

Purification of peroxidase from the medicinal herb *Andrographis paniculata* causing fungal hyphal distortion

MODHUMITA GHOSH

Division of Plant Biotechnology
Institute of Forest Genetics and Tree Breeding
Forest Campus
R.S. Puram, Coimbatore – 641 002
India

*corresponding author: phone: +91422 2484123, 2484100, fax: +91422 2430549,
e-mail: ghoshm@icfre.org; modhumitaghosh@hotmail.com

Summary

Peroxidases are ubiquitous in plants and their pattern of expression depends on tissue, development stage and environmental stimuli. They are involved in numerous physiological functions, including defense against pathogens and insect pests. The present study was conducted to isolate and purify a constitutive leaf peroxidase from the medicinal herb, *Andrographis paniculata*. The enzyme was purified through a series of purification steps and a 35kDa peroxidase was purified to homogeneity with pI value of 6.0. *In vitro* hyphal extension inhibition assay against the forest pathogen *Trichosporium vesiculosum* revealed significant morphological distortion of the protein treated hyphae.

Key words: antifungal protein, defense, hyphal inhibition, oxido reductases, pathogenesis-related protein

INTRODUCTION

Peroxidases (E.C. 1.11.1.7) are a super family of oxido-reductases ubiquitous in the plant, fungi and vertebrates. They are reported to accumulate specifically during infection and several roles have been attributed to the plant heme peroxidases in host – pathogen interactions [1, 2]. *In vitro* studies have indicated that peroxidases in the presence of H₂O₂ inhibit the growth of fungi [3, 4]. However, few

reports have suggested the antifungal nature of specific peroxidases in absence of H_2O_2 as in wheat WP1 [5], French bean legume [6] *Hibiscus esculentus* and *Vigna sinensis ssp. sesquipedalis* [4] and *Acorus calamus* [7].

Andrographis paniculata (Burm.f) Nees (Acanthaceae) native to India and Sri Lanka is used in traditional system of medicine. The extract has clinical applications as antihepatotoxic, antibiotic, antimalarial, antihepatitic, antithrombogenic, anti-inflammatory, anti-snake venom and antipyretic. In the present study a constitutively expressed peroxidase was purified from the leaves of *Andrographis paniculata* and characterized for its antifungal activity.

MATERIALS AND METHODS

Plant material and fungal culture

Seeds of *Andrographis paniculata* obtained from Tropical Botanical Garden and Research Institute, Palode, Kerala, India and the fungal culture of *Trichosporium vesiculosum* (*Casuarina* isolate) were grown and maintained in potato dextrose agar medium.

Extraction and fractionation of peroxidase

Fifty grams of leaf tissues from *Andrographis paniculata* was homogenized in liquid Nitrogen and extracted in three volumes of phosphate buffer (25 mM Na_2PO_4 ; 250 mM NaCl; 10 mM EDTA; 5 mM DTT; 1 mM PMSF; 1.5% PVPP; 0.2% activated charcoal and 100 mM ascorbic acid). The extract was filtered and centrifuged at 9,000 rpm for 30 minutes at 4°C and the supernatant (F1) was subjected to 60% ammonium sulphate precipitation overnight. Subsequently, the protein pellet was recovered after centrifugation and suspended in 50 mM sodium phosphate buffer, pH 6.0 (F2). The crude protein was then subjected to gel filtration using sepharose 6B column (Sigma Aldrich Ltd., USA) pre equilibrated with 50 mM sodium phosphate buffer, pH 6.0. Proteins were eluted using a 1M NaCl gradient in phosphate buffer, pH 6.0. Ten ml fractions were collected with flow rate of one ml/min and fractions showing maximum peroxidase activity with 3,5,3',5'- tetra methyl benzidine (TMB) and H_2O_2 were pooled as F3. The pooled fractions were further separated by Superose 12 10/300 GL column in the FPLC system (GE Healthcare, Piscataway, NJ, USA). The column was initially equilibrated with 50mM sodium phosphate buffer, pH 6.0 prior to protein injection. The protein fractions were eluted using 50 mM sodium phosphate, pH 6.0 with 100 mM to 1M NaCl gradient. One ml fractions were collected and fractions showing maximum peroxidase activity were pooled as F4. The pooled F4 fraction was further separated through activated DEAE cellulose column and eluted using phosphate buffer, pH 6.0 with

