfamily have been again subdivided into three classes; peroxidases of prokaryotic origin are in class I, secretory fungal peroxidases from class II and class III consist of extracellular plant peroxidases (Welinder, 1992). In most divergent cases less than 20% similarity in amino acid composition was found between class III peroxidases, but they have highly conserved residues in the haem pocket playing a key role in the catalytic cycle. In plant, peroxidases are mainly located in the cell wall (Chen *et al*., 2002). Scialabba *et al*., in 2002 assayed peroxidase activity in crude extracts of integument cotyledons and embryo axis of raddish seeds. It is one of the key enzyme that controls plants growth and development and also takes part in various cellular processes such as protection of plant cell from damage by H_2O_2, phenol oxidation (Bratkovskaja *et al*., 2004), salt stress tolerance (M'barek, 2007) protection of tissue from damage and infection by microorganisms (Sat, 2008), lignification (Quiroga *et al*., 2000) etc. Hoang A. in 2010 suggested the role of peroxidase in defense response of buffalograss to chinch bug. Apart from biological functions peroxidases received extensive attention for its potentially interesting application in different field such as medicinal, chemical, biotechnological and food industries (Kwak *et al*., 1996, Van *et al*., 1997; Greco *et al*, 2001). Being one of the most thermostable enzyme peroxidases are used for special applications like waste water treatment specially in oxidation and removal of industrial textile and non textile dyes (Hidalgo *et al*., 2011, Khan and Husain, 2006), in food industry as an indicator of vegetable bleaching (Rodrigo *et al*., 1997) , as a potential tool in biocatalysis and bioelectrolysis (Knutsen *et al*., 2005; Belcarz *et al*., 2007), as immunoconjugates in immunoassay techniques (ELISA kit) and also incorporated in biosensors (Yang *et al*., 2009; Alonso-Lomillo *et al*., 2011) and removal of carcinogenic aromatic amines from industrial aqueous effluents (Klivanov and Morris, 1981). They are also used for production of pharmaceuticals and for the synthesis of conductive polymers (Ford *et al*., 2004; Sakharov *et al*., 2003). Theses peroxidases are widely distributed in nature and can be easily extracted from different plants and animal tissues. Despite the numerous sources of peroxidases their present commercial uses are limited.

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Till date Horseradish Peroxidase is the most widely studied peroxidase and also used in various biotechnological fields. But search for other potential peroxidase sources are going on and different peroxidases such as soybean POX, cabbage POX have shown promising characteristics for biotechnological applications. The suitability of peroxidase for biotechnological application can be investigated through its kinetic characterization. Various inhibitors and stimulators are persisted in the environment in relation to the peroxidase enzyme. A wide variety of compounds namely, metal cations and other inorganic species, organic compounds, pesticides, herbicides etc. 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 (Sariri *et al*., 2005). Again a wide variety of proteins and enzymes incorporate metal ions or metal complexes into their complex structure and trigger enhancement of their activity (Dubey *et al.*, 2007). Few research works has been reported on biological and physiological role tobacco peroxidase in plant (Lagrimini *et al*., 1990; Marco *et al*., 1999; Gazarian *et al*., 1996), but the detail biochemical characterization of tobacco peroxidase is lacking. The present study was aimed for kinetic characterization of tobacco peroxidase and also to determine the effect of various amino acids on tobacco peroxidase activity.

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 *N. tabaccum* were 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^oC and the supernatant was labeled as crude extract (Nagaraja *et al*., 2009). The extract was heated at 65 ºC 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 Km and Vmax 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 amino acids

To study the effect of different amino acids on the enzymatic activity of tobacco peroxidase, the concentrations of all compounds, i.e. H_2O_2, O-dianisidine and enzyme were kept constant and two different concentrations of each amino acid (0.2 mM and 0.8mM) were assayed. The reaction rate was measured at various concentration of H_2O_2 in the presence of various amino acids. The types of inhibitions were 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.

RESULTS AND DISCUSSION

The enzyme extract of native tobacco peroxidase was prepared from tobacco leaf using sodium phosphate buffer of pH 6. Tobacco peroxidase showed optimum activity at pH 6 and showed a significant decrease in activity with increasing and decreasing pH.

The result was corroborated with the results of Civello *et al*. (1995), Halpin *et al*. (1989), Diao *et al*. (2011) and Rehman *et al*. (1999) who reported optimum pH 6.0 to 6.5 for different vegetable and fruit sources. The effect of temperature on tobacco 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 60°C. The result was substantiated with the results of Kumar *et al*. (2011). However the enzyme showed considerable activity in the temperature range between 40 to 70°C.

