Journal of Molecular Catalysis B: Enzymatic 68 (2011) 30–36

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Isolation and partial characterization of phenol oxidases from *Mangifera indica* L. sap (latex)

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ARTICLE INFO

Article history:
Received 18 March 2010
Received in revised form 23 July 2010
Accepted 15 September 2010
Available online 22 September 2010

Keywords:
Mangifera indica
Anacardiaceae
Mango sap
Peroxidase
Polyphenol oxidase
Laccase

ABSTRACT

Mango sap (latex), a by-product in mango industry, was separated into upper non-aqueous phase and lower aqueous phase. Aqueous phase contains very low protein (4.3 mg/ml) but contains high specific activities for peroxidase and polyphenol oxidase. The aqueous phase of sap was subjected to ion-exchange chromatography on DEAE-Sephacel. The bound protein was separated into three enzyme peaks: peak I showed peroxidase activity, peak II showed polyphenol oxidase activity and peak III showed activities against substrates of peroxidase as well as polyphenol oxidase. On native PAGE and SDS-PAGE, each peak showed a single band. Based on the substrate specificity and inhibitor studies peak III was identified as laccase. Although they showed variations in their mobility on native PAGE, these enzymes showed similar molecular weight of 100,000 ± 5000. These enzymes exhibited maximum activity at pH 6 however, polyphenol oxidase showed good activity even in basic pH. Peroxidase and polyphenol oxidase showed stability up to 70 °C while laccase was found to be stable up to 60 °C. Syringaldazine was the best substrate for laccase while catechol was the best for polyphenol oxidase. Thus, mango sap a by-product in mango industry is a good source of these phenol oxidases.

1. Introduction

Polyphenol oxidase is a generic term used for the group of enzymes that catalyses the oxidation of different phenolic compounds. Based on substrate specificity these polyphenol oxidases have been named as tyrosinase or monophenol monooxidase or cresolase (EC 1.14.18.1), catechol oxidase or diphenol oxidase (EC 1.10.3.1), laccases or *p*-diphenol oxygen oxidoreductase (EC 1.10.3.2) and these enzymes are present in various fungi and plant tissues [1,2]. The presence of laccase in the exudates of Japanese lacquer tree, *Rhus vernicifera*, was discovered as early as 1883 [2,3]. Peroxidase (EC 1.11.1.7; donor: hydrogen-peroxide oxidoreductase) is an another oxidative enzyme present in different tissues of plants and animals. Peroxidase oxidises a variety of phenolic and amines in the presence of hydrogen peroxide while polyphenol oxidases oxidizes phenolic compounds in the presence of molecular oxygen. There is a considerable overlap in their substrate specificities of these three phenol oxidases. However, these enzymes may be differentiated by their specificities to certain substrates and inhibitors [4,5]. Some of the attributed functions of these three groups of enzymes are similar. These oxidases have been implicated in plant senescence, fruit ripening and defensive role against insects [6–8] and are also involved in protein cross-linking [9]. Due to wide substrate specificity, laccase and peroxidase are used for a wide variety of applications in food processing and biological applications, analytical chemistry, immunochemical studies, water purification, biotransformation of various valuable chemicals and biosensors [2,10–12].

Mango is one of the major tropical fruits and India produces about 40% of the world production. Mango fruit has duct system, which continues into the stalk but ends before the abscission zone. These fruit ducts contain a viscous liquid referred to as mango sap (latex) [13,14]. During harvesting of mango fruits, sap initially spurts and then oozes out onto the surface of the fruits and causes sap-injury. Sap-injury is characterised as darkening or browning of the peel due to contact with the sap. Sap-injury not only reduces consumer acceptance of the fruit, but also lowers shelf-life of the fruit as the injured regions of the peel tend to be more susceptible to fungal or bacterial infections. De-sapping of the mangoes is one of the methods practiced to control sap-injury and the sap thus obtained is currently being wasted.

Sap can be separated into two phases, aqueous and non-aqueous. Earlier, we have reported that non-aqueous phase has high specific activities of polyphenol oxidase (catechol oxidase) and peroxidase [15]. In the present study we report the purification of sap enzymes and their properties. The presence of PPO/laccase has been reported by few workers [15–17] while presence of peroxidase in sap has been reported only by Saby John et al. [15]. In the

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doi:10.1016/j.molcatb.2010.09.004

present study we have purified these enzymes in a single step by ion-exchange chromatography on DEAE-Sephacel and studied their biochemical properties.

2. Materials and methods

2.1. Plant materials and chemicals

Raspuri mango variety grown in CFTRI campus, Mysore, Karnataka, India, was used in the present study. Sephadex G-200 was obtained from Pharmacia Biochemicals, Uppsala, Sweden. DEAE-Sephacel, SDS, β-mercaptoethanol, acrylamide, *N*,*N*′ bisacrylamide, Tris, protein markers, *o*-dianisidine, syringaldazine, *p*-phenylenediamine, guaiacol, catechol were obtained from Sigma, USA. All other chemicals used in this study were of analytical reagent grade chemicals. Glass double distilled water was used throughout this research.

