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Purification and characterization of peroxidase from *Leucaena leucocephala*, a tree legume

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ABSTRACT

Peroxidase was purified to homogeneity from a tree legume *Leucaena leucocephala*. On SDS-PAGE the purified enzyme exhibited two distinct subunits each of 66 and 58 kDa. Determination of native molecular weight of the purified peroxidase revealed a size of ~200 kDa suggesting a heterotrimeric structure (consisting of two subunits of 66 kDa and one subunit of 58 kDa) for native peroxidase. Purified peroxidase was found to be a glycoprotein (0.09 mg carbohydrates per mg purified peroxidase protein). Purified enzyme exhibited pH optimum of 5.0 and temperature optimum of 55 °C. The Michaelis–Menten constants (Km) for guaiacol, H_2O_2, were found to be 2.9 and 5.6 mM, respectively. Divalent cations namely, Ca^{2+} and Mn^{2+} activated peroxidase at lower concentration (up to 50 mM) while inhibited at higher concentration. Monovalent cation namely Na^+ did not inhibit peroxidase at concentration as high as 4 M. *L. leucocephala* peroxidase was found to be unique as it was not inhibited by azide.

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1. Introduction

Peroxidases are major H_2O_2 decomposing enzymes which catalyze one electron oxidation of wide range of substrates at the expense of H_2O_2 [1]. Guaiacol peroxidases (GPX) (EC 1.11.1.7) which oxidize guaiacol (o-methoxyphenol), belongs to class III peroxidase and are widely distributed in plant kingdom. GPXs are located in cytosol, vacuole, cell wall, apoplast and extracellular medium, but not in organelles and are assumed to be involved in a wide range of physiological processes related to plant growth and development [2], such as cell wall metabolism [1], lignification [3], suberization [4], auxins metabolism [5], wound healing [6], reactive oxygen species (ROS) metabolism [7], defense against pathogens [8], fruit ripening, fruit growth [9], seed germination [10] and detoxification of heavy metals and other toxic molecules (e.g. 2,4-dichlorophenol) [11,12]. Though peroxidases have been purified and characterized from a number of sources like wheat grass, *Allium sativum*, *Solanum melongena* fruit juice, buckwheat seed, *Lycopersicon esculentum*, *Beta vulgaris*, *Roystonea regia*, etc. [13–19], their isolation and characterization from a legume tree is lacking. Peroxidases are glycoproteins [20] and are glycosylated to varying degree leading to generation of multiple molecular forms. In *Arabidopsis thaliana,* the majority of peroxidases have been shown to have one to two putative glycosylation sites. *A. sativum* peroxidase is reported to be monomeric protein of 36.5 kDa [14], while that of *R. regia* is a homodimeric protein of 51 kDa subunit size each [19]. Peroxidase from oil palm fruit is reported to be a homotetrameric protein of 200 kDa containing 48 kDa each subunit size [21]. A heterodimeric peroxidase consisting of subunits of 63 and 57 kDa is also reported from tomato [17].

Michaelis–Menten constant (Km) for guaiacol is reported to vary greatly among the guaiacol peroxidases from different sources. Thus, peroxidases from wheat grass, *A. sativum*, *S. melongena* fruit juice, buckwheat seed and *B. vulgaris* show Km for guaiacol as 3.8, 9.5, 6.5, 0.288 and 98.61 mM, respectively [13–16,18]. Km for H_2O_2 is reported to be 2, 0.33 and 0.133 mM in *A. sativum* [14], *S. melongena* [15] and *B. vulgaris* [18] peroxidase, respectively.

Temperature and pH optima for peroxidases from different sources are reported to vary greatly. Thus, buckwheat seed peroxidase shows temperature optimum of 10–30 °C [16] while that of *S. melongena* shows a temperature optimum of 84 °C [15]. The pH optimum for most of the peroxidases such as *A. sativum*, *S. melongena*, and *B. vulgaris* were found to be about 5 [13,14,18] whereas buckwheat seed peroxidases and *L. esculentum* have their pH optima 9 [16,17].

