

PURIFICATION AND CHARACTERIZATION OF PEROXIDASE FROM *MORINGA OLEIFERA* L. LEAVES

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Peroxidase catalyzes the oxidation of various electron donor substrates such as phenol and aromatic amines in the presence of hydrogen peroxide. In this study, peroxidase was purified 164-fold from the leaves of *Moringa oleifera* L. with a recovery of 28% by ammonium sulphate precipitation, DEAE-cellulose column chromatography, Sephadex G-200 column chromatography, and Con-A column chromatography. SDS-PAGE showed a polypeptide band with molecular weight of 43 kDa. The enzyme was found to be a single subunit in nature. The purified enzyme displayed optimum activity at pH 6.0 and at a temperature of 50 °C with a K_m value of 0.2335 mM for guaiacol as best substrate. It is a glycoprotein that contains 9.05% sugar as estimated by the phenol sulfuric acid method. Some ions (Ni^{2+} , Pb^{2+} , Zn^{2+} , Al^{3+} , Mg^{2+} , Cu^{2+} , Co^{2+} , and Cd^{2+}) exhibited low inhibitory effect while Fe^{2+} , Fe^{3+} , and Hg^{2+} exhibited strong inhibitory effects. EDTA markedly inhibited the peroxidase activity.

Keywords: Drumstick; Peroxidase; *Moringa oleifera*; Enzyme purification; Characterization; Antioxidative

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INTRODUCTION

Peroxidase, an antioxidative enzyme, is widely distributed in microbes, plants, and animal tissues and represents a heme-containing enzymes family (Huystee and Cairns 1982). This oxidoreductase catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms (Rodrigo *et al.* 1996). In the presence of peroxide, peroxidase from plant tissues are able to oxidize a wide range of phenolic compounds, such as guaiacol, catechol, pyrogallol, chlorogenic acid, and catechin (Onsa *et al.* 2004). This enzyme can provide value for multiple industrial applications, of which the most important ones include decolorization of waste (Jadhav *et al.* 2009), treatment of waste water containing phenolic compounds (Lai and Lin 2005; Dalal and Gupta 2007), and synthesis of various aromatic chemicals and removal of peroxides from food stuffs and industrial wastes (Kim and Yoo 1996; Saitou *et al.* 1991). In the biological field, *e.g.* as diagnostic kits for enzyme immunoassays and as an important component of ELISA system, this enzyme has also been widely used (Castillo *et al.* 2002; Deepa and Arumughan 2002).

Horseradish (*Armoracia rusticana*) roots are used as a traditional source of peroxidase for commercial production. Numerous studies have been carried out in a search for an alternative source of peroxidase with higher stability, availability, degree of purification, and substrate specificity. Peroxidase enzyme has been purified and characterized from many sources, *e.g.* sweet potato tubers (Castillo *et al.* 2002), oil palm leaf (Deepa and Arumughan 2002), rice (Ito *et al.* 1991), tea leaves (Kvaratskhelia *et al.* 1997), okra (Yemenicioglu *et al.* 1998), *Ipomoea palmetto* leaves (Srinivas *et al.* 1999), broccoli (Thongsook and Barrett 2005), *Copaifera longsdorffii* leaves (Maciel *et al.* 2007), apple (Dubey *et al.* 2007), vanilla bean (Marquez *et al.* 2008), turnip roots (Motamed *et al.* 2009), and soybean hulls (Gillikin and Graham 1991).

Moringa oleifera Lam. Syn. *Moringa pterygosperma* Gaerth (Family: Moringaceae, English Name: Drumstick) is a medium sized tree species. It is native to the sub-Himalayan tracts of Northwest India, Afghanistan, Bangladesh, China, Nepal, and Pakistan but now is also widely cultivated in Malaysia, Sri Lanka, tropical America, tropical Africa, Malabar, and the Philippines. This tree is important mainly for its wood, bark, stems, fruits, flowers, and leaves. The wood of this tree is suitable for pulp production for newsprint (Singh *et al.* 1983). The bark of this tree yields fiber, which is used in manufacturing cordage, paper, and mats. Mucilaginous gum exuded from its stems is used in calico printing and leather tanning (Nautiyal and Venhataraman 1987). The fruits and flowers are rich in nutrient content (Ramachandran *et al.* 1980). Its leaves are very inexpensive and easily available. It is one of the most important vegetables in terms of wide adaptability and food value. In our study, an appreciable activity of peroxidase in *M. oleifera* L. leaves was observed. According to the literature review, data on the purification and characterization of *M. oleifera* L. leaves peroxidase are not available. To address this gap, the present article describes the purification and characterization of peroxidase from *M. oleifera* L. leaves.

