Thermostability assessment, profiling and localization of peroxidase activity in stem tissues of *Leptadenia pyrotechnica*: a defensive enzyme for survival in high temperature conditions

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Plants are known to overcome the biotic and abiotic stresses through various adaptive measures. Lignification is one such process that fortifies the cell wall wherein peroxidases play an important role. Here, we studied the thermostability and optimum pH of peroxidase activity, isozyme profile and its localization in stem tissues of the perennial desert shrub, *Leptadenia pyrotechnica* (Forsk.) Decne, locally called ‘kip’, belonging to the family Apocynaceae (subfam. Asclepiadaceae). *L. pyrotechnica* is an important component of the extreme arid environment of the Thar Desert. Apart from lignifications, peroxidase plays vital role in suberization, detoxification of hydrogen peroxides, fruit ripening, cross-linking of cell wall components and salt tolerance. The highest (72%) and lowest (8.5%) relative activity of peroxidase was observed at pH 6 and pH 3, respectively. Peroxidase of *L. pyrotechnica* was highly active within a broad range of pH 6-8. The maximum (81.5%) relative activity was observed at 40°C for 25 min. Relative activities of 52.3-23.1% were observed at 60 and 80°C, respectively, for 5 min of treatment. Five isozymes of peroxidase were observed in the zymogram. The bright amber and red colour of peroxidase and lignin were observed, respectively, in the stem tissues. Co-localization of peroxidase and lignin in xylem tissues of stem in *L. pyrotechnica* can be correlated with the role of peroxidase in lignin biosynthesis and for adaptive modification of arid zone plants under extreme climatic conditions.

**Keywords**: Abiotic stress, Adaptation, Kheemp, Khip, Lignification, Thar Desert

Peroxidases are involved in many biological processes and adaptive modifications of plants, animals and microbes under biotic and abiotic stresses. It is an important ancestral antioxidant enzyme evolved in bacteria for the protection of oxidative stress. Peroxidase (EC 1.11.1.7) is a heme containing glycoprotein enzyme of the oxidoreductase group and catalyses oxidation of organic and inorganic substrates using H$_2$O$_2$ (hydrogen peroxide). It is a novel sensing material for detection of hydrogen peroxide for living cells sensitivity and reporter enzyme for diagnostics and histochemistry. Peroxidase plays an important role in lignification, seed germination, suberization, detoxification of hydrogen peroxides, fruit ripening, cross-linking of cell wall components and salt tolerance. It can also be used as an indicator in the study of growth alterations, environmental stress conditions, rigidification of cell wall due to lignification and other biological processes. Lignification is a cell wall fortifying process in xylem tissue during tissue differentiation and lignin is the most abundant organic compound in the biosphere second to cellulose. Specific isoenzymes of cell wall-localized peroxidases are widely believed to be responsible for the final enzymatic step in lignification. Besides their important biochemical properties in plants, animals and microbes, peroxidases also have important practical applications. The optical and electrochemical properties of porphyrin, metalloporphyrin and heme group of peroxidase enzyme are useful in preparing the reusable catalytic coating of biosensors and other biomedical devices. In the present study, we investigated (i) peroxidase activity under extreme temperatures and pH regimes; (ii) resolution of isozymes using native PAGE; (iii) peroxidase localization in stem tissues in relation to lignification in a perennial Asclepiadaceae shrub (Fam. Apocynaceae) *Leptadenia pyrotechnica* (Forsk.) Decne from the Indian Thar Desert, locally called Kheemp or Khip, is an important component of vegetation in this sandy arid ecosystem (Fig. 1). It has immense economic and pharmacological importance and is a potential source of abiotic resistant genes for genetic modification of *Leptadenia pyrotechnica* in natural habitat on sand dunes of Jaisalmer region of India.
economically important plants to resist abiotic stresses.

Materials and Methods

Extraction and assay of peroxidase activity

Shoot apices of three field grown *Leptadenia pyrotechnica* were collected from Jodhpur region of Rajasthan, India during July-August months. Shoot apices were frozen in liquid nitrogen and then stored at −20°C. One gram of plant material was rinsed with 90% ethanol, homogenized in chilled mortar and pestle with 2 mL of 0.2 M sodium phosphate buffer (pH 6). The homogenate was centrifuged at 15000 rpm for 45 min at 4°C. The clear supernatant was used as a crude extract for peroxidase activity assay.

Peroxidase activity was determined by the method as described in the Worthington Enzyme Manual. In a cuvette, 2.5 mL of 0.2 M of sodium phosphate buffer (pH 6), 0.3 mL of 0.02 M hydrogen peroxide (H₂O₂) and 0.1 mL of crude extract were added and the absorbance was set to zero after which 0.1 mL of 0.002 M o-dianisidine (dissolved in 30% methanol) was added and mixed quickly. The rate of decomposition of hydrogen peroxide by the enzyme peroxidase with o-dianisidine as hydrogen donor was determined spectrophotometrically by measuring the rate of colour development at 460 nm. The absorbance was recorded up to 3 min at an interval of 15 s. The peroxidase activity is expressed as change in absorbance at 460 nm per min per gram of fresh weight (A₄₆₀ₙ₅/min/gram fresh wt.).

