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Ficus sycomorus latex: A thermostable peroxidase

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Peroxidase from sycamore fig *Ficus sycomorus* latex (POLI) was purified by heat treatment, anion exchange chromatography and molecular exclusion chromatography. The purity was determined from high specific activity (9166 units/mg protein), purification fold (28), RZ value 3.1 and a single band in native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE and visualized peroxidase activity on the PAGE. POLI had molecular mass of 43 kDa. Substrates commonly used in immunodiagnostic kits as 2,2-azino-bis [3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS), 4-chloro-1-naphthol (4C-1N), *o*-phenylenediamine (OPD) and 3,3`,5,5`- tetramethylbenzidine (TMB) were found to be the best substrates for the enzyme. The K_m for catalysis of H_2O_2 was 1.2 mM. The catalytic efficiency (Vmax/Km) for POLI was found to follow the order: ABTS, 4C-1N, OPD, TMB, guaiacol, *p*-aminoantipyrine, *o*-dianisidine and pyrogallol. The enzyme showed a broad pH optimum ranged from pH 5.5 to 7.0. The optimal temperature for the enzyme was 35 to 40°C. POLI showed highest thermal stability. No loss of enzyme activity was recorded up to 60°C, whereas only 20% of enzyme activity was lost at 70 to 90°C. The thermal inactivation profiles of POLI demonstrated that the enzyme had higher thermal resistance. The peroxidase activity was slightly enhanced by low concentration of Ca^{2+}, Ni^{+2} and Mg^{2+} and high concentration of Mn^{2+}. Fe^{+3}, Zn^{+2}, Hg^{+2} caused slightly inhibitory effects. In conclusion, sycamore fig latex will be a new and potential source for a peroxidase enzyme.

Key words: *Ficus sycomorus*, latex, peroxidase, purification, characterization.

INTRODUCTION

Latex is a complex emulsion secreted by specialized cells named laticifers. In some plants, the latex is a form of stored food, while in other plants the latex contains specialized metabolites which represent biochemical end products that do not re-enter the primary metabolism such as terpenoids, cardiac glycosides, alkaloids, lignin, cannabinoids and tannins (Evert, 2006). Latex not only serves as a repository for natural products but also exhibit unique proteomes, including proteases, esterase, glucanases, lipase, peroxidase and chitinase (Domsalla and Melzig, 2008; Mura et al., 2005; Van der Horn and Jones, 2004). The primary function of latex is to protect the plant in case of injuries and drying by forming a protective film that prevents the entry of fungi and bacteria. Both proteins and metabolites within latex have been shown to protect plants against feeding insects (Hagel et al., 2008). Latex has been reported in 12500 plant species representing 22 families (Evert, 2006).

Ficus, the fig genus, consists of over 800 species are widely scattered over the tropical and subtropical region of the world. The parts of *Ficus carica* and *Ficus sycomorus* used for the treatment of tumors and diseases associated or characterized by inflammation include the fruits in different stages of ripening, fresh or dry, tree bark, leaves, twigs and young shoots, and also latex from the bark, fruit and young branches (Lansky et al., 2008). The latex released on picking the fruits of fig *Ficus carica* was used to treat skin tumors and warts (Rubnov et al., 2001). Fig latex and its component (R3) have been shown to inhibit the growth of transplanted and spontaneous tumors in mice (Ullman et al., 1952; Ullman, 1952), while *in vitro* data showed that the extract from fig

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latex (resin) inhibited the proliferation of some human cancer cells (Rubnov et al., 2001; Wang et al., 2008). Figs also have been shown to possess antioxidant, antibacterial, hypolipidemic, and hypoglycemic activities (Lansky et al., 2008; Abdel-Hameed, 2009; Ao et al., 2008).

Peroxidases (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) are widely distributed in organisms, and plants have an abundance of peroxidase isozymes which have been implicated in a wide variety of cellular functions, including lignification (Quiroga et al., 2000), suberization (Bernards et al., 1999), auxin oxidation (Gazaryan et al., 1996), and plant defense (McLusky et al., 1999). Peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) are a group of heme-containing oxidoreductases that act on peroxide as electron donors. They are present in all known organisms and their biological function is related to the removal of the toxic hydrogen peroxide which is a product of cell metabolism. Peroxidases are generally reported to participate in the protection of plant tissues from damage and infection by pathogenic microorganisms, and in wound healing (Dunford, 1999). *In vitro*, this enzyme is widely employed in microanalysis (Gorton, 1995). Currently, peroxidases are used also in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals (Egorov et al., 2000). The presence of latex peroxidases and other antioxidant enzymes in several plants may confirm latex as having a protective role against possible oxidative damage which begins after plant is wounded (Freitas et al., 2007; Kawano, 2003; Mura et al., 2008).

Although, peroxidases are widely distributed in plant kingdom, at present commercially available peroxidase is horseradish peroxidase. However, there is increased interest to find alternative/additional peroxidases with novel properties. The present study focused on the purification and characterization of peroxidase from sycamore fig *F. sycomorus* latex.

MATERIALS AND METHODS

Plant materials

The latex samples were collected from the neck of cut fruit of Egypt sycamore fig *F. sycomorus*.

Peroxidase assay

Peroxidase activity was carried out according to Miranda et al. (1995). The reaction mixture containing in 1 ml: 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and least amount of enzyme preparation. Assays were carried out at room temperature. The change of absorbance at 470 nm due to guaiacol oxidation was followed at 30 s intervals. One unit of peroxidase activity is defined as the amount of enzyme which increases the O.D. 1.0 min^{-1} under standard assay conditions.

