Journal of Molecular Catalysis B: Enzymatic 71 (2011) 63–70

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

A novel cationic peroxidase (VanPrx) from a hemi-parasitic plant (*Viscum angulatum*) of Western Ghats (India): Purification, characterization and kinetic properties

Mrinal Kumar Das, Radhey Shyam Sharma, Vandana Mishra *

Bioresources & Environmental Biotechnology Laboratory, Department of Environmental Biology and the Centre for Environmental Management of Degraded Ecosystems University of Delhi, Delhi 110 007, India

ARTICLE INFO

Article history:
Received 31 December 2010
Received in revised form 18 March 2011
Accepted 24 March 2011
Available online 31 March 2011

Keywords:
Class III peroxidase
Substrate specificity
Parasitic plant
Viscum angulatum
Compounds I–III

ABSTRACT

Peroxidases are oxido-reductases that have industrial applications. Hemi-parasitic plants which experience continuous oxidative-stress may serve as a source for novel peroxidases. A wild endemic hemi-parasitic plant (*Viscum angulatum*) from the Western Ghats (India), was selected and the cationic peroxidase VanPrx (p*I* 9.6) was isolated and purified. Analyses of trypsin digested peptides by LC–MS/MS confirmed that VanPrx was a Class III peroxidase. Further, the matrix assisted laser desorption ionization-time of flight (MALDI-TOF) analysis showed its molecular weight as 46.42 kDa. The ultraviolet/visible absorption spectrum is characteristic of heme containing plant peroxidases with a soret peak at 403 nm and *R*/*Z* value of 3.1. Different redox states of VanPrx are not similar to those of Class III peroxidases. Unlike HRP, its Compound I was relatively unstable. Further, it did not show formation of Compound III on addition of high concentration of NADH (200 times the molar concentration of enzyme). In contrast to that of classical plant peroxidases, N-terminal sequence of VanPrx was neither conserved nor blocked by pyroglutamate. Kinetic studies with twelve electron donors showed that VanPrx possessed high activity towards substrates of different chemical nature (viz. guaiacol, ferulic acid, ABTS, etc.). Unlike most cationic peroxidases, a significantly high activity of VanPrx with hydroxycinnamic acid derivatives rather than hydroxycinnamyl alcohols indicates its involvement in suberization. Suberization is associated with seed germination and establishment of haustoria of parasitic plants on the host plant. Unlike other cationic peroxidases, lack of IAA oxidase activity in VanPrx may be associated with the absence of root system in *V. angulatum*. VanPrx is highly thermo stable and retains partial activity even after an 80 °C treatment for 10 min. Inhibition kinetics revealed that unlike HRP, VanPrx is not inhibited by EDTA. Furthermore, TEMED a competitive inhibitor of HRP inhibited VanPrx uncompetitively. The present study identifies an endemic hemi-parasitic plant as a source of a novel isoform of Class III peroxidase with distinct substrate affinity, redox states, inhibition kinetics of some of the inhibitors and high thermostability.

1. Introduction

Peroxidases are oxido-reductases ubiquitous in biological kingdoms. Based on the homologies in amino acid sequence, heme peroxidases (containing iron III protoporphyrin IX as the prosthetic group) have been classified as: (i) Class I (intracellular): ascorbate peroxidase, cytochrome c peroxidase, catalase-peroxidase, etc.; (ii) Class II (fungal secretory): manganese peroxidase, lignin peroxidase and versatile peroxidase; and (iii) Class III (plant secretory-peroxidase). In peroxidative cycle, peroxidases accept electrons from various donor molecules (phenolic compounds, auxin, lignin precursors, etc.) and catalyse the reduction of H_2O_2 [http://peroxibase.toulouse.inra.fr// 1,2]. The Class III peroxidases are secreted outside the cells or transported into vacuoles [3]. Amino acid sequence homology among members of Class III peroxidases varies from 50 to 95% [4].

In plants, Class III peroxidases play an important role in various developmental processes and environmental responses. For example, they are involved in: (i) formation of rigid structures in land plants for adaptation (lignification, suberization, etc.) [5,6], (ii) cell wall metabolism (cell elongation, cross-linking of cell wall structural proteins) [1,7], (iii) auxin catabolism [8], and (iv) tolerance to abiotic and biotic stresses (salt tolerance, ROS generation and oxidative stress, defense against pathogen attack, etc.) [9,10]. The ability of peroxidases to serve as indole acetic acid (IAA) oxidases in hormonal regulation, has made them an integral part of plant physiology research [11].

* Corresponding author. Tel.: +91 11 2766 6237; fax: +91 11 2766 6237.
E-mail address: mistletoe_h@hotmail.com (V. Mishra).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.03.010

Peroxidases have industrial and environmental applications. Class III peroxidases (HRP, soybean, turnip peroxidases, etc.) have been used in detoxification of water that is contaminated by phenols, cresols and chlorinated phenols [12]. Peroxidase-based diagnostic kits and antibody labeling kits are available in the market. Keeping in view their increasing applications, peroxidases from new sources with unique properties are in demand. Hemi-parasitic plants, which continuously experience osmotic- and oxidative-stresses, may also be used for such application purposes. Western Ghats (high altitude hill regions) in India, one of the hot spots of biodiversity is a centre for diversity of hemiparasitic plants, which have not yet been explored for peroxidases. It is for this reason, a hemi-parasitic plant (*Viscum angulatum*) flourishing in the Western Ghats region of peninsular India was selected and a novel peroxidase (VanPrx) was purified and characterized. In our opinion, this is the first detailed study on plant peroxidase enzyme of any hemiparasitic plant.

2. Materials and methods

2.1. Plant material

V. angulatum parasitic on *Olea dioica* was collected from the Western Ghats region of India and was stored at −20 °C till further use.

2.2. Chemicals

All the buffers and reagents were either of analytical grade or of the highest purity available. Ammonium persulphate, NaN_3, KCN from Merck (Darmstadt, Germany); β-mercaptoethanol, TEMED, acrylamide/bis from BioRad (CA, USA); acrylamide, BSA, Coomassie brilliant blue (CBB) R250, CBB G250, guaiacol, NADH, polyvinyl polypyrollidone (PVPP), dithiothreitol (DTT), SP Sephadex C25, DEAE Sephadex A50, Sepharose® 4B, gallic acid from Sigma (St. Louise, USA); protein markers from Pharmacia (Uppsala, Sweden); pyrogallol, ferulic acid, and diethylenetriaminepentaacetic acid (DTPA) from Sisco Research Laboratory (Mumbai, India); caffeic acid, p-coumaric acid from HiMedia Laboratories (Mumbai, India); Na_2HPO_4, $NaH_2PO_4{\cdot}H_2O$, NaCl, ammonium sulphate, Tris, CH_3COONa and CH_3COOH from Merck (Mumbai, India) and Milli Q water were used.