Analysis of optimum pH for peroxidase activity

The pH value for optimum peroxidase enzyme activity was determined by assaying the enzyme at a range of pH values (3-12). The peroxidase activity was calculated in percentage of relative activity. The relative activity was calculated by comparing the peroxidase activity with untreated enzyme.

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\text{% relative activity} = \frac{100 \times \text{Enzyme (Treated) OD Value}}{\text{Control (Untreated) OD Value}}
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Analysis of thermostability of peroxidase activity

The enzyme activity was also examined in relation to temperature and time of incubation. The effect of temperature on peroxidase activity was analyzed by incubating the reaction mixture in a water bath for 5-30 min at temperatures ranging 20-80°C. The peroxidase activity was calculated in percentage of relative activity.

Resolution of peroxidase isoenzymes

Vertical Poly Acrylamide Gel Electrophoresis was performed at 4°C with an ATTO AE-6530m system, Japan. Peroxidase isoenzymes were resolved on 90x80x1 mm 4.5% stacking and 7.5% separating gel using Tris/Glycine running buffer. The gels were washed twice with chilled distilled water and then incubated in saturated o-dianisidine (in 25% acetic acid) for 5 min and 3% hydrogen peroxide till development of green bands. The gels were washed with chilled distilled water and photographed on a white light illuminator.

Histochemical localization of lignin and peroxidase in stem tissues

Transverse sections of stem were treated with 1% aqueous solution of phloroglucin for 5 min followed by 50% HCl for 2 min and then mounted in glycerol for localization of lignin as described by Prasad. The peroxidase in stem tissues was localized using pyrogallol as described by Vance. The transverse section of stem was placed on a glass slide and covered with 2 drops of 50 mM pyrogallol solution (aq.) for 10 min. Two drops of 0.06% H₂O₂ were added on the stem transverse section for 3 min and then blotted away. The stem section was then rinsed with water and mounted in glycerol for visualization.

Results and Discussion

Effect of pH and temperature on peroxidase activity

In the present study, peroxidase of *L. pyrotechnica* was observed to be highly active within a broad range of pH 6-8. The highest relative activity (72%) of peroxidase was at pH 6.0 and the lowest at pH 3.0 (8.5%) (Fig. 2). Belcarz et al. also reported maximum peroxidase activity at pH 6.0 and up to 97% activity at pH 7.0 in the cool-season plant spring cabbage. The peroxidase activity in *Litchi chinensis* was found optimum at pH 6.5. The cation and anion peroxidase of *Aloe barbadensis* also showed optimum activity at pH 6.0. Various pH values in estimation of
secondary structural elements indicated that beta strands and beta-turns are significantly reduced at pH 5.5 causing the heme to be further exposed to the solvent and increasing conformation flexibility of the protein.27

The effect of temperature (20-80°C) and incubation time interval of 5 to 30 min on peroxidase activity was observed in L. pyrotechnica. A maximum of 80 to 30% peroxidase activity was observed between 40 and 60°C, respectively. The maximum relative activity (81.5%) was observed at 40°C for 25 min (Fig. 3). Dubey et al.28 also reported thermostability of peroxidase activity at high temperature in Golden delicious HP apple (Mallus pumilus) and Golden delicious HP JK apple. Peroxidase activity at 70°C in Chinese cabbage roots has been reported by Wang et al.29 Optimum activity at 55°C was observed in Gossypium hirsutum30 and at 40°C in sunflower root31. As temperature increased above 40°C to 60 and 80°C, the relative activity was decreased from 52.3 to 23.1%, respectively, in up to 5 min of treatment.

Resolution of isoenzymes and localization of peroxidase and lignin in stem tissues

The zymogram of peroxidase of L. pyrotechnica showed five distinct bands on the gel after staining (Fig. 4). Similarly, five isoenzymes have been observed in copper induced anionic peroxidase of sunflower root whereas only single band of isoenzyme was observed in Brassica oleracea.22 Kumar et al.32 reported two distinct bands in Satalum album leaf. Peroxidase activity and lignin were co-localized in the xylem tissues of the stem (Fig. 5). The histochemical localization of peroxidase activity and lignin in the stem tissues of L. pyrotechnica was used to support the role of peroxidase and lignin in tissue differentiation and adaptive modification. Co-localization of peroxidase and lignin in the xylem tissues can be correlated with the role of peroxidase in lignin biosynthesis and adaptive modifications. It is believed that specific isoenzymes of cell wall-localized peroxidases are responsible for the final enzymatic step in lignifications.15 These studies may help better understanding of basic and applied aspects of peroxidase enzymes and its role in stress tolerance management of plants.

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References