EXPERIMENTAL

Materials

The leaves of *M. oleifera* L. were collected from Rajshahi University campus, Bangladesh. Ammonium sulfate, DEAE-cellulose, Sephadex G-200, Concanavallin-A, bovine serum albumin, phenol, guaiacol, pyrogallol, o-dianisidine, catechol, and hydrogen peroxide were obtained from Merck, Germany. All other chemicals used in this study were of analytical grade and obtained from commercial sources. The chemicals and other reagents were used without any further purification.

Crude Extract

The crude extract of leaves was prepared in the following process. The fresh, healthy leaves (50 g) were washed thoroughly with distilled water at room temperature. The leaves were homogenized with 200 mL of 100 mM Tris-HCl buffer, pH 7.5 containing 0.1% polyvinylpyrrolidone (PVP) in a blender for 5 min. The homogenate was filtered using a cheese cloth (arranged in four folds) to remove suspended particles. The

clear filtrate was then centrifuged at 9000 g for 25 min at 4 °C. The supernatant was collected and stored at 4 °C and used as crude soluble *M. oleifera* L. leaves enzyme.

Procedures

Protein and enzyme assay

Protein was determined by the method of Lowry (Lowry *et al.* 1951) using bovine serum albumin as the standard. Peroxidase activity was measured spectrophotometrically with guaiacol as substrate (Tonami *et al.* 2004).

Ammonium sulfate precipitation and dialysis

Ammonium sulfate precipitation of peroxidase was done in an ice bath by using the finely ground ammonium sulfate. The powder was weighed and added slowly to crude soluble leaf extract by constant stirring to ensure complete solubility, and the solution was kept at 4 °C for 2 h for complete precipitation. Different degrees of saturation were achieved by progressively adding the specified quantity of ammonium sulfate according to the relevant saturation chart. After each saturation step the precipitate was collected by centrifuging the enzyme extract at 10,000 g for 20 min at 4 °C. The collected fractions (0-20%, 20-40%, 40-60%, 60-80%, and 80-90%) were analyzed for enzyme activity and total protein content. The specific activity was calculated, and the values were expressed in terms of purification fold (Rudrappa *et al.* 2007). The fraction with maximum specific activity was dialyzed with 100 mM Tris-HCl buffer, pH 7.5 for 24 h by changing the buffer thrice. The dialyzed fraction was used for further purification by DEAE-cellulose chromatography.

DEAE-cellulose chromatography

The dialyzed fraction was loaded onto a DEAE-cellulose column (2.1×24 cm), pre-equilibrated with 100 mM Tris-HCl buffer, pH 7.5. Bound proteins were then eluted with 25-200 mM NaCl gradient in the same buffer. Absorbances at 280 nm and peroxidase activity were monitored (Tonami *et al.* 2004). The eluted fraction containing maximum enzyme activity were pooled and used for subsequent steps.

Gel filtration on Sephadex G-200 column

The maximum enzyme activity containing fraction from the DEAE-cellulose column chromatography step was loaded onto a Sephadex G-200 column. The column (2.7 × 40.0 cm) was equilibrated and eluted with same 100 mM Tris-HCl buffer, pH 7.5. Fractions of 3 mL each were collected throughout the elution. Absorbances at 280 nm and peroxidase activity were monitored.

Con-A affinity column chromatography

The fraction containing high enzyme activity from gel-filtration chromatography was loaded to the Con-A column (8.0×50.0 mm). The column was regenerated with the regeneration buffer containing 100 mM sodium acetate buffer, pH 4.5, 1M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, equilibrated by buffer containing 20 mM Tris-HCl buffer, pH 7.5 with 0.5 M NaCl, binding buffer, containing 20 mM Tris-HCl buffer, pH 7.5 with 0.5 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂. Peroxidase was then eluted with 25-200 mM sucrose,

α -methyl mannopyranoside gradient in 20 mM Tris-HCl buffer, pH 7.5, and 500 mM NaCl. Absorbances at 280 nm and peroxidase activity were monitored.

