Biochemical characterization of *Santalum album* (Chandan) leaf peroxidase

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Abstract The *Santalum* peroxidase was extracted from the leaves and precipitated with double volume of chilled acetone. The optimum percent relative activity for the *Santalum* peroxidase was observed at pH 5.0 and 50 °C temperature. The *Santalum* peroxidase per cent relative activity was stimulated in the presence of phenolic compounds like ferrulic acid and caffeic acids; however, indole-3-acetic acid (IAA) and protocatechuic acid act as inhibitors. All divalent cations Fe^{2+} , Mn^{2+} , Mg^{2+} , Cu^{2+} and Zn^{2+} stimulate the relative activity of the *Santalum* peroxidase at concentration of 2.0 μM . Amino acids like L-alanine and L-valine activate the per cent relative activity, while L-proline and DL-methionine showed moderate inhibition for the *Santalum* peroxidase. However, a very low a concentration of cysteine acts as a strong inhibitor of *Santalum* peroxidase at the concentration of 0.4 mM. Native polyacrylamide gel electrophoresis (Native-PAGE) was performed for isoenzyme determination and two bands were observed. K_m and $V_{\max}$ values were calculated from Lineweaver-Burk graph. The apparent $V_{\max}/K_m$ value for O-dianisidine and H_2O_2 were 400 and 5.0×105 Units/min/mL respectively.

Keywords *Santalum album* · Peroxidases · Precipitation · Purification · Heat stability · Native-PAGE

Introduction

Peroxidases (E.C. 1.11.1.7) are present in plants in multiple forms and are known for a long time to be involved in several biophysical functions (Malabadi and Nataraja 2002). Plant peroxidases are found in tonoplast and plasmalemma, inside and outside the cell wall (Vallejos, 1983) and can be subdivided into three subgroups (acidic, neutral and cationic) according to their isoelectrophoretic mobilities. Class III peroxidases have been found in crude plant extracts by staining for activity after separation by gel electrophoresis (Hiraga 2001). These enzymes catalyze the oxidation of a variety of electron donor substrate (e.g. phenols, aromatic amines) in the presence of hydrogen peroxide (Passardi et al. 2004). Microbes and animal tissues represent a huge family of heme containing enzymes (Dunford 1999). Many studies have been done on amino-acid sequencing and heme structure of peroxidases (Welinder and Mazza 1977; Silva et al. 1990). Peroxidase has been implicated in metabolic processes such as ethylene biogenesis, cell development and membrane integrity (Welinder and Mazza 1977), building and rigidification of cell walls (Passardi et al. 2004), auxin catabolism (Delannoy et al. 2004), senescence (Faivre-Rampant 1998), defense mechanism towards pathogens (Choi et al. 2007; Passardi et al. 2005) and various abiotic stresses, including metal ions and UV stress (Marcel et al. 2001), salt (Yi and Lee 2003), air pollution damage (Lee 2002), cold tolerance (Tao et al. 1998), control of cell elongation, polymerization of extension (Ahmed et al. 1995), generation of reactive oxygen species (Bestwick et al. 1998), hydrogen peroxide scaveng-

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ing (Kawaoka et al. 2003), lignin polymerization (Fagerstedt et al. 2010) and the suberization processes (Quiroga et al. 2000; Kolattukudy 2001).

