PERGAMON

Phytochemistry 61 (2002) 503–511

PHYTOCHEMISTRY

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Purification and characterization of soluble peroxidase from oil palm (*Elaeis guineensis* Jacq.) leaf

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Received in revised form 22 March 2002; accepted 2 May 2002

Abstract

Soluble peroxidase (POD) from oil palm leaf was purified by $(NH_4)_2SO_4$ precipitation, anion exchange chromatography and molecular exclusion chromatography. The purification grade obtained was 429 yielding 54% of the enzyme activity. Electrophoresis of purified enzyme under denatured conditions revealed M_r of 48 ± 2 kDa. It has an optimum pH of 5 and it exhibited very high pH and thermal stabilities. K_m for guaiacol, ABTS and pyrogallol were 3.96, 1 and 0.84 mM, respectively. Immunocytochemical localization studies showed that soluble POD was mainly located in the vascular bundles and epidermis of leaf.

Keywords: *Elaeis guineensis* Jacq.; Oil palm; Leaflet; Enzyme purification; Peroxidase; Vascular bundle; Xylem; Phloem

1. Introduction

Peroxidase (EC 1.11.1.7) (POD) is widely distributed among higher plants and isoenzymes of peroxidase occur in a variety of tissues of several species. It is an oxidoreductase that catalyzes the oxidation of various electron donors with H_2O_2 and carries a 'b'-type haem as prosthetic group. Even though they are ubiquitous in higher plants, their true physiological function and control remain unclear. Several POD isoenzymes occur in each species studied so far, differing in molecular and catalytic properties. Due to the variety of reactions they catalyze in vitro and the large number of isoenzymes found, it has not yet been possible to assign an in vivo function for a particular isozyme (Gaspar et al., 1991). Some of the physiological functions of POD in plants involve participation in lignification of the cell wall, metabolism of indole-3-acetic acid, biosynthesis of ethylene, defence against infection and wound healing (Gazaryan et al., 1996; Kobayashi et al., 1996; Christensen et al., 1998).

POD has been isolated and characterized from a large number of sources like fruits, leaves, tubers, etc. Some of the leaf sources from which POD was purified were barley (Saeki et al., 1986), rice (Ito et al., 1991), cotton (Triplett and Mellon, 1992), etc. POD is of great interest to biological researchers and has been widely used as an important component of reagents for clinical diagnosis of laboratory experiments such as in 'ELISA' and enzyme immunoassay kits. POD is an enzyme having wide industrial applications. POD is capable of catalyzing the polymerization of phenols in organic solvents (Dordick et al., 1987), degrading toxic phenols from wastewater (Dec and Bollag, 1994), decolourizing synthetic dyes (Spadara and Renganathan, 1994) and now POD is introduced into a relatively new area of detergent formulation (Showell, 1998).

Palm oil is the second largest source of edible oil in the world and is derived from oil palm fruit. POD present in the fruit is known to initiate oxidation of oil. Here we report that oil palm leaf is one of the richest sources of peroxidase hitherto not studied. In this paper, the purification and characterization of soluble peroxidase from oil palm leaf including kinetic and catalytic properties of the purified enzyme were studied. Immunocytochemical localization of POD using antibodies raised against purified leaf POD was also done and the physiological significance of POD in oil palm is also discussed.

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PII: S0031-9422(02)00167-X

2. Results and discussion

2.1. Purification of oil palm leaf peroxidase

Oil palm leaf POD was purified 429-fold to homogeneity (confirmed by SDS–PAGE) with a recovery of 54% (Table 1). POD was found to be precipitated with very high concentrations of $(NH_4)_2SO_4$ in the range of 60–95%. The 60–95% $(NH_4)_2SO_4$ precipitate was concentrated, dialyzed against 5 mM Tris–HCl, pH 8.4 and applied to the DEAE-cellulose column. POD bound to the DEAE-cellulose and was eluted with a linear gradient of 0–0.5 M NaCl in 5 mM Tris–HCl, pH 8.4. The enzyme was eluted at a salt concentration of 0.125 M NaCl along with the major protein peak (Fig. 1). This step also facilitated the removal of colour (probably due to phenolics), which was retained in the column yielding a virtually colourless enzyme preparation.

The final step of purification was done by SG-100 column chromatography. Active fractions from the above column were concentrated, dialyzed against 50 mM phosphate buffer, pH 6.0 containing 0.5 M NaCl and applied to SG-100 column. On eluting the column with the same buffer (Fig. 2), POD came out with the void volume, which indicated a very high molecular weight for the protein probably due to aggregation.

