Chapter II

Purification and characterization of peroxidase from leaves of *E. teriticornis*
In spite of extensive studies, the precise physiological function(s) of plant peroxidase remain poorly understood. On the other hand, there are numerous reports in literature that point to an important role for peroxidases in plant metabolism. Peroxidases have been described as critical enzymes in the lignification process (Brunow & Ronnerberg, 1979; Harkin & Obst, 1973; Mader & Amberg-Fisher, 1982; Mader & Fussl, 1982; Stich & Ebermann, 1988; Christensen et al., 1998; Lee et al., 2001), as a predominant enzyme operating under stress conditions including salinity, temperature extremes and systemic acquired resistance (Salin & Bridges, 1981; Patton et al., 1986; Klumpp et al., 1989; Martinez et al., 2000), as indoleacetic acid oxidase and also as a NADH oxidase (Grambow et al., 1983; Kobayashi et al., 1984; Nakajima & Yamazaki, 1979; Srivastava et al., 1977; Korori et al., 1989; Levitt, 1956; Levitt 1958) and as a mediator in the cross linking of cell walls and several other functions (Gibson et al., 1978). It has been suggested that lignification by peroxidase requires H₂O₂ as co-substrate and may be formed in situ by peroxidase acting as NADH oxidases (Gross et al., 1977; Polle and Chakrabarti, 1994).

Lignin is a polymeric constituent of the plant cell wall and, second to cellulose, is the most abundant organic compound in the biosphere. It is a complex aromatic polymer derived mainly from the polymerization of three different hydroxycinnamyl alcohols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Christensen et al., 1998). Specific isoenzymes of cell wall-localized peroxidases are widely believed to be responsible for the final enzymatic step in lignification: the oxidative dehydrogenation of monolignols which leads to free radical polymerization (Higuchi, 1985). Extensive studies (Christensen et al., 1998; Vitali et al., 1998; Yoo et al., 2002) have been made to define a link between a single peroxidase/multiple peroxidases and developmentally regulated lignification. The activity of
plant peroxidase(s) with specific substrates has also received widespread interest, especially in view of the relationship with lignin formation and its implication in various crop and forage plants.

On the basis of their amino acid sequence and metal binding properties, peroxidases from higher plants, fungi and bacteria have been categorized into three classes (Welinder, 1992). Class I includes yeast cytochrome C-peroxidase, chloroplast and cytosolic ascorbate peroxidases, and bacterial peroxidases; class II comprises the extracellular fungal lignin and manganese peroxidases, and class III contains the classic secretory peroxidases from higher plants. Classical secretory plant peroxidases (Class III; EC 1.11.1.7; donor, hydrogen peroxide oxidoreductase) are heme-containing enzymes of approximately 300 amino acids. The majority of them are N-glycosylated and are believed to be localized in the cell wall or the vacuole (Welinder, 1992). Most peroxidases can oxidize a wide range of substrates at the expense of H$_2$O$_2$, albeit at somewhat different rates. Classical peroxidases have been implicated in several primary and secondary metabolic processes, including hormone catabolism (Kenten, 1955), pathogen defense (Moerschbacher, 1992), phenol oxidation (Lagrimini, 1991), cross-linking of cell wall-structural proteins and polysaccharides (Fry, 1986; Lamport, 1986), and particularly in lignin polymerization (Mader, 1992; McDougall, 1992; Bajer et al., 1993). The major reasons it has been difficult to assign a specific function to any particular peroxidase have been the very high redundancy found in peroxidase genes, the broad spectrum of substrates accepted by these enzymes, and the very similar immunological properties of different isozymes. The Arabidopsis genome has been recently estimated to encode more than 40 different ER-targeted peroxidases grouped in families of high homology (Welinder, 1996). Additionally, because the down-regulation of specific peroxidases did not always lead to phenotypes, it was suggested that other isoenzymes were
compensating for reduced expression levels (Sherf et al., 1993). This phenomenon has further complicated the determination of physiological roles for peroxidases, when using a molecular biology approach. At present the methodological approaches target the study of these enzymes at different levels (e.g. transcription, translation, enzymatic properties, temporal expression), without interference from gene redundancy.