Electrophoresis

Purity of the purified peroxidase and the molecular weight were determined by native polyacrylamide gel electrophoresis (native PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively, by the use of Laemmli (Laemmli 1970) gel method on 10% polyacrylamide slab gel containing 1% SDS. Gels were stained with Coomassie Brilliant Blue. Standard proteins such as lysozyme (14 kDa), trypsin inhibitor (20 kDa), pepsin (36 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), and phosphorylase b (97 kDa) were used for the calibration.

Kinetic constants

Different concentrations of guaiacol, a suitable amount of purified enzyme, and H₂O₂ of fixed saturated concentrations were mixed and incubated to determine the effect of substrate, guaiacol, and H₂O₂. From the data obtained, the values of Michaelis constant (K_m) was determined from Lineweaver-Burk double reciprocal plots.

Optimum pH and pH stability

The optimum pH value for the peroxidase activity was determined by assaying enzyme activity at different pH values, using the following buffers: 0.1 M glycine-HCl buffer (pH 2.0), 0.1 M acetate buffer (pH 3.0 to 5.0), 0.1 M phosphate buffer (pH 5.5 to 7.5) and 0.1 M Tris-HCl buffer (pH 8.0 to 9.5). Stability of the enzyme at different pH values was determined by measuring the residual activity after incubating the enzyme at pH 2.0 to 9.5 for 24 h at 35 °C. The assay conditions were otherwise similar to that given above.

Optimum temperature and temperature stability

The optimum temperature for the peroxidase activity was determined by assaying enzyme activity at different temperatures from 10 to 90 °C at pH 6.0. Heat stability was measured by incubating the enzyme at 10 to 90 °C for 30 min in 0.1 M phosphate buffer at pH 6.0. After heat treatment, the enzyme solution was cooled and the residual activity assayed under standard assay conditions.

Glycoprotein test and sugar estimation

Glycoprotein and sugar in the enzyme molecules were estimated according to the previous work (Dubois *et al.* 1956). Phenol in the presence of sulfuric acid can be used for quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharides and polysaccharides. The method was also applied for the detection and estimation of sugar in protein.

Substrate specificity

For the determination of the substrate specificity of the enzyme, four well known peroxidase substrates: pyrogallol, o-dianisidine, catechol, and guaiacol were used as substrate during the assay by the standard procedure, as mentioned earlier. When

studying substrate specificity of peroxidase, the activity was measured under optimal conditions determined for each substrate. Temperature was controlled by using a water bath. The changes in absorbance were read for 3 min using a spectrophotometer. The following wavelengths were used in the assays: at 470 nm for guaiacol, 420 nm for pyrogallol and o-dianisidine, and 290 nm for catechol.

Chemicals and metal ions effect

By pre-incubating the enzyme (0.25 to 0.3 mg/mL) with 0.5 mL of individual metal ion or reagent at specified concentrations for 30 min, followed by incubation under the standard assay conditions and then assaying the enzyme activity, the effects of various metal ions and the reagent, ethylene diamine tetra acetic acid (EDTA) on enzyme activity were determined. The activity of the enzyme assayed in the absence of reagents or metal ions was taken as 100%.

Data Analysis

The values reported are the mean of at least three independent determinations.

RESULTS AND DISCUSSION

Purification of *M. oleifera* L. Leaves Peroxidase

Purification of *M. oleifera* L. leaves peroxidase is summarized in Table 1. Ammonium sulfate fractionation was done by using the finely ground ammonium sulfate. The fraction obtained in 80 to 90% interval showed the maximum activity. This primary purification step resulted in about 3.78-fold purification of peroxidase from the crude extract. Following ammonium sulfate precipitation, the enzyme extract was dialyzed.

Table 1. Summary of Purification of the Peroxidase Enzyme from *M. oleifera* L. Leaves

Purification steps	Total enzyme activity (unit)	Total protein (mg)	Specific activity (unit mg ⁻¹)	Recovery (%)	Purification fold
Crude extract	685.75	325.0	2.11	100	1.00
(NH ₄) ₂ SO ₄ Precipitation	402.04	50.34	7.98	58.62	3.78
DEAE-cellulose chromatography	304.9	13.22	23.06	44.46	10.9
Gel filtration by Sephadex G-200	229.84	1.99	115.49	33.51	54.73
Con-A column chromatography	190.54	0.55	346.43	27.78	164.18
One unit of peroxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol per minute at room temperature.					