2.3. Purification process of the peroxidase

Peroxidase was purified by a two step ion-exchange chromatography using ActaPrime fraction collector (Amersham Biosciences, Uppsala, Sweden). All the steps of purification process were carried out at 4 °C. Crude protein extract was prepared from 300 g of frozen plant tissue by grinding it into fine powder in liquid nitrogen. The powdered tissue was suspended in phosphate buffer saline (PBS, 0.1 M sodium phosphate, 0.14 M NaCl, pH 7.6) containing 2.0% PVPP. The suspension was continuously stirred overnight at 4 °C. Crude extract was then filtered and centrifuged at 20,000 × *g* for 30 min and the total protein in the supernatant was fractionated into two parts by the process of ammonium sulphate precipitation. The total protein was subjected to 0–25% ammonium sulphate saturation by gradual addition of salt with continuous stirring at 4 °C and incubated for 8 h. The protein precipitate was collected by centrifuging at 12,000 × *g* for 15 min (fraction I). The supernatant was again saturated with 26–85% ammonium sulphate and incubated for 12 h. The protein precipitate was collected by centrifuging at 12,000 × *g* for 15 min (fraction II). Both the fractions were resuspended in 25 mM Tris buffer, pH 8.3 (TB) and dialysed against same buffer for 48 h with 4 changes a day [13]. The fraction II (84 ml) was loaded (24 ml/h) onto DEAE Sephadex A50 column (25 mm × 100 mm) that was previously equilibrated with TB. The unbound fractions (fraction numbers: 11–33; fraction size: 3 ml) possessing peroxidase activity were pooled and concentrated by ultrafiltration (Stirred Ultrafiltration Cell, model 8050, Amicon, Millipore, MA, USA) using 10 kDa YM membrane (Amicon, Millipore, MA, USA). Peroxidase activity was measured using guaiacol as a substrate as discussed in Section 2.11 [one unit is defined as the amount of enzyme needed to produce 1 μmol colored product ($OD_{470\,nm}$) from guaiacol in 1 min at 25 °C]. The protein was suspended in 25 mM sodium acetate buffer (pH 4.5) (SAB) and 14 ml concentrated sample was loaded onto SP Sephadex C25 column (25 mm × 100 mm). The bound protein was eluted (36 ml/h) with salt gradient (0–1 M NaCl in SAB) after washing (36 ml/h) the column with SAB. Eluted fractions (fraction numbers: 17–27; fraction size: 3 ml) were tested for the protein content and peroxidase activity. The fractions forming a peak having the desired activity were pooled, concentrated and desalted by ultrafiltration using 10 kDa YM membrane.

2.4. Protein estimation

The protein content of crude extract, fractionated protein samples and purified protein was estimated by standard Bradford's dye binding assay using BSA as standard [14]. The elution profiles were prepared based on the absorbance (280 nm) of eluted fractions measured automatically by the UV lamp installed in the fraction collector (Acta Prime, GE Healthcare, Uppsala, Sweden).

2.5. MALDI-TOF and LC–MS/MS analysis

The exact molecular weight of VanPrx was determined by matrix assisted laser desorption ionization-time of flight (MALDI-TOF). Electrospray ionization ion trap mass spectrometry for protein identification was performed using an Agilent 1100 series 2D Nano LC–MS. Both these analyses were carried out at the Centre for Genomic Applications, New Delhi, India. Sample for MALDI-TOF analysis was prepared using dried droplet method. One microliter sample solution (1 μg) and 1 μl of sinnapinic acid in 1:2 (v/v) of acetonitrile (ACN):0.1% trifluoro acetic acid (TFA) were mixed nicely. One microlitre of this mixture was spotted on a MALDI target plate and allowed to air dry at 25 °C. Calibration standard (Bruker) was also prepared in the same way. MALDI target plate was loaded into Ultraflex MALDI-TOF-TOF for subsequent peptide spectra acquisition and analysis. A LASER power of 337 nm wavelength was used for ionization of the samples spotted on the target plate. Sample peaks were calibrated with peaks obtained from the calibration standard. After spectra were obtained, MS analysis was carried out using Flex analysis software (ver 2.2, Bruker).

For 2D Nano LC–MS anlysis, excised 1D gel piece containing protein band of interest was destained and in gel digestion was carried out by trypsin (Gold Mass Spectroscopy Grade, Promega, Madison, USA) in 50 mM NH_4CO_3 pH 7.8 at 37 °C for 16 h (as per manufacturer's protocol). Digested protein sample was applied to the HPLC to separate the peptides using Agilent 1100 NanoLC-1100 system (Agilent, Palo Alto, CA, USA) combined with a microwell-plate sampler and thermostatted column compartment for preconcentration (LC Packings, Agilent). A 6 μl of sample was loaded on the column (Zorbax 300SB-C18, 150 mm × 75 μm, 3.5 μm) using a pre-concentration step in a micro pre-column cartridge (Zorbax 300SB-C18, 5 mm × 300 μm, 5 μm) at a flow rate of 5 μl/min. After 5 min, the pre-column was connected with the separating column, and multi step gradient (3% till 5 min, 15% for 5–8 min, 45% for 8–50 min, 90% for 50–55 min, 90% for 55–70 min, then again 3% for 71 min) was started. The buffers used were 0.1% HCOOH in water and 0.1% HCOOH in 90% ACN. An LC/MSD Trap XCT with a nano-electrospary interface (Agilent) operated in the positive ion mode

was used for MS. Ionization (1.5 kV ionization potential) was performed with a liquid junction and a non-coated capillary probe (New Objective, Cambridge, USA). Calibration of the instrument was performed using the standard Agilent tune mix. Peptide ions were analyzed by the data-dependent method. The scan sequence consists of 1 full MS scan followed by 4MS/MS scans of the most abundant ions. Data was analyzed using Agilent Ion trap Analysis software version 5.2 and the protein was identified by database search against the MASCOT database (www.matrixscience.com).

2.6. Electrophoretic analyses

The protein samples were denatured using Laemmli buffer and electrophoresed on 4–10% SDS–polyacrylamide slab gel system. Polypeptides were made visible by Coomassie brilliant blue R250 (CBB) staining and their molecular weights were determined using Quantity One software. Glycoproteins were identified by using periodic acid Schiff's (PAS) reagent staining [15].

2.7. Determination of sugar content

Sugar content of the protein was estimated using orcinol–sulphuric acid method [16]. The protein sample was added to freshly prepared ice cooled orcinol solution. Absorbance was measured at 420 nm after a treatment of 80 °C for 15 min. D-Glucose (Sigma) was used as the standard.

2.8. N-terminal sequence analyses

The sequence of the first ten amino acids from N-terminal end was determined by Edman degradation method using an automated protein sequencer (Applied Biosystem). The percentage sequence similarity with some other Class III peroxidases was determined by using a general-purpose multiple sequence alignment program ClustalW [17].

2.9. Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing was done in Bio-Rad 111 mini-IEF chamber using 7 cm IPG strips (BioRad, CA, USA). Protein sample (100 μg) was dissolved in rehydration buffer (150 μl) and single IPG strip was soaked for 12–16 h. Protein in the rehydrated strip was focused and subjected to electrophoresis (as per manufacturer's protocol). The strip was loaded on a 10% SDS-PAGE and stained with coomassie brilliant blue R250 to identify the protein spot and isoelectric point was determined by comparing with IEF markers (IEF mix 3.6–9.3, Sigma, St. Louis, USA) using PDQuest software (BioRad, CA, USA).