Purification of peroxidases from *Ficus sycomorus* latex

Unless otherwise stated, all preparation steps were carried out at 4 to 7°C. 4 ml of *F. sycomorus* latex was incubated in a 60°C water bath for 30 min and subsequently chilled in ice bath. The denatured proteins were removed by centrifugation at 12,000 rpm for 10 min. The supernatant was collected and dialyzed against three changes of 20 mM Tris-HCl buffer, pH 7.0. The dialyzate was applied directly to a diethylaminoethyl (DEAE)-sepharose column (10×1.6 cm i.d.) pre-equilibrated with the same buffer. The adsorbed material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.5 M prepared in the same buffer at a flow rate of 30 ml/h, and 3-ml fractions were collected. The pooled fractions (0.2 M NaCl) with the highest peroxidase activity was concentrated through dialysis against solid sucrose and applied on a Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with the same buffer and developed at a flow rate of 20 ml/h, and 3.5 ml fractions were collected.

Protein determination

Protein was quantified by the method of Bradford (1976). Bovine serum albumin was used as the protein standard.

Polyacrylamide gel electrophoresis

Electrophoresis under non-denaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis (1964) using a Tris-glycine buffer, pH 8.3. Protein bands were located by staining with coomassie brilliant blue.

Molecular weight determination

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90 x 1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for the calibration curve.

Detection of peroxidase activity on the gel

Peroxidase activity was detected on the polyacrylamide gel according to Gottlieb (1973). After electrophoresis, the gel was immersed for 2 to 4 h in the dark at room temperature in 50 mM sodium acetate buffer, pH 5.5 containing 250 mM hydrogen peroxide and 2 mM guaiacol. After visualization for the enzymes, the gel was photographed immediately.

RESULTS AND DISCUSSION

The purification of peroxidases from *F. sycomorus* latex is summarized in Table 1. The initial heat treatment (60°C for 30 min) offered an efficient means of partial purification, thus 40% of total protein of latex was removed and the most of peroxidase activity (98%) was

Table 1. Purification scheme of peroxidases from *F. sycomorus* latex.

Purification step	Total protein (mg)	Total activity (unit*)	Specific activity (units mg^{-1} protein)	Fold purification	% Recovery
Crude extract (latex)	6.5	2120	326	1	100
Heat treatment	4	2080	520	1.6	98
Dialysis	2	2336	1168	3.6	110
Chromatography on DEAE-Sepharose					
0.2M NaCl (POLI)	0.48	2000	4166	13	94
0.3M NaCl (POLII)	0.136	195.6	1438	4	9.2
Gel filtration on Sephacryl S-200 (POLI)	0.18	1650	9166	28	78

*One unit of peroxidase activity is defined as the amount of enzyme that increases the optical density 1.0 min^{-1} under standard assay conditions.
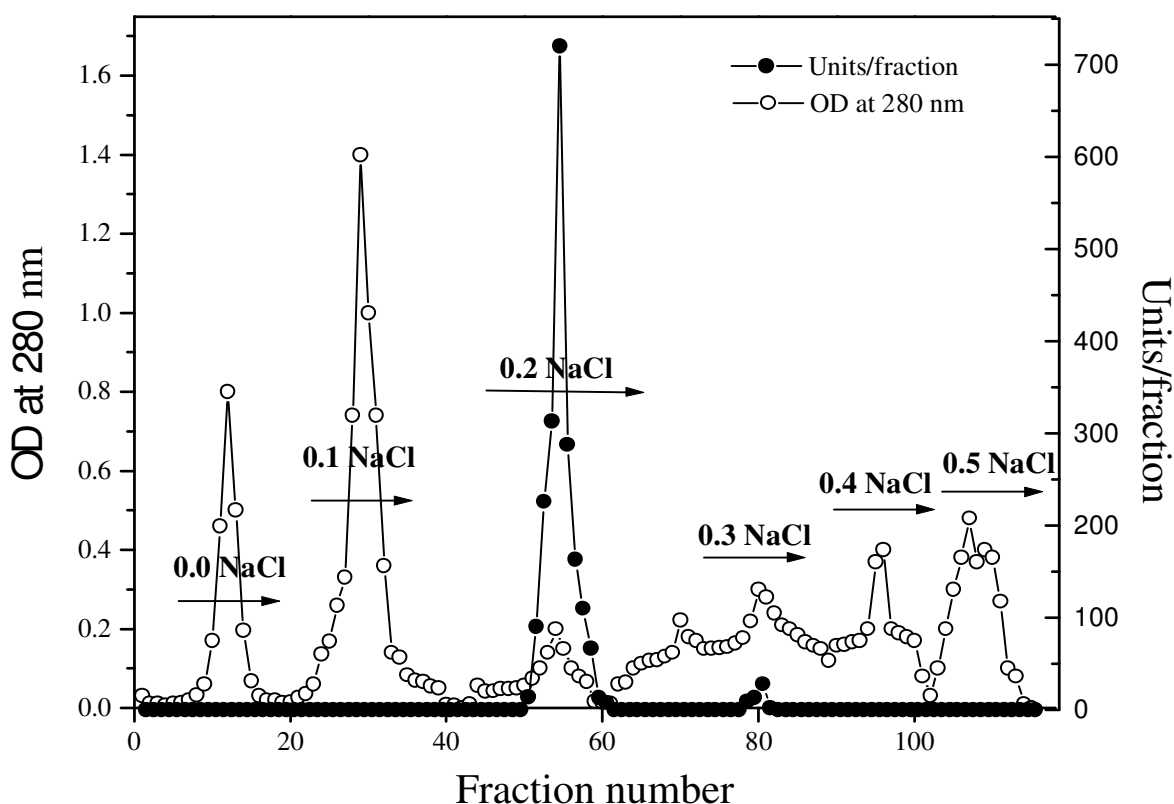