The dialyzed enzyme extract was applied to the DEAE-cellulose column and the chromatography gave a peak (F-1) containing enzyme activity before addition of NaCl (Fig. 1). This active peak (F-1) was pooled, and its purity was checked by slab gel electrophoresis (Fig. 4), that gave more bands, indicating impurities of the protein. The purification fold of this step was 10.9. The active peak was then applied to the Sephadex G-200 column at 4 °C. The proteins were eluted as one major (F-1a) and one minor peak (F-1b) (Fig. 2). It was found that only the major fraction contained the peroxidase activity. Active peak fraction was pooled and concentrated. The purification fold of this step was 54.73. Pooled fraction (F-1a) was checked by electrophoresis (Fig. 4), which gave multiple bands indicating more than one protein. Finally, the concentrated active peak fraction from gel filtration was applied to the Con-A affinity column chromatography (Fig. 3). This final step gave a single active peak (F-1a³) at 100 mM sucrose and provided about 164.18-fold purification of the enzyme. The purified enzyme was homogeneous on SDS-slab gel electrophoresis, giving a single protein band (Fig. 4). Pandey and Dwivedi (2011) also purified *L. leucocephala* peroxidase by the same procedure.

Table 2. Purification of Peroxidase by Different Researchers

Sources	No. of steps	Purification fold	Recovery (%)	Specific activity (unit mg ⁻¹)	References
<i>Ipomoea palmetto</i> leaves	4	48.60	75.30	349.8	Srinivas <i>et al.</i> 1999
Vegetable sources (horseradish legumes)	3	14.08	1.82	15.21	Rehman <i>et al.</i> 1999
<i>Copaifera langsdorffii</i> leaves	2	46.86	3.50	135.44	Maciel <i>et al.</i> 2007
<i>Armoracia rusticana</i> (horseradish)	3	41.00	28.00	N/A	Miranda <i>et al.</i> 2004
<i>Armoracia rusticana</i> (horseradish)	4	80.00	46.00	86.00	Regalado <i>et al.</i> 1996
<i>Moringa oleifera</i> L. leaves	4	164.18	27.78	346.43	Present study

N/A: Data not available.

This research on the purification of peroxidase improves and extends further the earlier research (Table 2). The peroxidase was purified to a high degree (164.18 fold), and purity level was significantly higher than those values reported by another researcher (Srinivas *et al.* 1999; Rehman *et al.* 1999; Maciel *et al.* 2007; Miranda *et al.* 2004; Regalado *et al.* 1996).

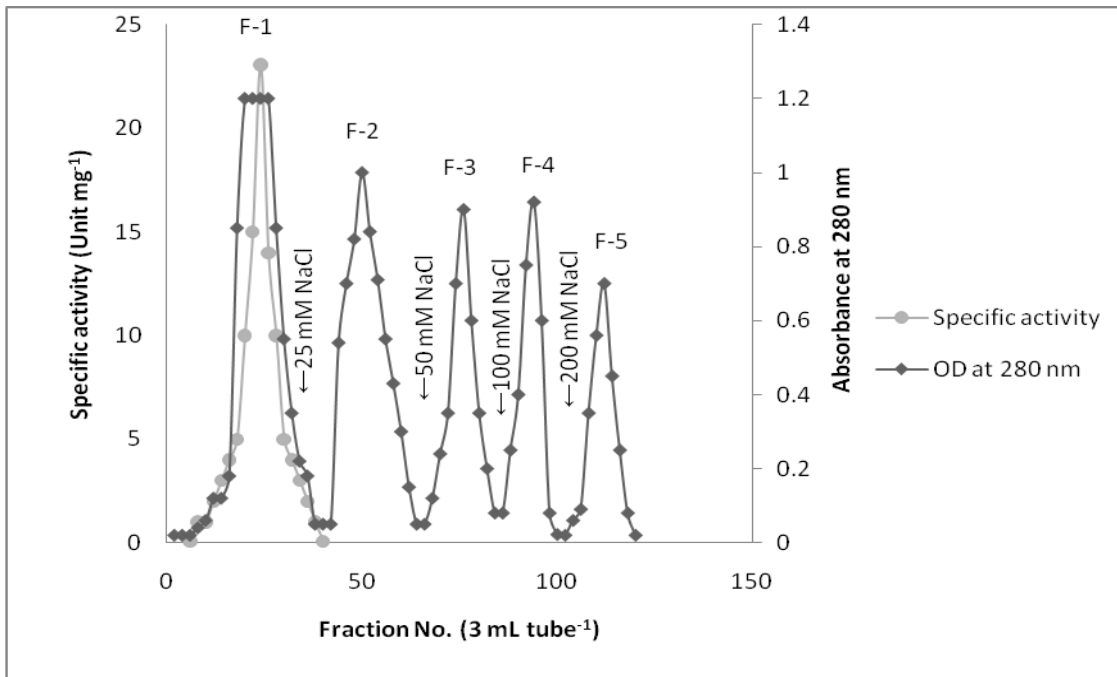


Fig. 1. Elution profile of DEAE-cellulose column (2.1x24 cm) for *M. oleifera* L. leaves peroxidase

