

A Defense Associated Peroxidase from Lemon Having Dye Decolorizing Ability and Offering Resistance to Heat, Heavy Metals and Organic Solvents

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Abstract

Peroxidases have ability to catalyze redox reaction of a wide range of phenolic as well as non-phenolic compounds and exhibited various physiological roles in plant life cycle. From the point of view of industrial applications of peroxidases, the isolation and characterization of peroxidases offering resistance to higher pH and temperature, salts, organic solvents is highly desirable. In this direction, a partial cDNA clone of peroxidase from lemon was isolated and characterized. The peroxidase was found to be defense associated as evident by the higher expression in diseased condition than that of healthy one at both the transcript and enzymatic activity levels. This defense related peroxidase from lemon leaves was purified to homogeneity using quick two step processes of heat treatment and affinity chromatography. The native peroxidase was found to be a heterotrimer of 200 kDa, consisting of two subunits each of 66 kDa while, one subunit of 70 kDa. The purified peroxidase was found to be stable towards heat (retained 92% activity at 80°C for 1 h) and organic solvents, namely ethanol, methanol and isopropanol (retained 30-50% activity in the presence of 50% (v/v) of these solvents for 1 h). Purified peroxidase also exhibited tolerance to heavy metal ions such as Cd²⁺, Ni²⁺ and Cs²⁺. The purified lemon peroxidase was found to efficiently oxidize the industrial dyes in the order of aniline blue>methyl orange>indigo carmine >trypan blue > crystal violet, such that 40-54% dye decolorization was observed within 4 h. Thus, the properties exhibited by purified lemon peroxidase make it a promising candidate enzyme for industrial exploitation.

Keywords: Defense response; Dye decolorization; Peroxidase; Thermo-stability

Introduction

Peroxidases are one of the major H₂O₂ decomposing enzymes and have ability to catalyze redox reaction of a wide range of phenolic as well as non-phenolic compounds. On the basis of presence or absence of heme, the peroxidases have been classified into heme and non-heme peroxidases [1]. Heme peroxidases have further been assigned into two superfamilies, namely peroxidase-cyclooxygenase (PCOX) superfamily and the peroxidase-catalase (PCAT) superfamily [2,3]. The peroxidases belonging to PCOX superfamily exclusively contain animal peroxidases which have been suggested to be involved in the innate immunity, defense responses etc. [4]. The myeloperoxidase, eosinophil peroxidase, lactoperoxidase, etc. are examples of peroxidases belonging to this family. The PCAT superfamily is the most intensively studied superfamily of non-animal heme peroxidases. The non-animal peroxidases have further been sub-divided into three classes namely, class I, II and III [5]. Class I peroxidases exhibited major role in oxidative stress i.e. detoxification of ROS [6]. They include cytochrome c peroxidase, ascorbate peroxidase and catalase peroxidase. The class II peroxidases, exclusively containing fungal peroxidases, have major role in lignin biodegradation [7]. They include lignin peroxidases, manganese peroxidases and versatile peroxidases. The class III peroxidases are widely distributed in plant kingdom [8]. They include horseradish peroxidase (HRP), peanut peroxidase, soybean peroxidase, etc.

The class III plant peroxidases have been reported to play crucial roles in the plant life cycle [13] such as cell wall metabolism [9], lignification [10], suberization [11], auxins metabolism [12], wound healing, reactive oxygen species (ROS) and reactive nitrogen species (RNS) metabolism [13,14], fruit growth and ripening [15], defense against pathogens [16] etc.

Peroxidases are significant enzymes from the point of view of their medicinal, biochemical, immunological, biotechnological and industrial applications [17]. Thus, peroxidases have been shown to

have great potential in the decolorization process to decrease textile industry pollutant residues [18]. HRP is reported to degrade phenol and substituted phenols via a free radical polymerization mechanism and can be effective in degrading and precipitating industrially important azo dyes [19]. Subramaniam, et al, [20] have also suggested the Bitter Gourd peroxidase, as a dye decolorizing peroxidase that could be used in treatment of effluents with dyes from tanning industries. Uses of some peroxidases, such as horseradish, *Brassica campestris*, turnip, tomato etc. for degrading and detoxifying polyaromatic hydrocarbons, polychlorinated biphenyls, have been attempted [21,22]. Some peroxidases have also been reported for removal of phenolic compounds from synthetic model effluents and also from real industrial effluents [19,23]. Peroxidases have also been reported to have immense potential and wide spread application as biosensors [23]. The importance of peroxidases in polymer chemistry is based on their ability to oxidize a variety of phenolic molecules which provide ideal sites for cross-linking leading to polymerization reactions. The grafting applications become an important industrial application of peroxidases [24].

Peroxidases have been purified and characterized from a number of sources like tea, horseradish, soybean, *Lycopersicon esculentum*, *Beta vulgaris*, etc. [25-29]. Native structure of plant peroxidases are reported to vary from being monomeric to multimeric (either homo- or hetero-

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meric). Thus, the well characterized class III peroxidase, i.e. HRP is a monomeric protein of 40 kDa. Peroxidase from *Roystonea regia* is a homodimeric protein consisting of 51 kDa subunit each, while the tomato peroxidase is reported to be a heterodimeric protein consisting of 63 and 57 kDa subunit each [28,30]. The *Leucaena leucocephala* peroxidase, on the other hand, is reported to be a heterotrimeric protein consisting of two subunits each of 67 kDa and one subunit of 58 kDa [31]. Peroxidases are reported to have broad substrate specificity exhibiting wide variation in Michaelis–Menten constant (K_m) values for their substrates. Thus, the peroxidases isolated from different sources such as *Carica papaya*, *Leucaena leucocephala*, lettuce, exhibited K_m for guaiacol as 0.8, 2.9, 4.74 mM etc., respectively [31,32]. Similarly, the K_m for H_2O_2 are reported to be 0.25 mM for *Carica papaya* peroxidase [32], 1.2 mM for *Ficus sycomorus* latex peroxidase [33] and 5.6 mM for *Leucaena leucocephala* peroxidase [31]. With regards to optimum pH for plant peroxidases, the source as well as tissue specific variability have been reported in literature. Thus, for example, peroxidase from potato, *Vigna sp.*, exhibited optimum pH of 5.0 [34,35], while the peroxidases isolated from carrot, radish exhibited optimum activity at pH 6.0 [34,36]. Similarly, the optimum temperature for most of the peroxidases varied greatly. For example, the optimum temperature of peroxidases from *Carica papaya*, cabbage, *Leucaena leucocephala* have been reported to be 40°C, 50°C and 55°C, etc. respectively [31,32,36].

In literature, some thermostable peroxidases have been reported. For example, the African oil palm tree peroxidase which exhibited stability at 70°C for 1 hr at neutral pH [37], while, the *Ficus sycomorus* peroxidase retained 80% activity at 70°C after 15 min incubation [33] and the peroxidase isolated from vigna species retained its 65% activity for 1 hr at 70°C [35]. The horseradish peroxidase (HRP), the industrially most exploited peroxidase, exhibited loss of activity at 70°C within 5 min [25]. Thus, the availability of novel peroxidases having tolerance towards heat, organic solvents, salts, heavy metals etc, is scarce.