Concerted efforts are now being made to isolate different peroxidases and their isozymes at various stages of cellular life. Typically, such purification entails the fractionation of plant tissue extract with ammonium sulfate followed by repeated chromatography of the peroxidase enriched fractions on CM and DEAE cellulose (Paul, 1958; Kon & Whitaker, 1965; Morita et al., 1970; & Asada & Takahashi, 1971). In this manner and occasionally, through additional fractionations with organic solvents (Takahashii & Asada, 1971; Keilin & Hartree, 1951) chromatography on hydroxyapatite (Yasunobu et al., 1966; Asada & Takahashi, 1971; Keilin & Hartree, 1951) and electrophoretic separation (Hosova, 1960; Delincee & Radola et al., 1975) peroxidase has been isolated from various plant species.

The fast-migrating cationic peroxidase isozyme, RC3, was purified from rice callus. The molecular mass of the enzyme was about 34 kDa as determined by SDS-PAGE and 38 kDa by Sephacyl S-100 gel filtration. The pI value of the enzyme was 8.9 (Yoo & Lee, 2002). A basic peroxidase (HR 2) was secreted into the medium of turnip hairy root cultures. The purified enzyme had a pI of 9.6, a molecular mass of 39.3 kDa and showed a high thermo stability (Agostini et al., 2002). Four peroxidase isozymes were isolated from rice leaves (Ito et al., 1991). The molecular weights estimated by SDS –PAGE were 48, 48, 40 and 39.5 Kda and their isoelectric points were 5.4, 8.1, 9.3 and 9.2 respectively. Sesto & van Huystee (1989) purified a cationic peroxidase from peanut suspension cell culture. The procedure
started with sequential acetone and ammonium sulphate precipitation. At this point the extracted medium proteins were applied to a cationic exchange column to separate the cationic and anionic proteins. Analysis by gel electrophoresis showed a single peptide corresponding to the major cationic peroxidase. The peptide at 66 kDa stained quite heavily for carbohydrates. The RZ values increased to a maximum of 3.5. An acidic peroxidase secreted into culture medium by cell suspension culture of *Cassia didymobotrya* was purified. The enzyme was shown to be a glycoprotein with a molecular mass of about 43kDa by SDS-PAGE and 50kDa by gel filtration (Vitali *et al*., 1998). Soybean peroxidase was isolated and purified from the seed coat of “Montsew” Chinese soybeans. The soybean peroxidase was purified by perfusion anion exchange high performance liquid chromatography on poros DEAE strong anion 4.6mm/100mm (Zhichen *et al*., 1999). A basic peroxidase isozyme was isolated from pepper fruits (Bernal *et al*., 1994). The isoenzyme was purified, by preparative isoelectric focusing in glycerol-stabilized pH gradients between 3.0-10. Singh *et al*., (2002) isolated a peroxidase from turnip root using metal affinity chromatography. The molecular weight of the purified enzyme was found to be 37-39 kDa. The enzyme showed maximum activity in phosphate buffer pH 6.0 and lowest activity in borate buffer at the same pH. Turnip peroxidase also contains an iron moiety, which is found to be about 0.28%. Deépa & Arumughan (2002) purified soluble peroxidase from oil palm leaf by (NH₄)₂SO₄ precipitation, anion exchange chromatography and molecular exclusion chromatography. The molecular weight of the purified enzyme was in the range of 48 ± 2kDa. The enzyme had an optimum pH of 5 and exhibited very high pH and thermal stability. Immunocytochemical localization studies demonstrated that the peroxidase was mainly located in the vascular bundle of epidermis of leaf. A basic heme-containing peroxidase isoenzyme was purified from artichoke flowers (*Cynara scolymus*) by Lopez-Molina *et al* (2003). The enzyme was shown to be a monomeric glycoprotein,
\[ M_r = 42300 \pm 1000 \] with an isoelectric point > 9. Gray & Montgomery (2003) purified a major cationic peroxidase from corn steep water by a combination of acetone and ammonium sulfate precipitation and sequential chromatography on CM-cellulose, Phenyl-Sepharose and Sephadex G-75. The final enzyme showed a 36400 fold purification with a 12% recovery and had a pI value of approximately 8.9. A plant peroxidase localized in the root tissue of *Raphanus sativus* was purified by precipitation using a reversible soluble/insoluble ion exchange polymer system comprising of carboxymethyl cellulose, calcium and polyethylene glycol. The enzyme was further purified and concentrated by thiophilic chromatography (Aruna & Lali, 2001). Nair & Showalter (1996) isolated a novel cell wall bound cationic peroxidase from the roots of the carrot plant. The purified enzyme had a molecular mass of 45 kDa and RZ value of 2.3. The temperature and pH optima for the enzyme (with guaiacol as substrate) were found to be 32 °C and 4.9 respectively.