The above three steps resulted the purification of POD to homogeneity resulting in a fold purification of 429 with a yield of 54%. The enzyme thus obtained was used for further studies.

2.2. Optimum pH

By using guaiacol as substrate, POD showed a pH optimum of 5.0. An optimum pH of 5 suggests that the enzyme can function in an acidic environment, such as in the vacuole (Takahama and Egashira, 1991). PODs purified from various sources have their pH optimum mostly in the region of 4.5–6.5. The pH optimum for rice is 5.0 (Ito et al., 1991), for tomato (Jen et al., 1980), soybean (Sessa and Anderson, 1981) and coconut PODs (Mujer et al., 1983) it is 5.5, and for strawberry it is 6.0 (Civello et al., 1995).

2.3. pH stability

POD showed greater stability with respect to H^+ ion concentration in the alkaline range. In the pH range 5–10, activity of the enzyme was more or less similar to the original activity. But as the pH was lowered below 5, a sharp decline in stability occurred and at highly acidic conditions the enzyme lost all it's activity. The loss of enzyme activity at low pH was reported to be due to the detachment of haem prosthetic group from the polypeptide chain (Vamos-Vigyazo, 1981).

2.4. Thermal stability

Oil palm leaf peroxidase exhibited very high resistance to heat even at temperature as high as 80 °C. A major portion of the enzymatic activity was retained at this temperature even after 60 min of incubation. As the temperature was increased from 30 °C, the resistance exerted by the peroxidase also increased up to 70 °C after which the temperature declined from 80 °C onwards (Fig. 3).

POD is reported to be one of the most heat stable enzymes in plants. It was observed that 6 min at 121 °C is needed to inactivate POD in green peas (Vamos-Vigyazo, 1981). However, the resistance to treatment depends on the source of the enzyme as well as the assay conditions, especially pH and nature of substrate employed. Present study shows that the thermal stability of oil palm leaf POD is greater than that reported for cotton (Triplett and Mellon, 1992), strawberry (Civello et al., 1995) and coconut (Mujer et al., 1983). It has been shown that the thermal stability of POD is due to the presence of a large number of cysteine residues in the polypeptide chain. It was also suggested that the non-linear inactivation curves are due to the formation of new complexes of higher thermostability formed

Table 1
Purification protocol of oil palm leaf POD

Fraction	Volume (ml)	Total activity units	Total protein (mg)	Specific activity (units/mg protein)	Fold purification	Yield (%)
1. Crude extract[a]	500	20325	34.740	585.06	1	100
2. 60–95% $(NH_4)_2SO_4$ precipitate[b]	9	16476	0.288	57208.30	98	81.06
3. DEAE-cellulose column[c]	22	12510	0.065	192461.50	359	61.54
4. Sephadex G-100 Gel filtration[d]	20	11048	0.044	251090.90	429	54.35

[a] Twenty-five grams of the leaf acetone powder was suspended in 500 ml of 0.1 M acetate buffer, pH 4.0 containing 1% PVP and stirred for 1 h at 4 °C. Homogenate filtered through two layers of cheesecloth, centrifuged at 10,000 rpm for 20 min at 4 °C.

[b] $(NH_4)_2SO_4$ saturation to 60%, stirred for 1 h and centrifuged. Supernatant was further saturated to 95% with $(NH_4)_2SO_4$ and stirred for 15 h at 4 °C.

[c] POD elution in 5 mM Tris–HCl, pH 8.4 using a linear gradient of 0–0.5 M NaCl.

[d] Gel filtration in Sephadex G-100 in 50 mM phosphate buffer, pH 6.0 containing 0.5 M NaCl.

from thermally denatured enzyme protein and groups of POD that remain active (Vamos-Vigyazo, 1981). The thermal stability of cotton leaf peroxidase was reported to be due to the carbohydrate components of the glycoenzyme (Triplett and Mellon, 1992).

2.5. Effect of substrate concentration

Substrate concentration studies carried out using Lineweaver–Burk plot showed an apparent K_m of 3.96 mM for guaiacol and 1.3 mM for H_2O_2. K_m values for