2.2. Separation of aqueous phase from mango sap (latex)

As described earlier [18], mango fruits were harvested with pedicel intact and subsequently, the pedicels were detached from the fruit at the abscission zone and the sap was collected in to the glass tube for about 1 min. The sap thus collected was separated into aqueous and non-aqueous phases by centrifugation at 3000 × *g* at room temperature. Aqueous phase thus collected was stored at 4 °C until further use.

2.3. Protein estimation

Protein was estimated by the dye binding method described by Bradford [19] using bovine serum albumin as a standard.

2.4. DEAE-Sephacel ion-exchange chromatography

Mango sap proteins were diluted with equal volume of 50 mM Tris–HCl buffer, pH 8.0 and loaded onto DEAE-Sephacel column (3.0 cm × 16.5 cm) which was pre-equilibrated with the same buffer. The bound protein was eluted using 0.025 M, 0.05 M, 0.075 M and 0.1 M sodium chloride in 0.05 M Tris–HCl buffer, pH 8.0, in a step-wise manner. The flow rate was maintained at 10 ml/h and 2 ml fractions were collected, monitored for protein by determining the absorbance at 280 nm and assayed for PPO and POD in the individual peaks. The enzyme fractions, 85–100 (peak I); 120–130 (peak II); and 150–165 (peak III) were pooled separately and used for further studies.

2.5. Electrophoresis

2.5.1. Polyacrylamide gel electrophoresis of purified fractions (PAGE)

The fractions obtained from the chromatography were subjected to electrophoresis on 7.5% native gel and the protein loaded in each lane was 10 μg. The gel composition, running buffer composition, staining and destaining procedures were followed according to the method described by Laemmli [20] without SDS and β-mercaptoethanol. The protein and enzyme staining was done as per the procedures described by Saby John et al. [15].

2.5.2. SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on 10% gel in the presence of 0.1% sodium dodecyl sulphate according to the method of Laemmli [20]. A mixture of molecular weight markers containing carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (66,000), rabbit muscle phosphorylase b (97,400), *Escherichia coli* β-galactosidase (116,000) and rabbit muscle myosin (205,000), as well as the enzyme samples prepared in 1% SDS and 5% mercaptoethanol, were boiled for 5 min. The pH of the running buffer was 8.5 and a constant voltage of 50 V was employed. An amount of 10 μg of protein was loaded in each lane. Following the run, the proteins were stained with 0.25% Coomassie brilliant blue for 5 h and destained with 7% acetic acid, 10% methanol and 83% water. The molecular weights of the enzymes were calculated from the standard graph obtained using R_f values of standards vs. their molecular weight.

2.6. Determination of molecular weight of the purified enzymes using gel filtration chromatography on Sephadex G-200

The molecular weights of the purified enzymes were determined using Sephadex G-200 gel filtration column which was calibrated using protein standards. The void volume was determined using blue dextran.

2.7. Peroxidase assays

The peroxidase assay was carried out using 0.25% *o*-dianisidine as substrate as described by Saby John et al. [15]. One unit of activity was defined as that amount of enzyme which produced an increase in absorbance of 1/min at 460 nm. For substrate specificity studies of peak I enzyme increase in absorbance was monitored at 460 nm for diaminobenzidine and *p*-phenylenedaimine; 470 nm for guaiacol, 650 nm for tetramethylbenzidine. All these substrate were solubilized in water except guaiacol which was solubilized in ethanol.

2.8. Effect of inhibitors on peak I (POD)

The effect of varying concentrations of inhibitors on the activity of enzyme in peak I was determined. The compounds tested were sodium azide, potassium cyanide, hydrazine, dithiothrietol, ethylenediaminetetracetic acid and cetyltrimethylammonium bromide. With the exception of hydrazine which is alcohol-soluble, all the compounds were solubilized in water. Appropriately diluted enzyme (100 μl; 50–60 U) was incubated with 100 μl of varying concentrations of inhibitor in 600 μl of 50 mM sodium acetate buffer, pH 6.0 at room temperature for 5 min. Hydrogen peroxide (1%; 100 μl) and *o*-dianisidine (0.25%; 100 μl) were then added to the reaction mixture and the absorbance was recorded at 460 nm for 3 min. The enzyme unit was defined as mentioned earlier.