Figure 1. A typical elution profile for the chromatography of *F. sycomorus* latex peroxidase on DEAE-Sepharose column (10×1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7. Fractions of 3 ml were collected at flow rate 30 ml/h.

recovered with specific activity of 520 units/mg protein and 1.6 purification fold over the crude extract. The heat treatment represents a method of purification of thermal stable enzymes (Winans, 1981). Most of the enzymes especially proteases isolated from plant latex are heat labile and autocatalytic in nature (Dubey and Jagannadham; 2003; Sundd et al., 1998). Some proteins in *Synadenium grantii* latex were coagulated at high temperature without affecting some enzymes activities (Rajesh et al., 2006). Heat treatment has been employed

in the purification of several enzymes from different sources such as phospholipase in serum, plasma and extra cellular exudates (Kuslys et al., 1996; Vishwanath et al., 1996). The peroxidase activity from *F. sycomorus* latex was increased after dialysis (110% recovery) with 3.6 fold purification over the crude extract. The chromatographic profile of DEAE-sepharose column showed seven protein fractions, two of them were eluted at 0.2 and 0.3 M NaCl with peroxidase activity (Figure 1). Most peroxidase activity was recovered in POLI which
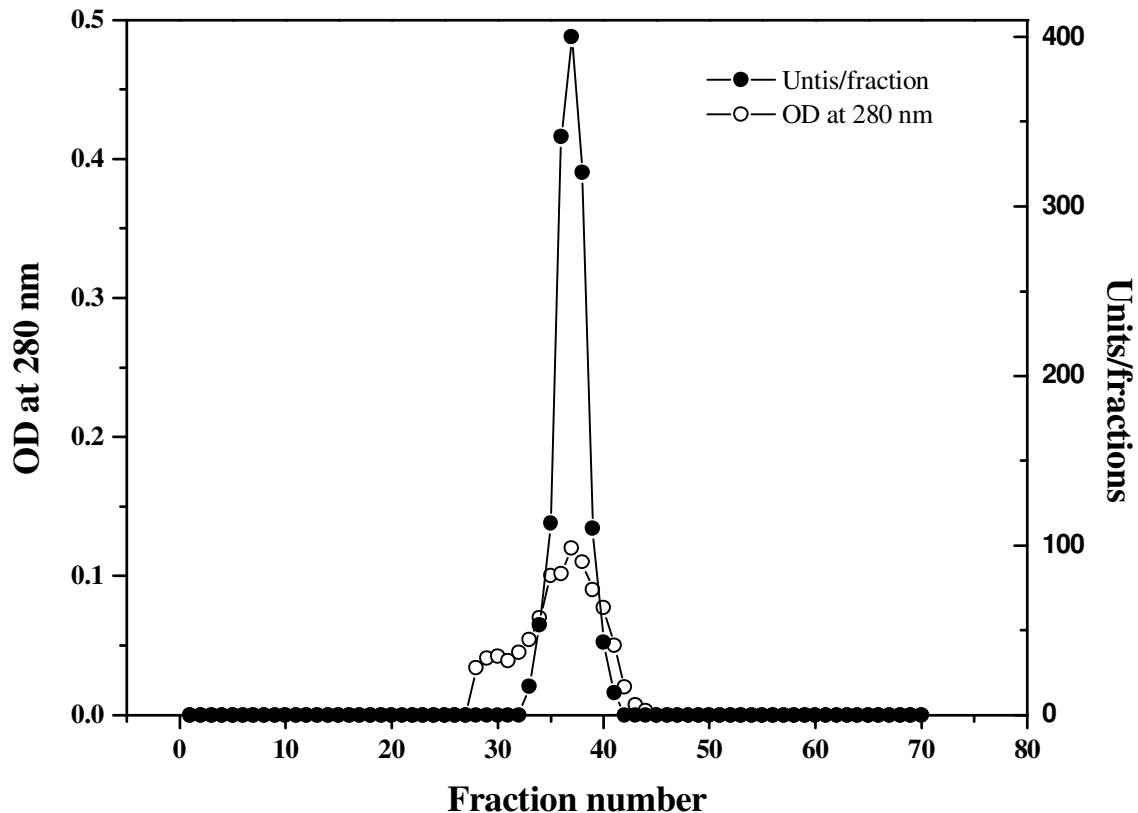
Figure 2. A typical elution profile for the chromatography of *F. sycomorus* latex peroxidase of DEAE-Sepharose fraction 0.2 M NaCl on Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7. Fractions of 3.5 ml were collected at flow rate 20 ml/h.

considered anionic peroxidase. However, a cationic peroxidae was isolated from latex of *Euphorbia characias* (Floris et al., 1984; Medda et al., 2003). Most plants had cationic and anionic peroxidases (Clemente, 1998; Mohamed et al., 2008; Valderrama and Clemente, 2004; Das et al., 2011; Kumara et al., 2011). The resolution of POLI on Sephacryl S-200 showed one major protein with peroxidase activity (Figure 2). The recovery percent of POLI was 78% with specific activity of 9166 units/mg protein and 28 purification fold over the crude extract. The RZ value (A400/A280) of POLI, which is a good criterion of purity and heme content, was 3.1. After Sephacryl S-200 column analysis, the purity of POLI was proved by Native-PAGE (Figure 3a). POLI is a monomeric protein with a molecular mass of 43 kDa as estimated by both Sephacryl S-200 chromatography and SDS-PAGE (Figure 3b). A nearly value of molecular weight (48 kDa) was detected for latex peroxidase from *E. characias* (Floris et al., 1984). The similar molecular weights (40 to 44 kDa) were detected for several plant peroxidases (Clemente, 1998; Hu et al., 1989). One band of visualized peroxidase activity was detected on the polyacrylamide gel after Sephacryl S-200 chromatography (Figure 3c).