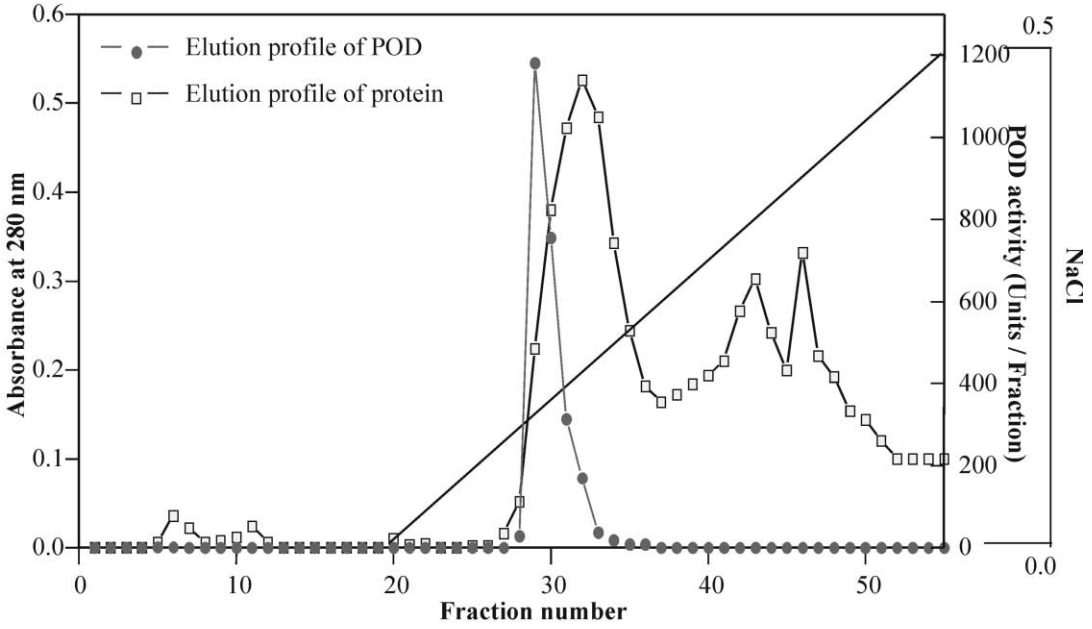

Fig. 1. *Ion-exchange chromatography of leaf POD on DEAE-cellulose*: DEAE-cellulose column was equilibrated with 5 mM Tris–HCl, pH 8.4 and the 60–95% $(NH_4)_2SO_4$ precipitate after dialysis against the same buffer was applied to the column and the fractions were eluted at a flow rate of 1 ml/min. Retained proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Fractions of 3 ml each were collected and their absorbance at 280 nm and POD activity were checked.