Plant peroxidase has also been isolated purified and characterized from various woody plants including *Populus trichocarpa* (Christensen et al., 1998), *Pinus pinaster* (Karmali & Santos, 1988), and *Araucaria araucana* (Riquelme & Cardemil, 1995).

In an attempt to identify lignin specific peroxidase isozymes Christensen et al (1998) analyzed the anionic peroxidase in the xylem of Poplar. Purification and characterization yielded five different peroxidases namely, PXP 1,PXP 2, PXP 3-4,PXP 5, and PXP 6. All five peroxidases were strongly glycosylated (3.6% to 4.9% N-glucosamine), with apparent molecular masses between 46 and 54 kDa and pI values between pH 3.1 and 3.8. Two of the five isolated peroxidases (PXP 3-4,PXP 5) could oxidize the lignin monomer analog syringaldazine, an activity previously correlated with lignification in poplar.
Fossdal et al (2001) have isolated a cDNA encoding the putative defense related and basic plant peroxidase (Spruce pathogen-induced2), with an estimated molecular mass of 34 kDa, from roots of Norway spruce (Picea abies) seedlings. This report was the first description of the isolation of a complete cDNA encoding a putative peroxidase from a gymnosperm.

Riquelme & Cardemil (1995) have purified and partial characterized two cationic peroxidases from the cell walls of seeds and seedlings of Araucaria araucana. They have determined the amino acid composition and NH$_2$-terminal sequence of both the enzymes. The two peroxidases were similar in their amino acid composition and both have identical NH$_2$-terminal sequences, indicating that the two proteins were genetically related and probably isoforms of the same kind of peroxidase.

Peroxidase was purified from needles of Pinus pinaster. The purified enzyme showed a single protein band on SDS-PAGE and native PAGE with Mr. of 37,000 and 151 respectively. The pl of the purified enzyme was found to be 3.2. The enzyme had an optimum pH of activity 5.0 and temperature optimum of 30 °C (Karmali & Santos, 1998).

The genus Eucalyptus is one of the most important forest trees. It has grown to the position of a major plantation tree in India. Several species of eucalypts are grown for varied uses like pulpwood, firewood, poles, windbreaks and timber. It has already been discussed that plant peroxidase is the major enzyme for the formation of wood. No studies have been carried out on the properties of purified peroxidase (s) enzyme from Eucalyptus tereticornis sp. Based on the above facts this investigation has been made to find out the nature of peroxidase in Eucalyptus leaves. To understand the nature of peroxidase in details the enzyme was purified and its temperature, pH stability, substrate specificity, isozyme pattern, molecular weight determination etc. have been studied.
Materials & Methods
Purification of peroxidase from Eucalyptus leaves

(All steps shown below were carried out at 4°C.)

Step 1. Crude extract preparation: 50 gm of thoroughly washed and blotted leaf material was cut and homogenized using 100mM Na-phosphate buffer (pH-6.5) containing, 0.2% polyvinyl pyrrolidone and 0.4% Triton X-100. The homogenate was then centrifuged at 12000g for 15 minutes and the clear supernatants were used as the enzyme source.