Although peroxidases are widely distributed in the plant kingdom, the major source of commercially available peroxidases is roots of horseradish. On the other hand, availability of peroxidases with different specificity would promote the development of new analytical applications (Paul 1986) such as clinical diagnosis (blood sugar and cholesterol), immunoassays (ELISA kit), biosensor construction (Abelskov et al. 1997), food processing and food storage, treatment of waste water containing phenols and aromatic amines (Wu et al. 1998), bio-bleaching processes, lignin degradation in fuel, production of dimeric alkaloids, oxidations, and bio-transformation of organic compounds (Ryan et al. 1994) and potential industrial process (Torres et al. 1997) because of its high sensitivity. These enzymes can participate in a number of oxidation and biodegradation reactions associated with changes of flavor, color, texture, and the nutritional quality of food (Clemente and Robinson 1995; Clemente and Pastore 1998). Enzymatic browning results mostly from polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (Šukalović et al., 2010; Slatnar et al., 2010). Substantial uses of isoenzyme (peroxidase) based markers have been reported by several workers for the crop improvements programmes in several crops (Vallejos 1983; Glaszmann et al. 1989) and genetic diversity study in Indian sandal (Suma and Balasundaran 2003), sugarcane (Manjunatha et al. 2003). The present study was performed using leaf peroxidases of *Santalum album*. The objective of this study was to determine the isolation, partial purification, biochemical characterization and kinetic properties of *Santalum* peroxidases.

Materials and method

Experimental material The plant materials used in experiment are the leaves of *Santalum album*. The fresh, healthy and young leaves were collected, (Botanical garden of D. D. U. University, Gorakhpur, U. P., India) washed, cut into small pieces and used for enzyme (peroxidase) extraction.

Preparation of crude enzyme To assess the total peroxidases activity from wild *Santalum* plant, crude extract was prepared by homogenization of 25 grams leaves in mortar and pestle added with 100 mM sodium acetate buffer of pH 5.0 and Polyclar aT (1.0 g/10 g of tissue) as phenolic scavenger (Kumar et al. 2008; Singh et al. 2010). The homogenate was filtered through four layers of cheese cloth and centrifuged for 20 min at 16,000 rpm at 4 °C temperature. The clear supernatant obtained was used for peroxidase activity assay (Kumar et al. 2008).

Partial purification of peroxidase Semi-purified peroxidase was obtained from the crude extract, which was precipitated with pre-cooled acetone (−18 °C). Using a burette the acetone was added slowly down the sides of the container held in an ice bath. The homogenate was kept at 4 °C for 12 to 14 h and centrifuged at 11,000 rpm for 15 min at 4 °C. The supernatant was discarded, and the acetone powder was dried at a temperature of 10–15 °C and dissolved in deionized water (Dubey et al. 2007; Kumar et al. 2008; Singh et al. 2010) and it is used for the peroxidase assay and the estimation (Dubey et al. 2007). The partial purified peroxidase was stored at −18 °C until use (Maciel et al. 2007).

Enzyme assay Peroxidase activity was measured by a change in absorbance at 470 nm, due to oxidation of o-dianisidine (250 µl) in the presence of hydrogen peroxidase (500 nM) in 1 ml reaction mixture (Dubey et al. 2007). One unit of enzyme activity is defined as the amount of enzyme producing a 0.001 absorbance change per minute under the standard assay conditions (Kumar et al. 2008; Dalal and Gupta 2010).

Study of pH optima The optimum pH value (the point where the enzyme is most active) for *Santalum* peroxidase activity was found by assaying enzyme activity at different pH levels. Enzyme activity as a function of pH was determined using buffers of different pH such as sodium acetate buffer (pH 4.0–5.0), Sodium phosphate buffer (pH 6.0–7.0) and Tris–HCl buffer (pH 8.0–9.0). *Santalum* peroxidase activity was assayed under standard conditions (Sakharov et al. 2002; Koksai and Gulcin 2008a, b; Singh et al. 2010) and calculated the per cent relative activity. Percent relative activity is described in term of the change of absorbance per minute or per second amount of sample added to the extract or to the mixture.

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

Effect of temperature and thermal stability The effect of temperature on *Santalum* peroxidase was tested by heating the standard reaction solutions (buffer and substrates) to the appropriate temperatures before introduction of the enzyme. The enzyme was incubated for 10 min at different temperatures ranges from 30 °C to 80 °C and aliquots were withdrawn at regular time intervals and assayed. The percentage relative *Santalum* peroxidase activity was calculated by comparison with unheated enzyme (Koksai and Gulcin 2008a, b).