In view of array of industrial applications of peroxidases, search for peroxidases having novel properties such as tolerance to heat, organic solvents, salts etc., as good alternative to microbial peroxidases, is of prime concern for both fundamental studies on their structure and catalytic mechanism as well as their application in biotechnology / bioremediation / industrial sectors. Thus, in this direction, in the present paper, a cDNA of peroxidase was cloned and characterized. The expressional analyses of the lemon peroxidase at transcript as well as activity level were investigated in healthy and diseased lemon leaves to find out its role in defense response. Furthermore, the peroxidase from lemon leaves was purified through successive steps of heat treatment (70°C) and con-A affinity column chromatography and its physicochemical properties, namely molecular weight, subunit composition, effect of substrates, pH, temperature, salts, and organic solvents has been investigated. In addition, the industrial applicability of the purified lemon peroxidase has also been demonstrated by investigating industrial dye decolorizing ability and efficacy.

Material and Methods

Plant material

Young leaves of lemon (*Citrus limon*), a small evergreen tree native to Asia and used for various culinary and non-culinary purposes throughout the world, was collected from the garden of Department of Biochemistry, University of Lucknow, India and used as plant material for gene cloning, enzyme extraction and purification. The expressional analyses were carried out using healthy and diseased leaves. On the 3rd day of physical appearance of the disease, the diseased leaves, were

taken from the plants infected with *Xanthomonas axonopodis*, causing citrus canker disease.

Preparation of crude enzyme extract

A 25% homogenate of *C. limon* leaves was made in Tris-HCl buffer (100 mM, pH-7.5), containing PVP (polyvinylpyrrolidone) insoluble (0.1% w/v) and β -mercaptoethanol (7 mM) using ice cold blender. Homogenate was centrifuged at 8422 x g for 30 min at 4°C using Sigma 4K15 centrifuge and clear supernatant was collected and used as crude enzyme extract.

Peroxidase activity assay and protein estimation

Peroxidase activity was measured by Putter's protocol (Pütter, 1974) with slight modification, using guaiacol as substrate. The 3 ml assay system consisted of Tris-HCl (50 mM, pH 7.0), H_2O_2 (20 mM) and guaiacol (3.33 mM). Reaction was started by adding a suitable amount of enzyme aliquot in the assay system. The 3, 3'-Dimethoxy-4, 4'-biphenol formation was monitored spectrophotometrically by measuring increase in absorbance at 470 nm at 30°C using ELICO SL-159 UV-Vis spectrophotometer [38]. The molar extinction coefficient of 3,3'-dimethoxy-4,4'- biphenol was used as 6.39 $mM^{-1} cm^{-1}$. One unit of enzyme activity was defined as the enzyme catalyzing the production of 1 mmol of 3,3'-dimethoxy-4,4' biphenol per min at reaction condition. Protein was estimated by Bradford's method using BSA as standard [39].

Cloning and characterization of lemon peroxidase

Total RNA was isolated using total RNA isolation kit (Merk GeNei, India) following the protocol of manufacturer. In order to isolate a cDNA clone of peroxidase gene, the first strand cDNA was prepared using Revert Aid First strand cDNA synthesis kit (MBI Fermentas) following the manufacturer's protocols and subjected to PCR using lemon peroxidase specific forward primer (binding toward 5' end) (FP1: 5'CTTATATGTGTTTTGTTC AAGGGTG 3') and reverse primer (binding towards 3' end) (RP1: 5' CTCCTGAGAGTGC AAC AAGATC-3') with the thermal parameter of initial denaturation at 94°C for 5 min; 35 cycles of cyclic denaturation at 94°C for 45 s, annealing at 54°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min in DNA Engine (PTC 200, MJ research). The obtained amplicon was ligated in pGEM T-Easy vector (Promega; Madison, USA) using PCR based cloning approach and transformed in *E. coli* DH5 α cells. The transformants were screened out using blue white selection on LB agar plate containing ampicillin (100 μ g/mL), following the protocol as described by Green and Sambrook [40]. The plasmid was isolated from the positive recombinant colony and sequenced commercially.

The nucleotide sequence of the cDNA clone was analyzed using Basic Local Alignment Search Tool (BLASTn) tool of NCBI and converted into its deduced amino acid sequences using online Translator tool on ExPASy. The sequence similarity as well as conserved domain analysis of the amino acid sequence was done using BLASTp and CD (conserved domain) search tool of NCBI to confirm the clone as peroxidase.

Biochemical and molecular expressional analyses

In order to investigate the role of lemon peroxidase in defense response, the relative expressional analyses, under healthy and diseased conditions, at transcript level, was performed. Relative expressional analyses of transcripts of the lemon peroxidase was measured by quantitative Reverse Transcriptase PCR (qRT-PCR) with the cDNA

samples, prepared from RNA samples, isolated from healthy and diseased lemon leaves, using qRT-PCR master mix kit (Stratagene). For qRT-PCR, the same primers (FP1 and RP1) were used in real time PCR machine (Stratagene) followed by the protocol as described by the manufacturer. The thermal conditions of the reaction as: 1 cycle at 95°C (5 min); 40 cycles at 95°C (30 s), 54°C (20 s) and 72°C (60 s) and finally a denaturation cycle at 95°C (60 s), 54°C (30 s) and 95°C (30 s). The fold expression was calculated by the $2^{-\Delta\Delta Ct}$ method as described by Pfaffl [41].

For Biochemical expressional analyses, the homogenates were prepared separately from healthy and diseased lemon leaves and peroxidase activity was measured as described earlier.

Purification of peroxidase

For purification, the crude enzyme extract was incubated at 70°C for 1 h in a water bath and subsequently allowed to cool at room temperature and centrifuged at $8422 \times g$ for 10 min at 25°C using Sigma 4K15 centrifuge. The supernatant obtained was subjected to purification through Con-A affinity column chromatography as described by Pandey and Dwivedi [31]. The different fractions were collected and the protein and activity were analyzed as described earlier. The fractions showing higher specific activity were used for further biochemical characterization.

To check the homogeneity of preparation, native PAGE was done by making 10 % resolving gel with 3 % stacking gel as described by Davis [42]. Enzyme samples, containing equal amount of protein, were loaded into the wells of gel and electrophoresis was run at 4°C at constant voltage of 120 V using Tris-glycine running buffer (pH 8.3).

Physicochemical characterization of purified peroxidase

Molecular weight determination

The native molecular weight of the purified peroxidase was determined by gel filtration chromatography using Sephadex G-200 matrix. The void volume (V_0) of the column was determined using blue dextran. Catalase (240 kDa), alcohol dehydrogenase (150 kDa), phosphorylase β (97.4 kDa), bovine serum albumin (67 kDa) and lysozyme (14.3 kDa) were used as standard proteins. The elution volume (V_e) of each standard protein as well as purified peroxidase was determined. The molecular weight of the purified peroxidase was calculated from a calibration curve (log molecular weight of the standard proteins versus V_e/V_0).

The native molecular weight of the purified peroxidase was also determined by running native-PAGE as described [31] with native molecular weight markers namely soybean trypsin inhibitor (20.1 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and catalase (240 kDa) along with purified peroxidase separately. The relative mobilities (R_m) of marker proteins were plotted against their log molecular weight. The molecular weight of purified peroxidase was calculated with the help of the calibration curve.

Subunit molecular weight of the purified peroxidase was determined using SDS-PAGE as described by Laemmli [43]. After electrophoresis, the gel was stained with silver staining as described by Switzer and co-workers [44].

Effect of substrates

The effect of substrates (guaiacol and H_2O_2) was determined by incubating a suitable amount of purified enzyme aliquots with varying concentrations of guaiacol and a fixed saturating concentration of H_2O_2 or vice versa. The K_m was determined using the Lineweaver–Burk plot.

