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ORIGINAL RESEARCH PAPER

Partial Purification and Biochemical Characterization of Peroxidase (E.C. 1.11.1.7) from *Catharanthus roseus*

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Key words
Periwinkle, Peroxidase, Purification, Ion-exchange chromatography, SDS-PAGE

Abstract
The Madagascar periwinkle *Catharanthus roseus* is a tropical plant belonging to family Apocynaceae which produces the dimeric monoterpenoid indole alkaloids vincristine and vinblastine. These compounds are also powerful cytostatic drugs used in cancer chemotherapy but are produced by the plant leaves in very low amounts (0.0003% dry weight). A crucial step in the synthesis of vincristine and vinblastine is the coupling of catharanthine and vindoline to produce the dimeric precursor α-3',4'-anhydrovinblastine (AVLB). Though a putative peroxidase bearing an AVLB synthase activity has been implicated to be involved in the coupling reaction, direct proof of the existence of a peroxidase in leaves able to couple catharanthine and vindoline has not been produced. The present study reports the purification and characterization of enzyme peroxidase from leaves of *C. roseus*. For characterization of peroxidase from *C. roseus*, kinetic properties viz. Km value with respect to substrate H2O2 as well as the optimum pH, optimum temperature etc. were determined.

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INTRODUCTION

The Madagascar periwinkle *Catharanthus roseus* is a tropical plant belonging to family Apocynaceae which produces the dimeric monoterpenoid indole alkaloids vincristine and vinblastine. These compounds are also powerful cytostatic drugs used in cancer chemotherapy but are produced by the plant leaves in very low amounts (0.0003% dry weight). A crucial step in the synthesis of vincristine and vinblastine is the coupling of catharanthine and vindoline to produce the dimeric precursor α-3',4'-anhydrovinblastine (AVLB). Though a putative peroxidase bearing an AVLB synthase activity has been implicated to be involved in the coupling reaction, direct proof of the existence of a peroxidase in leaves able to couple catharanthine and vindoline has not been produced. The present study reports the purification and characterization of enzyme peroxidase from leaves of *C. roseus*. For characterization of peroxidase from *C. roseus*, kinetic properties viz. K_m value with respect to substrate H_2O_2 as well as the optimum pH, optimum temperature etc. were determined.

MATERIALS AND METHODS

Materials

The leaves of Catharanthus roseus were obtained in and around Gwalior. All the reagents used in the present study were of AR grade and were purchased from SD Fine Chemicals, Mumbai (India).

Determination of POD Activity

POD activity was assayed in crude enzyme preparation (supernatant) as described by Putter (1974), with some modification. POD activity in crude homogenate of leaves of *C. roseus* was determined colorimetrically at 470 nm with substrate H_2O_2 and dye o-dianisidine (DAS). The oxidation of the reduced form of dye produced a brick red color readable at 470 nm in a colorimeter maintained at 37°C.

Salt Fractionation of *C. roseus* leaf homogenate as a step towards partial purification of POD from *C. roseus*

Homogenate of *C. roseus* leaves was fractionated to 35%, 45%, 55%, 65% and 75% saturation of ammonium sulfate. All ammonium sulfate fractions were analyzed for POD activity using the method of Putter (1974).[1]

Cation-exchange Chromatography of POD Enriched Fractions

Solvents A (0.05 M citrate Buffer, pH 5.5) and B (0-3.0 M NaCl in 0.05 M citrate buffer, pH 5.5) were degassed for 5 min. Carboxymethyl-Cellulose (CM-Cellulose) preswollen in 20% ethanol was prepared by decanting 20% ethanol solution and replacing it with starting buffer (0.05 M citrate buffer pH 5.5). The dissolved 65 and 75 % ammonium sulfate pellets (1.0 ml) were pooled and loaded on exchanger bed without disturbing the top surface. The flow-rate was adjusted between 10-15 ml/hr (0.3ml/min) and 1.0 ml fractions were collected in different tubes. The column was subsequently washed with citrate buffer and 0.5 ml fractions were collected in different tubes. Finally, the bounded proteins were eluted by applying a linear gradient of NaCl from 0-0.3 M in starting buffer.