Peroxidases are metalloproteins containing heme (Fe porphyrin IX) center that is proposed to be stabilized by certain metal ions like Ca^{2+} [22]. In the native stage the iron is present in +3 oxidation state and linked to four pyrrol nitrogen of heme and nitrogen of proximal histidine. The sixth co-ordination position is free [23]. Thus, metal ions like Ca^{2+}, Mn^{2+} and Na^+ are reported to activate peroxidase from wheat grass, peanut and cabbage [13,24,25]. On the other hand, *A. sativum* peroxidase was reported to be inhibited by Ca^{2+} and Mn^{2+} [14]. Sodium azide was reported to inhibit

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S. melongena, *B. vulgaris* and corn peroxidases [15,18,26]. Guaiacol peroxidases (GPX) has also been shown to be involved in salicylic acid (SA) mediated defense responses. In SA mediated hypersensitive resistance (HR) and systemic acquired resistance (SAR), SA inhibits peroxidase thereby increasing the level of H_2O_2, which acts as second messenger to activate defense gene expression [27,28].

Peroxidases are very commonly used in the construction of biosensors and other analytical applications such as ELISA and also used for developing convenient and quick methods for the determination and quantification of hydrogen peroxide in both the biological and industrial samples [29]. Some of the other applications of the peroxidases including determination of lipid peroxidation in cell membranes and in meat food products, in catalyzing the polymerization and precipitation of aqueous phenols and decolorization of bleach plant effluent by hydrogen peroxide. In modern organic synthesis, uses of purified peroxidases, immobilized in various matrices or peroxidase rich microorganisms provide better way of downstream processing for product quality and recovery and become an important emerging applications of this enzyme [30].

The peroxidases can be purified from native sources by using conventional downstream processing techniques such as homogenization, ammonium sulphate fractionation, ion exchange, gel filtration, Con-A affinity as well as two-phase partitioning chromatographies [13,26,31,32]. Thus, there is a great need to utilize the natural sources for the exploitation for enzymology through which we can fulfill our local requirements.

In the present paper we report purification and characterization of peroxidase from soft stem tissue of *Leucaena leucocephala*, a tree legume of significance to fodder and the paper and pulp industry in India. Peroxidases from tree legumes are not well-characterized.

2. Materials and methods

2.1. Plant material

Soft stem of *L. Leucocephala*, growing in the garden of Department of Biochemistry, University of Lucknow, was used as a source of plant material.

2.2. Enzyme assay

Peroxidase activity was measured by Pÿtter's protocol [33], with slight modifications, using guaiacol as substrate. The assay system consisted of sodium phosphate pH 7.0 (50 mM), H_2O_2 (0.067%) and guaiacol (3.33 mM). Reaction was started by adding a suitable amount of enzyme aliquot in a final volume of 3 ml. 3,3′-Dimethoxy-4,4′-biphenoquinone formation was monitored spectrophotometrically by measuring increase in absorbance at 470 nm at room temperature [34]. The molar extinction coefficient of 3,3′-dimethoxy-4,4′-biphenoquinone (earlier considered as tetraguaiacol) was taken 6.39 mM^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 mmol of 3,3′-dimethoxy-4,4′-biphenoquinone formed per min at room temperature.

2.3. Preparation of enzyme extract

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

2.4. Purification of peroxidase

Crude extract was brought to 50% saturation by gradually adding solid ammonium sulphate. Afterwards it was centrifuged at 12,000 rpm for 30 min at 4 °C using Sigma 4K15 centrifuge. Pellet was suspended in minimum volume of Tris–HCl, pH 7.5 (100 mM). Enzyme preparation was dialyzed against same buffer at 4 °C overnight by changing the buffer thrice.

The dialyzed enzyme sample was loaded on to glass column, packed with equilibrated DEAE (diethyl amino ethyl)–cellulose matrix (Tris–HCl, pH 7.5). Elution was done with NaCl gradient (50–500 mM) in Tris–HCl buffer, pH 7.5. The eluted fractions (having high enzyme activity) from DEAE column were pooled and again loaded on to the glass column packed with equilibrated Sephadex G-200 (Tris–HCl, pH 7.5) matrix and elution was done with same Tris–HCl buffer, pH 7.5. The fractions (having high enzyme activity) collected from Sephadex G-200 column was pooled and loaded on to the Concanavallin-A (Con-A) column regenerated by regeneration buffer, containing sodium acetate, pH 4.5 (100 mM), $MnCl_2$ (1 mM), $CaCl_2$ (1 mM), NaCl (1 M), equilibrated by equilibration buffer, containing Tris–HCl buffer, pH 7.5 (20 mM) and NaCl (0.5 M) and binding buffer, containing Tris–HCl buffer, pH 7.5 (20 mM), NaCl (0.5 M), $MnCl_2$ (1 mM), $CaCl_2$ (1 mM). Elution was done with sucrose gradient (50–500 mM) in Tris–HCl buffer, pH 7.5 (20 mM) and NaCl (500 mM).