Effects of pH, temperature and thermostability

The effect of pH on enzyme activity was determined using 50 mM buffer of different pH such as sodium tartrate (pH 3.0), sodium acetate (pH 4.5 and 5.0), sodium phosphate (pH 6.0, 6.5 and 7.0), Tris–HCl (pH 7.5, 8.0, 8.5 and 9.0) at fixed concentration of guaiacol and H_2O_2 .

The optimum temperature was determined, by incubating the whole reaction mixture along with enzyme at various temperatures ranging from 20°C to 80°C for 5 min and peroxidase activity was measured spectrophotometrically as described earlier.

Furthermore, in order to explore the thermostability of the purified enzyme at 70°C, the enzyme aliquot was incubated at 70°C for 120 min and activity was measured at every 20 min interval as described earlier. In addition, the thermostability of purified peroxidase was also determined by incubating the enzyme at 75°C, 80°C and 85°C for 1 hr and activity was measured at 20 min interval as described earlier.

Effect of metal ions

The effect of different metal ions such as Na^+ , Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} on purified peroxidase was determined by performing the enzyme assay with different concentration of their salts (5-500 mM). The effect of heavy metals namely cadmium, cesium, nickel and mercury were also determined with varying the concentration of their salts (1-20 mM).

Effect of organic solvents

Effect of different organic solvents namely ethanol, methanol and isopropanol on purified peroxidase activity was investigated by incubating the enzyme for 1 h in presence of various concentration of the organic solvents (0-80%) and activity was measured after 30 min and 60 min of incubation as described earlier.

Dye decolorization

Dyes, namely methyl orange (azo group), congo red, trypan blue (diazo group), aniline blue, malachite green, crystal violet (triphenylmethane) and indigo carmine (indigoid group) were used to determine the efficacy of the purified peroxidase in dye decolorization as described by Parshetti and co-workers with slight modification. Reaction mixture (3 ml), consisted of Tris-HCl (50 mM, pH 7.0), H_2O_2 (20 mM), dye (50 μg) and purified peroxidase (40 μg), was incubated at 30°C for 4 hrs. The untreated dye (dye without enzyme and H_2O_2) was used as control. Since H_2O_2 is a well-known bleaching agent and thus, in order to investigate the effect of H_2O_2 , a parallel control (dye with only H_2O_2 and no enzyme) was also used. After 4 hrs incubation, the absorbance was measured at 500, 596, 617, 590, 457, 595 and 610 nm for congo red, aniline blue, malachite green, crystal violet, methyl orange, trypan blue and indigo carmine, respectively as described by Chanwun and coworkers [45]. The efficacy of peroxidase on dye decolorization as a function of dye concentration (10-100 μg) was also investigated.

The percentage dye decolorization was calculated using the formula:

$$\% \text{ dye decolorization} = (A_0 - A) \times 100 / A_0$$

Where A_0 is absorbance of untreated dye, A is absorbance of treated dye after desired time interval. The net % dye decolorization was calculated by subtracting the % dye decolorization in presence of H_2O_2 alone (parallel control), from % dye decolorization in presence of both enzyme and H_2O_2 .

Statistical Analyses

All the experiments were performed in triplicates and accordingly

the statistical analyses such as calculation of mean and standard deviation were done.

Results and Discussion

Cloning and validation of lemon peroxidase

A partial cDNA of peroxidase from lemon leaves was isolated and cloned following the PCR based approach. The nucleotide sequence (408bp) of this clone, designated as *LPRX* (lemon peroxidase), was analyzed through BLASTn tool which exhibited homology with peroxidase from various sources. The deduced amino acid sequence of the clone, obtained by translator tool of ExPASy, was again subjected to BLASTp analysis which also exhibited homology with various peroxidases thereby validating the clone to be of peroxidase. The conserved domain analysis of the *LPRX* protein was done using CD search tool of NCBI. The analyses revealed that the *LPRX* protein exhibited similarity with secretory peroxidases (cd00693) belonging to plant heme-dependent peroxidase superfamily. The nucleotide and protein sequence of the partial peroxidase gene along with conserved domain analyses is shown in Figure 1.

Functional validation of lemon peroxidase in defense response

The role of the cloned lemon peroxidase in defense response was investigated by performing relative transcript expressional analyses using qRT-PCR. Results are shown in Figure 2. The data revealed about three folds' higher expression of the lemon peroxidase, at transcript level, under diseased condition as compared to that of healthy (normal) one, suggesting the role of this peroxidase in defense response. Further investigation at the level of enzyme activity, the peroxidase activity was found to be about 4 folds more under diseased condition as compared to that of healthy one. Thus, based on these analyses, the role

of lemon peroxidase in defense response was explicitly demonstrated. Involvement of peroxidases in defense response under fungal and viral infections has been demonstrated in a number of plants such as maize, peaches and bean [46-48]. A ripening associated peroxidase from papaya has also been shown to exhibit a role in defense response [49].

Purification of peroxidase

The defense related peroxidase from the leaves of *Citrus limon* (lemon) was isolated and purified through successive steps of heat treatment (70°C) and con-A affinity column chromatography. The purification chart for lemon peroxidase is presented in Table 1. Thus, the lemon peroxidase was purified to 34.40 folds with overall recovery of 54.22% and specific activity 420.67 Units/mg. Homogeneity of the purified enzyme was established by running native PAGE where a single band was obtained (Figure 3B).

Molecular weight determination

Native molecular weight of purified peroxidase was determined by gel filtration column chromatography as well as by running native PAGE with standard molecular weight marker proteins. The results are presented in Figures 3A and 3B. The native molecular weights of the purified peroxidase from both the methods were found to be ~200 kDa. The purified peroxidase was further analyzed for subunit size and composition by running SDS-PAGE. Results are shown in Figure 3C. SDS PAGE analyses revealed that the purified lemon peroxidase exhibited two different sized subunits each of about 66 and 70 kDa. Thus, based on the subunit size of the purified peroxidase, a heterotrimeric structure (consisting of two subunits of 66 kDa, one subunit of 70 kDa) is suggested for purified lemon peroxidase.

In literature, wide variability in the size of peroxidases isolated from various sources has been reported. For example, *Leucaena leucocephala* peroxidase is reported to be a heterotrimeric protein 200 kDa



Figure 1: (A) Nucleotide (red) and protein (black) sequence of lemon peroxidase (B) conserved domain analyses.

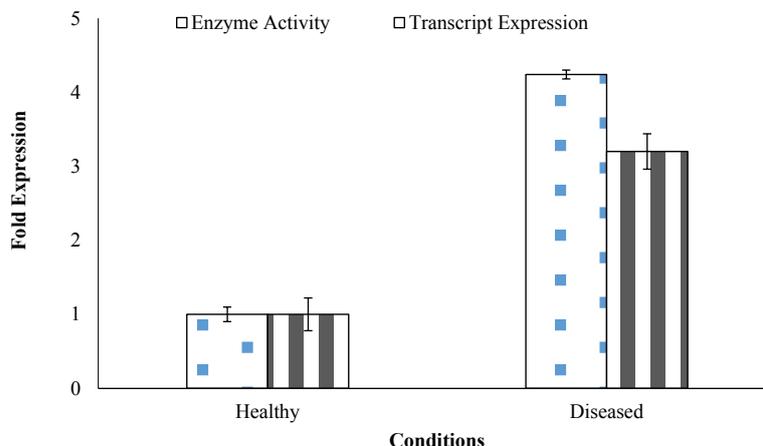


Figure 2: Expressional analyses of lemon peroxidase at transcript and activity levels.