2.10. Substrate specificity, peroxidase activity and determination of optimum pH

Substrate specificity was determined by assaying peroxidase activity using a range of substrates including phenols (guaiacol—A_{470}, $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$ [18]; pyrogallol—A_{420}, $\varepsilon = 2.4\ mM^{-1}\ cm^{-1}$ [19]), hydroxycinnamic acids (ferulic acid—A_{318}, $\varepsilon = 15.2\ mM^{-1}\ cm^{-1}$ [20]; caffeic acid—A_{320}, $\varepsilon = 15.3\ mM^{-1}\ cm^{-1}$ [20]; p-coumaric-acid—A_{308}, $\varepsilon = 19.2\ mM^{-1}\ cm^{-1}$ [20]), hydroxycinnamyl alcohols (coniferyl alcohol—A_{265}, $\varepsilon = 7.5\ mM^{-1}\ cm^{-1}$ [18]; sinapyl alcohol—A_{270}, $\varepsilon = 7.5\ mM^{-1}\ cm^{-1}$ [21]), natural ([ascorbic acid—A_{290}, $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$ [19]; indole acetic acid—A_{261}, $\varepsilon = 3.2\ mM^{-1}\ cm^{-1}$ [18]) and synthetic substrates (ABTS—A_{405}, $\varepsilon = 36.0\ mm^{-1}\ cm^{-1}$ [20]; tetramethyl benzidine—A_{652}, $\varepsilon = 39.0\ mM^{-1}\ cm^{-1}$ [18]; o-phenylenediamine—A_{440}, $\varepsilon = 11.1\ mM^{-1}\ cm^{-1}$ [22]). To determine the maximum catalytic activity, different concentrations of electron donors were used in 3 ml of reaction mixture containing appropriate buffer and 0.5 mM of H_2O_2. Absorbance of reaction mixture was recorded for 3 min with a time interval of 10 s. Oxidation rates of the electron donors were measured in terms of decrease or increase in absorption at their respective λ_{max} of substrate degradation or product formation. Maximum enzymatic activity (V_{max}) and Michaelis constant (K_m) were calculated with the help of a hyperbolic regression analysis program (Hyper32.exe ver 1.0.0, 2003) [23]. Enzyme activity was also calculated for hydroxycinnamic alcohols and hydroxycinnamic acids to compare their respective activities at 100 μM.

To identify the pH optima for each substrate, the enzyme activity was assayed in appropriate buffer with varying pH ranging from 2.6 to 10.5 (Table 3). Three replicas were kept for each measurement. Buffers in pH range between 2.6 and 5.5 were prepared by mixing 0.1 M Na_2HPO_4 and 0.5 M citric acid. For pH range 6.0–7.5, 0.05 M sodium phosphate buffer was used. For pH range of 8.0–9.0 and 9.5–10.5, 0.05 M Tris buffer and 0.05 M sodium carbonate buffer were used, respectively. Absorption spectra were recorded and optimum pH was identified for each substrate.

2.11. Determination of temperature optima and thermal stability of the VanPrx

Enzyme activity was determined using guaiacol as a substrate at a temperature ranging from 20 to 85 °C. VanPrx was incubated at different temperatures for 10 min each and activity was determined at 25 °C. Thermal stability was also tested up to 12 h at 65 °C. The resulting samples were collected after 10, 20, 30 min, and then after every hour till 12 h and then simultaneously subjected to standard peroxidase activity measurement. Three replicas were kept for each measurement.

2.12. Spectroscopic analyses of VanPrx and identification of its different redox states

Soret–vis absorption spectrum analysis was performed with VanPrx (0.5 mg/ml) in 0.05 M sodium phosphate buffer, pH 6.5. Wavelength scanning (250–700 nm) was done (quartz cuvette: 1 cm path length; spectral bandwidth: 1 nm; scan speed: 480 nm min^{-1}) using UV–visible spectrophotometer (*Lambda* 25, Perkin Elmer). Compounds of different redox states of VanPrx (Compounds I–III) were prepared using various reducing substrates and analyzed spectrophotometrically. Different concentrations of H_2O_2 were added to the protein (stoichiometric amount for Compound I; 10 times the molar concentration of protein for Compound II; and 100 times for Compound III) [19]. Ability to form Compound III was also tested by addition of NADH (200 times the molar concentration of protein) [24] and DTT [25].

2.13. Inhibitors of VanPrx and their kinetics

Inhibitory effect of different compounds (citric acid, oxalic acid, CTAB, EDTA, TEMED, DTPA, sodium azide (NaN_3), potassium cyanide (KCN), gallic acid and IAA on peroxidase activity was determined by combining reaction mixture (3 ml) containing guaiacol (10 mM) as substrate with appropriate amount of enzyme, inhibitor and 0.5 mM H_2O_2 in 50 mM sodium acetate buffer, pH 4.5. The above reaction mixture was incubated at room temperature for 5 min for observing the enzyme inhibition. The inhibitory effect on peroxidase activity was also determined by using different concentrations of guaiacol in a solution of 0.1 mM NaN_3, 0.02 mM KCN and 5 mM TEMED in same buffer mentioned above [26]. The results were plotted in Lineweaver–Burk plot and compared with double

Table 1
Summary purification of a cationic peroxidase from *Viscum angulatum* (VanPrx).

Fraction	Volume (ml)	Total activity units[a] (μmol min^{-1})	Total protein (mg)	Specific activity (units/mg protein)	Fold purification	Yield (%)	*R*/*Z* value
Crude extract	874	17,600	606	29.04	1	100	nd
Fraction 1	23	nd	12.6	nd	–	–	nd
Fraction 2	84	20,400	561	36.6	1.26	115.9	nd
DEAE Sephadex A50	14	8116	16.9	480	16.52	46	2.8
SP-Sephadex C25	4.3	5850	7.3	801.3	27.6	33	3.1

nd: not determined. *R*/*Z* value represents ratio of $A_{403\,nm}/A_{280\,nm}$.
[a] One unit is defined as the amount of enzyme needed to produce 1 μmol colored product ($OD_{470\,nm}$) from guaiacol in 1 min at 25 °C.

reciprocal curve of the enzyme activity. Inhibition constants (K_i) for NaN_3 and KCN were calculated by using the following formula:

$$\frac{V'_{max}}{V_{max}} = 1 + \frac{I_{nc}}{K_i}$$

where V_{max} is maximum activity without inhibitor, V'_{max} is maximum activity in the presence of inhibitor and I_{nc} is the concentration of inhibitor.

3. Results and discussion

3.1. Purification of peroxidase from V. angulatum plant

A 46.42 kDa peroxidase (VanPrx) from *V. angulatum* inhabiting Kolhapur region (16°41′0″N 74°14′35″E) of Western Ghats, India was purified. Using ammonium sulphate fractionation, the total extracted protein was separated into fraction I, rich in contaminants; and fraction II containing peroxidase activity. The protein-rich fraction II (561 mg) was loaded onto an anion-exchange column (DEAE Sephadex A50). Although the bulk of peroxidase activity was detected in the unadsorbed protein fraction, this step facilitated the removal of dark brown colored contaminants which were retained by the matrix. Unadsorbed fraction was subjected to cation-exchange chromatography (SP Sephadex C25). Elution with linear gradient of NaCl (0–1 M) gave a major protein peak which also corresponds to a peroxidase peak (Fig. 2a). Pooling and concentrating the fractions forming a peak of peroxidase activity yielded purified peroxidase VanPrx (~28-fold) (Fig. 2b; Table 1). Reinheitszahl value ($A_{403\,nm}/A_{280\,nm}$) of 3.1 also indicated that VanPrx was in a highly purified state [23,27].