Protein was determined by Bradford's method using BSA as standard [35].

2.5. In-gel activity staining using native polyacrylamide gel electrophoresis (PAGE)

For separation and in gel activity staining of peroxidase, a 10% resolving gel with 3% stacking gel was made as described [36]. Enzyme samples (mixed with loading buffer having Tris–HCl pH 6.8 (100 mM), glycerol (30%), bromo phenol blue (0.25%)) containing equal protein were loaded into the wells of gel. The gel was run at 4 °C at 120 constant voltage mode using Tris–glycine buffer, pH 8.3. After the run, the gel was transferred to an in-gel enzyme staining solution containing sodium phosphate buffer, pH 7.0 (50 mM), guaiacol (10 mM), H_2O_2 (0.2%) till brick red colored band appeared.

2.6. Molecular weight determination

The native molecular weight of the purified peroxidase was determined by gel filtration chromatography [37]. The void volume (Vo) of the Sephadex G-200 column was determined by eluting blue dextran. Catalase (240 kDa), alcohol dehydrogenase (150 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (67 kDa) and lysozyme (14.3 kDa) were used as standard protein.1.0 mg/ml of each standard protein was applied on to the column and protein in column eluent was determined by Bradford's [35] method. The elution volume (Ve) of each standard protein as well as purified peroxidase was calculated. The molecular weight of the peroxidase was calculated from a calibration curve, where log of the molecular weights of the standards were plotted against the ratio of the elution volumes of the standards and the void volume of the column.

Native molecular weight of the purified peroxidase was also determined by native-PAGE. Polyacrylamide gel electrophoresis under native condition was done with native molecular weight marker in the order soyabean 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 (Rm) were plotted against their log molecular weight. Molecular weight of peroxidase was calculated with the help of calibration curve.

Table 1
Purification chart for peroxidase isolated from soft stem tissue of *Leucaena leucocephala*.

Steps	Total activity (Units[a])	Total protein (mg)	Specific activity (Units[a]/mg)	Recovery (%)	Fold purification
Crude	121.52	172	0.71	100	–
Ammonium sulphate fraction (dialyzed)	75.02	28.6	2.62	61.73	3.6
DEAE ion-exchange column chromatography	53.45	8.7	6.14	43.98	8.64
Sephadex G-200 gel filtration chromatography	52.5	1.6	32.8	43.2	46.2
Con-A column chromatography	31.7	0.55	63.4	26.09	89.3

[a] One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 mmol of 3,3′-dimethoxy-4,4′-biphenoquinone formed per min at room temperature.

Subunit molecular weight of the purified peroxidase was determination by using SDS-PAGE as described [36]. Enzyme sample containing equal protein, prepared in gel loading dye, was loaded into the wells of gel. After electrophoresis, the gel was stained with Coomassie brilliant blue G-250 in 40% methanol and 10% acetic acid for 60 min and then de-stained in 40% methanol and 10% acetic acid solution for 2–4 h.

2.7. Physicochemical characterization of purified peroxidase

2.7.1. Effect of substrates, temperature and pH

The effect of substrates guaiacol and H_2O_2 were determined by incubating a suitable amount of purified enzyme aliquots with different concentrations of guaiacol and a fixed saturated concentration of H_2O_2 or vice versa. The Km was determined from double reciprocal plots from the data obtained. Since veratryl alcohol is the substrate of lignin peroxidase, thus, the tetraguaiacol formation was also studied in presence of veratryl alcohol along with guaiacol.