2.9. Polyphenol oxidase assays

The polyphenol oxidase assays were carried out using 0.5 M catechol substrate as described by Saby John et al. [15]. One unit of activity was defined as that amount of enzyme which produced an increase in absorbance of 1/min at 420 nm. For substrate studies of peaks II and III enzymes, increase in absorbance was monitored at 420 nm for catechol, 4-methyl catechol, *p*-quinol, and *o*-dianisidine; 460 nm for β-napthol; 525 nm for syringaldazine; 410 nm for *p*-phenylene diamine; 280 nm for tyrosine. All these substrate were solubilized in water except syringalazine and β-napthol which were solubilized in ethanol.

2.10. Effect of inhibitors on peaks II and III (polyphenol oxidases)

The effect of varying concentrations of inhibitors on the activities of enzymes in peaks II and III was determined. The compounds tested were ascorbic acid, cinnamic acid, citric acid, sodium chloride, sodium thiosulphate, cysteine, cetyltrimethylammonium bromide and polyvinylpyrrolidone. With the exception of cinnamic

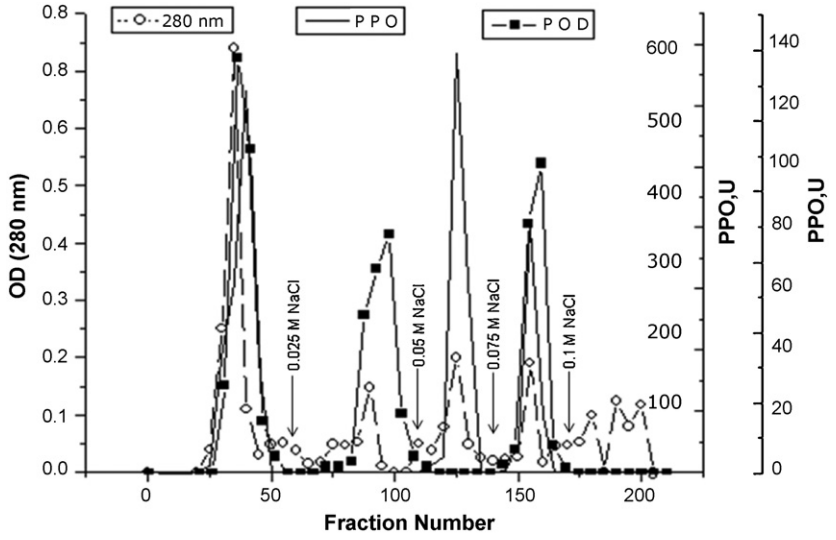

Fig. 1. Anion-exchange chromatography of mango sap enzymes on DEAE-Sephacel column (3.0 cm × 16.5 cm). Fraction size, 2 ml. Peak I, peroxidase; peak II, polyphenol oxidase; peak III, showed both peroxidase and polyphenol oxidase activities (laccase).

acid which was solubilized in methanol, all the other compounds are solubilized in water. Appropriately diluted enzyme (100 μl; 50–60 U) was incubated with 100 μl of varying concentrations of inhibitor in 700 μl of 50 mM sodium acetate buffer, pH 6.0 at room temperature for 5 min. Catechol (0.5 M; 100 μl) was then added to the reaction mixture and the absorbance was recorded at 420 nm for 3 min. The enzyme unit was defined as mentioned earlier.

2.11. Effect of substrate concentration on the purified enzymes

The enzyme activities of PPO and laccase were determined at varying concentrations of catechol (5–100 mM) and that of POD was determined at varying concentrations of *o*-dianisidine (0.2–2.5 mM) and H_2O_2 (3–50 mM) in the 1 ml reaction mixture. All other assay conditions were maintained the same as described earlier.

2.12. Effect of pH

The effect of pH on the enzymes PPO, laccase and POD was studied at pH values ranging from 4.0 to 6.0 (50 mM sodium acetate buffer); 6.0 to 8.0 (0.05 M sodium phosphate buffer) and 8.0 to 9.0 (0.05 M Tris–HCl buffer). The assays were carried out as described earlier.

2.13. Temperature stability of enzymes

The purified enzymes were incubated in the sodium acetate buffer (pH 6.0) at 30, 40, 50, 60, 70, 80 and 90 °C for 15 min and assayed for enzyme activity as described earlier.

3. Results and discussion

3.1. Composition of mango sap

Raspuri variety mango sap was separated into upper non-aqueous phase and lower aqueous phase by simple centrifugation. The aqueous phase content was 110 ml/100 kg of mango and the non-aqueous phase was 14 ml/100 kg mango. The aqueous phase was rich in non-starchy carbohydrate (309 mg/ml sap). It contained very little protein (4.3 mg/ml) but has very high specific activities for peroxidase (POD; 808 U/mg protein) and polyphenol oxidase (PPO; 218 U/mg protein).