3.2. Biochemical characterization

MALDI-TOF analyses suggested that the molecular weight of VanPrx is 46.42 kDa. Presence of a single band on SDS-PAGE (Fig. 1b) indicated its monomeric nature like most plant peroxidases [28]. Appearance of pink stained VanPrx band on PAS staining suggested its glycoprotein nature, which is one of the characteristics of Class III peroxidases (Fig. 1c). The sugar content of VanPrx was 12.6%, which is less than that of HRP-C (18.0%).

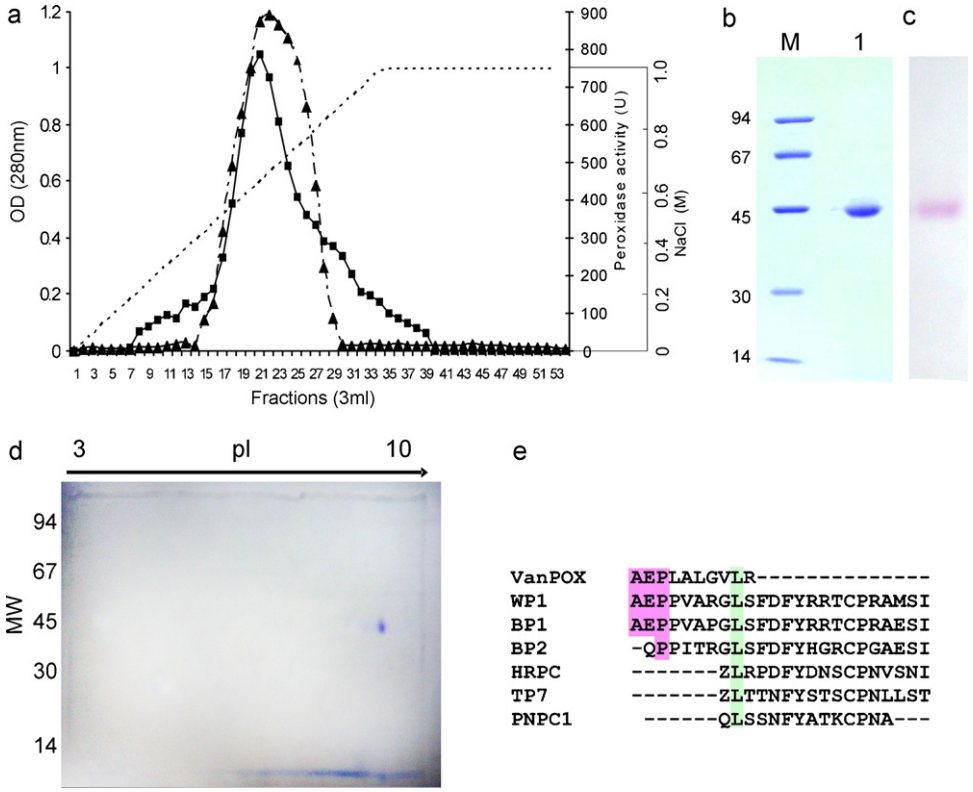

Fig. 1. (a) Elution profile of VanPrx after cation exchange chromatography (SP Sephadex C25) monitored at 280 nm (solid line with square) and assayed for peroxidase activity (dash with triangle). The NaCl gradient (0–1 M) is represented by dotted line. (b) SDS-PAGE profile of purified VanPrx (lane M: molecular weight marker; lane 1: VanPrx). (c) PAS stained SDS-PAGE profile of VanPrx. (d) 2D PAGE profile of VanPrx showing single spot. (e) N-terminal sequence of VanPrx and its comparison with Wheat peroxidase (WP1) Barley peroxidases (BP1 and BP2), Horse radish peroxidase (HRPC), turnip peroxidase (TP7) and cationic peanut peroxidase (PNC1). Z indicates pyroglutamate.

Table 2
Michaelis constant (K_m) and maximum catalytic rate (V_{max}) of VanPrx with different electron donors. Enzyme activity was calculated at 100 μM concentration for some substrates. Results shown as mean ± standard deviation of three replicas.

Electron donor	K_m (mM)	V_{max} (mM min^{-1} mg^{-1} protein)	Enzyme activity at 100 μM substrate concentration (mM min^{-1} mg^{-1} protein)
Guaiacol	5.83 ± 0.61	709.6 ± 77.3	nd
ABTS	0.34 ± 0.08	798.7 ± 29.4	nd
Pyrogallol	6.22 ± 0.91	294.1 ± 11.2	nd
Tetramethylbenzidine	0.32 ± 0.05	238.1 ± 7.8[a]	nd
o-Phenylenediamine	0.47 ± 0.07	333.0 ± 19.5	nd
Ferulic Acid	0.51 ± 0.11	731.2 ± 41.9	146.25 ± 6.1
Caffeic Acid	0.17 ± 0.07	191.6 ± 9.7[a]	86.42 ± 3.6
p-Coumaric Acid	0.11 ± 0.01	77.2 ± 12.3	61.36 ± 3.2
Coniferyl alcohol	0.23 ± 0.02	21.6 ± 1.1[a]	9.71 ± 0.8
Sinapyl alcohol	0.19 ± 0.02	66.3 ± 9.5[a]	31.27 ± 1.3
Ascorbic acid	0.39 ± 0.06	16.2 ± 0.2[a]	5.72 ± 0.6
Indole acetic acid	na	na	na

nd: not determined; na: no activity.
[a] V_{max} measured, substrates did not show typical saturation.

Glycoproteins often have N-linked sugar chains containing mannose residues [29]. The isoelectric focusing of VanPrx showed a single band with p*I* value 9.6 which confirmed its cationic nature. Presence of single spot in 2D PAGE profile (Fig. 1d) suggested that the VanPrx has been purified to homogeneity. LC–MS/MS analyses of VanPrx showed its homologies (similarity in molecular weight) with peroxidases from Spinach (T09166), *Populus* (Q50KB0_9ROSI), *Medicago* (Q1RXM7_MEDTR), *Pisum sativum* (Q18PR1_PEA), *Glycine* (Q9ZRG5_SOYBN) and *Oryza* (Q5Z7J2_ORYSA). Comparison of first 10 amino acid residues of VanPrx N-terminal sequence with that of other plant peroxidases (Fig. 1e) showed its similarity with wheat peroxidase (WP1, 40%) and barley peroxidase (BP1, 40%). The N-terminal sequence similarity with most Class III peroxidases (horse radish peroxidase HRPC, turnip peroxidase TP7, peanut peroxidase PNPC1, artichoke peroxidase) varied between 10 and 20%. Both BP1 and WP1 have diverse physiological roles [20,30]. However, longer sequence may give a clearer picture (Fig. 1e).

3.3. Substrate specificity, optimum pH and kinetic studies

Peroxidases have been suggested to be involved in the establishment of host–parasite relationship [31,32]. However, their exact role in parasitism remains largely unclear. They generate reactive oxygen species (ROS) which may have adverse effect on the infection process and development of the parasite [33,34]. Else they may facilitate penetration of haustoria by loosening the host cell wall [35]. Most of these studies have been carried out on holo parasitic plants (*Orobanche ramose*, *O. aegyptiaca*, *Cuscuta* sp., etc.) [35,36]. In our opinion, this is the first detailed study on the peroxidase enzyme of any hemi-parasitic plant.