Effect of temperature on enzyme activity was determined after incubation of whole reaction mixture at various temperatures ranges from 25 to 75 °C for 5 min. Aliquots were then measured spectrophotometrically for peroxidase activity. The dependence of pH on purified enzyme activity was determined by using 50 mM buffers of different pH such as sodium tartrate pH 3, sodium acetate pH 4.5, 5, sodium phosphate pH 7, Tris–HCl pH 7.5, 8, 8.8 at fixed concentration of guaiacol and H_2O_2.

2.7.2. Effect of metal ions, azide and salicylic acid

The effect of different metal ions (Na^+, Ca^{2+}, Mn^{2+}) on purified guaiacol peroxidase activity of soft stem of *L. leucocephala* was determined by performing the enzyme assay with different concentrations of metal salts. Effect of different concentrations of azide on purified peroxidase activity was also analyzed. The effect of different concentrations of salicylic acid on purified peroxidase was also studied.

2.7.3. Carbohydrate content determination of purified peroxidase

The peroxidase is a glycoprotein that is confirmed by Con-A matrix binding. Thus carbohydrate content was measured by phenol–sulphuric acid method [38] as follows. A suitable aliquot of enzyme was taken in water to make the final volume of 2 ml. To this 0.5 ml of 80% distilled phenol and 5 ml of concentrated sulphuric acid were added and contents were mixed by vortexing. After 20 min of incubation the absorbance was read at 490 nm using Elico SL159 UV–VIS spectrophotometer. Similarly, a calibration curve using glucose as standard was drawn.

3. Results and discussion

3.1. Purification of peroxidase

Peroxidase from the soft stem tissue of *L. leucocephala* was isolated and purified through successive steps of ammonium sulphate fractionation, DEAE cellulose, Sephadex G-200 and Con-A affinity column chromatographies. The purification chart for *L. leucocephala* peroxidase is given in Table 1. Peroxidase was purified to 89.3 folds with overall recovery of 26% and specific activity 63.4 Units/mg protein. The homogeneity of the purified peroxidase enzyme was established by running a native PAGE, where a single band was obtained (Fig. 1C).

3.2. Molecular weight determination

Native molecular weight of the purified peroxidase was determined using gel filtration method. Results are shown in Fig. 1A. We have also used native PAGE using standard molecular weight marker protein for determination of native molecular weight (Fig. 1B and C). It is worth mentioning that with both the methods native molecular weight of the purified peroxidase preparation was found to be same, i.e. ~200 kDa.

The purified peroxidase preparation was further analyzed for subunit composition by running SDS-PAGE. Results are shown in Fig. 2. It is notable that *L. leucocephala* peroxidase consisted of two different sized subunits each of 66 and 58 kDa. Thus based on the subunit size (Fig. 2) of the purified peroxidase a heterotrimeric structure (consisting of two subunits of 66 kDa and one subunit of 58 kDa) is suggested for purified peroxidase. To the best of our knowledge this is the first report of a heterotrimer peroxidase. *L. esculentum* peroxidase has been reported to be a heterodimer protein of size 142 kDa having two subunits of size 63 and 57 kDa [17]. On the other hand most of the peroxidases, reported in literature, show homomeric structure. For example, oil palm fruit has homotetrameric peroxidase of size 200 kDa containing 4 subunits of size 48 kDa each [21], *R. regia* peroxidase is found to be a homodimeric protein of 51 kDa subunit size each, while that of *A. sativum* peroxidase is reported to be monomeric protein of 36.5 kDa [14].

The existence of multiple molecular forms of the peroxidases is reported in *Zinnia elegans* [39]. The origins of multiple molecular forms in tobacco have been suggested to be post-translational modification (glycosylation) rather than their genetic origin [40]. In the present study, we have demonstrated that the two types of subunits are not arising due to post-translational modification (glycosylation) of purified peroxidase protein. Thus, when the deglycosylation of the purified peroxidase was done and the molecular weight of subunits were determined by SDS-PAGE, the size of the subunits were found to be same (data not shown) suggesting the origin of two different sized subunits (66 and 58 kDa) might be at the gene level. Thus, heteromeric nature of *L. leucocephala* peroxidase is suggestive of multigene family nature of peroxidase in this tree species as reported for rice and *Arabidopsis,* where, 138 genes reported from rice [41] and 73 genes from *Arabidopsis* [42].