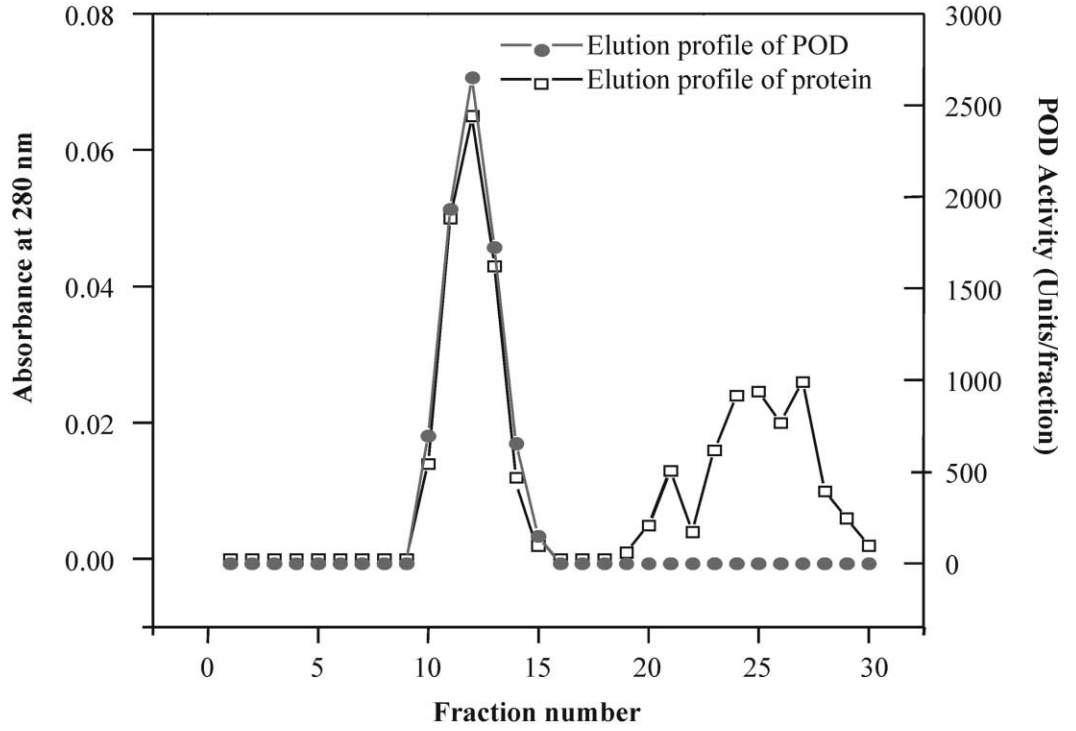

Fig. 2. *Molecular exclusion chromatography of leaf POD on Sephadex G-100*: SG-100 column was equilibrated with 50 mM phosphate buffer, pH 6.0 containing 0.5 M NaCl. The sample obtained from DEAE-cellulose column was dialyzed against the same buffer and applied to the column. Fractions (0.5 ml) at a flow rate of 20 ml/h were collected. The absorbance at 280 nm and POD activity of each fraction were determined.

the substrates ABTS and pyrogallol were 1 and 0.84 mM and the K_m values for H_2O_2 for ABTS and pyrogallol were 3.8 μM and 5.6 μM, respectively in the present study.

2.6. Effect of various inhibitors

Among the compounds tested, dithiothreitiol and sodium metabisulphite at 1 mM concentration inhibited POD activity completely. Sodium azide at 1 mM concentration exhibited only 25% inhibition. But as its concentration was increased to 20 mM about 98% inhibition was observed. Sodium thiocyanate also inhibited POD activity (~50%) at lower concentrations (1 mM).

2.7. Absorption spectrum of purified oil palm leaf POD

Purified enzyme was brown in colour. Enzyme in 0.1 M acetate buffer, pH 5.0 was scanned in the 250–500 nm range. The spectrum showed two peaks of absorption maxima, one at 403.5 nm (corresponding to ha) and the other at 278.5 nm (for protein). It has an RZ value of 2.3.

2.8. Electrophoresis

Purified enzyme upon native PAGE revealed a single band for POD activity (stained with benzidine) and a single band for protein (stained with Coomassie brilliant blue). This confirmed that the enzyme preparation was pure. Glycoprotein nature of POD was confirmed by staining the gel with PAS reagent after PAGE. A pink coloured band appeared at the region of enzyme activity (Fig. 4).

2.9. Molecular weight of oil palm leaf POD

Electrophoresis of purified enzyme under denaturing conditions revealed a band for POD corresponding to a M_r of 48±2 kDa (Fig. 5). The molecular weight of purified POD was also determined by gel filtration on Sephadex G-200 gel filtration column. POD was eluted as a single peak corresponding to an estimated M_r of about 200 kda. One of the probable reason for such high molecular weight may be the aggregation of protein under the present conditions employed. Most of the peroxidases reported to date are monomers. For example, rice peroxidase (48 kDa; Ito et al., 1991), and cotton peroxidase (48 kDa) (Tripplet and Mellon, 1992) are all monomers. One exception is coconut peroxidase, which was reported to exist as a tetramer having a subunit M_r of 55 kDa (Mujer et al., 1983).

2.10. Carbohydrate content of purified leaf POD

Staining the gels (after native PAGE) for glycoprotein indicated that the leaf POD contained sugar bound to protein moiety. On analysis it was found that leaf POD contained 37% carbohydrate bound to the protein molecule. HRP, turnip POD and Japanese radish POD have been reported to contain ca. 18, 12–18 and 20% carbohydrate, respectively (Kim and Kim, 1996).

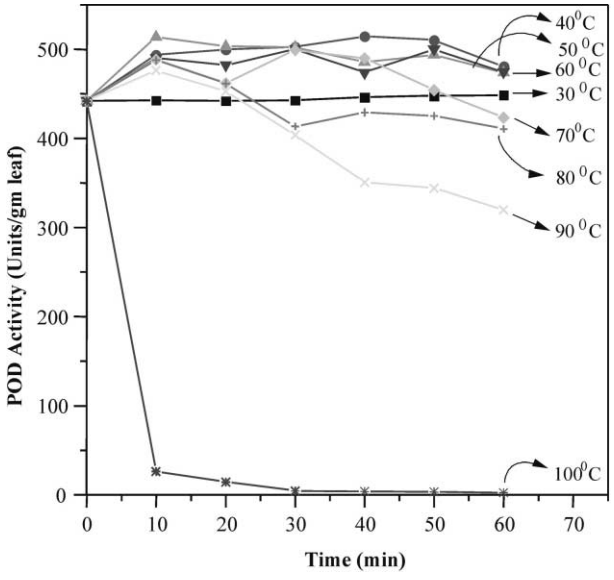

Fig. 3. *Thermal stability of purified POD*: enzyme extract was kept at respective temperatures (30–100 °C) in acetate buffer (pH 5.0) for 60 min. Aliquots were taken out at 10-min intervals, immediately cooled in ice and assayed for POD activity. Values plotted on the graph are the mean of three independent experiments. Variation was less than 2%.