Step 2. Ammonium sulfate fractionation: The supernatant fluid was brought to 80% saturation with respect to ammonium sulfate. After another centrifugation at 15000 g for 15 minutes the pellet was resuspended in 100mM Na-phosphate buffer (pH 6.5). The solution was then dialyzed versus 100mM Na-phosphate buffer (pH 6.5) overnight.

Step-3 Sephadex G-100 Chromatography: The dialyzed solution was loaded on to a Sephadex G-100 column (1.5 cm diameter, 60 cm long) previously equilibrated with 50 mM phosphate buffer (pH 6.5). The column was washed with the same buffer. Active fractions with high peroxidase activity were combined and concentrated by lyophilization.

Step 4. Ion exchange chromatography on DEAE Sepharose column: The lyophilized powder from step 3 was taken in phosphate buffer (pH 6.5), and subsequently loaded on to a DEAE Sepharose column (2.5 cm diameter 20cm long), previously equilibrated with 25mM Na phosphate buffer (pH 7.2). The column was washed with the same buffer, and eluted with a linear gradient of 200-800mM NaCl in 25mM Na phosphate buffer (pH 7.2). Active fractions were combined and concentrated by lyophilization.
Solution for Gel Electrophoresis

- SDS-PAGE gel Electrophoresis

a. 30 : 0.8 Acrylamide: Bisacrylamide 30 gm Acrylamide and 0.8 Solution Bisacrylamide dissolved in Water and the volume made Up to 100 ml.

b. Buffer for resolving gel 1.5 M Tris-HCl (pH=8.8)
c. Buffer for stacking gel 1.5 M Tris-HCl (pH=6.8)
d. 5X SDS-PAGE sample buffer 0.0625 M Tris-HCl (pH 6.8),
15% Glycerol, 0.1% SDS, 1%
β-Mercaptoethanol, 0.001%
Bromophenol blue.
e. SDS-PAGE running buffer 14.4 gm/ Glycine/lit, 3 gm Tris-
Cl/lit and 0.1% SDS.
f. Polyacrylamide gel staining solution 0.1 % Coomassie blue R-250
Solution, in 50:10:40 Methanol
Acetic acid: Water
g. Destaining solution 20:10:70 Methanol: Acetic acid
Water.

Effect of Temperature and pH on Enzyme activity

The stability of peroxidase was assayed by placing the enzyme samples (1 ml) in a water bath at the tested temperature for 30 minutes. The tubes were cooled in cold water (12 °C) and then assayed for peroxidase activity at 37 °C. The effect of pH on the enzyme activity was carried
out by 50 mM citrate buffer (pH 4) 50 mM Na-phosphate buffer (pH 6.5) and 50 mM borax buffer (pH 10).

**Molecular weight determination**

The molecular mass of the peroxidase was estimated by SDS/PAGE following the method of Laemmli (1970).

For the estimation of molecular weight of purified peroxidase, polyacrylamide gel electrophoresis of standard protein marker and purified samples was run on 10% concentration of SDS gel following the method of Laemmli (1970). For SDS/PAGE, BSA (Mr 66000), Ovalbumin (Mr 45000), Carbonic anhydrase (Mr 30000) Lysozyme (Mr 14000) and Cytochrome (Mr 12000) were used as reference standard proteins.

**Isoelectric focusing**

Isoelectric focusing was performed in a LKB 8101 Ampholine electro focusing apparatus, with an Ampholine range between 3 and 10. The gel was stained for protein content with coomassie Brilliant Blue R-250.

**Spectrophotometric and Spectrofluorometric study**

Spectrofluorometric study of purified enzyme was done with a Hitachi F4500 spectrofluorimeter, where excitation and emission slit widths were both 2.5 nm. A 700 µl quartz cuvette was used.
All spectrophotometric measurements were made using Hitachi U-2000 spectrophotometer with 1000 µl quartz cuvette.

**Lectin Affinity Chromatography:**

Partially purified leaf extracts were subjected separately to affinity chromatography using Concanavalin-A-Sepharose-4B (Sigma). The column was equilibrated with TBS buffer (0.02M Tris-HCl, 0.5 M NaCl, containing 1 mM CaCl₂, pH 7.4) and column bound proteins were eluted with TBS buffer containing 0.1 M methyl-α-D mannopyranoside (Sigma).