3.2. Purification of enzymes

Acetone precipitation $(NH)_2SO_4$ precipitation methods were used as a first step to purify enzymes. However, most of the activities of these enzymes were lost during precipitation. Gel filtration chromatography on Sephadex G-200, PPO and POD enzymes eluted as one peak. However, when aqueous phase of sap was subjected to chromatography on DEAE-Sephacel, nearly 35% of POD and 22% of PPO eluted from the column as unbound enzyme (Fig. 1). This may not be due to the over loading of the protein (enzyme) since only 6 mg of the protein was loaded on to the column which was much below the capacity of column. Therefore, the unbound PPO and POD activities may be due to different isozymes. The presence of several isozymes of PPO and POD has been reported by different workers [1,7,21–23]. However, as the specific activity of unbound peak was very low comparable to crude enzyme preparation, this fraction was not further studied for its properties.

The bound proteins were eluted using a stepwise sodium chloride gradient (0.025 M, 0.05 M, 0.075 M, 0.1 M NaCl) in 50 mM Tris–HCl buffer (pH 8.0) (Fig. 1). Eluting the column with 0.025 M NaCl in 50 mM Tris–HCl (pH 8.0) eluted a protein peak which showed only POD activity (peak I). Elution with 0.05 M NaCl resulted in the elution of another protein peak which showed only PPO activity (peak II). Elution with 0.075 M NaCl in Tris buffer eluted a protein peak, which showed both PPO and POD activities (peak III). Fractions eluted with 0.1 M NaCl did not show PPO or POD activity (Table 1; Fig. 1).

3.2.1. Electrophoresis

Enzyme peaks I, II and III obtained during elution with NaCl were subjected to native PAGE as well as SDS-PAGE for their purity evaluation. All the three peaks showed a single band on both native PAGE and SDS-PAGE indicating that the peaks obtained on ion-exchange chromatography were pure (Figs. 2 and 3). Following native PAGE, peak I stained only for POD (Fig. 2C, lane 3), peak II stained only for PPO (Fig. 2B, lane 1) and peak III stained for both PPO and POD (Fig. 2B, lane 2 and Fig. 2C, lane 2) and are in agreement with the enzyme activity observed in the chromatography. Thus, the results indicated that peak I is POD and peak II is PPO. Peak III enzyme showed a sharp band by enzyme and protein staining by both PAGE and SDS-PAGE and showed significantly different mobility on Native PAGE compared to other peaks. Based on the substrate and inhibition studies peak III was identified as laccase, which

Table 1
Purification of peroxidase and polyphenol oxidase of mango sap on DEAE-Sephacel column.

Enzyme fractions	Protein (mg)	PPO		Purification		POD		Purification	
		Total activity (U)	Specific activity	Fold	Yield (%)	Total activity (U)	Specific activity	Fold	Yield (%)
Crude	6.20	1350	218	1	100	5010	808	1	100
Peak I	0.48	–	–	–	–	1320	2750	3.4	26
Peak II	0.52	680	1308	6.0	50	–	–	–	–
Peak III	0.53	268	506	2.3	19.8	1154	2177	2.7	23

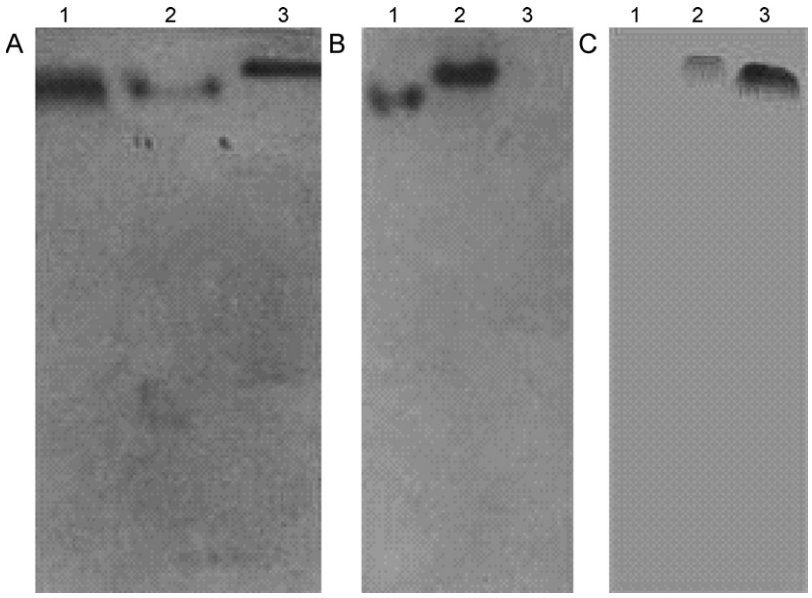

Fig. 2. Native PAGE of peroxidase, polyphenol oxidase and laccase. (A) Coomassie stain for proteins: lane 1, peroxidase (peak I); lane 2, polyphenol oxidase (peak II); lane 3, laccase (peak III); (B) polyphenol oxidase stain: lane 1, polyphenol oxidase (peak II); lane 2, laccase (peak III); lane 3, peroxidase (peak I); (C) peroxidase stain: lane 1, polyphenol oxidase (peak II); lane 2, laccase (peak III), lane 3, peroxidase (peak I).

oxidized some of the substrates of both PPO and POD as described later (results on substrate and inhibitor studies, Section 3.3.1).