1 M NaCl gradient. The fractions showing peroxidase activity was pooled (F5) and precipitated with 60% ammonium sulphate. The precipitated proteins were pelleted by centrifugation and suspended in buffer, subjected to diafiltration using microsep centrifugal device with 3KD cutoff (Pall Life Sciences, Ann Arbor, MI, USA) (F6). The protein sample was finally purified by affinity chromatography using concanavalin A sepharose column. Ten ml of Con A sepharose 4B (Sigma Aldrich Ltd., USA) was packed in column and washed with five volumes of binding buffer (100 mM acetate buffer, pH 6.0; 1M NaCl; 1 mM $MnCl_2$; and 1 mM $CaCl_2$). The protein was loaded in to the column and eluted with a gradient of methyl β -D-mannopyranoside (0–0.5 M) in binding buffer. The eluted fractions were tested for peroxidase activity and fractions showing the maximum activity were pooled. The fractions were concentrated and desalted by diafiltration (F7).

The protein concentration of all fractions was determined using Bradford's reagent (Sigma Aldrich Ltd., USA) as described by Bradford [8].

Enzyme assay and gel localization of peroxidase

Peroxidase assay was performed using TMB as the chromogenic substrate and hydrogen peroxide [9]. The chromogenic activity in each sample was recorded spectrophotometrically at A450 nm. The pH suitable for optimal peroxidase activity was determined by estimating its activity in buffers ranging from pH 3.0 to 9.0. The buffers used in the assay included 200 mM acetate buffer (pH 3.0–5.0) and 200mM phosphate buffer (pH 6.0–9.0). Gel localization of peroxidase was conducted by immersing the gel after electrophoresis in 1X solution of TMB/ H_2O_2 (Bangalore Genei Ltd., India). The development of bands with blue coloration was documented.

Determination of molecular weight and pI of peroxidase

The protein fraction F7 was resolved in gradient 10–15 native precast gel in Phast automated electrophoretic system (GE Healthcare, Piscataway, NJ, USA) along with standard molecular weight markers (Bangalore Genei Ltd, India) and stained with silver nitrate as described by the manufacturer. The pI value of the protein was determined by isoelectric focusing in IEF 3 – 9 precast gel using the Phast automated electrophoretic system (GE Healthcare, Piscataway, NJ, USA) along with broad range (pH 3 – 10) IEF marker (GE Healthcare, Piscataway, NJ, USA) and the gel was stained with silver nitrate as described by the manufacturer.

In vitro hyphal extension inhibition assay

Agar blocks from actively growing plate of *Trichosporium vesiculosum* were inoculated in petri plate containing potato dextrose agar and incubated at 32°C

for 48 hours. Subsequently, sterile Whatman filter paper discs loaded with different protein fractions were placed towards the periphery of the growing hyphae. A control with sterile water was also added. The plate was incubated for 24 to 48 hours and observations were made for the appearance of a crescent shaped inhibition zone. Subsequently, the changes in hyphal morphology was recorded for the hyphal mass actively growing in the centre of the plate and also the mass growing towards the protein fractions using Nikon UFX-DX microscope.

RESULTS

Extraction, purification and bioassay of peroxidase

The total leaf protein extracted from *A. paniculata* and precipitated using ammonium sulphate (F2) was sequentially purified through sepharose 6B column and four peaks were observed (fig. 1). Fractions 19, 20, 21 and 22 showing maximum enzyme activity were pooled as fraction F3. The pooled fraction was further separated by Superose 12 10/300 GL column and three major peaks were documented (fig. 2). The fractions 21, 22 23 showing maximum peroxidase activity were pooled as fraction F4. The F4 pooled fraction was separated through anion exchange chromatography using DEAE cellulose and fractions with enzyme activity was pooled as F5 which was further purified using affinity chromatography and designated as F7. All peroxidase assays were conducted at pH 6.0 since maximum enzyme activity was observed (data not shown at pH 6.0). Table 1 shows the sequential peroxidase enrichment in the fractions.