Protein Estimation

Protein was estimated in all fractions following the method of Lowry et al. (1951) using BSA as standard.[2]

Characterization of Peroxidase

1. SDS PAGE: SDS-PAGE was performed on a 12% gel according to the method of Laemmli (1970).[3]
2. Native PAGE: Native PAGE was performed on a 7.5% gel according to the method of Laemmli, (1970).[3]
3. Activity Staining: Gels were incubated for 5-10 min at 30ºC in a reaction mixture comprising 10 ml 0.1M potassium phosphate buffer pH= 7.0, 20 mg Benzidine (dissolved in methanol) and 0.2% H_2O_2. After incubation the gel was rinsed in distilled water and kept for observation. The sites on the gel where the enzyme peroxidase was present was stained blue turned brown confirming the presence of native peroxidase.
4. Kinetic studies: The source of enzyme protein used for kinetic studies was the fraction found to be most enriched in peroxidase after CEC. The apparent K_m value of enzyme peroxidase for substrate

H_2O_2 was determined using varying H_2O_2 concentrations from 30 to 90 mM and a fixed o-dianisidine (DAS) concentration of 1.0 mM. Data were plotted as double reciprocal Lineweaver-Burk plots to determine the apparent K_m value.

5. Effect of pH: The optimum pH of *C. roseus* POD was determined within the pH range of 5.7 to 8.0 of 0.1M-phosphate buffer.
6. Effect of Temperature: Effect of temperature on the stability of the enzyme protein was determined in the range from 40 to 70ºC.

RESULTS AND DISCUSSION

POD activity in crude homogenate of leaves of *C. roseus* was calculated to be around 0.156 µmol/min and the rate of formation of the oxidized form of dye (o-DAS) by peroxidase was found to be fairly linear up to 5 min (Fig 1).

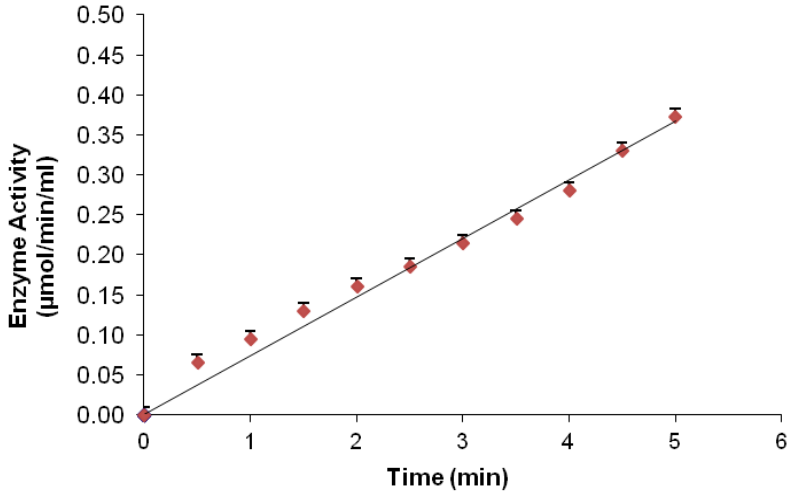

Fig 1. Linearity of leaf POD assay with respect to time (Values are mean ± SD of 3 separate experiments)

Fig 2 shows the specific activity profile of *C. roseus* leaf POD after salt fractionation. POD activity was found to be localized mainly in 75% ammonium sulfate fraction.

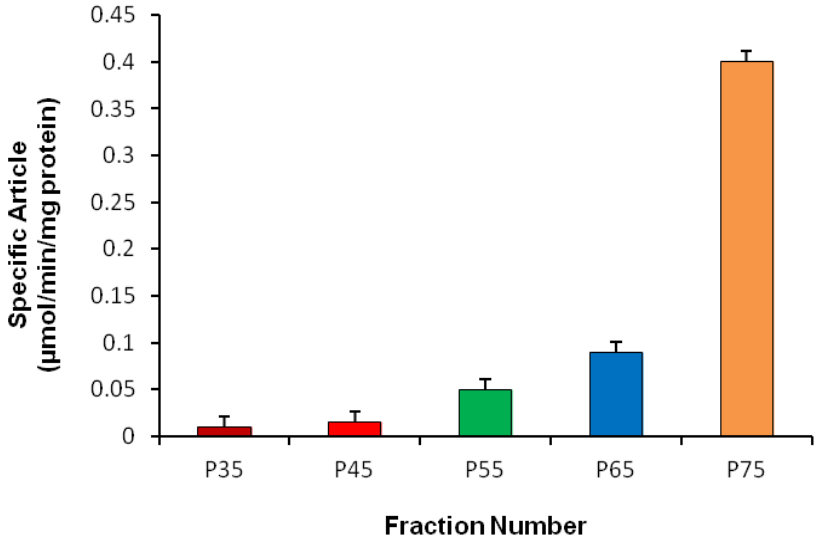

Fig 2. Specific activity profile of *C. roseus* leaf POD after salt fractionation

Fig 3 depicts the elution profile for total activity and total protein of enzyme POD from CM-Cellulose column. The enzyme was eluted in fractions 1-4, thereby indicating that the enzyme carried an overall positive charge at pH 5.5.