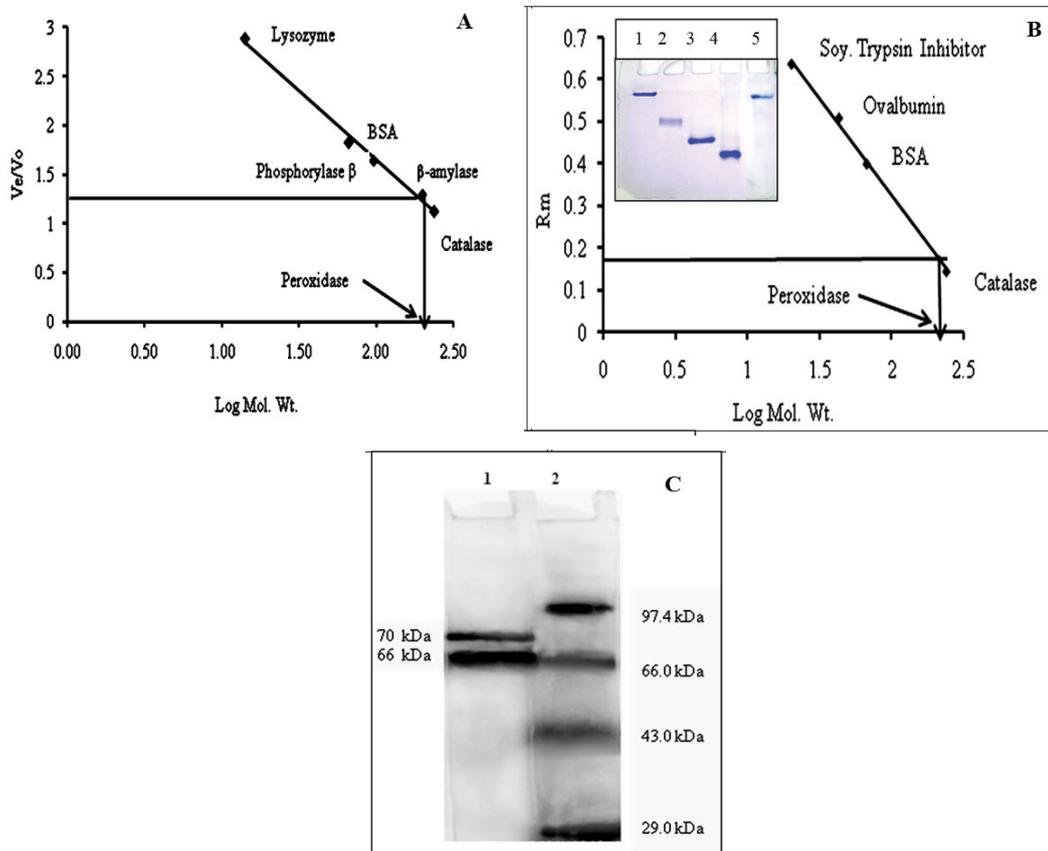


Figure 3: (A) Calibration plot (V_e/V_o versus log molecular weight) for molecular weight determination of purified peroxidase by gel filtration Chromatography. The standard molecular weight marker proteins used were lysozyme (14.3kDa), bovine serum albumin (67 kDa), phosphorylase B (97.4 kDa), β amylase (200kDa) and catalase (240 kDa). (B) Calibration plot (relative mobility (R_m) versus log molecular weight) for molecular weight determination of purified peroxidase by Native-PAGE. The standard molecular weight marker proteins used were soybean trypsin inhibitor (20.1 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and catalase (240 kDa). The picture of native-PAGE gel stained with Coomassie brilliant blue staining is presented in inset. (Lanes 1, 2, 3, 4, 5: catalase (240kDa), bovine serum albumin (67 kDa), ovalbumin (43kDa), soybean trypsin inhibitor (20.1 kDa), purified lemon peroxidase, respectively) (C) SDS-PAGE analysis of purified peroxidase (Lane 1: purified peroxidase enzyme Lane 2: molecular weight marker. The protein bands were visualized by silver staining).

Steps	Total activity (Units)	Total protein (mg)	Specific Activity (U/mg)	Recovery (%)	Fold Purification
Crude	60078.20	4911.52	12.23	100	1
Heat Treatment (70°C)	49056.69	787.23	62.32	81.65	5.10
Con-A Affinity Column Chromatography	32576.83	77.44	420.67	54.22	34.40

Table 1: Purification chart for peroxidase isolated from lemon.

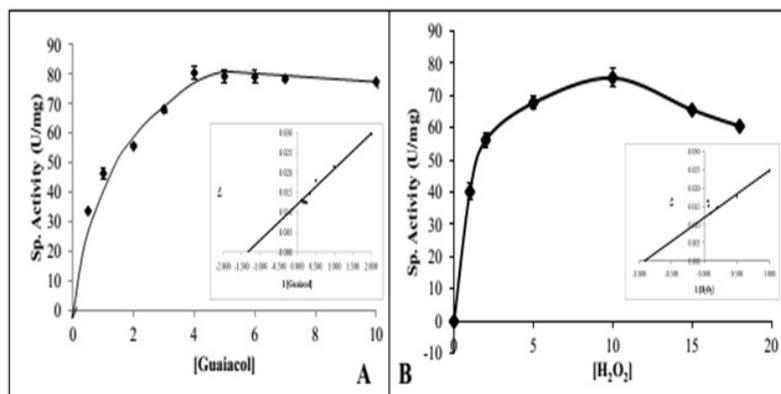


Figure 4: Effect of substrates, guaiacol (A): H₂O₂ (B): On the activity of purified peroxidase. (Inset shows Line-weaver–Burk plot of the data).

(consisting of two subunits of 66 kDa and one subunit of 58 kDa) [31]. The tomato peroxidase is reported to be a heterodimeric protein with 63 and 57 kDa subunit size [28] while, *R. regia* peroxidase is reported to be a homodimeric structure with 51 kDa subunit size each [30]. The peroxidases, purified from lettuce, horseradish, cauliflower are reported to be monomeric protein of size 35, 40, 44 kDa, respectively [26,32,50].

Effect of substrates on purified peroxidase

Effect of guaiacol on the purified peroxidase activity was determined by varying the concentration of guaiacol at the fixed and saturating concentration of second substrate H₂O₂. Results are presented in Figure 4A. The K_m for guaiacol was found to be 0.77 mM. Furthermore, the effect of H₂O₂ on the purified peroxidase activity was determined by varying the H₂O₂ concentration at fixed and saturating concentration of guaiacol. Results were presented in Figure 4B. Inhibition of the peroxidase activity at high H₂O₂ concentration was observed. Apparent K_m for H₂O₂ was found to be 1.09 mM.

Thus, the purified lemon peroxidase exhibited higher substrate affinity towards guaiacol than those of other peroxidases isolated from *Leucaena leucocephala*, lettuce, *Ficus*, cauliflower which have been reported to exhibit the K_m values for guaiacol as 2.9, 4.74, 9.5, 141.61 mM, respectively [31,32,33,50]. The purified Lemon peroxidase was also found to exhibit lower affinity for H₂O₂ ($K_m=1.09$ mM) than those of the peroxidases isolated from *Vigna* species ($K_m=0.45$ mM), tobacco ($K_m=0.250$ mM), radish ($K_m=0.277$ mM), cabbage ($K_m=0.370$ mM) [35,36]. The K_m close to our peroxidase has been reported for *Ficus sycomorus* peroxidase ($K_m=1.2$ mM) [33].

