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Antifungal Properties of Haem Peroxidase from *Acorus calamus*

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• *Background and Aims* Plants have evolved a number of inducible defence mechanisms against pathogen attack, including synthesis of pathogenesis-related proteins. The aim of the study was to purify and characterize antifungal protein from leaves of *Acorus calamus*.
• *Methods* Leaf proteins from *A. calamus* were fractionated by cation exchange chromatography and gel filtration and the fraction inhibiting the hyphal extension of phytopathogens was characterized. The temperature stability and pH optima of the protein were determined and its presence was localized in the leaf tissues.
• *Key Results* The purified protein was identified as a class III haem peroxidase with a molecular weight of approx. 32 kDa and pI of 7·93. The temperature stability of the enzyme was observed from 5 °C to 60 °C with a temperature optimum of 36 °C. Maximum enzyme activity was registered at pH 5·5. The pH and temperature optima were corroborated with the antifungal activity of the enzyme. The enzyme was localized in the leaf epidermal cells and lumen tissues of xylem, characteristic of class III peroxidases. The toxic nature of the enzyme which inhibited hyphal growth was demonstrated against phytopathogens such as *Macrophomina phaseolina*, *Fusarium moniliforme* and *Trichosporium vesiculosum*. Microscopic observations revealed distortion in the hyphal structure with stunted growth, increased volume and extensive hyphal branching.
• *Conclusions* This study indicates that peroxidases may have a role to play in host defence by inhibiting the hyphal extension of invading pathogens.

Key words: *Acorus calamus*, haem peroxidase, antifungal, hyphal inhibition, thermal stability, localization, epidermal cells, xylem lumen.

INTRODUCTION

Plants have evolved a number of inducible defence mechanisms against pathogen attack. Some of the responses are constitutive and pathogen non-specific, but the majority of them are induced after recognition of the pathogen. Recognition results in the activation of a variety of defence responses, including rapid localized cell death (the hypersensitive response) (Hammond-Kosack and Jones, 1996), synthesis of pathogenesis-related (PR) proteins and induction of systemic acquired resistance (Schneider *et al.*, 1996; Selitrennikoff, 2001). Systemic acquired resistance is characterized by the activation of a broad spectrum of host defence responses, locally at the site of the initial pathogen attack and systemically in distal tissues, providing resistance against widely diverse organisms such as fungi, bacteria and viruses (Ryals *et al.*, 1996; Sticher *et al.*, 1997; Van Loon, 1999; Durrant and Dong, 2004).

Seventeen classes of antifungal proteins/PR proteins have been described, comprising four families of chitinases (Graham and Sticklen, 1994; Kasprzewska, 2003; Shenoy *et al.*, 2006), one each of 1,3-glucanases (Simmons, 1994; Leubner-Metzger and Meins, 1999; Saikia *et al.*, 2005), proteinase inhibitors (Mosolov *et al.*, 2001; Valueva and Mosolov, 2004), one specific peroxidase (Lagrimini *et al.*, 1987; Kawano, 2003), a PR-1 family with unknown biochemical properties (Niderman *et al.*, 1995; Rauscher *et al.*, 1999), the thaumatin-like PR-5 family (Van Loon, 1982; Trudel *et al*., 1998), the birch allergen Betv1-related PR-10 family (McGee *et al.*, 2001; Hashimoto *et al.*, 2004), defensins (Terras *et al.*, 1992; Thomma *et al.*, 2002), lipid-transfer proteins (García-Olmedo *et al.*, 1995; Regente and de la Canal, 2000), thionins (Bohlmann and Apel, 1991; Epple *et al.*, 1995; Peligrini and Franco, 2005) and other proteins including 2S storage albumins (Terras *et al.*, 1993; Barciszewski *et al.*, 2000) and ribosome inactivating proteins (RIPs) (Nielsen and Boston, 2001; Sharma *et al.*, 2004). The role of PR proteins, their classification, mechanism of action, their role in defence mechanisms and generation of transgenics with increased resistance have been extensively reviewed (Van Loon, 1985; Kitajima and Sato, 1999; Selitrennikoff, 2001; Punja, 2001; Edreva, 2005; De Lucca *et al.*, 2005).

In the present study, a constitutively expressed protein with antifungal property was purified from leaves of *Acorus calamus* and was characterized as a peroxidase belonging to the PR9 family of pathogenesis-related proteins.

MATERIALS AND METHODS

Plant material

Rhizomes of *Acorus calamus* were obtained from Munnar (Iddukki district) and the Tropical Botanical Garden and Research Institute, Palode, Kerala, India. The rhizomes were propagated and maintained in vermiculite (grade 3), coir pith and river sand (1 : 1 : 1) in shady conditions.