Determination of effect of Inhibitors (Phenolic compounds, metal ions and amino acids) The 10 µl/ml enzyme was incubated for 10 min with derivatives of hydroxycinnamic

acid such as ferulic acid (0.02–0.08 μM), caffeic acid (1.0–4.5 μM), a hydroxybenzoic acid derivative like protocatechuic acid (1.0–4.5 μM) and growth hormones like indol 3-acetic acid (1.0–4.5 μM). The effects of metal cations Fe^{2+} , Cu^{2+} , Mn^{2+} , Mg^{2+} and Zn^{2+} (1.0–2.0 μM), and different amino acids (D-alanine, DL-valine, DL-methionine, L-proline and L-cysteine) were also examined with concentrations of 1 and 2 mM/ml.

Determination of kinetic constants 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. Under optimal conditions, the efficiency of catalytic oxidation of o-dianasidine by hydrogen peroxide in the presence of POD was evaluated. K_m and V_{max} values were calculated for peroxidase reactions with each of the five substrates, using the Lineweaver-Burk plot transformation of the Michaelis-Menten equation. Michaelis-Menten constant (K_m), is the substrate concentration required for an enzyme to reach one-half its maximum velocity. V_{max} (maximum velocity) is only one kinetic constant of enzymes, where all the enzyme active sites are bound to substrate. K_m and V_{max} values were determined for o-dianasidine/ H_2O_2 substrate pair. For this, the enzyme activity was measured at five different concentrations of o-dianasidine at constant H_2O_2 concentration. In addition, this measurement was performed at a constant concentration of o-dianasidine at five different H_2O_2 concentrations.

Native PAGE electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed under native conditions for separating POD

Fig. 1 Effect of pH on the relative activity of *Santalum* peroxidase

Fig. 2 Effect of temperature on the per cent relative activity of *Santalum* peroxidase

isoenzyme under natural conditions (Laemmli 1970; Koksai and Gulcin 2008a, b). The experiment was conducted with the electrode buffer Tris/Glycine (pH 8.3) using 5 % stacking gel and 6 % separating gels. The 5 μl enzyme samples were loaded on to each well of the stacking gel and the gel was run initially at 60 V followed by 100 V later. After running, gels were incubated in 4.5 mM guaiacol and 22.5 mM H_2O_2 in 100 mM phosphate buffer (pH 7.0) at 37 °C until appearance of the enzyme bands (Koksai and Gulcin 2008a, b; Kumar et al. 2008).

Result and discussion

Effect of pH

Santalum album peroxidase showed the 100 % relative activity at pH 5.0 and it decreased with increase in pH and showed the minimum relative activity at pH 9 (25 %). The Similar observations were reported in case of *Ricinus communis* leaf peroxidase (Kumar et al. 2008). The results of present investigations (Fig. 1) are corroborated with the results of apple peroxidases i.e. acidic pH ranged 5–7 in four different varieties of apple (Dubey et al. 2007; Dalal and Gupta 2010; Singh et al. 2010). Optimum pH of peroxidases was also reported in case of vegetable sources

Fig. 3 Effect of divalent cations on the per cent relative activity of *Santalum* peroxidase

Table 1 Effect of metals ions on the relative activity of *Santalum* peroxidase

Metal ions	Concentration (mM)	Relative activity (%)
control	0	100
CuSO ₄	0.2	140
	0.4	180
FeSO ₄	0.2	290
	0.4	533
MnCl ₄	0.2	150
	0.4	215
MgSO ₄	0.2	130
	0.4	190
ZnSO ₄	0.2	135
	0.4	140

(Vamos-Vigyazo 1981), apoplastic peroxidases from several plant species (Hendricks et al. 1991; Nair and Showalter 1996) at acidic pH. The optimum pH for peroxidase of *Cassia didymobotrya* 5.5 (Vitali et al. 1998), peach 5.0 (Neves 2002), *Ricinus* 5.0 (Kumar et al. 2008), cotton 4.7 (Akhunov et al. 2004) and sunflower 4.0 (Hager et al. 2008). Estimation of secondary structural elements at various pH values indicated that there is a significant reduction of beta-strands and beta-turns at pH 5.5 causing the heme to be further exposed to the solvent and increasing the overall conformational flexibility of the protein (Kamal and Behere 2003).