3.3. Effect of guaiacol, H_2O_2 and veratryl alcohol on the peroxidase activity

Effect of guaiacol on the peroxidase activity was determined by varying the concentration of guaiacol while keeping a fixed and saturated concentration of second substrate H_2O_2. For determination of the Km, double reciprocal plot of the data was plotted (data

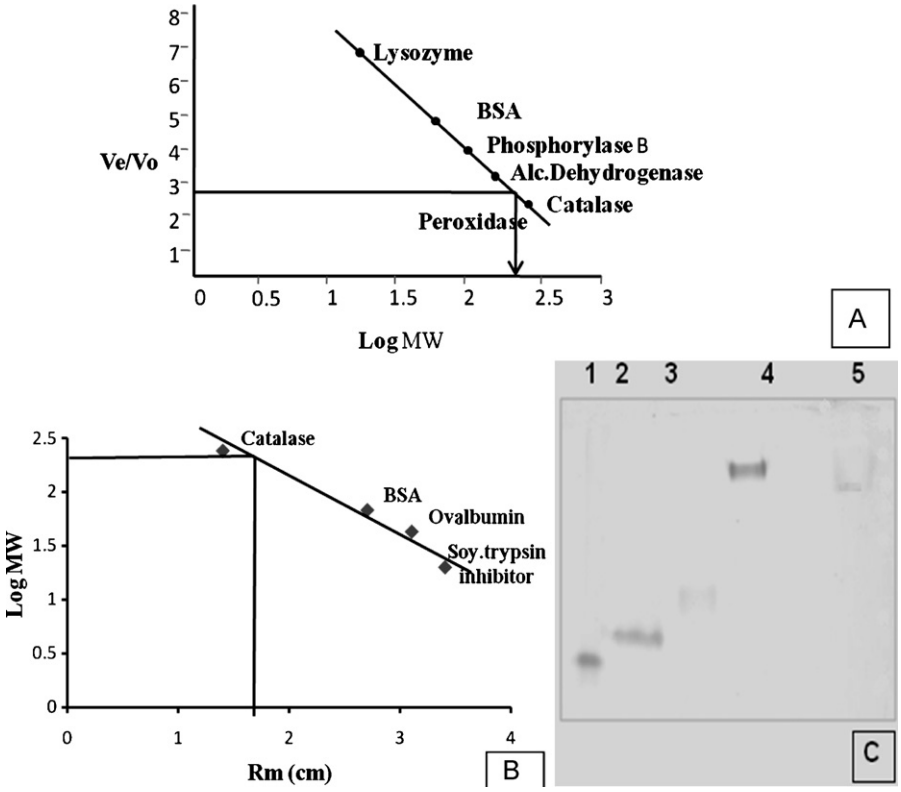

Fig. 1. (A) Calibration plot for the determination of molecular weight of purified peroxidase by gel filtration chromatography. The standard proteins used were catalase (240 kDa), alcohol dehydrogenase (150 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (67 kDa) and lysozyme (14.3 kDa). (B) Calibration plot 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). (C) Native-polyacrylamide gel electrophoresis of standard molecular weight marker proteins along with purified peroxidase; Lanes 1, 2, 3, 4: soybean trypsin inhibitor (20.1 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), catalase (240 kDa), respectively. Lane 5: purified peroxidase enzyme. The gel was visualized by Coomassie brilliant blue staining.

not shown). Km was found to be 2.9 mM. Km for guaiacol for peroxidase from various sources is reported to vary widely. Thus for example, wheat grass, *S. melongena*, *A. sativum* and *B. vulgaris* peroxidase show different Km values for guaiacol such as 3.8, 6.5, 9.5, and 98.61 mM, respectively [13–15,18]. Thus, *L. leucocephala* peroxidase shows lower Km value for guaiacol suggesting higher affinity of enzyme for guaiacol.

Effect of H_2O_2 on the peroxidase activity was determined by varying the H_2O_2 concentration keeping the guaiacol concentration constant and saturating. Inhibition of the peroxidase activity at high H_2O_2 concentration (beyond 25 mM) was observed. For determination of the Km, a double reciprocal plot was plotted (data not shown), excluding the data range in which inhibition at high substrate was obtained. Apparent Km for H_2O_2 was found to be 5.6 mM. Km for H_2O_2 has been shown to vary depending upon the source of the peroxidase. Thus, Km for H_2O_2 for peroxidase from *A. sativum* [14], *S. melongena* [15] and *B. vulgaris* [18] were found to be 2, 0.33 and 0.133 mM, respectively. The *L. leucocephala* peroxidase was found to have relatively higher Km for H_2O_2 than those of other peroxidases reported in literature.