VanPrx showed affinity for an array of substrates (Table 2). Its rate of reaction (V_{max}) was comparable for guaiacol (709.6 mM min^{-1} mg^{-1} protein), ferulic acid (731.2 mM min^{-1} mg^{-1} protein) and ABTS (798.7 mM min^{-1} mg^{-1} protein). However, its relative affinity for ferulic acid and ABTS (K_m) was more than 10-fold higher as compared to guaiacol. Feruloyl residues which provide rigidity to the cell wall can form covalent bonds with each other by oxidative coupling mediated by peroxidase–H_2O_2 [37]. Accumulation of oligomers of ferulate strengthens the cell wall thereby restricting cell expansion, pathogen invasion and susceptibility of cell wall to digestion [38]. Among the naturally occurring phenylpropanoids, VanPrx showed higher V_{max} for ferulic acid, caffeic acid and p-comaric acid (hydroxycinnamic acid derivatives) than for coniferyl alcohol and sinapyl alcohol (hydroxycinnamyl alcohols), in spite of the fact that sinapyl alcohol is present in *Viscum* spp. Hydroxycinnamic acids containing aliphatic moieties are incorporated into suberin whereas hydroxycinnamyl alcohols are involved in lignification. Role of cationic peroxidases in lignification and anionic peroxidases in suberization has been shown [39–41]. Some of the basic peroxidases are also shown to be involved in suberization although, studies in this area have been limited [39,42]. Further, a wound-induced basic peroxidase from potato was shown to catalyse ferulic acid and coniferyl alcohol equally well [39]. However, VanPrx, despite being highly basic isoform shows contrasting preference for hydroxycinnamic acid derivatives, thereby suggesting its role in suberization. Suberization is a desired change during seed germination, and also in establishment of haustorial connection by the parasitic plant with the host [43,44]. Further, suberin deposition is associated with wound healing and resistance to the hyperosmotic stress [9]. It cannot be overlooked that the hemi-parasitic plants often experience hyperosmotic stress due to dependency on the host for their water requirement. Therefore, eco-physiological significance of high preference of VanPrx (cationic peroxidase) for hydroxycinnamic acid derivatives may be an interesting phenomenon for further investigation.

In most of the substrates, except for pyrogallol, enzyme activity was found to be the highest below pH 5.0 (Table 3). Loss of enzyme activity at high pH might be due to loss of hemin group [45]. Cationic peroxidases are localized in plant vacuoles [46] and are involved in indole acetic acid (IAA) oxidation, whereas anionic peroxidases are localized in cell wall and intercellular spaces and are involved in cell wall rigidity. In contrast, despite its cationic nature, VanPrx did not oxidize IAA and appeared to be involved in suberization. Further, IAA oxidation has been associated with rooting in plants [11]. Being a stem parasitic plant, *V. angulatum* lacks the root system and attaches itself to the host with the help of haustoria. Unlike most Class III peroxidases, VanPrx can also utilize ascorbate as a substrate although its enzyme activity was relatively low.

3.4. Optimum temperature and thermal stability

Due to high activity (V_{max}) on phenolic substrates (Table 2), VanPrx may have potential application in bioremediation of phenols present in contaminated waters. It may be noted that relatively low thermostability of most plant peroxidases is the major limiting factor for their application in wastewater treatment [12]. Interestingly, VanPrx is highly thermostable. It retains ~80% of the activity after incubation at 65 °C for 10 min (Fig. 2). There is a steep and continuous drop of enzyme activity beyond this temperature. Atleast 30% of its activity is retained after 12 h of incubation at 65 °C. One-fourth of its activity is maintained after

Table 3
Relative enzyme activity (ratio of actual highest activity expressed as percentage) of VanPrx with different substrates across a pH range (2.6–10.0).

Substrate	Relative activity (%) at pH														
	2.6	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
Guaiacol	35.4 ± 4.1	42.5 ± 3.7	64.16 ± 2.4	62.5 ± 3.5	100 ± 5.1	83.3 ± 5.7	89 ± 3.7	76.6 ± 4.5	66.6 ± 2.1	54.1 ± 2.6	45.8 ± 3.2	35.2 ± 2.2	14.5 ± 1.7	2.4 ± 0.6	1.25 ± 0.1	0.58 ± 0.1
ABTS	44.3 ± 1.9	61.6 ± 3.5	77.3 ± 2.9	100 ± 4.7	89.2 ± 6.3	85.1 ± 4.6	77.3 ± 5.8	68.5 ± 3.4	63.9 ± 4.3	54.2 ± 2.4	41.6 ± 2.7	33.4 ± 1.9	13.3 ± 0.5	9.1 ± 0.2	6.8 ± 0.2	2.2 ± 0.1
Pyrogallol	13.4 ± 1.1	17.6 ± 1.4	28 ± 1.1	38.2 ± 3.1	44.5 ± 2.6	49.5 ± 3.3	53.4 ± 2.4	60.7 ± 2.8	93.1 ± 4.5	100 ± 3.8	71.5 ± 2.2	32.4 ± 5.1	21.4 ± 2.3	7.7 ± 0.6	4.6 ± 0.3	2.8 ± 0.1
Tetramethyl-benzidine	19.3 ± 2.1	28.8 ± 1.6	46.1 ± 2.2	51.4 ± 4.3	84.0 ± 6.7	100 ± 4.7	91.3 ± 4.5	78.6 ± 2.5	54.2 ± 3.4	41.7 ± 1.7	23.4 ± 2.3	11.1 ± 0.4	6.3 ± 0.1	3.9 ± 0.1	3.1 ± 0.1	3.2 ± 0.1
o-Phenyle-nediamine	22.5 ± 1.5	32.2 ± 1.9	58.7 ± 2.8	77.3 ± 2.2	100 ± 4.1	96.2 ± 4.6	88.4 ± 4.8	64.1 ± 2.7	51.6 ± 3.8	35.6 ± 2.1	28.0 ± 1.1	19.2 ± 1.4	15.5 ± 1.8	8.4 ± 0.8	4.5 ± 0.2	1.5 ± 0.1
Ferulic acid	38.5 ± 2.2	49.2 ± 2.6	87.8 ± 5.3	100 ± 4.1	74.6 ± 2.7	82.1 ± 2.3	84.9 ± 5.9	94.6 ± 5.3	83.2 ± 4.1	79.8 ± 3.7	52.7 ± 5.4	27.4 ± 0.5	16.2 ± 0.4	7.8 ± 0.1	4.5 ± 0.1	1.1 ± 0.1
Caffeic Acid	46.2 ± 1.9	55.2 ± 3.2	100 ± 5.6	98 ± 6.1	74.3 ± 4.5	52.9 ± 2.8	43.0 ± 1.7	38.9 ± 2.1	34.5 ± 3.6	26.4 ± 3.7	20.3 ± 1.3	14.8 ± 1.7	12.8 ± 1.5	8.1 ± 1.1	4 ± 0.2	2.7 ± 0.2
p-Coumaric acid	39.1 ± 1.8	61.7 ± 3.6	100 ± 7.4	87.4 ± 6.2	67.4 ± 3.4	51.9 ± 6.8	56.6 ± 3.2	47.2 ± 4.4	41.5 ± 2.1	33.7 ± 2.3	20.1 ± 0.9	18.6 ± 1.1	11.5 ± 1.3	8.7 ± 0.3	3.5 ± 0.2	1.7 ± 0.1
Coniferyl alcohol	37.3 ± 2.1	52.5 ± 3.8	77.1 ± 8.1	100 ± 7.3	82.4 ± 3.4	65.7 ± 6.5	54.3 ± 6.2	47.6 ± 5.8	42.1 ± 5.3	31.5 ± 4.7	23.8 ± 4.5	18.5 ± 2.1	9.7 ± 1.1	5.5 ± 0.3	2.1 ± 0.1	0.9 ± 0.1
Sinapyl alcohol	45.7 ± 5.6	59.2 ± 5.8	87.5 ± 7.2	100 ± 6.7	85.8 ± 4.7	73.2 ± 8.8	63.1 ± 3.9	50.4 ± 3.7	44.3 ± 5.1	37.9 ± 1.7	29.5 ± 1.4	22.6 ± 2.1	17.5 ± 1.5	11.2 ± 0.8	5.4 ± 0.2	3.1 ± 0.1