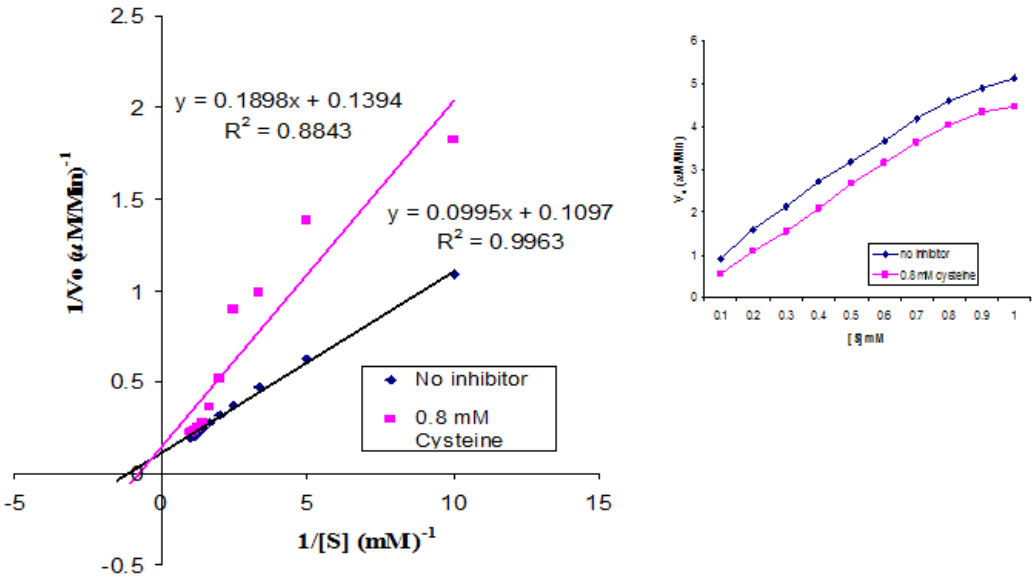

Fig. 1. Lineweaver-Burk plot of Tobacco peroxidase activity on hydrogen peroxide in the presence and absence of 0.8 mM cysteine. The unit of X and Y axes are $(mM)^{-1}$ and $(\mu M/min)^{-1}$. The inset curve is Michaelis-Menten plot

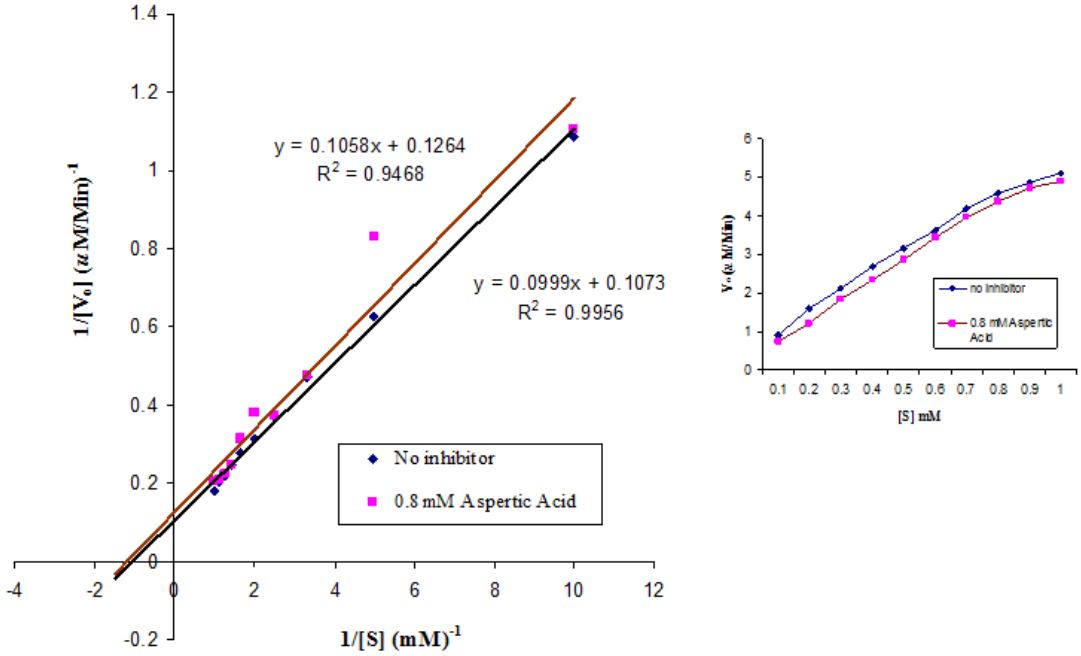

Fig. 2. Lineweaver-Burk plot of Tobacco peroxidase activity on hydrogen peroxide in the presence and absence of 0.8 mM Aspertic Acid. The unit of X and Y axes are $(mM)^{-1}$ and $(\mu M/min)^{-1}$. The inset curve is Michaelis-Menten plot