In the presence of H_2O_2, POLI catalyzes the oxidation of many potential natural electron donor substrates. The activity with guaiacol is regarded as 100% activity. The POLI had highest activity toward substrates commonly used in immunodiagnostic kits as ABTS, 4C-1N, OPD and TMB (Jiang et al., 2005) compared to guaiacol, which suggest that POLI may be used as a marker in enzyme immunoassay. While *p*-aminoantipyrine and guaiacol had moderate affinity toward POLI, *o*-dianisidine and pyrogallol had low affinity (Table 2). As the enzyme showed wide substrate specificity, POLI may belong to class III of the plant peroxidase subfamily with EC 1.11.1.7 (donor: hydrogen peroxide oxidoreductase) similar for horseradish and black gram *Vigna mungo* husk peroxidase (Ajila and Prasada Rao, 2009; Veitch, 2004). *E. characias* latex peroxidase oxidized some substrates in the order of *o*-dianisidine > pyrogallol > *p*-aminoantipyrine (Floris et ai, 1984).

The K_m values for various substrates of POLI were obtained by a typical reciprocal Lineweaver-Burk plot (Figure 4 and Table 3). The K_m for catalysis of H_2O_2 in the presence of 40 mM guaiacol was 1.2 mM. The lower K_m value of the enzyme indicated its higher affinity toward H_2O_2. The significance of low K_m values for H_2O_2 reflects a high number of H_2 or hydrophobic interactions between the substrate and the heme group at the enzyme active
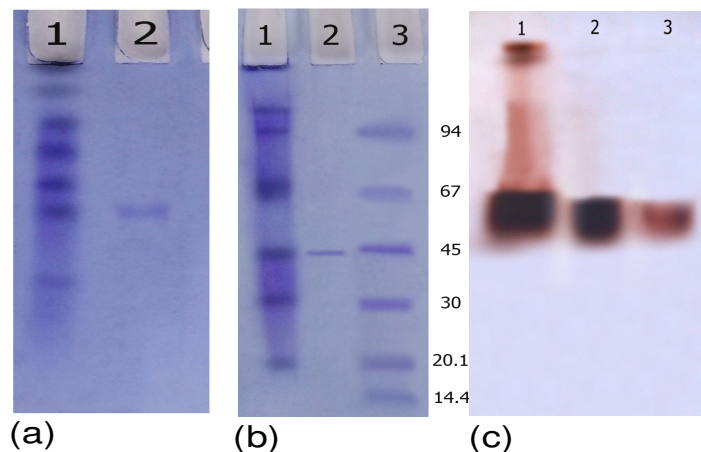
Figure 3. (a), Native-PAGE of *F. sycomorus* latex POLI during purification steps; lane 1 = Crude extract and lane 2 = Sephacryl S-200 POLI. (b) SDS-PAGE for molecular weight determination of *F. sycomorus* latex POLI; lane 1 = Crude extract, lane 2 = Sephacryl S-200 POLI and lane 3 = Standard proteins. (c) The peroxidase activity patterns of *F. sycomorus* latex POLI during purification steps; lane 1 = Crude extract, lane 2 = DEAE-Sepharose POLI and lane 3 = Sephacryl S-200 POLI.

Table 2. Relative activity of *F. sycomorus* latex POLI toward substrates.

Substrate	% Relative activity
Guaiacol	100
ABTS	244
4C-1N	238
OPD	230
TMB	165
p-Aminoantipyrine	96
O-Dianisidine	46
Pyrogallol	38

The reaction mixture contained in 1 ml: 8 mM H_2O_2, 40 mM of each substrate, 50 mM sodium acetate buffer, pH 5.5, and suitable amount of enzyme. The activity with guaiacol was taken as 100% activity. Each value represents the average of two experiments.

site (Ajila and Prasada Rao, 2009). This affinity of POLI with H_2O_2 was higher than that reported for peroxidases from other plants, example pear (K_m 1.5 mM) (Richard-Forget and Gauillard, 1997), chicory cell suspension culture (K_m 2.4 mM) (Boeuf et al., 2000) and *Brussels sprouts* (K_m 11.4 and 6.2 mM) (Regalado et al., 1999). However, Duarte-Vazquez et al. (2000) reported lower affinity for H_2O_2 interaction with turnip peroxidase Cl, C2 and C3 at K_m values of 0.04, 0.25 and 0.85 mM (Onsa et al., 2004). ABTS, 4C-1N, OPD, TMB, guaiacol, and *p*-aminoantipyrine showed higher affinity toward POLI, where their K_m values are 4.7, 5, 5.4, 6.8, 9.5 and 11.7 mM respectively. Both *o*-dianisidine and pyrogallol showed relatively higher K_m values, 16.6 and 26 mM, respectively indicated their relatively lower affinity to POLI. However, in terms of catalytic efficiency (V_{max}/K_m), POLI was found to follow the order: ABTS, 4C-1N, OPD, TMB, guaiacol, *p*-aminoantipyrine, *o*-dianisidine and pyrogallol. The lower K_m values of 2.85 mM *o*-phenylenediamine and 5 mM guaiacol were reported for citrus peroxidase (Mohamed et al., 2008). Although, mPOD isoenzymes from *Metroxylon sagu* showed a high V_{max} for guaiacol, their ability to oxidize this substrate is low, due to their low affinity for the enzyme, with high K_m values of 32.2 and 22.9 mM for mPOD-I and mPOD-II, respectively (Onsa et al., 2004).