treatment at 80 °C for 10 min. High glycosylation and cationic nature of VanPrx may be attributed to its stability at high temperature [21,38,39,47,48].

3.5. Spectral properties

The UV/vis absorption spectrum of native VanPrx in 50 mM sodium phosphate buffer, pH 6.5 was typical of heme-containing Class III plant peroxidases [22]. It showed a Soret peak at 403 nm with additional maxima at 495 and 638 nm. Addition of stoichiometric amount of H_2O_2 leads to the formation of Compound I. The soret band was shifted from 403 to 404 nm. There was also a significant decrease in the intensity of the soret peak and also the two peaks at 583 and 645 nm (Fig. 3). In contrast to HRP and most other peroxidases [24], Compound I was found to be more unstable as it got converted into Compound II soon after 2 min of its formation. An absorption spectrum recorded after addition of 10 molar equivalents of H_2O_2 to the native enzyme showed the formation of Compound II (Fig. 3). The Compound II was found to be stable and it was characterized by a shift in the soret peak to 419 nm along with an increase (5%) in intensity compared to that of the native form. Two characteristic peaks at 530 and 549 nm were similar to that of HRP [24]. An addition of NADH (200 molar concentration of VanPrx) did not reduce the native enzyme to ferro form to produce Compound III within any point of 30 min reaction, whereas in HRP it was formed after 5 min of addition [24]. Compound III was also expected after addition of high concentration (200 times of the molar concentration of protein) of H_2O_2 [19]. Its absorption spectrum is characterized by a shift in soret peak from 403 nm to 417 nm and two minor peaks at 534 nm and 556 nm. This spectrum was similar to that of Compound II than Compound III. Compound III was also formed by reduction of enzyme by DTT (2 mg for 2 μM protein in a 600 μl reaction mixture in 50 mM phosphate buffer pH 7.0) [25]. It leads to shift in soret peak to 417 nm after 5 min of DTT addition. However, unlike HRP it did not revert to its native form after exhaustion of O_2 in the reaction mixture [25]. After 90 min, the soret peak was decreased in intensity (~40%) and permanently shifted to 428 nm as in tomato peroxidase [25]. Therefore, Compound I of VanPrx was more unstable than that of HRP. Addition of 200 times molar concentration of H_2O_2 lead to the formation of a compound with peaks intermediate to that of Compound II and Compound III. However, addition of 200 molar concentration of NADH did not lead to the formation of Compound III in VanPrx unlike HRP. These observations indicate that the redox states of VanPrx are distinct. Detailed study of the oxidation states might reveal the structural changes affecting spectral properties of VanPrx.

3.6. Effect of various inhibitors

In inhibition kinetics, classical peroxidase inhibitors like potassium cyanide and sodium azide decreased the VanPrx activities by more than 90% (Table 4). Sensitivity to these inhibitors is common among a large number of hemocatalyzed reactions, thereby indicating heme nature of VanPrx. In the presence of NaN_3 and KCN, lack of change in K_m and decrease in V_{max} in Lineweaver Burk plot (Fig. 4) suggest that both are non-competitive inhibitors of VanPrx. The values of inhibition constants (K_i) for NaN_3 and KCN were 1.24 mM and 1.68 mM, respectively. Enzyme activity was partially inhibited (68%) by CTAB (20 mM). Citric acid and oxalic acid are known to inhibit peroxidase activity [26] but VanPrx showed relatively more tolerance to these two acids (Table 4). Interestingly, VanPrx showed no inhibition with EDTA unlike HRP which competitively inhibits guaiacol oxidation [49]. Instead it shows enhancement of activity by addition of EDTA (5 mM: 121.2%; 10 mM: 116.7%; and 20 mM: 109.8%). Due to structural analogy,

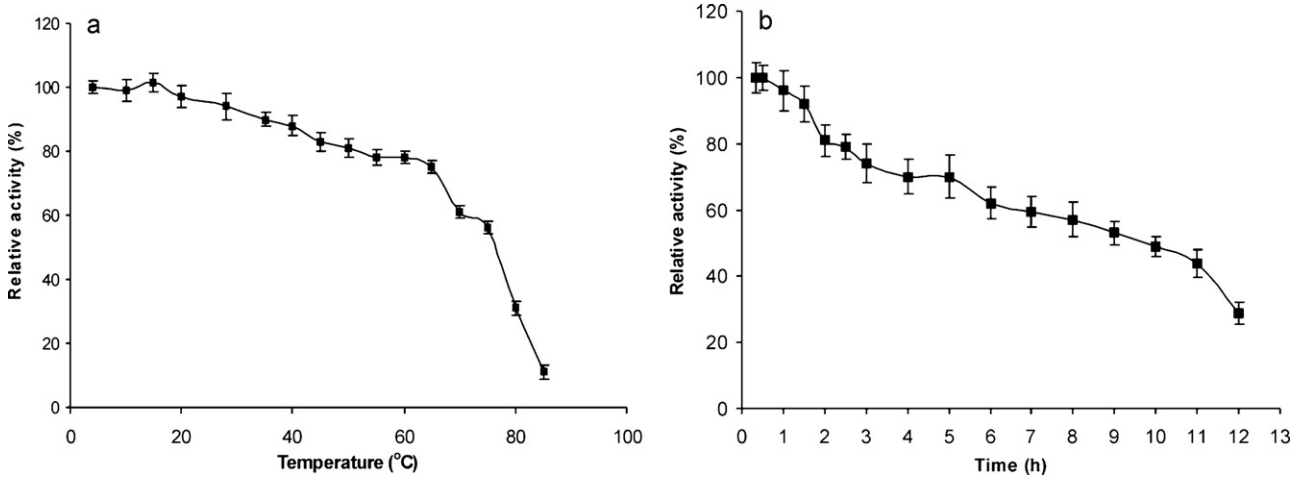

Fig. 2. Relative activity (ratio of actual highest activity expressed as percentage) of VanPrx treated: (a) at a range of temperature for 10 min; and (b) at 65 °C for different time intervals.

Table 4
Details of inhibitions (%) of VanPrx activity by different known inhibitors tested. Results shown as mean ± standard deviation of three replicas.