The mobilities of these three enzyme peaks on SDS-PAGE were similar, indicating similarity in their molecular weights (Fig. 3). The molecular weights of these enzymes were found to be 105,000. The molecular weight of the three peaks as determined by gel filtration chromatography on Sephadex G-200 was estimated to be 100,000.

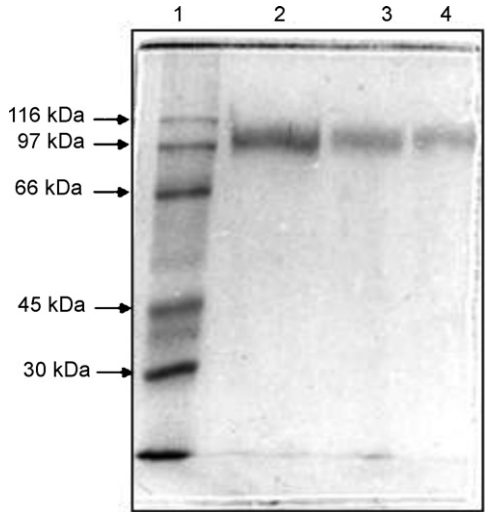

Fig. 3. SDS-PAGE of purified mango sap enzymes: lane 1, standard protein markers; lane 2, peroxidase (peak I); lane 3, polyphenol oxidase (peak II); lane 4, laccase (peak III).

Thus, all these three enzymes had showed single subunit with a molecular weight around 100,000.

There are no reports regarding the molecular weight of mango sap PPO or laccase. Although Joel et al. [16] and Rabinson et al. [17] have reported some of the properties of crude laccase enzyme from mango sap, they have not purified the enzyme, and therefore, the molecular weight of this enzyme was not reported. However, laccase from other exudates like *Schinus molle*, *R. vernicifera* are reported and their molecular weights are either comparable or little higher than mango sap laccase reported in the present study. Bar-Nun et al. [24] reported molecular weight of a laccase enzyme isolated from *S. molle* exudate by using SDS-PAGE and ultracentrifugation methods, and they were found to be 96,000 and 105,000, respectively. Yun-Yang et al. [25] reported the molecular weight of laccase and its isoform isolated from sap of Chinese lacquer tree (*R. vernicifera)* using SDS-PAGE, and they reported be 109,000 and 103,000, respectively. Normally molecular weights reported for fungal laccases varies between 60 and 80 kDa, but molecular weights as high as 390 kDa also reported and these laccases consists of either single subunit or multiple subunits [2,10,26]. However, the molecular weights of mango sap laccase, and laccases from *S. molle* and *R. vernicifera* laccase have closer molecular weights. Moreover, all these sap laccases have single subunit. For PPOs, molecular weights ranging from 33 kDa to more than 200 kDa was also reported in higher plants [27,28].

Molecular weights of peroxidases from plant sources have been reported to range from around 35 kDa to 105 kDa. Horseradish peroxidases and turnip peroxidase were reported have molecular weights of 40–46 kDa and 49 kDa, respectively [29], for black gram husk and wheat bran peroxidases it was reported as 35 kDa and

Table 2
Substrate specificity of peak I (POD) of mango sap.

Substrate	Concentration (mM)	Activity (%)
o-Dianisidine	1	100
Tetramethyl benzidine	1	12.5
Diaminobenzedine	1	20.8
p-Phenylenediamine	1	70.8
Guaiacol	50	16

Table 3
Effect of inhibitors on peak I (POD) of mango sap.

Inhibitor	Concentration (mM)	Inhibition (%)
NaN_2	10	72.8
KCN	0.02	85.7
Hydrazine	10	87.7
DTT	10	56.6
EDTA	10	38.4
CTAB	10	66.4

44 kDa, respectively [30,31]. The molecular weights of isoperoxidases isolated from wheat seedlings were reported to be 85 kDa and 24 kDa [32], for potato isoperoxidases it was reported to be between 48.5 and 105 kDa [33]. The molecular weights of animal peroxidases like eosinophil peroxidases were reported as 77 kDa and 118 kDa [34], human term placental peroxidase was estimated to be 119 kDa [35] and that of uterine peroxidase was 92 kDa [36].