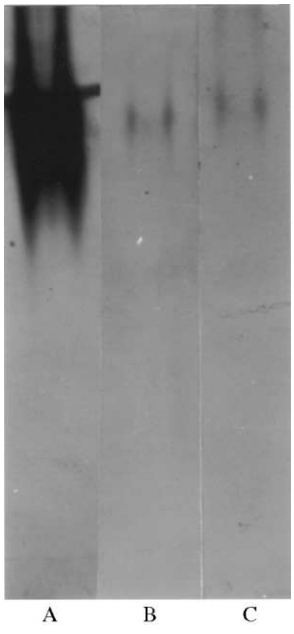

Fig. 4. *Native PAGE of purified POD stained for (A) POD activity, (B) protein & (C) glycoprotein*: gels after electrophoresis under non-denaturing conditions were stained for (A) POD activity using benzidine and 0.3% H_2O_2 in acetate buffer, pH 5 (B) protein with Coomassie Brilliant Blue (C) glycoprotein using PAS reagent.

The physiological significance of high carbohydrate content of leaf POD is not clear. It is reported that carbohydrate is an essential component of all products synthesized for export and that the carbohydrate provided a means for the cell to identify materials (Karp, 1979). The high thermal stability exhibited by leaf POD may be due to its higher carbohydrate content. Reports are available regarding the transport of various enzymes from leaf to various parts of the plant. For such transport and delivery to target sites, enzymes should posses some signals. So the carbohydrate components of glycoprotein have an important role in molecular recognition, sorting and transport (Nishimura et al., 1997).

2.11. Production and testing of antibody against POD

In order to examine the distribution of soluble POD in oil palm leaf, antibodies against purified leaf POD was raised in rabbits. The production of antibody was tested by immunodiffusion in which the antiserum formed precipitin bands against leaf POD.

2.12. Immunocytochemical localization of POD in oil palm leaf

The distribution of soluble POD in leaf was examined by immunocytochemical localization using antibodies raised against purified leaf POD. Blue staining in the sections indicated positive reaction. In leaf, staining for POD was observed in vascular bundles as well as areas adjacent to the epidermis. Inside the vascular bundle, staining was restricted to immature sclerenchymatous cells, xylem elements and phloem (Fig. 6a). Staining was also observed in the epidermis as well as in the hypodermis (Fig. 6d).

In leaf vascular bundles, the presence of POD with sclerenchymatous cells and xylem indicated that they are associated with lignification in these cells (Christensen et al., 1998). Lignin is a complex aromatic polymer derived mainly from the polymerization of three different hydroxy cinnamyl alcohols. Specific isoenzymes of PODs are believed to be responsible for the final enzymatic step in lignification (Higuchi, 1985). Presence of POD in phloem indicated that they might be transported. The epidermis of the leaf also showed staining for POD. This might be due to the presence of pectin and suberin in the epidermis which is capable of binding PODs (Espelie et al., 1986; Barcelo et al., 1988).

According to Akazawa and Hara-Nishimura (1985) all PODs are secretory proteins. The basic PODs may be secreted into the vacuoles, the acidic ones into the cell wall (Mader, 1992). In plants, PODs are mainly localized in cell walls, cytoplasm and vacuoles depending on the nature of cell and its development. Acidic PODs are located in cell walls and free intercellular spaces (Barcelo et al., 1987). This location is in accordance with their proposed role in cell wall rigidity (Fry, 1986). Basic PODs are present in vacuoles and are believed to participate in the oxidation of IAA in cells.

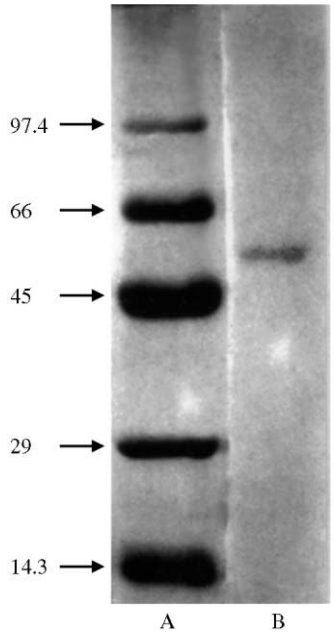

Fig. 5. *SDS–PAGE of purified POD*: purified leaf POD was subjected to SDS polyacrylamide gel electrophoresis using 10% gel. The gels were silver stained after electrophoresis. Lane A represents the MW markers (phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66 kDa; Ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa and lysozyme, 14.3 kDa) and lane B represents the purified leaf POD under reducing conditions.