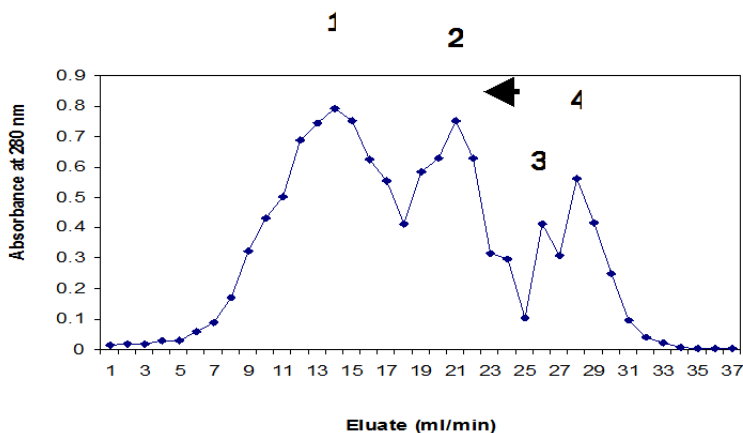


Figure 1. Chromatogram of fraction F2 using Sepharose 6B

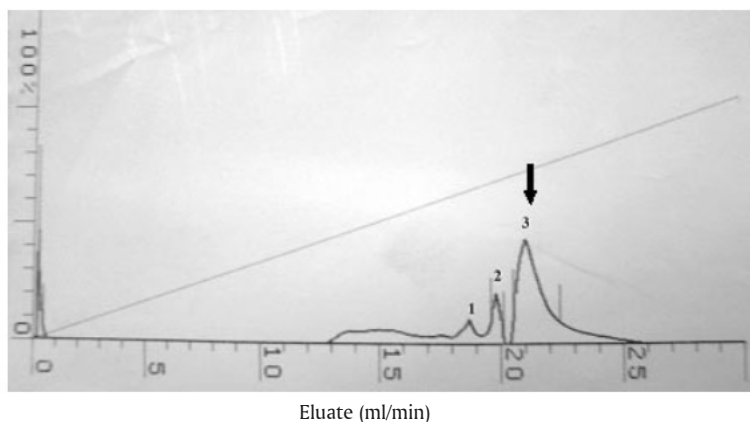


Figure 2. Gel filtration of fraction F3 using Superose 12 10/300 GL column

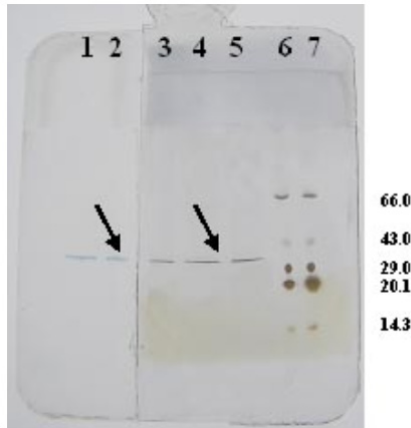
Table 1.

Enrichment of protein sample with peroxidase enzyme

protein fraction	protein concentration in 100 μ l sample	peroxidase activity given as absorbance at 450 nm/100 μ l sample
F1 (total proteins)	52	0.012
F2 (total proteins after 60% ammonium sulphate precipitation)	128	0.070
F3 (pooled fractions from Sepharose 6B through gel filtration)	8.4	0.037
F4 (pooled fractions from Superose 12 10/300 GL through gel filtration)	2.8	0.074
F5 (pooled fractions from DEAE cellulose through anion exchange chromatography)	3.4	0.026
F6 (precipitated F5 with 60% ammonium sulphate)	4.8	0.072
F7 (pooled fractions from Concanavalin A sepharose through affinity chromatography)	2.4	0.156