3.3. Characterization of enzyme activities in different peaks

3.3.1. Substrate specificity and inhibitor studies

Different compounds were tested as hydrogen donor substrates for the peak I enzyme. The enzyme showed activity with *o*-dianisidine, *p*-phenylenediamine, tetramethyl benzidine, diaminobenzidine and guaiacol in the presence of H_2O_2. However, 1 mM *o*-dianisidine was found to be the best substrate for this enzyme followed by *p*-phenylenediamine. Tetramethylbenzidine, guaiacol and diaminobenzidine, gave only 12.5–21% of activity obtained with *o*-dianisidine, indicating that these are poor substrates for POD (Table 2). Potassium cyanide inhibited the peak I enzyme activity up to 85% at a concentration of 0.02 mM. Hydrazine and sodium azide also inhibited the enzyme activity more effectively (Table 3). Thus, the peak I enzyme was found to be peroxidase.

As can be seen from Table 4, enzymes in peaks II and III showed very good activity with catechol as substrate followed by 4-methyl catechol. β-Napthol was found to be a better substrate for peak III compared to peak II enzyme. The enzyme in peak III oxidized syringaldazine, *p*-quinol and phenylenediamine and these substrates were not oxidized by peak II enzyme. Peak III enzyme had also oxidized *o*-dianisidine in the absence of hydrogen peroxide. Tyrosine was not a substrate to any of the enzymes in these peaks.

Joel et al. [16] reported that *p*-quinol and 4-methyl catechol as substrates for mango sap laccase, however, they reported that

Table 4
Substrate specificity of peak II (PPO) and peak III (laccase) of mango sap.

Substrate	Concentration (mM)	Peak II Relative activity (%)	Peak III Relative activity (%)
Catechol	5	100	100
4-Methyl catechol	50	48.1	52
β-Napthol	50	18.5	90.7
Tyrosine	1	0	0
p-Quinol	20	0	7.2
p-Phenylenediamine	0.5	0	8
Syringaldazine	0.1	0	204
o-Dianisidine	2	0	96

Table 5
Effect of inhibitors on peak II (PPO) and peak III (laccase) of mango sap.

Inhibitor	Concentration (mM)	Inhibition (%)	
		Peak II	Peak III
Ascorbic acid	5	80.4	100
Citric acid	100	77.6	82
NaCl	200	74.8	77.5
Sodium thiosulphate	0.5	94.4	39.9
Cysteine	5	77.4	95.5
PVP	30	52.3	45.9
CTAB	20	77.6	77.5
Cinnamic acid	5	30.0	100

syringaldazine was not oxidized by mango sap laccase. Sap PPOs of Raspuri variety from India showed more activity with catechol than 4-methylcatechol whereas Rabinson et al. [17] reported that PPO (laccase) of Kensington variety from Australia showed more activity with 4-methyl catechol than catechol. In the present study, syringaldazine was found to be the best substrate for peak III enzyme and the oxidation of *o*-dianisidine by this enzyme was comparable to that of catechol and the enzyme activity with *o*-dianisidine in the presence of hydrogen peroxide increased by 8-fold. Ratcliffe et al. [37] reported that *Agaricus bisporus* mushroom laccase oxidised *o*-dianisidine in the absence of hydrogen peroxide and a moderate increase in the laccase activity was reported in the presence of hydrogen peroxide.

The commonly used PPO inhibitors like ascorbic acid, cysteine, citric acid, sodium chloride, potassium cyanide inhibited the activities of peaks II and III enzymes (Table 5). Ascorbic acid and cysteine more effectively inhibited the peak III enzyme activity compared to that of enzyme of peak II. Several workers have used ascorbic acid and cysteine for PPO inhibition [38–41]. Ascorbic acid, cysteine are reported to prevent browning by reducing the oxidized product, quinone to diphenol. In addition, it was also reported that ascorbic acid irreversibly inhibits the PPO activity by affecting the histidine residues present at the active site [42]. Polyvinylpyrrolidone (PVP) and cetyltrimethylammonium bromide (CTAB) had shown similar inhibitory effects on both peaks II and III enzymes. Earlier, it was reported that PVP inhibited catechol oxidases but not laccases and it acted as a competitive inhibitor on catechol oxidases [5]. On the other hand, Walker and McCallion [43] reported that CTAB selectively inhibited laccase. However, in the present study, PVP and CTAB inhibited both type of PPOs. It is to be noted that inhibition by different compounds may be depends on the type of substrate employed in the enzyme assays. For example, CTAB inhibited Agaricus laccase up to 50% when syringaldazine was used as substrate, while no inhibition was observed with *p*-cresol or phenylene diamine [37]. Cinnamic acid completely inhibited the peak III enzyme activity whereas in case of peak II, only 30% of peak II activity was inhibited. Rabinson et al. [17] also reported that mango sap crude laccase enzyme was completely inhibited by cinnamic acid.