3. Experimental

3.1. Material

Fresh mature leaflets from oil palm (*E. guineensis* Jacq.) of '*tenera*' variety were collected from the oil palm plantation at Regional Research Laboratory, Trivandrum. DEAE-cellulose, Sephadex G-100, Sephadex G-200, chemicals for electrophoresis, molecular weight markers for electrophoresis and gel filtration, dextran blue, agarose, Freund's complete and incomplete adjuvants, etc. were purchased from Sigma Chemical Company, USA. Anti-rabbit IgG alkaline phosphatase assay kit was obtained from Genei Pvt. Ltd., Bangalore, India. All other chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., India.

3.2. Preparation of acetone powder

The leaflets were cut into small pieces after removing midribs. Fifty grams of the leaflet was homogenized using a mortar and pestle and then extracted with 1 l of acetone cooled to −20 °C. Acetone extract was filtered and the extraction of the residue continued (about five

times) until the filtrate was free from chlorophyll. Residue obtained after the filtration of acetone was dried at room temperature for 4 h and stored desiccated at 4 °C.

3.3. Extraction of POD

Twenty-five grams of acetone powder was suspended in 500 ml of 0.1 M acetate buffer, pH 4.0 containing 1% polyvinylpyrrolidone (PVP) and stirred for 1 h at 4 °C. The extract obtained was filtered through two layers of cheesecloth and centrifuged at 10,000 rpm at 0–4 °C for 20 min. The clear supernatant after centrifugation was used as the crude enzyme preparation.

3.4. Assay of POD

POD activity was determined using an assay system consisting of 20 mM guaiacol (0.5 ml), 0.1 M acetate buffer, pH 5.0 (2.1 ml), 40 mM H_2O_2 (0.2 ml) and the enzyme extract (0.2 ml) with a final volume of 3 ml (Chance and Maehly, 1955). Oxidation of guaiacol was measured by the increase in absorbance at 470 nm. One