**Reagents:**

1. **Suspension Buffer:** 0.1 M Acetate buffer, pH 6.0, containing 1M NaCl, 1M CaCl₂, 2H₂O, 1mM MnCl₂, 1mM MgCl₂, 6H₂O and 0.01 % thimerosol.

2. **Starting Buffer:** 0.02 M Tris-HCl buffer, pH 7.4, is containing 0.5 M NaCl, 1mM each of CaCl₂ and MgCl₂.

3. **Elution Buffer:** 0.02 M Tris-Buffer -Saline (TBS) containing (0.1 M) methyl-α-D mannopyranoside.

**Preparation of Lectin Affinity column:**

Concanavalin-A-Sepharose-4B was taken as a column for affinity chromatography. 14 mg/ml of packed gel of Concanavalin-A-Sepharose-4B was suspended in suspension buffer. 1 ml matrix was taken in 5 ml disposable syringe having dimension 0.5 x 5 cm. Glass wool was used to plug the bottom. The column was equilibrated with 0.1M TBS buffer. After
packing of gel, it was washed with at least 10- column volume of TBS before use. The column of immobilized lectin was stored at 4 °C.

*Elution of non-bound peroxidase:*

0.1 ml of aliquot (0.7mg/ml) of semi purified peroxidase from Sephadex G-75 was loaded on the column. Once the sample was applied, the column was washed with TBS- buffer at a flow rate of 1.0 ml/min. Fractions were collected and monitored at 280 nm until the absorbance reached the base line. Peroxidase activity was monitored at 460nm in the assay buffer as described earlier and expressed as A460/min. In each fraction 25 µl sample was used as the enzyme source.

*Elution of bound peroxidase:*

Adsorbed proteins were eluted with TBS buffer containing 0.1 M methyl-\(\alpha\)-D mannopyranoside. Absorbance of each eluted fraction was quantitated in terms of A280 and A460/min. Active fractions were combined and concentrated and specific activity of peroxidase was calculated.
Results
&
Discussion
As indicated in Figure 2a Eucalyptus peroxidase activity was extractable with Triton X-100 (TX-100). At increasing concentration of TX-100 there was an increase in the specific activity of the extracts. Specific activity of peroxidase was increased about 200% (as compared to extraction with the buffer only) when 0.2% TX-100 was added to extraction buffer. This increase was about 300% when 0.4% TX-100 was used. Increasing the concentration of TX-100 from 0.4-0.6 % did not change the specific activity of the extract any further. The results indicated that peroxidase was probably present as a bound form in the Eucalyptus leaves. Solubilization with a detergent could release this cryptic activity, which suggested that this binding was probably not covalent in nature. The stability of the peroxidase activity in the extract was further studied and results are shown in Figure 2b. After 96 hours of storage at 4 °C the extracts retained about 85% of the activity present at the start (0-hour) of the experiment. Thereafter peroxidase activity decreased sharply with time and after 144 hours of storage the loss in the activity was found to be 50%. The abrupt fall in activity may have been due to the oxidation of essential thiol groups in the enzyme (Liu et al., 1999) or for some other reason. e.g. the presence of proteases in the extract.

The purification procedure is summarized in Table 2a. The crude extract contained peroxidase at a specific activity of 0.79 units /mg protein. Ammonium sulfate fractionation followed by gel exclusion chromatography on Sephadex G-100 gave about 13.6 fold purification. There was a loss of about 62% activity after the ammonium sulfate fractionation step. Ion-exchange chromatography on diethyaminoethyl Sepharose(DEAE-Sepharose) resulted in 187-fold purification and 26% total activity was recovered as compared to starting material. The special challenges in the purification of proteins from plant material include the removal of phenolic substances, tannins and various coloured constituents (Hatti-kaul & Mattiasson,
A quick and convenient separation procedure is therefore essential especially when enzymes are involved as the end product. The major advantage of the purification procedure described here was its relative simplicity. The crude extract contained high amounts of pigmented material and phenolics. After the ion exchange chromatography, phenolics and pigmented material were retained in the column and a clear solution was obtained.