pH is a determining factor in the expression of an enzyme activity as it alters the ionization state of amino acid chains or the ionization of the substrate. The effect of pH on the activity of POLI was shown in Figure 5, the enzyme showed a broad pH optimum ranged from 5.5 to pH 7.0, and it decreased sharply with increase in pH. The
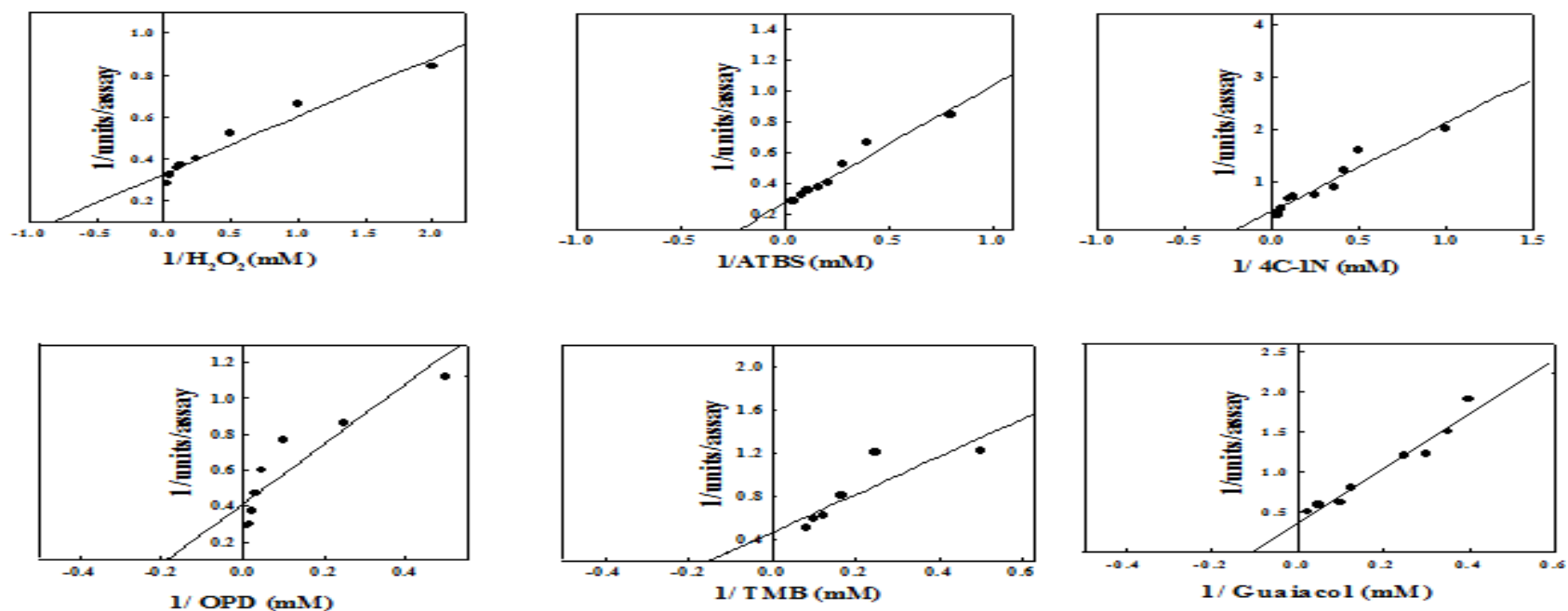
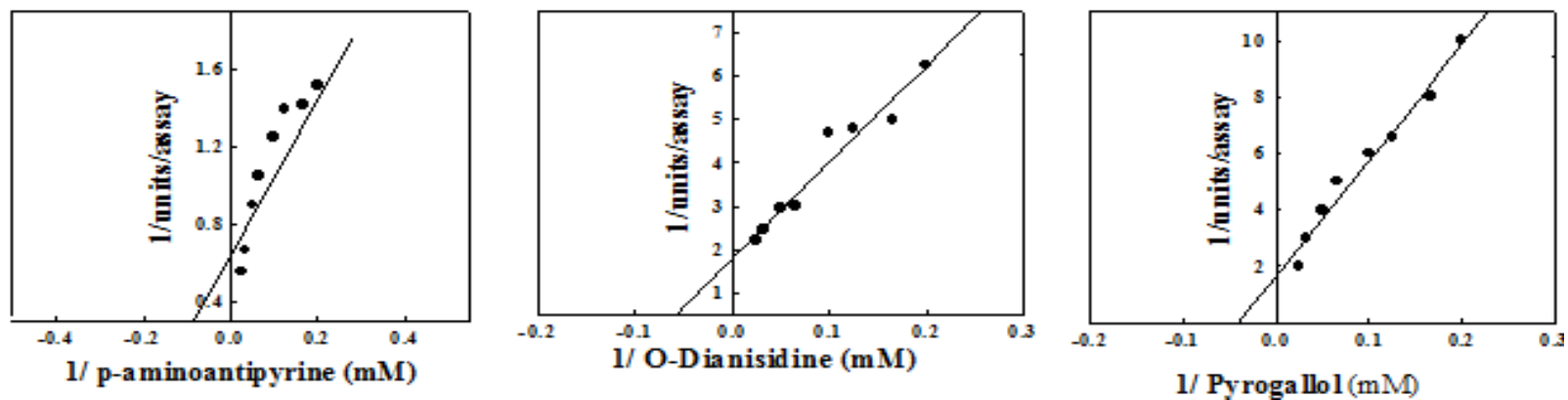**Figure 4.** A typical lineweaver–Burk plots of the *F. sycomorus* latex POLI.

Figure 4. Contd

Table 3. Kinetic parameters of *F. sycomorus* latex POLI.