Determination of molecular weight and pI of the antifungal protein

The purified peroxidase (F7) was separated on native precast gel and the presence of a single band was observed at approximately 35 kDa. The peroxidase was also localized on the gel and confirmed the presence of a single band (fig. 3). The pI value of the purified peroxidase was determined by isoelectric focusing on pre-cast gel and was determined to be approximately 6.0 and the activity staining of the enzyme confirmed the above results (fig. 4)

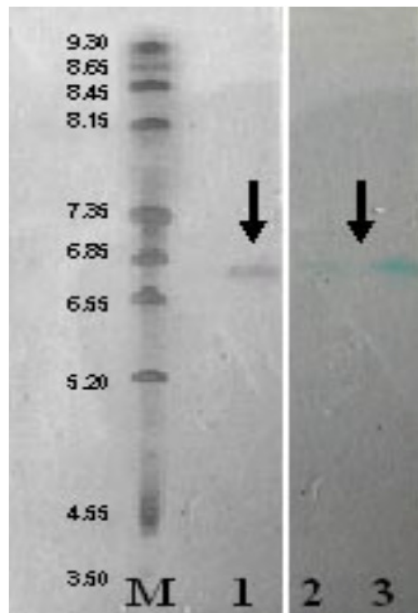


Lane 1-2: gel localization of peroxidase using TMB/H₂O₂

Lane 3-5: purified peroxidase stained with silver nitrate

Lane 6-7: molecular weight marker

Figure 3. Determination of molecular weight of peroxidase in native precast gel



Lane M: IEF markers (pI 3.50: amyloglucosidase; pI 4.55: soybean trypsin inhibitor; pI 5.20: b-lactoglobulin A; pI 5.85: bovine carbonic anhydrase; pI 6.55: human carbonic anhydrase B; pI 6.85: horse myoglobin-acetic band; pI 7.35: horse myoglobin-basic band; pI 8.65: lentil lectin- basic band and pI 9.30: trypsinogen)

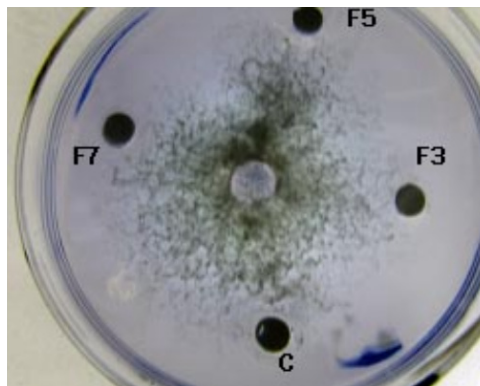
Lane 1: purified peroxidase stained with silver nitrate

Lane 2-3: gel localization of peroxidase using TMB/H₂O₂

Figure 4. Determination of pI of purified peroxidase

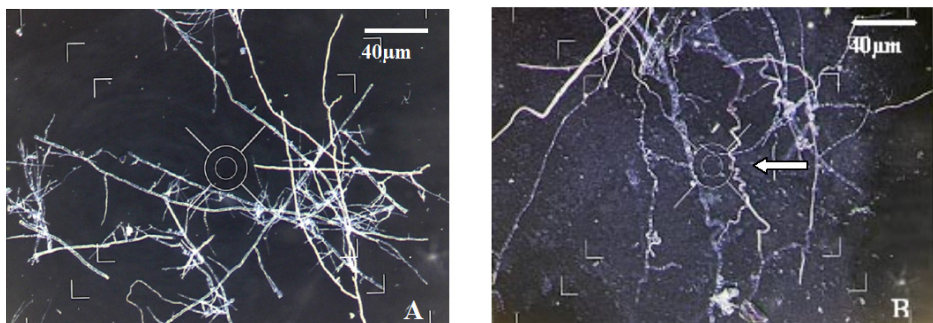
In vitro antifungal assay

All purified fractions were tested for antifungal activity against the wilt pathogen *Trichosporium vesiculosum* using hyphal extension inhibition assay. None of the fractions showed a distinct inhibition zone. The purified fraction F7 limited the hyphal extension at a concentration of 20 μg (fig. 5) revealing a low antifungal efficacy of the purified peroxidase. However, the hyphal mass growing towards the purified protein showed distinct morphological distortions including increased vacuolation and abnormal branching patterns when compared to actively growing hyphae towards the centre of the plate and towards the water soaked control filter discs (fig. 6A, 6B).