Though peaks II and III showed good activity with catechol and 4-methyl catechol indicating that they are PPOs but they showed lot of differences with regard to substrate specificities and inhibitors. Catechol oxidases, tyrosinases, laccases and peroxidases are present in many plant tissues and cause browning in many fruits and vegetables [7,8]. It is difficult to differentiate these enzymes because these enzymes oxidize similar substrates. Peroxidases require H_2O_2 as another substrate apart from phenols, and for tyrosinases, monophenols are specific substrates. However, differentiation between diphenol oxidases such as catechol oxidases and laccases is difficult because they use similar substrates and also inhibited by common compounds. On the other hand, laccases have wide substrate specificity. Laccases oxidize most of the *o*-diphenols that are oxidized by catechol oxidases. In addition, laccases are reported to catalyze the oxidation of syringaldazine,

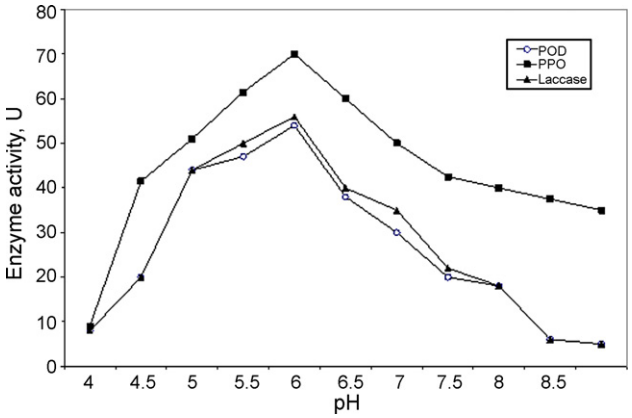

Fig. 4. Optimum pH for activities of peroxidase, polyphenol oxidase and laccase of mango sap.

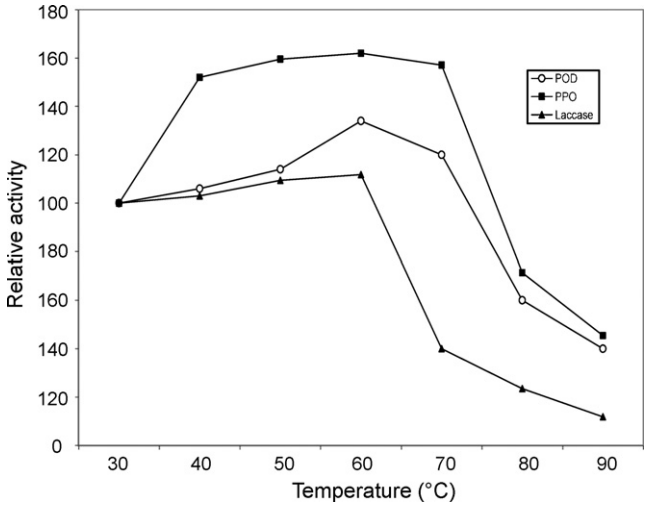

Fig. 5. Temperature stability of peroxidase, polyphenol oxidase and laccase of mango sap.

p-phenylenediamine, *o*-dianisidine [5,37,44]. Thus, selective substrates and inhibitors are reported to differentiate these two types of PPOs [5,37,45]. Ratcliffe et al. [37] reported that *A. bisporus* mushroom laccase oxidised both syringaldazine and *o*-dianisidine. In the present study, the results indicated that though both peaks II and III enzymes showed similarity with regard to the oxidation of certain substrates and inhibition by some compounds, they showed differences between peak II PPO and peak III PPO with respect to substrate specificities, and also activity inhibition by different compounds. Peak III enzyme oxidized more effectively syringaldazine and *o*-dianisidine, whereas peak II enzyme did not react with any of these two substrates. Based on these studies it can be inferred that peaks II and III enzymes are PPOs but peak II is catechol oxidase type PPO and peak III is laccase type PPO.

3.3.2. Other properties of mango sap POD, PPO and laccase

All these three enzymes showed good activity between pH 5 and 7 with pH optima of 6.0 (Fig. 4). POD and laccase showed decrease in activity in basic pH whereas PPO showed good activity even at basic pH, about half of the maximal activity was seen at pH 9.0. Mango pulp PPO also exhibited 46% of the maximal activity at pH 9.0 [41]. The pH optimum of POD from grape was 5.4, banana, 4.5–5.0, pineapple 4.2, HRP, 4.5–5.8, potato, 5.0–5.4 [7,21]. The pH optimum of PPO from mango peel was reported to be 5.4 [46], whereas that of the enzyme from pine apple ranged from 6 to 7 [47] while in DaChauna grapes it was 6.0 [48]. Joel et al. [16] reported laccase activity in mango sap, exhibited optimum activity at pH 6.0. Rabinson et al. [17] reported that sap PPO (laccase) was active between 4 and 7 with a pH optimum of 5.3. Bar-Nun et al. [24] reported a pH optimum of 6.2 for the laccase from *S. molle*.