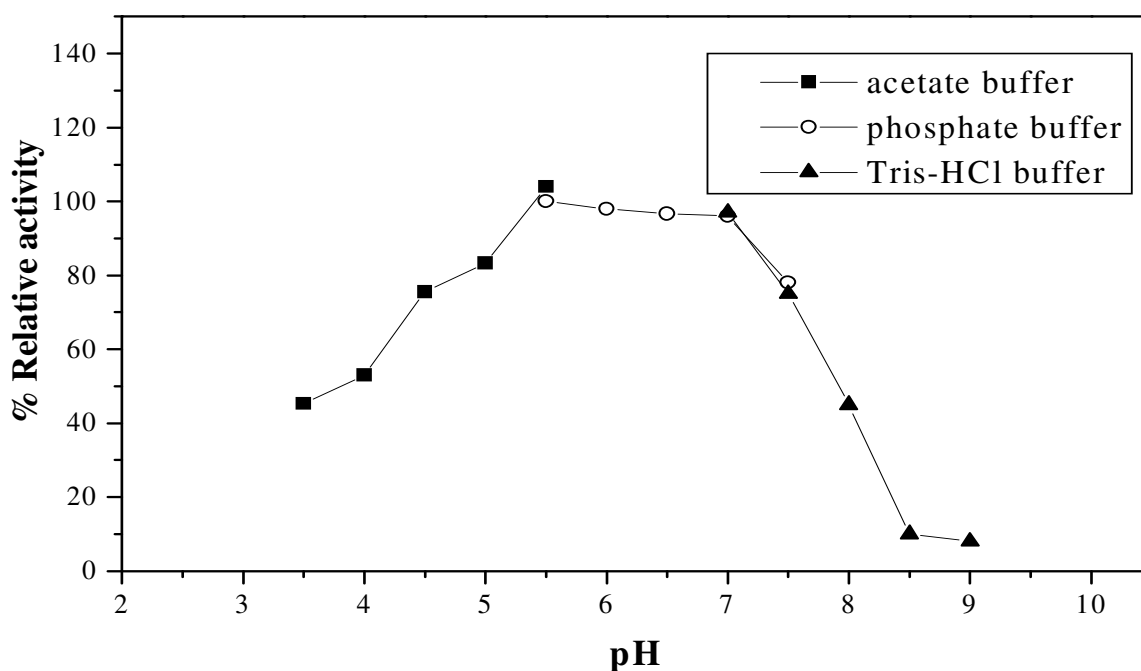| Substrate	K_m (mM substrates)	V_{max} (units/assay)	V_{max}/K_m (units/mM substrate)
H2O2	1.2	3.1	2.6
ABTS	4.7	3.8	0.8
4C-1N	5	2.3	0.46
OPD	5.4	2.46	0.45
TMB	6.8	2.2	0.32
Guaiacol	9.5	2.9	0.3
p-aminoantipyrine	11.7	1.57	0.134
o-Dianisidine	16.6	0.57	0.034
Pyrogallol	26	0.6	0.023

Figure 5. pH optimum of the *F. sycomorus* latex POLI. The reaction mixture contained in 1.0 ml: 8 mM H_2O_2, 40 mM guaiacol, a suitable amount of enzyme, and 20 mM sodium acetate buffer (pH 3.5 to 5.5), sodium phosphate buffer (pH 5.5 to 7.5) and Tris–HCl buffer (pH 7.5 to 9.0). Each point represents the average of two experiments.

pH optima of peroxidase from grape were 5.4, banana 4.5 to 5.0, pineapple 4.2, horseradish peroxidase (HRP) 4.6 to 5.8, potato 5.0 to 5.4 (Vamos-Vigyazo, 1981), black gram 5.5 (Ajila and Prasada Rao, 2009) and *E. characias* latex 5.5 (Floris et al., 1984), wheat germ 5.5 to 6.3 (Billaud et al., 1999). Lopez and Burgos (1995) reported that the release of heme group from the enzyme active site was pH dependent and occurred most rapidly at lower and higher pH and lead to the loss in activity. The active site of the enzyme is mainly composed of ionic groups (prosthetic groups) that must be in the proper ionic form in order to maintain the conformation of the active site of enzyme for substrate binding or reaction catalysis (Lopez and Burgos, 1995).