Effect of different amino acids

The effect of a number of amino acids 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 (60^{o}C) conditions. The tested amino acids of two different concentrations (0.2 mM and 0.8 mM) showed variable responses against the peroxidase catalyzed reaction Table 1. The native tobacco peroxidase (without amino acids) showed a specific activity of 77.5±3.61 U/mg. Certain amino acids like D-alanine, L-proline, DL-valin, DL-Tryptophan were found to stimulate the activity of peroxidase catalyzed reaction under given condition while certain other amino acids like L-lysine, Arginine, Histidine, Glutamic acid etc. showed no significant effect. Moderate inhibition was observed in the case of aspartic acid against tobacco peroxidase activity at high concentration (0.8 mM). However, a very low concentration of L-cysteine (0.2mM) acts as a strong inhibitor of Tobacco Peroxidase activity.

The K_m and V_{max} for native tobacco peroxidase (controlled) was found to be 0.91 mM and 9.25μM/min respectively. The amino acids like aspartic acid and cysteine showed inhibition against tobacco peroxidase. Kinetic studies revealed that the type of inhibition shown by Cysteine and Aspartic Acids were mixed inhibition for cystein and noncompetitive inhibition for aspartic acid. The inhibition types of peroxidase oxidation of o-dianisidine in the presence of each inhibitor were determined from the Line weaver-Burk plot.

The values of K_m for aspartic acid and cysteine were 0.91 mM and 1.3 mM respectively. The Km value did not change for tobacco Peroxidase activity in presence of Aspertic acid whereas remarkable increase in K_m value (1.3 mM) was seen in the Lineweaver-Burk plot in presence of cysteine. Both the inhibitors markedly decreased the V_{max} for peroxidation of O-dianisidine by Tobacco Peroxidase (V_{max} in presence of cysteine = 6.66 μM/min and V_{max} in presence of Aspertic Acid =8.33 μM/min).

Table 1. Effect of amino acids on tobacco peroxidase activity:

Sample	Enzyme Activity (U/mg)		
	MEAN, SEM, SD	Amino acid/ [Tobacco Peroxidase]= 0.2mM	Amino acid/ [Tobacco Peroxidase]= 0.8mM
Native Tobacco POX (Without amino acid)	Mean= 77.5 SEM= ± 1.20 SD= ± 3.61		
D-Alanin/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	83.77 1.781 5.95	92.41 3.76 4.71
L-Prolin/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	80.71.71 3.13 6.26	105.71 3.22 10.04
DL-Valin/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	88.94 1.522 6.08	100 3.335 11.98
DL-Tryptophan/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	80.75 1.23 5.00	90.07 1.88 5.36
Cystein/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	42.65 2.45 4.71	17.69 2.86 6.41
Aspertic acid/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	76.28 2.55 6.47	66.17 2.34 7.77
Glutamic acid/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	77.04 1.490 7.473	78.33 0.947 3.89
Lysine/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	79.04 2.72 5.72	78.21 1.107 4.057
Arginine/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	78.09 3.29 9.88	76.54 3.79 11.39
Histidine/ Tobacco Peroxidase	Mean= SEM= ± SD= ±	77.14 2.50 7.50	78.19 2.35 7.07

Kinetic Studies

The values of K_m and V_{max} for tobacco peroxidase were determined for the substrate hydrogen peroxide in the presence and absence of inhibitors like Cysteine and aspartic Acid (Fig.1 and Fig.2). The enzyme activities were measured at ten different concentrations of substrate and constant concentration of o-dianisidine.

From the investigation it was found that the sensitivity of tobacco peroxidase can be increased in the presence of certain amino acids which stimulate the enzyme activity whereas few amino acids inhibit the same. Tobacco peroxidase also showed tolerance to a wide range of temperature (40^{o}C- 70^{o}C) and pH (6.0-7.0). All these specificities of Tobacco peroxidase helps determining suitability of the enzyme in Biotechnology and various other applications and would promote the development of new analytical applications.

The inhibitory amino acids Cysteine and Aspertic acid might interfere with the tobacco peroxidase activity by competing with its normal substrate for their binding sites, binding to free substrate or reducing their oxidized form. Further studies are needed to define a possible physiological role for amino acids on tobacco peroxidase activity.

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