PAGE of the purified preparation revealed a single band when the zymogram was developed with o-dianisidine as the substrate (Plate 2a). SDS-PAGE of the purified protein gave a single band when stained with Coomassie Blue or upon silver staining (Plate 2b). When analyzed for the molecular weight the band corresponded to 58 kDa (Figure 2c).

To attempt a partial characterization of sugar composition of the enzyme the purified peroxidase was applied to a Concanavalin A coupled to Sepharose 4B column. Only a small fraction of activity was eluted initially, the majority was released with a (0.1M) α-methyl-mannoside solution (Figure 2d, 2e & Table 2b). The strong affinity of the enzyme for Concanavalin A can be explained by the mannose chains on the surface of the enzyme (Vitali et al., 1998). It has been found from various reports that peroxidases are glycosylated. Horseradish peroxidase (HRP) has been known to be glycoprotein for many years (Shannon et al., 1996; Theorell & Akeson 1943). Melo et al (1997) reported for the first time the presence of human Lewis (a) type of glycoprotein secreted by the cell suspension culture of Vaccinium myrtillus L. Five different peroxidases that were isolated from the xylem of poplar showed strong glycosylation (Christensen et al., 1998) It was reported that cationic peroxidase was also a glycoprotein Van Huystee & Maldonado 1982; Sesto & Van Huystee 1989). Gray et al., (1998) detected traces of high mannose type glycans in horseradish peroxidase isozymes c (HRP c). High mannose types of some oligosaccharides were also
observed in soybean hull peroxidase (Gray et al., 1996). Vitali et al., (1998) observed the presence of mannose chain on the leaf surface of peroxidase enzyme in the cell suspension culture of Cassia didymobotrya.

Figure 2f and Figure 2g show the spectrofluorometric and spectrophotometric scans of the purified peroxidase. A summary of spectrofluorometric scanning data is presented in Table 2c. It was evident from the data, that, for the excitation wavelength at 280 nm (for tyrosine and tryptophan) and at 295 nm (tryptophan) fluorescence intensity was reduced by about three fold but there was no change in emission maxima (340.8nm). It was apparent from the results that purified enzyme contained very low amount of tyrosine. This finding is keeing with the date of other workers (Lakowicz et al, 1982; Udenfriends, 1962).

An initial study with the crude extract from Eucalyptus leaves showed that this enzyme exhibited high specific activities with the guaicyl compound coniferyl alcohol, where as syringaldazine was not acted on as a substrate (Figure 2h). The peroxidase activity was further estimated with coniferyl alcohol syringaldazine and NADH as substrates using the purified peroxidase (Figure 2i). Interestingly however, the purified peroxidase exhibited activity with syringaldazine as substrate. This may be indicative of some natural inhibitor present in the crude extract. However the activity was feeble (4.5 U/mg protein) when compared to e.g. jute (13.5 U/mg protein), or rice (8.2 U/mg protein) as reported in earlier publication (Sinha, 2002)

The key property of lignin is determined by the ratio of two structural components syringyl and guaiacyl and this can vary substantially in both intraspecies and interspecies manner. It has been observed that in needles of Norway spruce (Miksche & Yasuda, 1977) syringyl
residues constitute only 4% of total lignin monomers. Temperate eucalypts generally have higher syringyl to guaiacyl ratios than tropical species. Of the two widely planted temperate species *Eucalyptus globulus* and *E. nitens*, the former generally has higher syringyl content (Wallis, 1999/2000). Goldberg et al., (1983) suggested that a peroxidase with affinity for syringaldazine was present in the cell walls during the phase of active lignification in *Poplar*. However, Western blots of crude extracts of Eucalyptus trees (in October) showed only one band corresponding to the peroxidase (Chapter 3). As reported earlier lignification of Eucalyptus stem was highest in October and was concomitant with highest peroxidase activity. Since the purified enzyme was sourced from Eucalyptus in October, it is highly probable that Eucalyptus peroxidase does not exhibit the “typical” syringaldazine oxidase activity associated with lignification in other species (Christensen et al., 2001). The results with the purified enzyme also indicated that NADH oxidase activity was very low. It has been reported that lignification by peroxidase requires H\(_2\)O\(_2\) as co-substrate. H\(_2\)O\(_2\) has been localized histochemically in lignifying tissue (Olson & Verner, 1993 and Polle & Chakrabarti, 1994) and may be formed *in situ* by peroxidase acting as NADH oxidases (Gross et al., 1977). Thus NADH oxidase has been reported to be the main H\(_2\)O\(_2\) donor in the plant. However, several workers have suggested that, the H\(_2\)O\(_2\) may be generated from other sources such as oxidases as reported by Schopfer (1994). The finding viz. low NADH oxidase activity seems to support the latter group of workers.