The optimal temperature and the effect of heat on the stability of the *Ficus sycomorus* latex POLI activity were examined using guaiacol as substrate (Figures 6 and 7). The optimal temperature for the enzyme was 35 to 40°C. More than 70% of the activity of the enzyme was retained in the temperature range of 10 to 50°C. POLI showed highest thermal stability. No loss of enzyme activity was recorded up to 60°C, whereas only 20% loss of enzyme activity was recorded at 70 to 90°C after the enzyme incubated at different temperatures for 15 min prior to substrate addition. The results appeared clear difference between the optimum temperature and thermal stability concerning with the residual activity. This may be attributed to H_2O_2 in the experiment; optimum
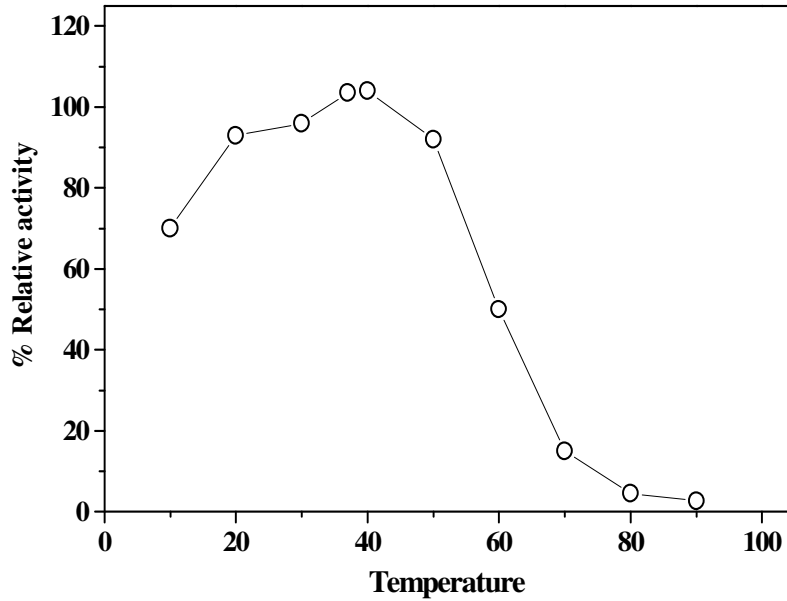
Figure 6. Optimum temperature of *F. sycomorus* latex POLI. The enzyme activity was measured at various temperatures using the standard assay method as previously described. Each point represents the average of two experiments.
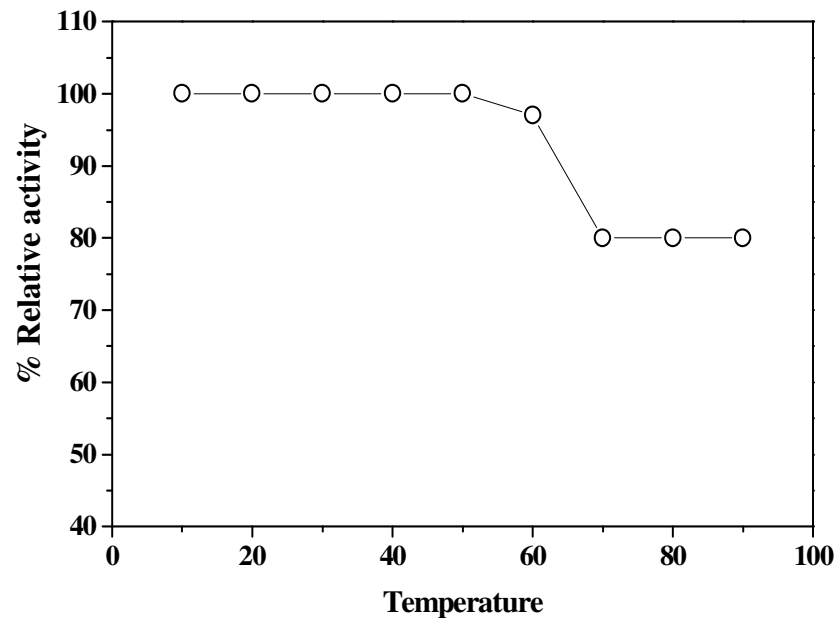

Figure 7. Effect of temperature on the thermal stability of *F. sycomorus* latex POLI. The reaction mixture contained in 1.0 ml: 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and a suitable amount of enzyme. The reaction mixture was pre-incubated at various temperatures for 15 min prior to substrate addition, followed by cooling in an ice bath. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments.

temperature was decayed at high temperature (70 to 90°C) and the enzyme activity was rapidly lost. Time dependent heat inactivation of POLI was studied in order to investigate its thermal resistance (Figure 8). The enzyme was incubated for different times at different temperatures (60 to 90°C) prior to substrate addition. The enzyme appeared thermal stability at 60°C during 30 min, whereas slightly lost in enzyme activity (25%) for 60 min.
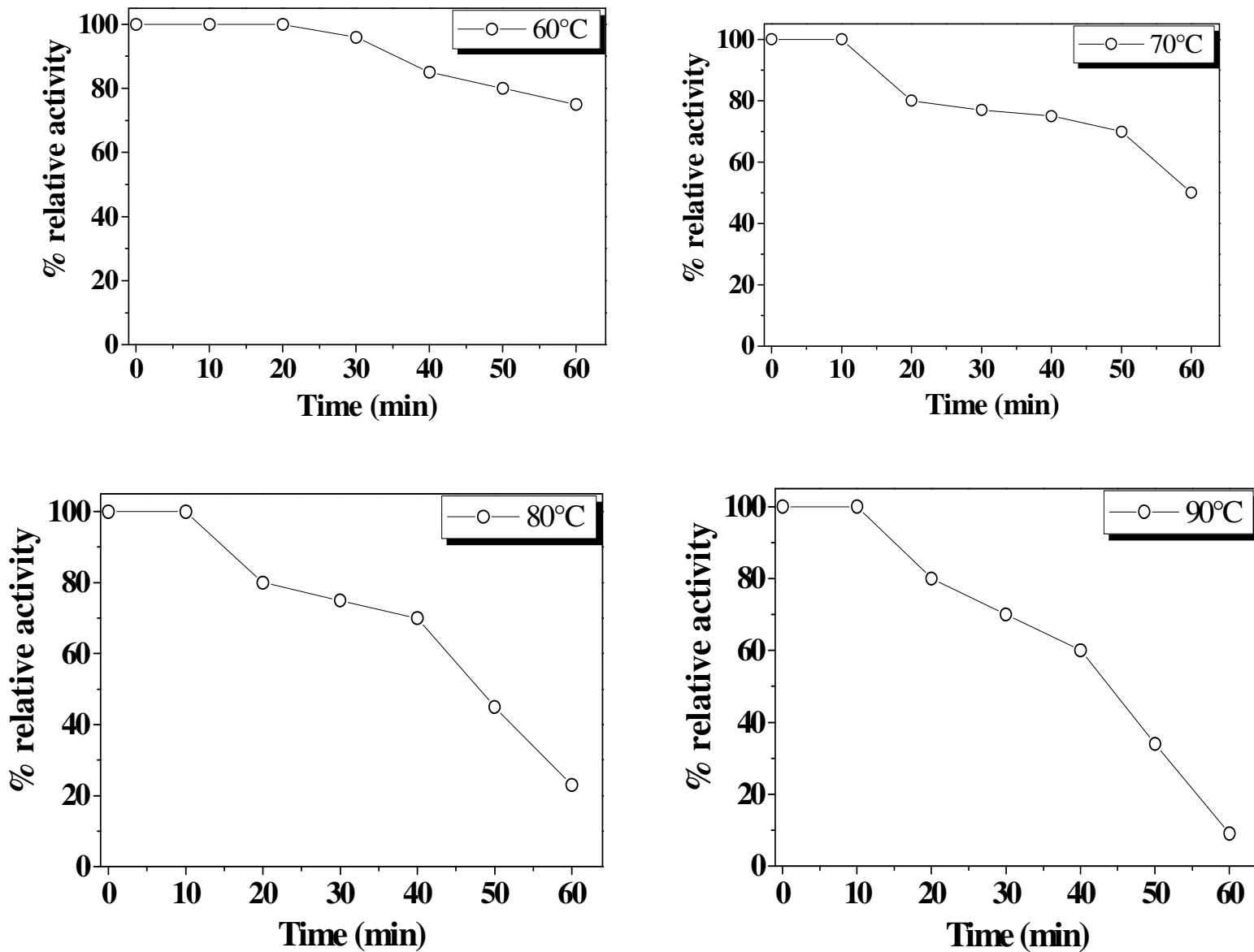
Figure 8. Time dependent heat inactivation profiles for *F. sycomorus* latex POLI. The enzyme was incubated for different times at different temperatures (60 to 90°C) prior to substrate addition and the residual peroxidase activity was assayed under standard assay conditions. Each point represents the average of two experiments.