Inhibitor used	Inhibition (%) at concentration (mM) of					
	0.5	1	2	5	10	20
Citric acid	nd	nd	nd	2.5 ± 0.6	9.7 ± 2.1	47.5 ± 1.8
Oxalic acid	nd	nd	nd	22.8 ± 1.7	59.4 ± 4.6	98.4 ± 3.1
CTAB	nd	nd	nd	27.5 ± 1.1	43.8 ± 1.6	68.1 ± 2.2
EDTA	nd	4 ± 0.3	5.5 ± 0.7	-12.2 ± 0.8	-16.7 ± 1.9	-9.8±.1.1
TEMED	nd	9.7 ± 0.8	29.5 ± 1.4	48.4 ± 3.4	87.6 ± 4.9	96.4 ± 3.4
DTPA	nd	3.2 ± 0.4	8.3 ± 1.1	31.8 ± 1.2	56.3 ± 3.3	79.2 ± 4.1
NaN_3	2.4 ± 2.7	81 ± 1.8	94.4 ± 2.4	100.0	nd	nd
KCN	94.2 ± 1.1	96 ± 1.6	100	nd	nd	nd
Gallic acid	9.3 ± 0.8	44.3 ± 2.2	64.3 ± 4.6	81.2 ± 2.1	89.2 ± 2.1	93.1 ± 3.2
IAA	ni	ni	ni	nd	nd	nd

nd: not determined; ni: no inhibition.

TEMED also competitively inhibits HRP like EDTA. In contrast, in our case TEMED is an uncompetitive inhibitor of VanPrx (Fig. 4) indicating that it does not bind to the enzyme but to the enzyme–substrate complex. Due to limited studies on three-dimensional structure of peroxidase–substrate/inhibitor complex, the details of their binding are not well understood. Therefore, no inhibition of VanPrx by EDTA and uncompetitive inhibition by TEMED are quite interesting and further research on this aspect will bring out new information on peroxidase–substrate/inhibitor binding interactions.

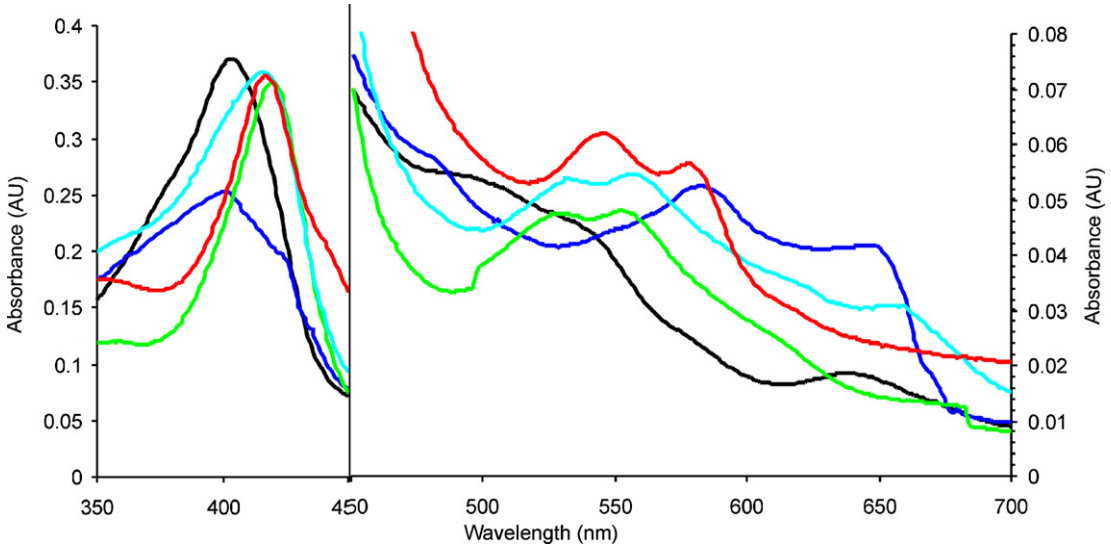

Fig. 3. Absorption spectra of native VanPrx (black) and Compound I (blue), Compound II (green) and Compound III (light blue) formed by addition of H_2O_2 and Compound III (red) formed by addition of DTT to native enzyme. Note the change in soret region and amplified (4×) region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

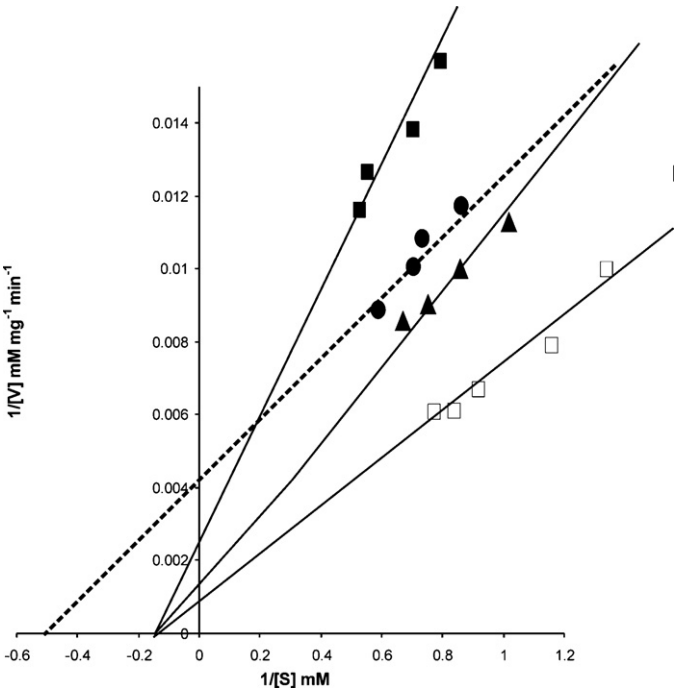

Fig. 4. Lineweaver plot of VanPrx without inhibitors (□) and inhibition of VanPrx after treatment with KCN (▲), NaN_3 (■) and TEMED (●) (note the noncompetitive inhibition by KCN and NaN_3, while inhibition by TEMED is uncompetitive).

4. Conclusion

In the present study, an endemic stem parasitic plant (*V. angulatum*) has been identified as a source of a novel peroxidase. Substrate affinity, LC–MS/MS analyses, glycoproteinaceous nature, identification of different redox states of VanPrx (Compounds I, II and III), inhibition kinetics by NaN_3 and KCN indicated that it is a Class III plant peroxidase. Unlike cationic peroxidases, VanPrx did not show IAA oxidase activity. However, like anionic peroxidases VanPrx appears to be involved in suberization. The spectral changes arising from oxidation indicate that it possessed distinct redox states. Further, high thermostability and distinct inhibition kinetics of some of the inhibitors as compared with other Class III peroxidases confirmed its uniqueness.

Acknowledgements

We acknowledge Dr. David Kothamasi, Department of Environmental Biology, University of Delhi, Dr. John Williams, Department of English, Satyawati College, University of Delhi and Ms. Promila Sharma, Blacksmith Institute, New Delhi, for careful reading of the manuscript. VM and RSS thank Department of Biotechnology, Government of India and University of Delhi for the research grant. MKD thanks Council of Scientific and Industrial Research, Government of India for fellowship.