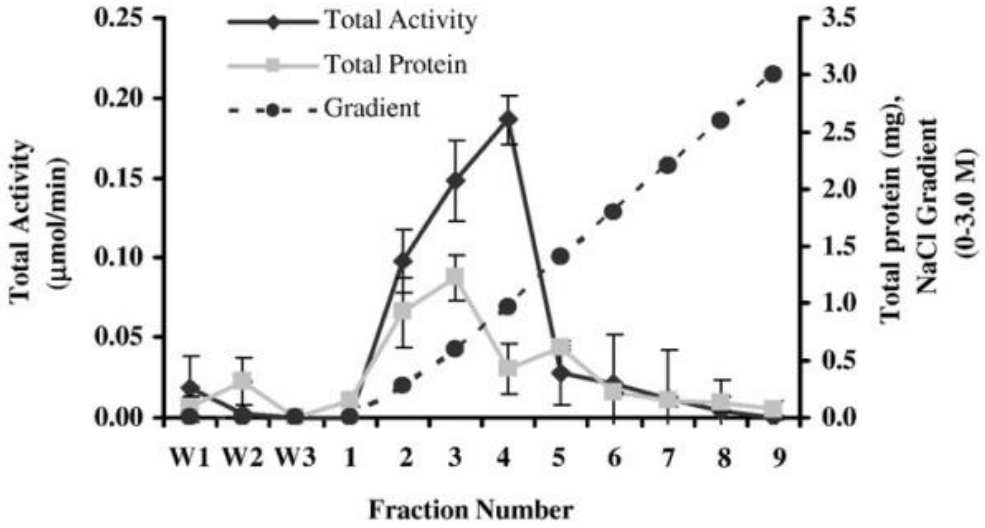

Fig 3. Cation Exchange Chromatography of POD enriched fractions

Using the above protocol, POD from *C. roseus* leaves was purified to around 19.1 fold with a total recovery of 4.7% from cation-exchange column (Table 1). The molecular weight of POD was estimated to be around 46-48 kDa by SDS-PAGE (Fig 4a). Enzyme activity was detected on non-denaturing PAGE by activity staining, thus proving that the isolation procedure yielded a biologically active (native) enzyme. Two bands were detected on native gel, thereby confirming the presence of two isoenzymes in leaves of *C. roseus* (Fig 4b).

Table 1. Summary of steps employed in the purification of POD from *C. roseus*

Purification step	Total Protein (mg)	Total Activity (µmoles / min)	Specific Activity (µmoles / min/mg)	Yield (%)	Fold Purification
Crude fraction	291.5	26.24	0.09	100	1
75% Ammonium sulphate cut	6.4	2.432	0.380	9.3	4.2
Cation exchange	0.17	1.232	7.25	4.7	19.1

Partially purified peroxidase displayed Michaelis-Menten behavior with regards to H_2O_2. The K_m value of the enzyme with respect to H_2O_2 was determined to be 0.08 mM (Fig 5). The enzyme was found to be maximally active at pH=6.5 (Fig 6). Peroxidase activity progressively decreased as temperature was increased from 40 to 70ºC due to denaturation of the protein at high temperature.

Discussion

In the present study, a peroxidase was purified from *C. roseus* leaves using a combination of salt precipitation and cation exchange chromatography, and some of its kinetic properties were studied. The purification scheme described here provides a convenient and efficient method for isolating POD from *C. roseus*. POD purification was optimized by inclusion of relatively fewer steps. The purification procedure also has an advantage of an experimental period as short as a day. The molecular weight of

POD was estimated to be around 46-48 kDa by SDS-PAGE which is in agreement with that of peroxidases studied from other sources. Detection of two bands bearing POD activity on native gel, might signify the existence of two distinct proteins with associated POD activity.