Effect of pH on purified peroxidase

The effect of pH on purified lemon peroxidase was investigated and results are presented in Figure 5A. It is noteworthy that the purified peroxidase was found to exhibit narrow pH optima in the range of 4.5–6.0, with maximum activity at pH 5.0. The enzyme activity steeply decreased beyond pH 6.5 and was completely lost at pH >7.0.

At very low pH, loss of activity may be due to instability of the heme binding to the enzyme, but loss of activity at high pH, may be due to chemical changes in the heme and protein denaturation. Similar reports of acidic pH optima (pH 5) for peroxidase of various plant species such as potato, tomato, *vigna* species, *Leucaena*, wheat grass and *Allium sativum* have been reported in literature [31,34,35,51]. Peroxidases having similar range of pH optima have also been reported.

Thus, pH optima in the range of pH 4.0–5.5 for lettuce [32] and pH 5.5–6.0 for papaya [52] have been reported. The peroxidases isolated from carrot, cabbage, radish and Turkish black radish have been reported to have optimum pH of 6.0 [21,34,36]. While, on the other hand, the peroxidases isolated from leaves of African oil palm, *Elaeis guineensis* are reported to exhibit their optimum pH of 7.0 [36,37].

Effect of temperature on purified peroxidase

The effect of temperature on purified peroxidase was investigated by assaying the enzyme activity at various temperatures (Figure 5B). The optimum temperature for *Citrus limon* peroxidase was found to be 40°C. About 73% activity was found to retain even at 60°C and beyond that the decline in activity was observed. The Q_{10} value of lemon peroxidase was found to be >1 upto optimum temperature, suggesting that the reaction rate is temperature-dependent.

Thermostability of the purified enzyme was investigated by incubating the enzyme at 70, 75, 80 and 85°C and the enzyme activity was measured as described earlier by withdrawing suitable aliquots at different time intervals. The results are presented in Figure 5C. The lemon peroxidase was found to be almost fully active upto 80°C for 1 h. However, at 70°C enzyme was fully active upto 2 hrs. At 85°C, however, enzyme lost 40% activity within 20 min and beyond that a progressive loss of activity was observed. Thus, it is noteworthy that the purified peroxidase was found to be quite stable up to 80°C.

With regards to temperature optima, wide variability has been reported for peroxidases isolated from various sources. The optimum temperature of red cabbage and lettuce peroxidase has been reported to be 45°C [32,53], whereas *Raphanus sativus* peroxidase exhibited the temperature optima of 30°C [54]. The radish and tobacco peroxidase have been reported to exhibit an optimum temperature of 40°C [36].

The purified peroxidase was found to exhibit better thermostability as compared to other reported thermostable peroxidases. Thus, for example the peroxidase from *Ficus sycomorus* was reported to exhibit 23% activity loss at 70°C after 30 min incubation [33], peroxidase isolated from *vigna* species, on the other hand, retained 65% activity at 70°C for 1 hr [35]. While, the palm tree peroxidase has been reported to be stable at 70°C for 1 hr [55]. In addition, the optimum temperature of the horseradish peroxidase (HRP) was reported to be 30°C which was found to rapidly denature at temperatures above 42°C [26]. Mogharrab, et al. [56] have also reported that the HRP exhibited up to 96.4% loss of activity at 70°C within 10 min. Thus, a comparison of the thermostability of the purified peroxidase, from the present study,

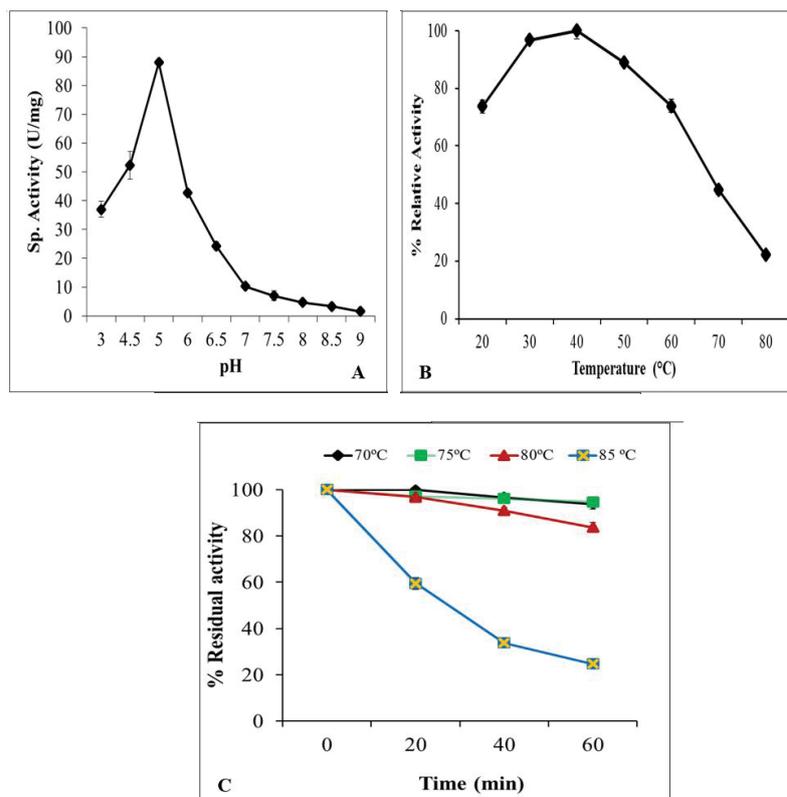


Figure 5: (A) Effect of pH on purified peroxidase (B) Effect of temperature on purified peroxidase (C) Thermo-stability of purified peroxidase.

Metal Salts	% Relative activity at different concentrations							
	Control	5 mM	10 mM	20 mM	50 mM	100 mM	200 mM	500 mM
NaCl	100	125.56 ± 0.74	152.34 ± 2.71	154.73 ± 1.36	137.72 ± 6.38	103.50 ± 0.77	92.66 ± 0.45	72.15 ± 3.56
CaCl ₂	100	131.68 ± 2.37	141.85 ± 1.99	149.83 ± 1.05	135.90 ± 2.32	110.52 ± 4.99	101.04 ± 1.35	83.29 ± 1.65
MnCl ₂	100	125.80 ± 1.41	137.94 ± 1.21	143.23 ± 2.10	152.61 ± 1.43	141.88 ± 2.24	110.52 ± 4.52	73.57 ± 8.25
MgCl ₂	100	101.11 ± 1.26	106.58 ± 1.53	120.92 ± 3.33	129.98 ± 1.35	123.48 ± 1.43	121.83 ± 2.43	101.89 ± 0.98
ZnCl ₂	100	136.75 ± 3.59	147.12 ± 1.36	151.41 ± 2.25	157.03 ± 2.10	165.41 ± 4.88	144.83 ± 1.78	134.21 ± 1.78
Heavy Metal Salts	% Relative activity at different concentration							
	Control	1 mM	2 mM	5 mM	10 mM	12 mM	15 mM	20 mM
CdCl ₂	100	101.10 ± 0.51	112.82 ± 6.06	111.05 ± 1.52	104.79 ± 1.18	103.10 ± 1.36	99.20 ± 1.47	94.78 ± 2.19
CsCl ₂	100	129.44 ± 4.77	143.24 ± 5.56	140.76 ± 1.08	133.51 ± 3.98	132.08 ± 2.99	116.53 ± 4.99	110.52 ± 1.03
NiSO ₄	100	101.88 ± 1.87	104.69 ± 2.61	103.63 ± 1.36	104.33 ± 1.73	101.15 ± 0.70	95.49 ± 2.67	90.54 ± 1.08
HgCl ₂	100	99.91 ± 2.94	94.08 ± 2.03	93.25 ± 3.99	88.42 ± 2.16	85.06 ± 2.90	84.70 ± 2.19	78.87 ± 1.18

Table 2: Effect of various metal salts on the purified peroxidase activity. The data were compared with control (no salts) and represented as % relative specific activity. The value of % relative specific activity represents mean ± S.D. of experiments done in triplicate for each set.

to those of majority of the peroxidases, including that of HRP which is industrially well-exploited, it is evident that the *C. limon* peroxidase exhibits moderate thermostability.