References

[1] S. Hiraga, K. Sasaki, H. Ito, Y. Ohashi, H. Matsui, Plant Cell Physiol. 42 (2001) 462–468.
[2] F. Passardi, D. Longet, C. Penel, C. Dunand, Phytochemistry 65 (2004) 1879–1893.
[3] J.M. Neuhaus, Plant Physiol. Biochem. 34 (1996) 217–221.
[4] K.G. Welinder, in: C. Penel, T. Gaspar, H. Greppin (Eds.), Plant Peroxidases, University of Geneva, Switzerland, 1992, pp. 1–24.
[5] R.W. Whetten, J.J. MacKay, R.R. Sederoff, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 585–609.
[6] C. Mathe, A. Barre, C. Jourda, C. Dunand, Arch. Biochem. Biophys. 500 (2010) 58–65.
[7] S.C. Fry, Annu. Rev. Plant Physiol. 37 (1986) 165–186.
[8] L.M. Lagrimini, R.J. Joly, J.R. Dunlap, T.Y. Liu, Plant Mol. Biol. 33 (1997) 887–895.
[9] S.S. Gill, N. Tuteja, Plant Physiol. Biochem. 48 (2010) 909–930.
[10] C.-F. Wang, L.-L. Huang, H.-C. Zhang, Q.-M. Han, Physiol. Mol. Plant Pathol. 74 (2010) 221–229.
[11] A.D. Vatulescu, A.S. Fortunato, M.C. Sá, S. Amâncio, C.P.P. Ricardo, P.A. Jackson, Plant Physiol. Biochem. 42 (2004) 609–615.
[12] C. Regalado, B.E. Garcia-Almendarez, M.A. Duarte-Vazquez, Phytochem. Rev. 3 (2004) 243–256.
[13] V. Mishra, R.S. Sharma, S. Yadav, C.R. Babu, T.P. Singh, Arch. Biochem. Biophys. 423 (2004) 288–301.
[14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[15] V. Mishra, R.S. Sharma, M. Paramsivam, S. Bilgrami, S. Yadav, A. Srinivasan, C. Betzel, C.R. Babu, T.P. Singh, Plant Sci. (2005) 615–625.
[16] C.A. White, J.F. Kennedy, in: M.F. Chaplin, J.F. Kennedy (Eds.), Carbohydrate Analysis: A Practical Approach, IRL Press, Oxford, 1986, pp. 37–54.
[17] V. Mishra, A.S. Ethayathulla, R.S. Sharma, S. Yadav, R. Krauspenhaar, C. Betzel, C.R. Babu, T.P. Singh, Acta Cryst. D60 (2004) 2295–2304.
[18] A. Mika, S. Luthje, Plant Physiol. 132 (2003) 1489–1498.
[19] M. Kvaratskhelia, C. Winkel, R.N.F. Thorneley, Plant Physiol. 114 (1997) 1237–1245.
[20] C.B. Rassmussen, A. Henriksen, A.K. Abelskov, R.B. Jensen, S.K. Rasmussen, J. Hejgaard, K.G. Welinder, Physiol. Plant 100 (1997) 102–110.
[21] U. Takahama, T. Oniki, Plant Cell Physiol. 38 (1997) 456–462.
[22] J.C. Leon, I.S. Alpeeva, T.A. Chubar, I.Y. Galaev, E. Csoregi, I.Y. Sakharov, Plant Sci. 163 (2002) 1011–1019.
[23] N.J. Price, C. Pinheiro, C.M. Soares, D.A. Ashford, C.P. Ricardo, P.A. Jackson, J. Biol. Chem. 278 (2003) 41389–41399.
[24] S.X. Chen, P. Schopfer, Eur. J. Biochem. 260 (1999) 726–735.
[25] D.M. Kokkinakis, J.L. Brooks, Plant Physiol. 63 (1979) 93–99.
[26] C.M. Ajila, U.J.S. Prasada Rao, J. Mol. Catal. B: Enzym. 60 (2009) 36–44.
[27] H.B. Dunford, Heme Peroxidases, Wiley-VCH, 1999.
[28] S.S. Deepa, C. Arumughan, Phytochemistry 61 (2002) 503–511.
[29] V. Mishra, S. Bilgrami, R.S. Sharma, P. Kaur, S. Yadav, R. Krauspenhaar, C. Betzel, W. Voelter, C.R. Babu, T.P. Singh, J. Biol. Chem. 280 (2005) 20712–20721.
[30] C. Caruso, G. Chilosi, L. Leonardi, L. Bertini, P. Magro, V. Buonocore, C. Caporale, Phytochemistry 58 (2001) 743–750.
[31] E.M. Estabrook, J.I. Yoder, Plant Physiol. 116 (1998) 1–7.
[32] W.J. Keyes, J.V. Taylor, R.P. Apkarian, D.G. Lynn, Plant Physiol. 127 (2001) 1508–2151.
[33] J. Foreman, V. Demidchik, J.H.F. Bothwel, P. Mylona, H. Miedema, M.A. Torres, P. Linstead, S. Costa, C. Brownlee, J.D.G. Jones, J.M. Davies, L. Dolan, Nature 422 (2003) 442–446.
[34] A. Liszkay, E. Van der Zalm, P. Schopfer, Plant Physiol. 136 (2004) 3114–3123.
[35] A.M. Mor, A. Mayer, Levine, Weed Biol. Manag. 8 (2008) 1–10.
[36] Veronesi, E. Bonnin, S. Calvez, P. Thalouarn, P. Simier, Biol. Plant. 51 (2007) 391–394.
[37] J. Ralph, M. Bunzel, J.M. Marita, R.D. Hatfield, F. Lu, H. Kim, P.F. Schatz, J.H. Grabber, H. Steinhart, Phytochem. Rev. 3 (2004) 79–96.
[38] J.H. Grabber, J. Ralph, R.D. Hatfield, J. Agric. Food Chem. 46 (1998) 2609–2614.
[39] M.A. Bernards, W.D. Fleming, D.B. Llewellyn, R. Priefer, X. Yang, A. Sabatino, G.L. Plourde, Plant Physiol. 121 (1999) 135–145.
[40] Ros Barcelo, L.V. Gomez Ros, C. Gabaldon, M. Lopez-Serrano, F. Pomar, J.S. Carrion, M.A. Pedreno, Phytochem. Rev. 3 (2004) 61–78.
[41] M.A. Bernards, D.K. Summerhurst, F.A. Razem, Phytochem. Rev. 3 (2004) 113–126.
[42] M. Quiroga, C. Guerrero, M.A. Botalla, A. Barcelo, I. Amaya, M.I. Medina, F.J. Alonso, S.M. deForchetti, H. Tigier, V. Velpuesta, Plant Physiol. 122 (2000) 1119–1128.
[43] H.S. Heide-Jorgensen, Bot. Gaz. 152 (1991) 321–334.
[44] S. Rümer, D.D. Cameron, R. Wacker, W. Hartung, F. Jiang, Flora 202 (2002) 194–200.
[45] S.S. Kim, D.J. Lee, J. Plant Physiol. 162 (2005) 609–617.
[46] D.A. Converso, M.E. Farnandez, Phytochemistry 40 (1995) 1341–1345.
[47] R. Pathirana, L. Watson, B. Chen, S. Leung, C. Voisey, T. Murray, M.T. McManus, Phytochemistry 66 (2005) 1869–1879.
[48] R.B. Van Huystee, M.G. Roig, V.L. Shnyrov, I.Y. Sakharov, Phytochem. Rev. 3 (2004) 19–28.
[49] D.K. Bhattacharya, S. Adak, U. Bandopadhyay, R.K. Banerjee, Biochem. J. 298 (1994) 281–288.