Table 4. Effect of metal ions on *F. sycomorus* latex POLI.

Metal	% Relative activity		
	1 mM	5 mM	10 mM
Ca+2	103	118.2	98.2
Mg+2	115.4	98.6	95.6
Fe+3	90	89	83
Zn+2	96.3	95	92
Ni+2	102.6	104	96.4
Hg+2	94	92	88.5
Mn+2	83.3	96.3	104
Cu+2	87	73.3	62

Enzyme was pre-incubated for 15 min at 37°C with 1, 5 and 10 mM of listed ions as a final concentration prior to substrate addition. Each value represents the average of two experiments.

At 70, 80 and 90°C, 23, 25 and 30% of POLI activity were lost during 30 min and after 1 h the residual activities of POLI were 50, 30 and 9%, respectively. The results show non-linear inactivation curves which is due to the formation of new complexes of higher thermostability formed from thermally denatured enzyme protein and groups of peroxidase that remain active (Vamos-Vigyazo, 1981). Peroxidase is reported to be one of the most heat stable enzymes in plants. It was observed that 6 min at 121°C is needed to inactivate POD in green peas (Vamos-Vigyazo, 1981). However, the resistance to treatment depends on the source of the enzyme as well as the assay conditions, especially pH and nature of substrate employed. Present study shows that the thermal stability of POLI is greater than that reported for strawberry (Whitaker, 1995) and black gram (Ajila and Prasada Rao, 2009). It has been shown that the thermal stability of peroxidase is due to the presence of a large number of cysteine residues in the polypeptide chain.

The activity of *F. sycomorus* latex POLI was variously affected by the presence of metal ions at 1, 5 and 10 mM (Table 4). The peroxidase activity was slightly enhanced by low concentration (1 and 5 mM) of Ca^{2+}, Ni^{+2} and Mg^{2+} and high concentration (10 mM) of Mn^{2+}. Fe^{+3}, Zn^{+2} and Hg^{+2} at all concentrations caused slightly inhibitory effects. The activity of enzyme was moderately inhibited by Cu^{+2} (87 to 62% inhibition). Fe^{3+} is considered essential for the activity of most plant POD enzymes as it is involved in binding of H_2O_2 and formation of compound I (Civello et al., 1995; Wong et al., 1995). Among the metal ions tested, Ca^{+2} is a cofactor that serves to maintain the conformational integrity of the enzyme's active site (Adams et al., 1996). Activation by Ca^{+2} ions was reported for avocado mPOD (Sanchez-Romera et al., 1994) and barley grain and wheat germ sPOD, where Ca^{+2} enhanced activities by ~2- and 6-fold, respectively (Billaud et al., 1999). The purified peroxidase from *E. characias* latex contained 1 mol of endogenous calcium per mol of enzyme; removal of this calcium ion resulted in almost complete loss of the enzyme activity. However,

when excess Ca^+ was added to the native enzyme the catalytic efficiency was enhanced by 3 orders of magnitude (Clemente, 1998).

Conclusion

POLI from sycamore fig *F. sycomorus* latex was found to be a rich source for a peroxidase enzyme. The enzyme showed high thermostability, broad pH optimum and the substrates commonly used in immunodiagnostic kits, were found to be the best substrates for the enzyme. Therefore, the enzyme may be used for several applications such as practical analytical applications.

Abbreviations:

SDS, Sodium dodecyl sulphate; **ABTS,** 2,2-azino-bis [3-ethyl-benzothiazoline-(6)-sulfonic acid]; **4C-1N,** 4-chloro-1-naphthol; **OPD,** o-phenylenediamine; **TMB,** 3,3`,5,5`tetramethylbenzidine; **DEAE,** diethylaminoethyl; **HRP,** horseradish peroxidase; **PAGE,** polyacrylamide gel electrophoresis.

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