Effect of metal ions on purified peroxidase

The effect of different metal ions (Na⁺, Ca²⁺, Mg²⁺, Mn²⁺, Zn²⁺) on purified lemon peroxidase was investigated. Results are presented in Table 2. Monovalent cation namely Na⁺ activated the peroxidase in a concentration dependent manner with maximum activation at 20 mM while it inhibited the peroxidase at 200 mM and above. Zn²⁺ activated the peroxidase in a concentration dependent manner with maximum activation at 100 mM and did not show inhibitory effect even at 500 mM. Similarly, Mg²⁺ activated the purified peroxidase maximum at

50 mM and did not inhibit peroxidase up to 500 mM. Ca²⁺ and Mn²⁺ activated the peroxidase maximum at 20 and 50 mM concentrations, respectively while inhibited at 500 mM concentration. Thus, Zn²⁺ was found to be the most potent activator of the purified lemon peroxidase. The effects of various heavy metal ions (Cd²⁺, Cs²⁺, Ni²⁺, Hg²⁺) on purified lemon peroxidase were also investigated. Results are shown in Table 2. The purified peroxidase was found to be activated by Cs²⁺ at all the concentration studied (upto 20 mM) with maximum activation at 2 mM. Cd²⁺ and Ni²⁺ were found to activate the peroxidase up to 12 mM with maximum activation at 2 mM. At concentration higher than 12 mM both the metal ions inhibited the peroxidase in a concentration dependent manner. On the other hand, Hg²⁺ exhibited slight inhibitory effect on the purified peroxidase at all the concentrations studied.

Concentration (%)	% Residual activity					
	Ethanol		Methanol		Isopropanol	
	30 min	60 min	30 min	60 min	30 min	60 min
Control	100.00	100.00	100.00	100.00	100.00	100.00
10	89.81 ± 0.9	69.34 ± 1.4	82.26 ± 1.2	80.90 ± 0.6	94.06 ± 0.9	83.95 ± 1.4
20	84.35 ± 1.4	59.47 ± 1.23	78.73 ± 0.56	75.04 ± 0.4	74.80 ± 0.5	71.83 ± 2.1
30	76.89 ± 1.6	58.27 ± 1.4	71.83 ± 0.6	65.33 ± 0.9	71.35 ± 1.1	67.90 ± 0.8
40	70.47 ± 1.54	43.50 ± 1.8	67.01 ± 0.2	59.39 ± 0.35	48.56 ± 0.5	46.31 ± 0.48
50	58.91 ± 0.98	41.17 ± 0.6	63.56 ± 0.9	50.88 ± 0.18	41.09 ± 0.89	31.94 ± 1.4
60	44.94 ± 0.84	37.32 ± 0.91	50.80 ± 0.79	35.47 ± 1.01	32.58 ± 1.4	22.39 ± 1.9
70	41.89 ± 0.54	13.64 ± 1.2	36.84 ± 1.3	24.56 ± 1.3	24.56 ± 1.7	12.76 ± 1.7
80	33.87 ± 0.12	14.57 ± 1.9	26.32 ± 1.09	18.46 ± 0.9	21.51 ± 2.1	11.40 ± 0.78

Table 3: Effect of organic solvents on purified peroxidase activity. The data were compared with control (no organic solvents) and represented as % residual activity. The value of % residual activity represents mean ± S.D. of experiments done in triplicate for each set.

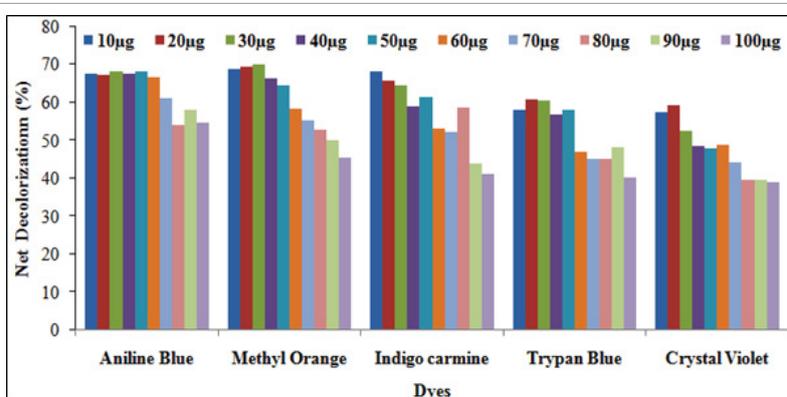


Figure 6: Effect of dye concentration on the efficacy of dye decolorization by purified peroxidase.

In agreement to our study, in the literature, the *Ficus sycomorus* peroxidase has been reported to be slightly activated by Ca^{2+} , Mg^{2+} , Ni^{+2} [33], the *Leucaena* peroxidase has also reported to be activated by Na^+ , Ca^{2+} and Mn^{2+} [40] and that of cotton peroxidases are also reported to be activated by Ni^{+2} [25]. On the other hand, other peroxidases such as the lettuce peroxidase was apparently inhibited by metal ions namely, Zn^{2+} , Ca^{2+} and Mn^{2+} [15], the Cs^{2+} and Ni^{2+} have reported to show inhibitory effect on *Ipomoea carnea* peroxidases [41]. Thus, the purified lemon peroxidase could be suggested to exhibit tolerance towards high concentration of these metal ions especially heavy metals such as Cs^{2+} , Cd^{2+} and Ni^{2+} . The modulation of enzyme activity by metals is suggested to be due to stabilization of the protein structure, responsible for catalysis [32].

Effects of organic solvents on purified peroxidase

Industrial applications of peroxidase become more versatile and efficient, if the enzyme offers resistance towards organic solvents. Depending upon the industrial applications of peroxidase, investigating enzyme activity in a reaction system consisted of organic solvents with aqueous phase (organo-aqueous phase) may be more beneficial than that of a system containing only aqueous phase. The organo-aqueous phase may provide a mimic environment to the enzyme to act on wide range of organic pollutants, which are more soluble in organic solvent and hydroorganic mixtures. Therefore, the effect of organic solvents such as ethanol, methanol and isopropanol (10-80%) on the purified peroxidase was investigated. Results are shown in Table 3. It is noteworthy that lemon peroxidase was found to retain 41-63% activity in presence of these organic solvents (at 50% (v/v) concentration) upto 30 min exposure. After prolonged exposure (60 min), 41, 51 and 32% residual activity was observed in presence of

50% (v/v) ethanol, methanol and isopropanol, respectively. At higher concentration (60-80 %) with prolonged incubation (60 min) steep decline in activity of purified peroxidase was observed. In presence of even higher concentration of methanol (80%), about 18% peroxidase activity was retained after 60 min of exposure. Thus, among these solvents, peroxidase exhibited maximum tolerance towards methanol that could be suggested as a better organic solvent followed by ethanol and isopropanol.