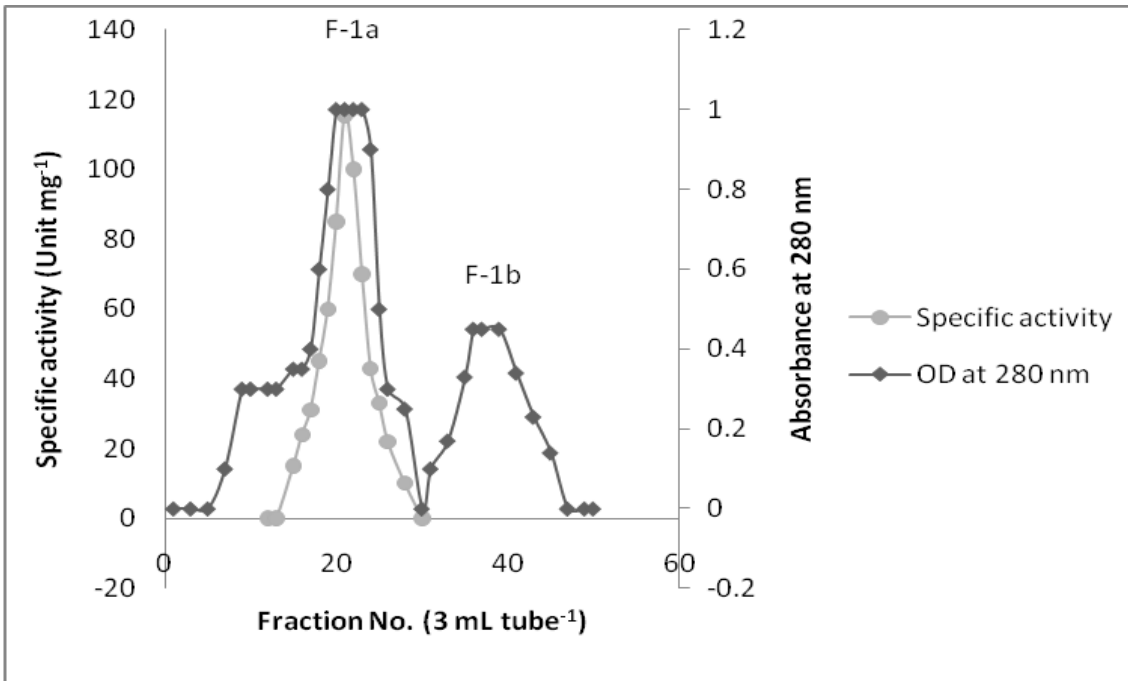


Fig. 2. Elution profile of Sephadex G-200 column (2.7 x 40.0 cm) *M. oleifera* L. leaves peroxidase

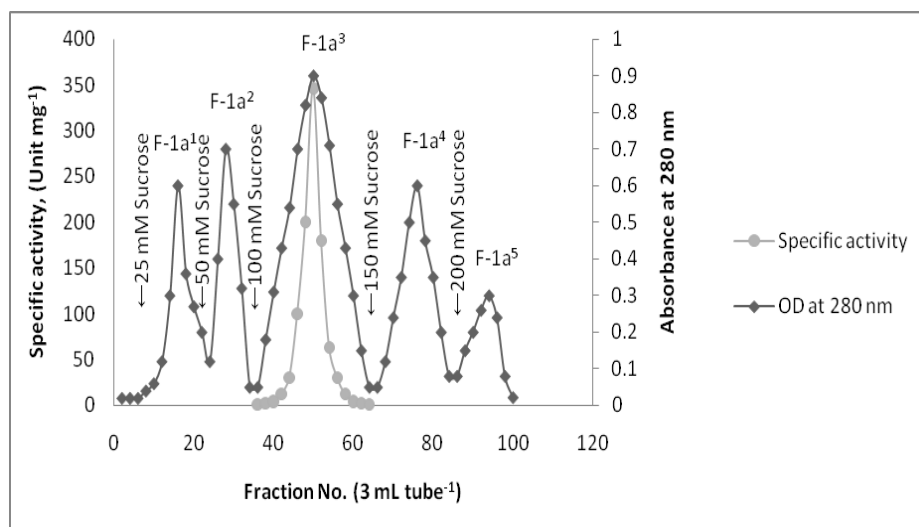


Fig. 3. Elution profile of Con A-Sepharose column (8.0×50.0 mm) for *M. oleifera* L. leaves peroxidase