The optimum temperature for the enzyme activity was 50°C. At 70°C the loss of peroxidase activity was about 90% of its maximum value and with further increase of temperature the activity was completely lost (Figure 2j). The temperature optima of peroxidase activity in Eucalyptus leaves suggested it's thermo stable nature. Thermostability of plant peroxidases has been supported by various studies. A peroxidase isoenzyme secreted by turnip hairy-root
cultures showed high thermo stability (Agostini et al., 2002). Purified peroxidase from palm leaf also showed thermostability (Deepa & Arumugan, 2002). A purified peroxidase isolated from root tissues of Raphanus sativus showed optimum activity at 40°C temperatures (Aruna & Lali, 2001).

The influence of pH on the activity of the purified enzyme is shown in Figure 2k. The enzyme showed activity over the pH range of 5-6.5 with an optimum activity at pH 6. At pH 7.5 the activity was only about 18% that of the optimum activity and at pH 8.5 the activity was totally lost. Various studies have indicated that peroxidase showed optimum activity in the pH range 5-6. Rice peroxidase showed maximum peroxidase activity at pH 5 (Ito et al., 1991). Peroxidase isolated from palm tree also showed an optimum at pH 5 (Deepa & Arumugan, 2002). The same pH optimum was found in Pinus sp needles (Karmali & Santos, 1998). Cationic peroxidase from carrot roots showed pH optima at 4.9 (Nair & Showalter, 1996). Eucalyptus peroxidase showed optimum pH at 6, which was similar to turnip peroxidase, isolated from turnip root (Singh et al., 2002) and the fast migrating cationic peroxidase from rice callus (Yoo & Lee, 2002).

Purified Eucalyptus peroxidase migrated as a single protein band when run on an analytical electric focusing gel. The isoelectric point of purified peroxidase was estimated to be 7.2 (Plate 2c). The pI value of peroxidase varies from plant to plant. The pI value of a fast migrating cationic peroxidase, isolated from rice callus, was 8.9 (Yoo & Lee, 2002). A basic peroxidase that was secreted into the medium of turnip hairy root culture had a pI of 9.6 (Agostini et al., 2002). The five different Poplar xylem peroxidases showed pI values between pH 3.1 and 3.8 (Christensen et al., 1998). The pI of the purified peroxidase enzyme from Pinus pinaster needles was found to be 3.2 (Karmali & Santos, 1998).
On the basis of the results obtained and the background literature, following conclusion was arrived, that the peroxidase enzyme isolated and purified (187 fold) from Eucalyptus leaves showed high specific activity (148 U/mg protein) and ran as a single protein band on SDS-PAGE and native PAGE. The pI of the purified enzyme was found to be 7.2. The enzyme has an optimum pH activity of 6.0 and temperature optimum of 30°C. The purified peroxidase had mannosyl moieties and a Mr of 58 kDa. Peroxidase activity in the crude extract utilized orthodianisidine (general substrate) and coniferyl alcohol but did not utilize syringaldazine the (typical substrate used to detect lignifying activity). Further the impure preparation of Eucalyptus peroxidase had very low to non-detectable NADH oxidase activity, which normally would supply H₂O₂ required for lignifications. However the active fractions collected from ion exchange chromatography could utilize all their substrates, though at different rates, with the highest rate being observed for coniferyl alcohol.
Tables, Figures
&
Plates
### Table 2a. Purification of peroxidase from Eucalyptus leaves