Effect of temperature

Santalum peroxidases showed maximum per cent relative activity at 60 °C. Similarly highest activities were shown in case of tobacco leaf at 70 °C, (Silva et al., 1990), in *Ricinus* at 60 °C (Ryan et al. 1994), in lima bean (*Phaseolus limensis*) seed at 55 °C (Wang et al. 2008), in sunflower at 50 °C (Hager et al. 2008) and in peach fruit at 40 °C (Neves 2002; Neves and Lourenço 1998) and a ranges of 40–70 °C

Fig. 4 Effect of phenolic compounds derivatives on the relative activity of *Santalum* peroxidase**Table 2** Effect of amino acids on the relative activity of *Santalum* peroxidase

Amino acids	Concentration (mM)	Relative activity (%)
Control	0	100
Cystein	0.2	16
	0.4	11
Alanine	0.2	116
	0.4	118
Valine	0.2	111
	0.4	100
Methionine	0.2	59
	0.4	77
Proline	0.2	104
	0.4	79

for four Indian apple varieties (Dubey et al. 2007; Singh et al. 2010). In the present investigation the relative activity (%) starts decreasing after 60 °C the decline in activity was observed upto (80 °C) (Fig. 2). The influence of temperature at 60 °C and above induces the protein globule unfolding and loosing of peroxidase activity (Moulding et al. 1987; Artiukhov et al. 2003).

Effect of metal ions

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). The divalent metal cations which are used in this study i.e. Fe²⁺, Mn²⁺, Mg²⁺, Cu²⁺ and Zn²⁺ were found to stimulate the relative activity of *Santalum* peroxidase 533, 215, 195, 180 and 140 respectively at 2.0 μM concentration (Fig. 3, Table 1). However, the divalent cation Fe²⁺ was found to be a potent stimulator for peroxidases relative activity of *Santalum* peroxidases. Similar kind of report for

Fig. 5 Effect of amino acids on the relative activity of *Santalum* peroxidase

Table 3 Effects of phenolic compound on the relative activity of *Santalum* peroxidase

Phenolic compounds	Concentration (μM)	Relative activity (%)
Control	0	100
Caffeic Acid	1.0	155
	2.0	175
	3.0	172
	4.0	170
	4.5	170
Protocatechuic Acid	1.0	22
	2.0	11
	3.0	05
	4.0	08
	4.5	01
Indole acetic acid	1.0	43
	2.0	31
	3.0	43
	4.0	26
	4.5	15
Ferulic acid	0.02	73
	0.04	116
	0.06	124
	0.08	131

the Cu^{2+} and Fe^{2+} were showed in case of *Ricinus* peroxidases (Kumar et al. 2008) and apples (Dubey et al. 2007). This activation of enzyme activity indicates that peroxidase require these cations as cofactors. Metals, whether tightly bound to the enzyme or taken up from solution along with the substrate, can participate in catalysis in several ways. Ionic interactions between an enzyme-bound metal and a substrate can help orient the substrate for reaction or stabilize charged reaction transition states. Metals can also mediate oxidation-reduction reactions by reversible changes in the metal ion's oxidation state. Nearly a third of all known enzymes require one or more metal ions for catalytic activity (Nelson and Cox 2007).