Electrophoresis

The SDS-PAGE analysis of the purified peroxidase, without and with treatment of 2-mercaptoethanol, revealed a single protein band, suggesting that the purified peroxidase enzyme consisted of a single polypeptide chain with molecular weight of 43 kDa (Fig. 4).

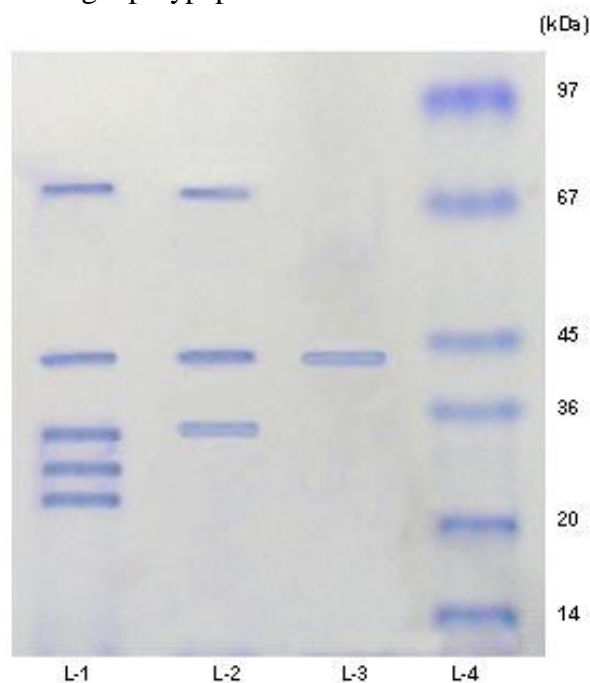


Fig. 4. Photographic representation of the SDS-PAGE of different fractions of peroxidase obtained during purification steps and standard proteins. L-1: Crude enzyme extract, L-2: extract after gel filtration on Sephadex G-100 column, L-3: purified peroxidase from *M. oleifera* L. leaves, L-4: Molecular weight markers

With some exceptions, the majority of peroxidases reported are monomers. Kokkinakis and Brooks (1979) and Vamos-Vigyazo (1981) reported the plant sources monomeric peroxidases, molecular weight varies from 30 to 60 kDa. Our results indicate that the *M. oleifera* L. leaves peroxidase monomer has a molecular weight in the same range (40 to 48 kDa) as previously reported for rice (Ito *et al.* 1991), broccoli (Thongsook and Barrett 2005), vanilla bean (Marquez *et al.* 2008), tomato (Jen *et al.* 1980), and cotton (Triplett and Mellon 1992).

Kinetic Constants

Kinetic studies were carried out under standard conditions. Apparent K_m and V_{max} values, determined from Lineweaver-Burk plots, were 0.2335 mM and 0.9346 U/mL.min, respectively for guaiacol as substrate. This lower K_m value indicating the higher affinity of *M. oleifera* L. leaves peroxidase for substrate guaiacol. It was reported that the K_m values of peroxidase activity from spring cabbage was 0.357 mM (Belcarz *et al.* 2008).

Optimum pH and pH stability

M. oleifera L. leaves peroxidase showed optimum activity at pH 6.0 (Fig. 5). A rapid decrease in activity was found on either the basic or acidic side of this optimum pH. Similar optimum pH (6.0) was observed for peroxidase from broccoli (Thongsook and Barrett 2005) and *Copaifera longsdorffii* leaves (Maciel *et al.* 2007). The pH of peroxidase purified from red delicious apple was 5.0 to 6.0, royal delicious apple 7.0 (Dubey *et al.* 2007), and turnip 4.0 (Motamed *et al.* 2009). The enzyme was stable over a narrow range of pH from 5.0 to 7.0 after 24 h incubation at 4 °C; the residual activity at pH 9.0 was 22 % (Fig. 5).