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity (Total units)</th>
<th>Activity (units/mg)</th>
<th>Fold purity</th>
<th>Yield (% Wt)</th>
<th>Yield (% Activity)</th>
</tr>
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<tbody>
<tr>
<td>Crude</td>
<td>3.069</td>
<td>76.72</td>
<td>61.06</td>
<td>0.7958</td>
<td>1</td>
<td>100</td>
<td>100</td>
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<tr>
<td>(NH₄)₂SO₄</td>
<td>2.486</td>
<td>24.86</td>
<td>22.83</td>
<td>0.9148</td>
<td>1.15</td>
<td>32</td>
<td>37</td>
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<td>Sephadex-100</td>
<td>0.559</td>
<td>2.795</td>
<td>20.17</td>
<td>10.828</td>
<td>13.60</td>
<td>3.6</td>
<td>33</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>0.0543</td>
<td>0.108</td>
<td>16.17</td>
<td>148.18</td>
<td>187.18</td>
<td>0.14</td>
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### Table 2b. Purification and recovery of semipurified Eucalyptus leaf peroxidase using Con A column

<table>
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<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units/mg)</th>
<th>Activity (Total units)</th>
<th>Fold purity</th>
<th>Recovery (% Activity)</th>
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<tr>
<td>Semi-purified</td>
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<td>0.35</td>
<td>8.46</td>
<td>3.1</td>
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<td>100</td>
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<tr>
<td>Con-A</td>
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<td>0.009</td>
<td>64.21</td>
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<td>7.58</td>
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### Table 2c. Spectrofluorometric characteristics of purified Eucalyptus peroxidase

**Excitation wavelength-280 nm**

<table>
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<tr>
<th>Quantity</th>
<th>Em *max</th>
<th>F.I. <strong>(A.U)</strong>***</th>
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</thead>
<tbody>
<tr>
<td>200μl in 1 ml buffer</td>
<td>340.8nm</td>
<td>3150</td>
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</table>

**Excitation wavelength-295 nm**

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<th>Quantity</th>
<th>Em max</th>
<th>F.I (A.U)</th>
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<tbody>
<tr>
<td>200μl in 1 ml buffer</td>
<td>340.8nm</td>
<td>1150</td>
</tr>
</tbody>
</table>

* Em max ———► Emission maxima

** FI ———► Fluorescence intensity

*** ———► Arbitrary units

Activity- (μ-mol o-dianisidine oxidized/min/ mg of protein).
Fig. 2a. Extractability of peroxidase from Eucalyptus leaves in Triton buffer

Fig. 2b. Time dependent Stability of Eucalyptus peroxidase in leaf extracts

Specific activity - (µ-mol o-dianisidine oxidized/min/ mg of protein).
Fig. 2c. Molecular weight of peroxidase isolated from Eucalyptus leaves (SDS-PAGE Analysis)
**Fig. 2d. Elution profile of semipurified Eucalyptus leaf peroxidase**

- O.D. at 280nm
- Number of fraction

**Fig. 2e. Activity profile of semipurified Eucalyptus leaf peroxidase on Con A column**

- Activity (A460 nm/min)
- Number of fraction
Fluorescence emission spectra of Eucalyptus peroxidase.

A-Excitation wavelength, 280nm
B-Excitation wavelength, 295nm
AU-Arbitrary units
Specific activity - (µ-mol o-dianisidine oxidized/min/ mg of protein).
Fig. 2j. Effect of temperature on purified peroxidase of *Eucalyptus* sp

Specific activity - (µ-mol o-dianisidine oxidized/min/ mg of protein).

Fig. 2k. Effect of pH on purified peroxidase of *Eucalyptus* sp
Plate 2a. Activity staining of purified peroxidase isozyme from Eucalyptus leaf

Plate 2b. Estimation of molecular weight of purified Eucalyptus leaf peroxidase by SDS-PAGE

Plate 2c. Isoelectric focusing of purified Eucalyptus peroxidase

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