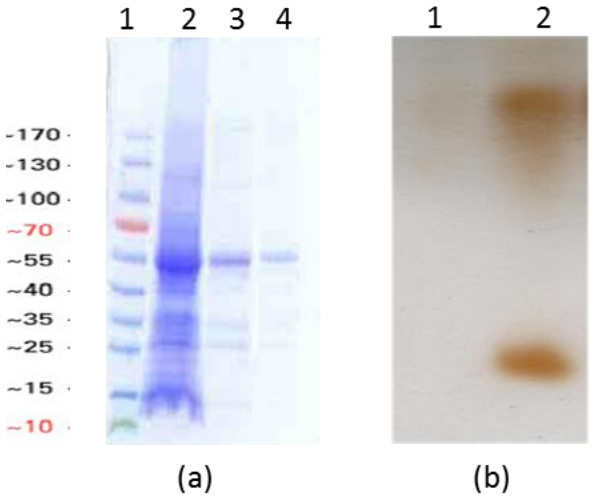

Fig 4. SDS PAGE and activity scanning of purified POD
(a) SDS PAGE analysis of purified POD: Lane 1-Marker; Lane 2-Crude Homogenate (10 µl), Lane 2- Purified enzyme (10 µl); Lane 4- Purified enzyme (5 µl).
(b) Activity staining of purified native POD: Lane 1- crude homogenate; (10 µl) Lane 2- P75 fraction (10 µl).

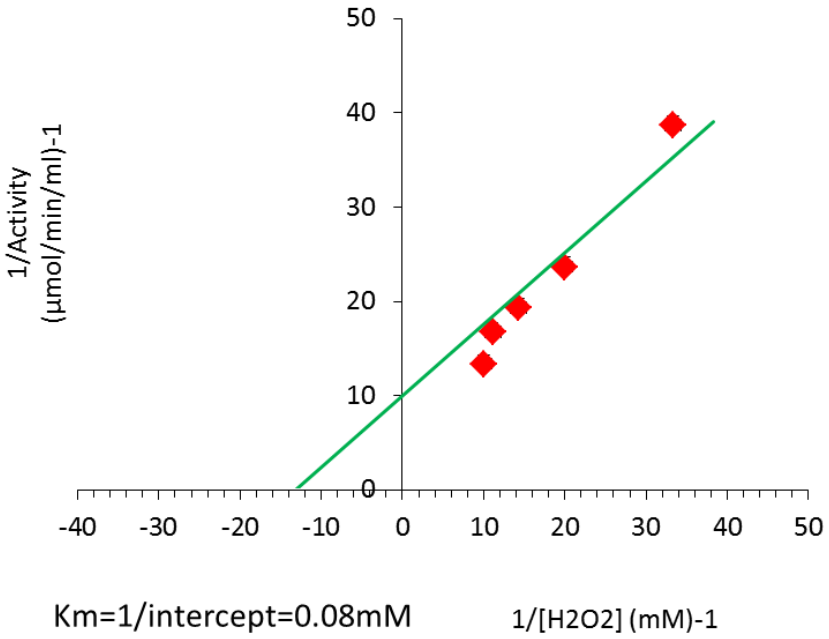

Fig 5. Line weaver Burk double reciprocal plot for K_m of partially purified POD from the leaves of *C. roseus* with respect to H_2O_2 (Values are mean ± SD based on experiments done in triplicates)

CONCLUSION

The peroxidase enzyme was isolated and partially purified from C. roseus. It was characterized for its activity. Further studies would focus on the role of both these proteins bearing POD activity in dimerization of monomeric alkaloids. Future studies would also involve efforts to enhance the levels of

alkaloids vincristine and vinblastine in *C. roseus* leaves under stress conditions due to possible increase in POD activity in leaves.

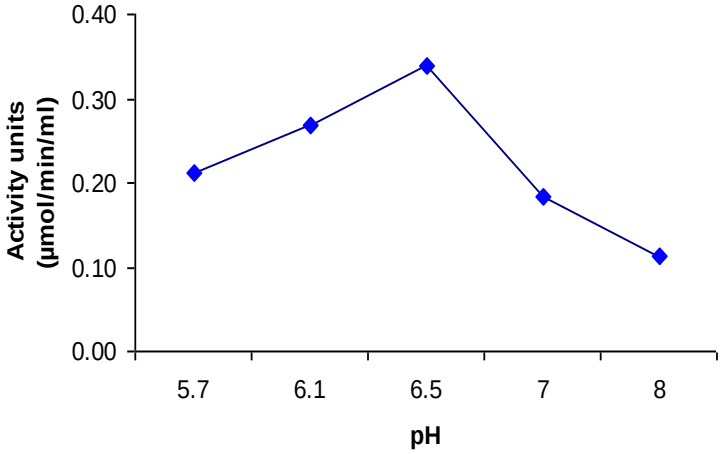

Fig 6. Effect of pH of the assay buffer on POD activity

DECLARATION OF INTEREST

It is hereby declared that this paper does not have any conflict of interest.

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