L. leucocephala peroxidase shows no activity with veratryl alcohol, a substrate used by class II fungal peroxidases involving in lignin degradation, confirming *L. leucocephala* peroxidase as a typical class III peroxidase which does not use veratryl alcohol as physiological substrate. Guaiacol peroxidase activity was found to be inhibited in presence of veratryl alcohol (data not shown). The basis of this type of inhibition may be due to competition of veratryl alcohol and guaiacol for binding to the active site [43]. Furthermore this inhibition of peroxidase by veratryl alcohol has been used to distinguish fungal peroxidase (class II peroxidase) from plant peroxidase (class III peroxidase).

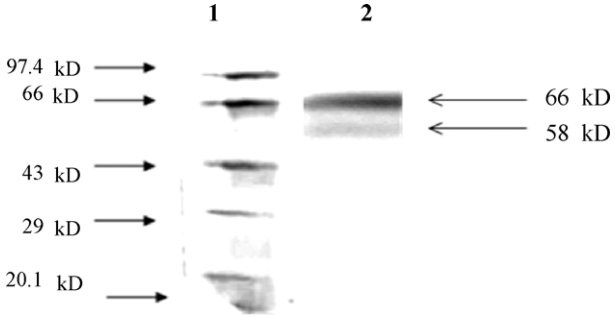

Fig. 2. SDS-polyacrylamide gel electrophoresis analysis of purified peroxidase stained with Coomassie brilliant blue. Lane 1: molecular weight marker. Lane 2: purified peroxidase enzyme.

3.4. Effect of temperature on the peroxidase activity

Effect of temperature on the peroxidase activity was investigated. The maximum peroxidase activity was found to be at 55 °C. It is remarkable that the *L. leucocephala* peroxidase was fairly stable to temperature and possessed considerable activity even at 75 °C. A wide variability with regards to temperature has been reported for peroxidase from various sources. *A. sativum*, *S. melongena*, peroxidases have temperatures optima 35 °C, 84 °C, respectively [14,15], whereas buckwheat seed peroxidase shows temperature optima of 10–30 °C [16].

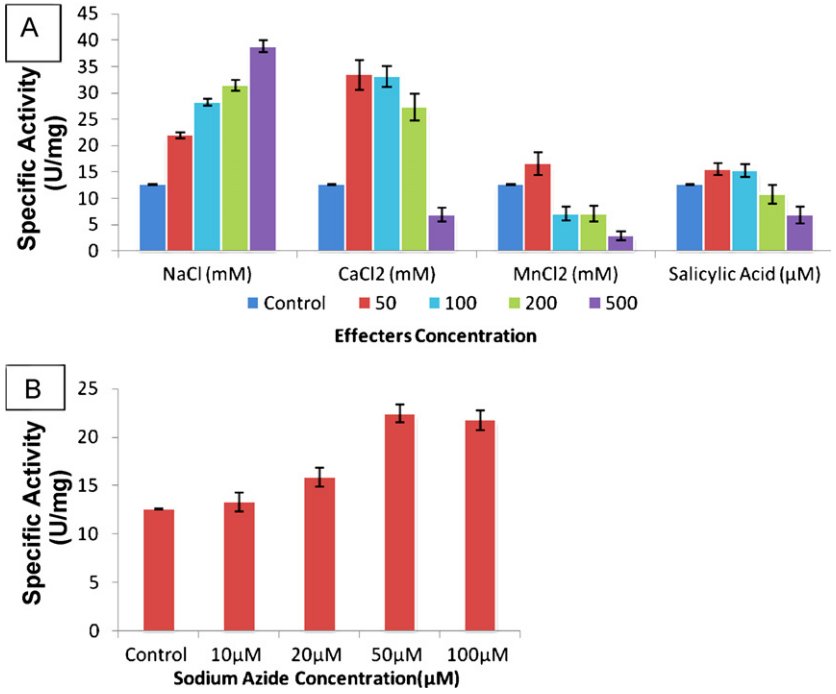

Fig. 3. (A) Effect of effectors namely, salts (NaCl, $CaCl_2$, $MgCl_2$) and salicylic acid on purified *Leucaena leucocephala* peroxidase activity. (B) Effect of azide on purified *Leucaena leucocephala* peroxidase activity.