Similar to our observation soybean seed hull peroxidase has been reported to retain considerable activity in the presence of methanol and ethanol [24]. Furthermore, immobilized *Momordica* peroxidase has been reported to retain 43% activity in presence of propanol (30%) [36]. In literature, the rice and horseradish peroxidases have also been reported to be activated by ethanol (40%) [54].

Dye decolorization by purified peroxidase

Now-a-days the interest is towards the application of enzymes for degradation of dyes, used for paper printing, color photography etc. and causing environmental problems. The enzymatic degradation process is less expensive and ecofriendly alternative [57]. Thus, the ability of the purified peroxidase to decolorize (oxidize) various dyes was investigated using seven dyes namely congo red, aniline blue, malachite green, crystal violet, methyl orange, trypan blue and indigo carmine which have been considered as the part of wastewater pollutants from textile, plastic and paper industries. The data revealed that out of seven, five dyes were quite efficiently decolorized by peroxidase in the order of aniline blue > methyl orange > indigo carmine > trypan blue > crystal violet. Efficiency of dye decolorization by the purified peroxidase as a function of dye concentration was also investigated. Data are presented

in Figure 6. The data revealed that the purified peroxidase exhibited considerable dye decolorization capability even at high concentration of dyes. About 40–54% dye decolorization of even 100 µg dyes were found to be achieved. In literature, the peroxidase from *Hevea brasiliensis* have been reported to decolorize crystal violet and aniline blue, while other dyes such as congo red, methyl orange, trypan blue, etc. were not decolorized by the *Hevea* peroxidase [58]. The peroxidases isolated from horseradish, tomato, turnip, bitter gourd have also been reported to decolorize various dyes but not very efficiently [23]. The bacterial and fungal peroxidases have also been reported for dye decolorization, but, their high cost, infection associated risk have been suggested to be limiting factors for their proper utilization in dye decolorization.

Thus, based on all above physicochemical characterization, it may be suggested that the purified *C. limon* peroxidase could be a promising candidate for industrial applications in view of its stability towards temperature, pH, high concentration of metal salts, heavy metals, organic solvents as well as dye decolorizing ability.

Conclusion

A defense associated partial cDNA clone of peroxidase from lemon was isolated and characterized. Based on qRT-PCR and activity analyses, higher expression was observed under diseased condition than that of healthy one, which is suggestive of its involvement in defense response. Furthermore, this defense associated peroxidase was purified to 34.40 folds with overall recovery of 54.22% through a quick purification protocol namely heat treatment and con-A affinity column chromatography. The purified peroxidase was found to be a heterotrimeric protein of size ~200 kDa (consisting of two subunits of 66 kDa, one subunit of 70 kDa). The lemon peroxidase was fully stable at 70°C upto 2 hrs while at 75°C and 80°C it was stable upto 1 hr. The purified peroxidases exhibited tolerance towards heavy metals namely, Cd²⁺, Cs²⁺ and Ni²⁺. On the other hand, Hg²⁺ showed mild inhibitory effect on the purified peroxidase. The purified peroxidase was quite stable towards organic solvents, as it retained 30–50% activity in the presence of 50% (v/v) organic solvents namely ethanol, methanol and isopropanol. The purified peroxidase was found to have significant dye decolorizing ability. Thus, the purified lemon peroxidase exhibited stability towards temperature, pH, salts, heavy metals, organic solvents and ability to oxidize a number of dyes, thereby making the lemon peroxidase, a promising candidate for its application in various analytical as well as bioremedial purposes. Furthermore, for the commercial exploitation of this novel peroxidase, in future, it can be the produced in bulk by over expression in a suitable host system.

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References

1. Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. *Plant Cell Reports* 24: 255–265.
2. Yves MEL, Mbassi MSEB, Mbacham W, Muluw JP (2011) Heat stable peroxidases from *Vigna* species (V). *African Journal of Biotechnology* 10: 3168–3175.
3. Passardi F, Theiler G, Zamocky M, Cosio C, Rouhier N, et al. (2007) PeroxiBase: the peroxidase database. *Phytochemistry* 68: 605611.
4. Diaz-Vivancos P, Rubio M, Mesonero V, Periago PM, Ros Barcelo A, et al. (2006) The apoplastic antioxidant system in *Prunus*: Response to long-term plum pox virus infection. *J Exp Bot* 57: 3813–3824.
5. Watanabe L, Nascimento AS, Zamorano LS, Shnyrov VL, Polikarpov I (2007) Purification, crystallization and preliminary X-ray diffraction analysis of royal palm tree (*Roystonea regia*) peroxidase. *Acta Cryst* 63: 780–783.
6. Passardi F, Bakalovic N, Teixeira FK, Pinheiro-Margis M, Penel C, et al. (2007) Prokaryotic origins of the peroxidase superfamily and organellar-mediated transmission to eukaryotes. *Genomics* 89: 567–579.
7. Pfaffl MW (2001) A new mathematical model for relative quantification real-time PCR. *Nucleic Acids Res* 29: 2002–2007.
8. Switzer RC, Merrill CR, Shifrin S (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem* 98: 231–237.
9. Parshetti G, Parshetti S, Kalyani D, Govindwar S (2012) Industrial dye decolorizing lignin peroxidase from *Kocuria rosea* MTCC 1532. *Annals of Microbiology* 62: 217–223.
10. Baldrian P, Snajdr J (2006) Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes. *Enzyme Microbial Technology* 39: 1023–1029.
11. Barceló AR, Pomer F (2001) Oxidation of cinnamyl alcohols and aldehydes by a basic peroxidase from lignifying *Zinnia elegans* hypocotyls. *Phytochemistry* 57: 1105–1113.
12. Fortea MI, Pellicer JA, Serrano-Martínez A, López-Miranda S, Lucas-Abellán C, et al. (2012) Red Cabbage (*Brassica oleracea*) as a New Source of High-Thermostable Peroxidase. *J Agri Food Chem* 60: 10641–10648.
13. Lavery CB, Macinnis MC, Macdonald MJ, Williams JB, Spencer CA, et al. (2010) Purification of peroxidase from Horseradish (*Armoracia rusticana*) roots. *J Agri Food Chem* 58: 8471–8476.
14. Marzouki SM, Limam F, Smaali MI, Ulber R, Marzouki MN (2005) A new thermostable peroxidase from garlic *Allium sativum*: purification, biochemical properties, immobilization, and use in H₂O₂ detection in milk. *Appl Biochem Biotechnol* 127: 201–214.
15. Hu Y, Wu J, Luo P, Mo Y (2012) Purification and partial characterization of peroxidase from lettuce stems. *African Journal of Biotechnology* 11: 2752–2756.
16. Bernards MA, Fleming WD, Llewellyn DB, Priefer R, Yang X, et al. (1999) Biochemical characterization of the suberization-associated anionic peroxidase of potato. *Plant Physiol* 121: 135–145.
17. Passardi F, Penel C, Dunand C (2004) Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci* 9: 534–540.
18. Liszkay A, Kenk B, Schopfer P (2003) Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. *Planta* 217: 658–667.
19. Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, et al. (2006) Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *Plant J* 47: 851–863.
20. Şişcioğlu M, Gülçin İ, Çankaya M, Atasever A, Şehitoğlu MH, et al. (2010) Purification and characterization of peroxidase from Turkish black radish (*Raphanus sativus* L.). *Journal of Medicinal Plants Research* 4: 1187–1196.
21. Huang R, Xia R, Hu L, Lu Y, Wang M (2007) Antioxidant activity and oxygen-scavenging system in orange pulp during fruit ripening and maturation. *Scientia Horticulture* 113: 166–172.
22. Bania I, Mahanta R (2012) Evaluation of peroxidases from various plant sources. *International Journal of Scientific and Research Publication* 2: 1–5.
23. Husain Q (2010) Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review. *Reviews in Environmental Science and Biotechnology* 9: 117–140.
24. Geng Z, Rao KJ, Bassi AS, Gijzen M, Krishnamoorthy N (2001) Investigation of biocatalytic properties of soybean seed hull peroxidase. *Catalyses Today* 64: 233–238.
25. Kouakou TH, Dué EA, Kouadio NE, Niameké S, Kouadio YJ, et al. (2009) Purification and Characterization of Cell Suspensions Peroxidase from Cotton (*Gossypium hirsutum* L.). *Appl Biochem Biotechnol* 157: 575–592.
26. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