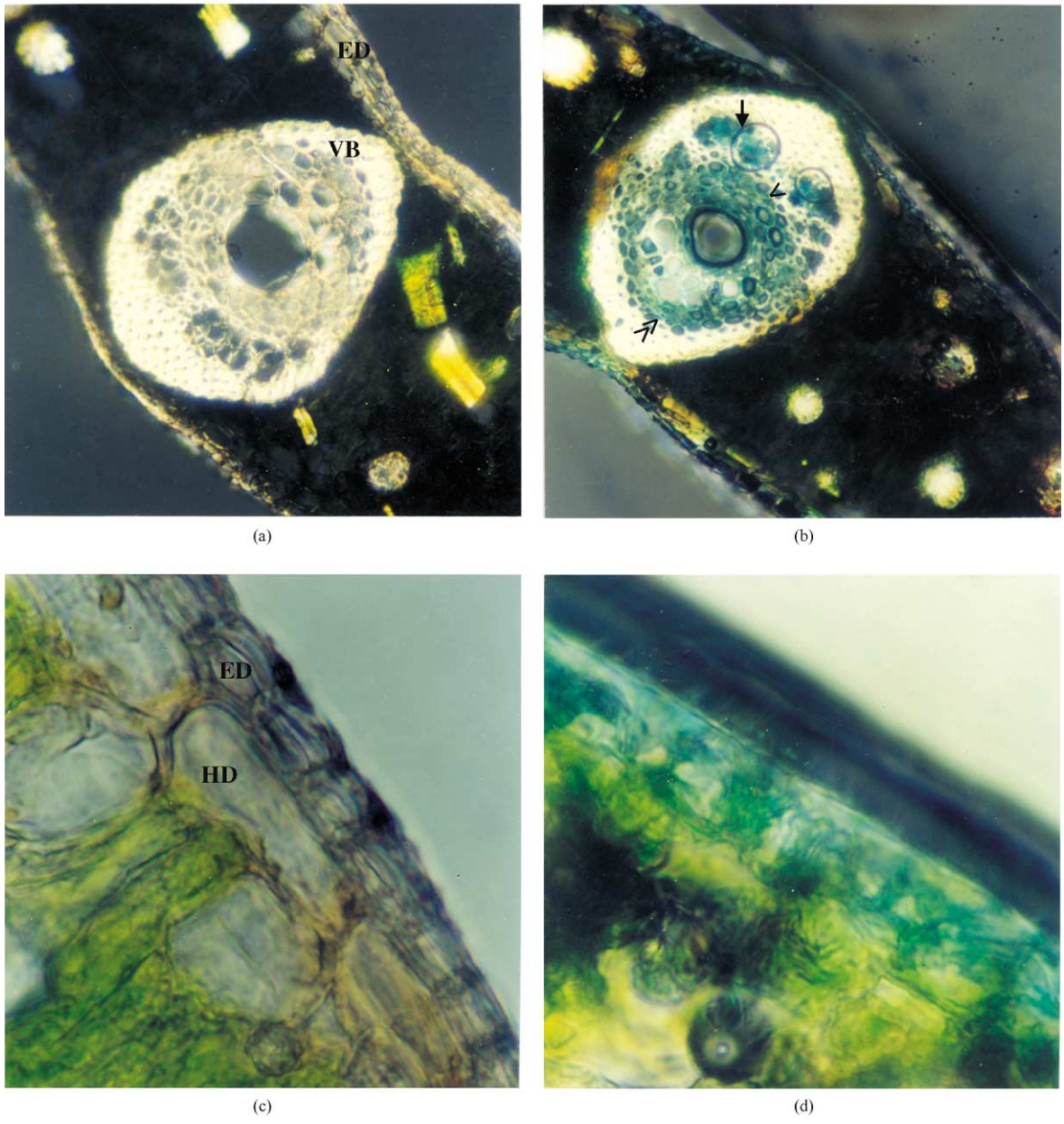

Fig. 6. *Immunochemical localization of POD in oil palm leaf*: cross-sections of oil palm leaf were treated with antibodies raised against leaf POD. Staining was done as described in materials and methods. (a) Untreated control (×100) of cross-section of leaf showing a single vascular bundle. (b) Test showing positive staining inside the vascular bundle and epidermis (×100). (c) and (d) were control and test of a portion of leaf showing epidermis and hypodermis (×400). ED, Epidermis; VB, Vascular bundle; HD, Hypodermis. → Phloem; >Xylem; > > Sclerenchyma.

unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.1/min.

3.5. Purification of POD

All operations were performed at 0–4 °C.

$(NH_4)_2SO_4$ precipitation: POD obtained from acetone powder extract was precipitated with $(NH_4)_2SO_4$ at 60–95% saturation. It was kept overnight at 4 °C and then centrifuged at 10,000 rpm for 30 min. The precipitate obtained was dissolved in 5 mM Tris–HCl buffer, pH 8.4 and dialyzed against the same buffer for 22 h with three changes.

DEAE-cellulose chromatography: a 2 cm column packed up to 15 cm bed height with DEAE-cellulose was equilibrated with 5 mM Tris–HCl buffer, pH 8.4. The dialyzed sample was applied to the column and washed with the same buffer at a flow rate of 1 ml/min. The retained enzyme was eluted with a linear gradient of 0–0.5 M NaCl in Tris–HCl buffer, pH 8.4 (150 ml + 150 ml) at the same flow rate. Three millilitre fractions were collected on which the absorbance at 280 nm and POD activity were determined. Fractions with POD activity were pooled together, dialyzed against 50 mM phosphate buffer, pH 6.0 containing 0.5 M NaCl.

Sephadex G-100 chromatography: a 2.5 cm column packed with Sephadex G-100 up to 40 cm bed height was equilibrated with 50 mM phosphate buffer, pH 6.0 containing 0.5 M NaCl. The dialyzed sample was applied to the column and 5 ml fractions at a flow rate of 20 ml/h were collected. Absorbance at 280 nm and POD activity of each fraction were measured. The fractions having POD activity were pooled together, dialyzed against 0.1 M acetate buffer, pH 5.0 and was used for further characterization studies.

3.6. Effect of pH

The optimum pH for POD activity was determined by using the following buffers: for pH 2 and 3, glycine–HCl buffer; pH 4 and 5, acetate buffer; pH 6 and 7, phosphate buffer; pH 8, Tris–HCl buffer and pH 9 and 10, glycine–NaOH buffer.

3.7. Effect of substrate concentration

Activity of POD at varying concentrations of guaiacol, pyrogallol, ABTS and H_2O_2 were determined and K_m values were calculated from Lineweaver–Burk plot.

3.8. Thermal stability

Thermal stability experiments were performed by subjecting the enzyme extract to heating at 30–100 °C. The enzyme extract (2 ml) was taken in separate test tubes and kept at respective temperatures for 60 min. From each tube, an aliquot of 0.2 ml was withdrawn at 5-min intervals and cooled by immersing in ice and was assayed immediately for residual POD activity.