3.5. Effect of pH on the peroxidase activity

The effect of pH on peroxidase activity was investigated. It is noteworthy that the *L. leucocephala* peroxidase was stable in the pH range of 4.5–7.0, with maximum activity at pH 5 (data not shown). The enzyme lost almost 90% of its activity at pH lower than pH 3 and higher than pH 8.8. 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 [44]. Similar reports of acidic pH optima (pH 5) for peroxidase of various plant species such as wheat grass and *A. sativum* have been given in literature [13,14].

3.6. Effect of metal ions (Na^+, Ca^{2+} and Mn^{2+}), azide and salicylic acid on the peroxidase activity

The *in vitro* effect of different metal ions on purified *L. leucocephala* peroxidase activity was investigated. Results are shown in Fig. 3A. Monovalent cation namely Na^+ activated peroxidase in a concentration dependent manner up to 3 M and did not inhibit peroxidase even at 4 M. On the other hand divalent cations namely Ca^{2+} and Mn^{2+} initially activated peroxidase up to 50 mM while inhibited peroxidase at higher concentration in a concentration dependent manner. *Allium* peroxidase was found to be inhibited 79% and 55% by Ca^{2+} and Mn^{2+}, respectively [14] while, wheat grass peroxidase were activated by Ca^{2+} as well as Na^+ [13].

Salicylic acid (SA) is a well known signaling compound in plant defense system against pathogen attack and it affects H_2O_2 catalyzing enzymes such as catalase, ascorbate peroxidase. Thus, the effect of salicylic acid on purified peroxidase activity was studied. Results are shown in Fig. 3A. Salicylic acid activated the *L. leucocephala* peroxidase up to 50 μM and then deactivates in a concentration dependent manner. Salicylic acid has been reported to inhibit guaiacol peroxidase [27,28]. Salicylic acid sensitivity of peroxidase is suggestive of its role in defense system in plants.

The effect of azide on purified *L. leucocephala* peroxidase was investigated. Results are shown in Fig. 3B. Peroxidase was found to be activated in presence of azide in concentration dependent manner. In contrary to our finding, azide was reported as an inhibitor for all peroxidases. Thus, for example, peroxidases from *B. vulgaris*, *S. melongena* and maize all found to be inhibited by azide [15,18,26]. It is important to note that *L. leucocephala* peroxidase was found to be unique in the sense that it was activated by azide rather than inhibition.

3.7. Carbohydrate content of peroxidase

Peroxidases are reported as glycoprotein [20]. The *L. leucocephala* peroxidase was also found to be a glycoprotein as evident by its ability to bind with Con-A, during purification. In order to confirm, we have determined the carbohydrate content of the purified peroxidase. *L. leucocephala* peroxidase was found to contain 0.09 mg carbohydrates per mg purified peroxidase protein. The glycoprotein nature of soybean root peroxidase and its significance in activity have been shown by [45]. Using tunicamycin, an inhibitor of glycoprotein synthesis, they have reported, reduced activity of peroxidase. Glycosylation of peroxidase has also been shown to be responsible for generation of multiple molecular forms of enzyme [39].

4. Concluding remarks

Peroxidases have gained a dominant position in areas such as biochemistry, biotechnology, physiology, histochemistry, cytochemistry, etc. because of their wide applicability. Peroxidase from *L. leucocephala*, an important tree legume for fodder and pulp and paper industry, was purified to homogeneity. Leucaena peroxidase was found to be unique in many ways, as compared to other peroxidases purified so far. Thus, the purified peroxidase was found to be a hetero-trimer. Purified enzyme was found to be an acidic and thermostable peroxidase. The peroxidase was activated by monovalent cation namely Na^+ up to a concentration as high as 4 M. Divalent cations such as Ca^{2+} and Mn^{2+} activated peroxidase at lower concentration (up to 50 mM) and inhibited at higher

concentration. Purified peroxidase was found to be activated by azide.

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