27. Cosio C, Dunand C (2009) Specific functions of individual class III peroxidase genes. *Journal of Experimental Botany* 60: 391-408.
28. Bhunia A, Durani S, Wangikar PP (2001) Horseradish peroxidase catalyzed degradation of industrially important dyes. *Biotechnol Bioeng* 72: 562-567.
29. Radwan DEM, Fayez KA, Mahmoud SY, Lu G (2010) Modifications of antioxidant activity and protein composition of bean leaf due to Bean-yellow mosaic virus infection and salicylic acid treatments. *Acta Physiologica Plantarum* 32: 891-904.
30. Wagner M, Nicell JA (2001) Peroxidase-catalyzed removal of phenols from a petroleum refinery wastewater. *Water Sci Technol* 43: 253-260.
31. Mohamed SA, Azza M, Abdel-Aty BH, Mohamed O, Mohamed El-Badry, et al. (2011) *Ficus sycamorus* latex: A thermostable peroxidase. *African Journal of Biotechnology* 10: 17532-17543.
32. Greppin H, Wiater RG, Ginalska G, Lobarzewski J (1999) The cabbage peroxidase isoforms changes influenced by Ca²⁺ and Mg²⁺ ions. *Plant Peroxidase Newsletter* 13: 129-135.
33. Mogharrab N, Ghourchian H, Amininasab M (2007) Structural stabilization and functional improvement of horseradish peroxidase upon modification of accessible lysines: experiments and simulation. *Biophys J* 92: 1192-1203.
34. Subramaniam S, Balasubramaniam R, Kuppuswamy K, Venkatesan G, Vasu K (2010) Bitter gourd (*Momordica charantia*) peroxidase in decolorization of dyes from tannery effluent. *Recent Research in Science and Technology* 01: 49-53.
35. Welinder KG (1992) Superfamily of plant, fungal and bacterial peroxidases. *Current Opinion of Structural Biology* 2: 388-393.
36. Ashraf H, Husain Q (2011) Application of immobilized peroxidase for the removal of pbromophenol from polluted water in batch and continuous processes. *Journal of Water Reuse and Desalination* 1: 52-60.
37. Sakharov IY (2004) Palm tree peroxidases. *Biochemistry (Moscow)* 69: 823-829.
38. Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R, et al. (2008) Genomic insights into Mn(II) oxidation by the marine alpha *Proteobacterium aurantimonas* sp. strain SI85-9A1. *Appl Environ Microbiol* 74: 2646-2658.
39. Bozzo GG, Raghobhama KG, Plaxton WC (2004) Structural and kinetic properties of a novel purple acid phosphatase from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures. *Biochem J* 377: 419-428.
40. Gross RA, Kumar A, Kalra B (2001) Polymer synthesis by *in vitro* enzyme catalysis. *Chem Rev* 101: 2097-2124.
41. Patel AK, Singh VK, Moir AJ, Jagannadham MV (2008) Biochemical and spectroscopic characterization of morning glory peroxidase from an invasive and hallucinogenic plant weed *Ipomoea carnea*. *J Agri Food Chem* 56: 9236-9245.
42. Da Silva ME, Franco TT (2000) Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. *J Chrom B Biomed Sci Appl* 23: 287-94.
43. Kvaratskhelia M, Winkel C, Thorneley RN (1997) Purification and characterization of a novel class III peroxidase isoenzyme from tea leaves. *Plant Physiol* 114: 1237-1245.
44. Suha OA, Babiker EM, Babiker EE (2013) Thermostability at different pH levels of peroxidase extracted from four vegetables. *Inter Food Res J* 20: 715-719.
45. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
46. Sakharov IY, Vesga Blanco MK, Sakharova IV (2002) Substrate specificity of african oil palm tree peroxidase. *Biochemistry (Mosc)* 67: 1043-1047.
47. Davis BJ (1964) Disc electrophoresis II. Method and application to human serum proteins. *Ann N Y Acad Sci* 121: 404-427.
48. Pütter J (1974) Method of enzymatic analysis, in: H.U. Bergmeyer (Ed) *New York: Verlag Chemie Weinheim* 685-690.
49. Pandey VP, Dwivedi UN (2011) Purification and characterization of peroxidase from *Leucaena leucocephala*, a tree legume. *J Mol Catal B Enzym* 68: 168-173.
50. Jia J, Wang B, Cheng G, Dong S (2002) A method to construct a third generation horseradish peroxidase biosensor: self-assembling gold nanoparticles to three-dimensional sol/gel network. *Anal Chem* 74: 2217-2223
51. Maciel HPFG, Gouvêa CMCP, Pastore GM (2007) Extraction and partial characterization of peroxidase from *Copaifera langsdorffii* Desf. *Leaves. Ciência e Tecnologia de Alimentos*. 27: 221-225.
52. Shimoni M, Bar-Zur A, Reuveni B (1991) The association of peroxidase activity and resistance of maize to *Exserohilum triticum*. *Journal of Phytopathology* 131: 315-321.
53. Doerge DR, Rao L, Divi M, Churchwell I (1997) Identification of the colored guaiacol oxidation product produced by peroxidases. *Anal Biochem* 250: 10-17.
54. Singh P, Prakash R, Shah K (2012) Effect of organic solvents on peroxidases from rice and horseradish: prospects for enzyme based applications. *Talanta* 97: 204-210.
55. Rudrappa T, Lakshmanan V, Kaunain R, Singara NM, Neelwarne B (2007) Purification and characterization of an intracellular peroxidase from genetically transformed roots of red beet (*Beta vulgaris* L.). *Food Chem* 105: 1312-1320.
56. Mc Innis SM, Desikan R, Hancock JT, Hiscock SJ (2006) Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signaling crosstalk? *New Phytol* 172: 221-228.
57. An SY, Min SK, Cha IH, Choi YL, Cho YS, et al. (2002) Decolorization of triphenylmethane and azo dyes by *Citrobacter* sp. *Biotechnol Lett* 24: 1037-1040.
58. Chanwun T, Muhamad N, Chirapongsatongkul N, Churngchow N (2013) *Hevea brasiliensis* cell suspension peroxidase: purification, characterization and application for dye decolorization. *AMB Express* 3: 14.