3.9. pH stability

Two millilitres of enzyme extract in different tubes were adjusted to pH 2–10 by adding 1 M HCl or 1 M NaOH. Each sample was kept for 30 min at ambient temperature (30–32 °C). At the end of the experimental period, the pH was adjusted back to initial pH and the residual enzyme activity was assayed as before.

3.10. Polyacrylamide gel electrophoresis

Electrophoresis was performed on polyacrylamide gels having a resolving gel composition of 7.5% and stacking gel composition of 5%. A constant power supply of 20 mA/plate was employed. The reservoir buffer was Tris–Glycine buffer having a pH of 8.8. POD bands were detected by immersing the gels in a solution of acetate buffer, pH 5.0 containing 1% benzidine and 0.3% H_2O_2 (Kokkinakis and Brooks, 1979). Colour development occurred within 2 min. Protein band pattern was visualized by staining with Coomassie Brilliant Blue. The gels were also stained for glycoprotein by the modified Periodic acid-Schiff (PAS) technique (Zacharius et al., 1969).

3.11. SDS–PAGE

Electrophoresis under denaturing conditions was performed at different polyacrylamide gel concentration of 10% according to the discontinuous buffer system of Laemmli (1970). Electrophoresis was carried out in vertical slab gels and the runs were performed at a constant current intensity of 15 mA/plate in the stacking and 30 mA/plate in the running gels. The molecular weight markers used were phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa). Ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa). The gels were silver stained for visualizing the protein bands (Oakley et al., 1980).

3.12. Sephadex G-200 chromatography

A 2.6 cm diameter Pharmacia column packed with Sephadex G-200 (Sigma) up to 70 cm bed height was used for the experiment. The column was pre-equilibrated with 50 mM Ki–PO_4 buffer, pH 6.0 containing 0.1 M KCl. 1 ml of purified enzyme was applied and eluted with the same buffer system at a flow rate of 15 ml/h. Fraction volume was 1 ml. The system was calibrated with the following M_r markers; β-amylase (200 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

3.13. Determination of protein

Protein content of the samples was quantified according to the method of Bradford (1976) using bovine serum albumin as standard.

3.14. Production of antibodies

Antibodies against purified leaf POD were produced in male albino rabbits (New Zealand white strain). The purified enzyme was administered in three injections. The first injection was given in Freund's complete adjuvant (500 μg of protein/1 ml adjuvant) and the second and third in Freund's incomplete adjuvant (500 μg of protein/500 μl of incomplete adjuvant and 1 mg of protein/500 μl of incomplete adjuvant) in 14 days interval. The hyperimmune rabbit was bled after 10 days from the last injection by ear vein puncture. The serum was separated and dispensed as 0.5-ml aliquots in microfuge tubes and kept at −20 °C. The cross-reaction was checked by immunodiffusion using 1% agarose gels (Ouchterlony and Nilsson, 1986).

3.15. Immunolocalization of leaf POD

Tissue preparation: microscopic slides were coated with 1% gelatin and dried at 37 °C for 1 h. Small tissue pieces (~2×4 mm) were cut from fresh leaf with razor blades and placed on the slide. The tissue sections were fixed with 10% formalin for 1 h and washed with PBS (3×10 ml).

Immunolabelling using streptavidin-ALP conjugate: the sections were blocked with blocking buffer and kept for 1 h. After removing excess block buffer, the antiserum (primary antibody) diluted with assay buffer (1:20) was added to fixed tissue sections and incubated for 15 h at 4 °C. The slides were rinsed thoroughly with wash buffer and incubated with goat anti-rabbit IgG–Biotin conjugate for 30 min. The slides were thoroughly washed with wash buffer and incubated with Streptavidin–ALP conjugate for 15 min. The slides were again washed with wash buffer and the sections were treated with BCIP/NBT substrate till blue colour was developed (3 min; Suresh et al., 1986; Nelson, 1993). The sections were washed with distilled water. Coverslips were mounted on top of the sections after adding a drop of glycerin and read on a Nikon Eclipse E 600 microscope. In the case of controls, tissue sections were treated with pre-immune serum instead of antiserum.

Acknowledgements

S.S.D. thanks CSIR, India for Senior Research Fellowship. The authors thank Director, Regional Research Laboratory (CSIR), Trivandrum for providing the laboratory facilities and Professor P.R. Sudhakaran, Department of Biochemistry, University of Kerala for providing necessary facilities for immunocytochemical work.

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