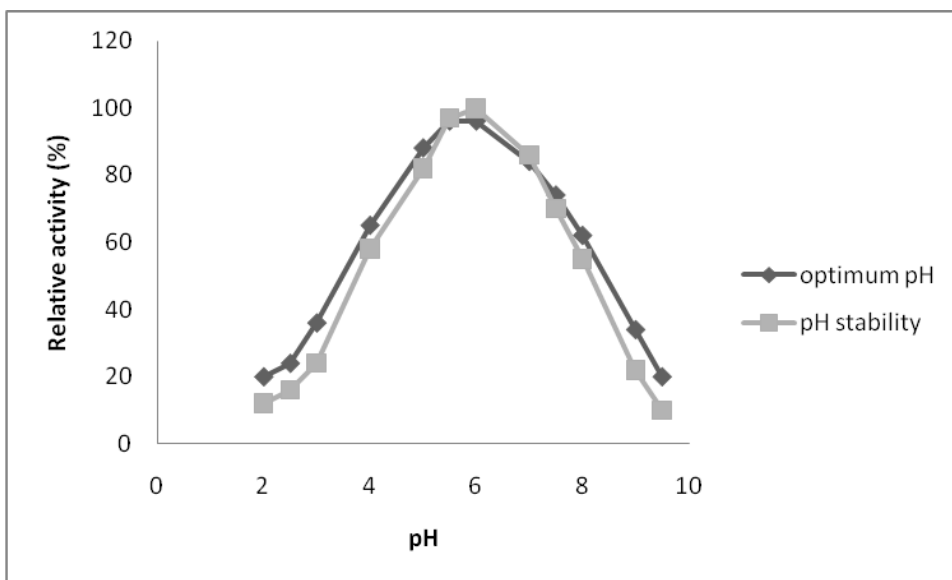


Fig. 5. Effects of pH on the activity and stability of *M. oleifera* L. leaves peroxidase

Optimum Temperature and Temperature Stability

M. oleifera L. leaves peroxidase maintained above 50% activity over a temperature range of 20 to 70°C with the optimum at 50 °C (Fig. 6). The enzyme activity increased sharply with a gradual increase in temperature up to 50 °C, while it gradually declined with further an increase in temperature, indicating a loss in the active conformation of the enzyme. The enzyme was only 18 % active at 80 °C. The optimal activity at 55 °C was reported from the soft stem of *Leucaena leucocephala* peroxidase (Pandey and Dwivedi 2011). On the other hand, maximum peroxidase activity of strawberry fruits was reported at 30 °C (Civello *et al.* 1995). The enzyme was stable at temperatures up to 60 °C for 30 min incubation (Fig. 6). Rapid inactivation occurred above 60 °C.

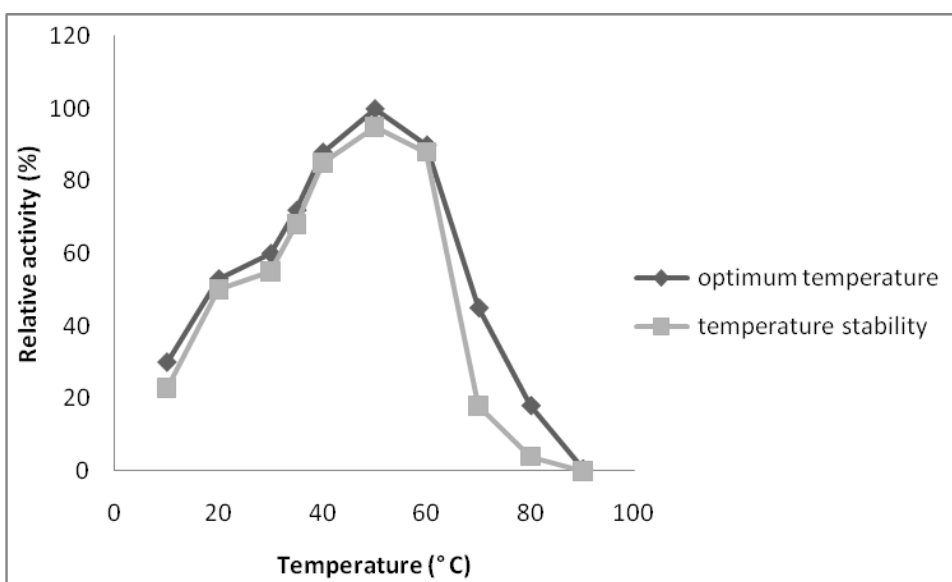


Fig. 6. Effects of temperature on the activity and stability of *M. oleifera* L. leaves peroxidase