The effect of substrate concentration on purified enzymes was studied by assaying their activity at different concentrations of substrates. For PPO and laccase catechol was used as substrate and for POD hydrogen peroxide and *o*-dianisidine were used as substrates. In the case of PPO and laccase, the activity of enzymes increased with increasing concentration of catechol and reached maximum at 33 mM concentration of catechol. However, in the case of laccase, beyond this concentration the enzyme activity decreased. The K_m values for PPO and laccase calculated based on Lineweaver–Burke plots of on initial velocity of the reaction were 12.5 mM and 8.33 mM respectively. POD activity also increased with increase in substrate concentration and reached maximum between 12.5 and 16.7 mM of hydrogen peroxide and 1.25 mM concentration of *o*-dianisidine and beyond this concentration incase of hydrogen peroxide, the POD activity gradually decreased whereas incase of *o*-dianisidine the activity decreased very sharply. The K_m values of H_2O_2 and *o*-dianisidine calculated from the Lineweaver–Burke plots was 2.22 mM and 0.9 mM for POD. The K_m value of mango peel PPO for catechol was reported to be 7.2 mM [46]. Bar-Nun et al. [24] reported a K_m of 1.25 mM for the laccase from *S. molle*.

Temperature stability studies indicated both POD and laccase activities gradually increased with increase in temperature incubated up to 60 °C. Heating above this temperature resulted in loss of activity and residual enzyme activity of POD and laccase observed on heat treatment at 90 °C for 15 min were 40% and 12% respectively (Fig. 5). Potato and cauliflower POD could be completely and irreversibly inactivated by heat treatment at 95 °C. One of the isoforms of laccase isolated from *Trametes versicolor* also showed temperature stability up to 60 °C [49]. The sap PPO showed temperature stability between 40 °C and 70 °C and after this the enzyme activity sharply decreased and it showed residual activity of 45% at 95 °C temperature for 15 min. Prabha and Patwardhan [46] reported temperature stability for PPO from mango peel up to 50 °C. Thus, mango sap PPO has a greater thermal stability than the peel enzyme.

4. General discussion and conclusion

Joel et al. [16] reported presence of laccase in mango sap and Rabinson et al. [17] reported some of the biochemical properties of crude PPO and concluded that sap PPO was laccase type PPO. Till date no reports on the presence of peroxidase and catechol oxidase type PPO in mango sap, and also for the first time sap enzymes are purified. We found that major protein band obtained on native PAGE showed both PPO and POD activities. Though these enzymes eluted together on gel filtration column, we were able to separate these enzymes on DEAE-Sephacel into three distinct peaks with different enzyme activities. The first peak showed POD activity whereas the second and third peaks showed PPO activities. The third peak enzyme also oxidized *o*-dianisidine, which is also a substrate for POD. On both native PAGE and SDS-PAGE each peak showed single band. These enzymes had similar pH optima and had single subunit with similar molecular weight. But, they showed little difference in mobility on native PAGE and they were separated on DEAE-Sephacel column with little change in the salt concentration in the eluent. They also showed some differences in their temperature stability.

All these enzyme peaks showed differences in substrate specificities and inhibition properties to different extents. The peaks (peaks II and III) showed PPO activity with catechol, and their activities were inhibited by ascorbic acid, citric acid, sodium chloride, sodium thiosulphate, cysteine, PVP, citric acid and CTAB to

different extents. However, peak III enzyme reacted with syringaldazine, a characteristic substrate of laccase, more effectively compared to catechol. Even this enzyme also reacted with *o*-dianisidine a characteristic substrate of peroxidase. Peak III PPO also showed activity with *p*-quinol, *p*-phenylene diamine whereas, peak II PPO did not show any activity with these substrates and also activity observed with β-napthol was less compared to peak III PPO. Cinnamic acid and ascorbic acid inhibited the peak III PPO activity completely. Thus, the evidences suggest that peak II is catechol oxidase type of PPO whereas peak III is a laccase type of PPO.

Physiological role of these phenoloxidases in sap is not known but these enzymes promote polymerization of quinones formed in the oxidation of the polyphenols which results in hardening of the tissue, thus protecting fruit from further attack by insects [27]. In mango industry sap-injury is a major problem, and several approaches have been used for the control of sap-injury and one of the approaches followed is desapping of mango and sap thus obtained is a by-product in mango industry. Earlier, we reported that organic phase of sap is a rich source of terpenoids which are used in various food products in ppm levels as a flavouring agent [18] and also it has antimicrobial properties [50]. Aqueous phase can be a good source of phenolases which have several applications in food processing and analytical biochemistry.

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