F3, F5 and F7: protein fractions
C: control with sterile water

Figure 5. *In vitro* Hyphal Extension Inhibition Assay of protein fractions



A: hyphal morphology of *Trichosporium vesiculosum* in control well
B: hyphal morphology of *Trichosporium vesiculosum* in protein treated well
Arrow indicates distorted hypha.

Figure 6. *In vitro* hyphal morphology studies

DISCUSSION

Peroxidases participate in a variety of plant defense mechanisms [1] in which H_2O_2 is often supplied by an oxidative burst, a common event in defense responses [10]. Chitin specific anionic and cationic isoforms were reported from several species [11-14]. Apoplastic peroxidases were reported to be overexpressed during hypersensitive reaction (HR) in cotton cotyledons [15], *Lactuca sativa* [16], *Picea abies* [17] and soybean [18]. Furthermore, in absence of H_2O_2 , specific peroxidases are reported to inhibit pathogen growth including WP1 from wheat [5], French bean legume [6], *Hibiscus esculentus* and *Vigna sinensis* [4] as well as *Acorus calamus* [7]. In the present study, the peroxidase purified from leaves of *Andrographis paniculata* did not reveal a strong antifungal activity as demonstrated in the above reports. However, the protein caused hyphal distortion revealing its affinity to chitin cell wall of the pathogen. This report reveals that constitutively expressed peroxidases may not act as a first line defense against invading pathogens, although, can synergistically delimit pathogen extension.

CONCLUSIONS

Peroxidases are reported to accumulate specifically during infection and are grouped under pathogenesis-related (PR) 9 proteins. Recent reports on antifungal property of the chitin binding peroxidases have revealed their role in basal defense in plant system. In the present study we report the purification of a constitutively expressed peroxidase from leaves of *Andrographis paniculata*. The protein had a limited antifungal activity but caused hyphal distortion revealing its affinity to chitin cell wall of the pathogen. This report reveals that constitutively expressed peroxidases may not act as a first line defense against invading pathogens but may synergistically delimit pathogen extension.

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OCZYSZCZANIE PEROKSYDAZY Z LECZNICZEJ ROŚLINY *ANDROGRAPHIS PANICULATA*,
POWODUJĄCEJ ZNIEKSZTAŁCENIA STRZĘPEK GRZYBNI

MODHUMITA GHOSH

Katedra Biotechnologii Roślin
Instytut Genetyki Leśnej i Hodowli Drzew
Kampus Leśny
R.S. Puram, Coimbatore – 641 002
Indie

*autor, do którego należy kierować korespondencję: tel.: +91422 2484123,
2484100, faks: +91422 2430549,
e-mail: ghoshm@icfre.org; modhumitaghosh@hotmail.com

Streszczenie

Peroksydazy są wszechobecne w roślinach. Forma ich ekspresji zależy od tkanki, stadium rozwojowego i wpływu środowiska. Są one związane z licznymi funkcjami fizjologicznymi, w tym z obroną przed patogenami i szkodnikami. Celem przedstawionej pracy było wyizolowanie i oczyszczenie peroksydazy z liści rośliny leczniczej *Andrographis paniculata*. Enzym był oczyszczany wieloetapowo i peroksydaza 35 kDa była oczyszczona jako jednorodna przy $pI=6,0$. W doświadczeniach prowadzonych *in vitro* metodą zahamowania wzrostu grzybni *Trichosporium vesiculosum* (patogen leśny) wykazano istotne zniekształcenia białek badanej grzybni.

Słowa kluczowe: białka przeciwgrzybicze, obrona, hamowanie wzrostu grzybni, oksydoreduktazy, białka związane z patogenezą