Glycoprotein Test and Sugar Estimation

The peroxidase purified from *M. oleifera* L. leaves showed a yellow-orange color in the presence of phenol-sulfuric acid, indicating that the enzyme contained sugar and hence was a glycoprotein. The sugar content of the enzyme was calculated to be 9.05%. Peroxidase purified from vanilla bean (Marquez *et al.* 2008), turnip roots (Duarte-Vazquez *et al.* 2001), Korean radish (Lee and Kim 1994), and Japanese radish (Kim and Kim 1996), have been reported to contain 15, 9.1, 9 to 14, and 20% sugar bound to the protein moiety, respectively. The presence of carbohydrate in broccoli stem peroxidase was also observed (Thongsook and Barrett 2005).

Substrate Specificity

The data on substrate specificity of the enzyme are summarized in Table 3.

Table 3. Substrate Specificity of *M. oleifera* L. Leaves Peroxidase

Substrate	Residual activity
Guaiacol	100
o-dianisidine	95
Catechol	95
Pyrogallol	99

The enzyme revealed similar specific activities against o-dianisidine and catechol; the result suggested that the better substrate for the enzyme was pyrogallol, and the best substrate was guaiacol for the peroxidase enzyme from *M. oleifera* leaves.

Effect of Chemicals and Metal Ions on the Purified Peroxidase

Table 4 shows the effects of chemicals and metal ions on the purified peroxidase determined at pH 6.0. One millimolar (mM) EDTA markedly inhibited the peroxidase activity. In this study, some metal ions (Ni^{2+} , Pb^{2+} , Zn^{2+} , Al^{3+} , Mg^{2+} , Cu^{2+} , Co^{2+} , and Cd^{2+}) exhibited a low inhibitory effect, while Fe^{2+} , Fe^{3+} , and Hg^{2+} exhibited a strong inhibitory effect. The inhibitory effects of Hg^{2+} on the activity of horseradish peroxidase were also reported (Einollahi *et al.* 2006). Marquez *et al.* (2008) reported similar inhibitory effects of EDTA on the activity of vanilla bean peroxidase. Sat (2008) and Marquez *et al.* (2008) also reported the inhibitory effect of some metals on the activity of peroxidase enzyme purified from Jerusalem artichoke tubers and vanilla bean, respectively. Hg^{2+} acts as a potent inhibitor of enzymatic reactions by binding to SH groups present in the active site of enzyme causing its irreversible inactivation (Vallee and Ulmer 1972). Debowska and Podstolski (2001) reported that EDTA, by reacting as a chelating agent of the Fe^{2+} atom found in the *Vanilla planifolia* shoot peroxidase active center, markedly inhibited the enzyme activity.

Table 4. EDTA and Metal Ions vs. *M. oleifera* L. Leaves Peroxidase Activity

Reagents	Residual activity					
	1 mM	2 mM	5 mM	10 mM	15 mM	20 mM
None	100	100	100	100	100	100
EDTA	70.1	62.6	54.1	35.3	25.1	15.2
Fe^{3+}	85.5	80.0	70.3	50.5	25.0	18.2
Fe^{2+}	93.1	89.5	80.0	60.5	40.3	25.4
Co^{2+}	97.4	94.9	86.3	65.4	42.3	26.2
Cd^{2+}	84.2	75.5	60.3	45.5	40.1	35.3
Ni^{2+}	96.4	92.1	82.5	60.3	37.5	10.3
Hg^{2+}	65.5	54.3	50.0	30.4	25	10.2
Pb^{2+}	90.1	80.3	70.5	40.5	30.3	12.5
Zn^{2+}	95.0	90.0	75.5	56.6	40.2	25.4
Al^{3+}	97.5	93.2	80.1	75.0	53.0	40.3
Mg^{2+}	93.8	85.0	77.2	69.12	58.0	43.3

CONCLUSIONS

Due to its wide applicability, the peroxidase enzyme has gained a dominant position in the area of biotechnology, biochemistry, and industrial technology. To use this enzyme in those areas, it is very important to have temperature stability and to maintain activity over a broad pH range. *Moringa oleifera* L. leaves are available in large quantities in almost all seasons, purified peroxidase from *Moringa oleifera* L. leaves is more stable and active in acid pHs, and the activity remains 90% at 60 °C for 30 min incubation. It has more substrate affinity according to its K_m values and it is possible to obtain highly purified peroxidase by these procedures.

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