Effect of phenolic compounds and growth hormone

The derivatives of hydroxybenzoic acid (Protocatechuic acid) and plant growth hormone (Indol 3-acetic acid) act as a potent inhibitor for *Santalum* peroxidase and shows

Table 4 Substrate specificity for the *Santalum album* peroxidase

Plant peroxidase	$V_{\max}$ (units/min/ml)	$K_m(\mu\text{M})$	$V_{\max}/K_m$
o-dianisidine as a substrate			
<i>Santalum</i> peroxidase	1×10^5	250	400
H_2O_2 as a substrate			
<i>Santalum</i> peroxidase	10×10^5	2.0	5,000,00

Fig. 6 Native-PAGE for *Santalum album* peroxidase isolated from the leaves

following relative activity 22, 11, 5.0, 8.0, 1.0 and 43, 31, 33, 26, 15 at different concentration 1.0, 2.0, 3.0, 4.0 and 4.5 μM . Protocatechuic acid have been also reported as strong inhibitor of peroxidase in *Ricinus* (Kumar et al. 2008) and peach (Neves 2002). Derivative of hydroxycinnamic acid, ferulic acid (0.02, 0.04, 0.06 and 0.08 μM) at very low concentration triggered the per cent relative activity of *Santalum* peroxidase i.e. 73, 116, 131 and 124. Caffeic acid derivative of hydroxycinnamic acid (concentration 1.0, 2.0, 3.0, 4.0 and 4.5 μM) also enhanced the activity of peroxidases in *Santalum* peroxidase which was recorded as 155, 175, 172, 170 and 170 of the *Santalum* peroxidase (Fig. 4, Table 2). The similar results were also showed for leaves peroxidase of *Ricinus* (Kumar et al. 2008). Peroxidase can exist in several oxidation states (Yamazaki and Yokota 1973). In the presence of NADH

and under aerobic conditions, peroxidase exists predominantly as compound III (oxyperoxidase) with its heme iron in the three equivalent oxidized state (Fe^{6+}) (Yamazaki and Yokota 1973). In the peroxidatic reaction, phenolic compounds are especially effective as electron donors for the intermediates compound I (Fe^{5+}) and compound II (Fe^{4+}) (Mader and Fussl 1982).

Effect of amino acids

Amino acids showed the variable responses against the *Santalum* peroxidases, some amino acids worked as stimulator while others are act as inhibitor of *Santalum* peroxidase percent relative activity. In the present investigation the tested amino acids D-alanine, L-proline and DL-valine showed the significant stimulation of the per cent relative activity by 118, 104 and 100 respectively at the concentration of 2 mM each. On the other hand, moderate inhibition was observed in the case of DL-methionine and L-cysteine, the per cent relative activity was 59 and 16 respectively (Fig. 5, Table 3). In case of apple methionine, proline, tryptophan, and valine stimulated all four apple peroxidases, but cysteine showed inhibition in Golden delicious JK (Dubey et al. 2007; Singh et al. 2010). It was observed that the L-cysteine, inhibit the enzyme activity in case of peach (Neves 2002). Similarly, *Ricinus* peroxidases were stimulated by amino acids like L-proline and L-alanine, while DL-methionine and L-valine showed strong inhibition (Kumar et al. 2008).

Determination of kinetic constants

K_m and V_{max} values were calculated from the plot of $1/V$ versus $1/[S]$ by the method of Lineweaver and Burk (1934) following the optimum pH and temperature conditions (Kumar et al. 2008). The V_{max}/K_m value for o-dianisidine was 400 units/min/mL and for H_2O_2 5.0×10^5 units/min/mL for *Santalum*, respectively. Table 1 shows the kinetic constants of *Santalum* peroxidase purified from *Santalum album* (Table 4). The ratio of V_{max}/K_m indicated a preferential action of the enzyme for hydrogen peroxide, compared to o-dianisidine, which is in contrast to peroxidases from apple (Clemente and Pastore 1998; Dubey et al. 2007; Singh et al. 2010), papaya (Silva et al. 1990), kiwifruit (Soda et al. 1991) and *Ricinus* (Kumar et al. 2008).

Native PAGE

The zymographic studies showed that the acetone precipitated *Santalum* peroxidase isolated from the leaves showed two distinct bands on the gel (Fig. 6). Similarly, Native-PAGE result observed i.e. seven distinct bands in case of *Ricinus communis* (Kumar et al. 2008) and three bands in

case of crude and two bands for purified peroxidase from peach fruit (Neves 2002), different banding pattern of enzymes proved that enzyme are present in more than one form (isozymes).

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