Fungal culture

The fungal isolates of *Fusarium moniliforme* (rice isolate), *Macrophomina phaseolina* (blackgram isolate)

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and *Trichosporium vesiculosum* (Panampally or Pan Kerala isolate and Salem or Sal, Tamil Nadu isolate) were maintained in potato dextrose agar medium.

Purification of antifungal protein

Twenty-five grams of young leaves from 25- to 30-d-old sprouting *A. calamus* were collected and washed thoroughly with distilled water. Leaves were homogenized in liquid nitrogen and extracted in 75 mL of extraction buffer (25 mM sodium phosphate, pH 7·0, 250 mM NaCl, 10 mM EDTA, 5 mM β-mercaptoethanol, 1 mM PMSF and 1·5 % PVPP). The extract was clarified through layers of cheesecloth and centrifuged twice at 8960 *g* for 20 min at 4 °C. The pellet was discarded and the supernatant was subjected to selective precipitation by adjusting the pH to 4·6 with glacial acetic acid. The precipitated protein was discarded after centrifugation at 8960 *g* for 10 min at 4 °C and the supernatant was passed through pre-packed desalting columns with an exclusion limit of 5000 Da (Bangalore Genei Ltd, India) for buffer exchange. The column was washed with five bed volumes of 50 mM sodium acetate buffer, pH 4·6. The protein sample was loaded and allowed to drain completely, followed by elution of the proteins using 50 mM sodium acetate buffer, pH 4·6.

The proteins present in the crude sample were further purified through cation exchange chromatography using a HiTrap SP FF column in an FPLC (fast protein liquid chromatography) system (GE Healthcare, Piscataway, NJ, USA). Prior to application of the protein sample, the column was washed with five column volumes of start buffer (50 mM sodium acetate buffer, pH 4·6) followed by activation with five column volumes of elution buffer (50 mM sodium acetate buffer, pH 4·6, + 1 M NaCl). The column was further equilibrated with ten column volumes of start buffer. The sample was injected into the column and washed with five column volumes of start buffer. Proteins were eluted with NaCl gradient of 50 mM sodium acetate pH 4·6 (0–1 M NaCl) with a flow rate of 1·0 mL min^{-1}. One-millilitre fractions were collected and the peak-giving fractions were pooled and dialysed. The proteins present in the fractions were precipitated with four volumes of ice-cold acetone after incubation for 20 min at –80 °C. The precipitated proteins were collected by centrifugation at 10 000 rpm for 15 min at 4 °C. The pellets were air dried and dissolved in 50 mM sodium phosphate buffer, pH 7·0. The precipitated protein was further purified by gel filtration using Superose 12 10/300 GL column in the FPLC system. The column was initially equilibrated with 50 mM sodium phosphate buffer, pH 7·0, prior to protein injection. The protein fractions were eluted using 50 mM sodium phosphate, pH 7·0, with 15 mM NaCl. One-millilitre fractions were collected and the peak-giving fractions were pooled, desalted and concentrated using a Microsep centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA) with a 3000-Da cut-off membrane. The protein concentration of each fraction was determined using Bradford's reagent (Sigma Aldrich Ltd, USA) as described by Bradford (1976).

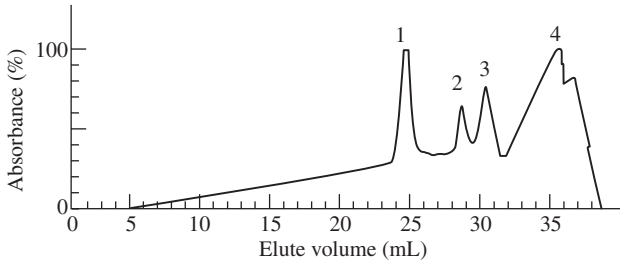

FIG. 1. Chromatogram of protein fraction through a Superose 12 10/300 GL column. Peaks from the eluted protein fractions are numbered 1–4.

Determination of antifungal activity of purified fraction

Hyphal extension inhibition assay. Agar blocks from actively growing plates of *F. moniliforme*, *M. phaseolina* and *T. vesiculosum* were inoculated in Petri dishes containing potato dextrose agar and incubated at 32 °C for 48 h. Subsequently, sterile Whatman filter paper discs (1 mm thickness) were placed towards the periphery of the growing hyphae and the purified protein fractions were added to the disc in varying concentrations. The plate was incubated for 24 h and observed for the appearance of a crescent inhibition zone.

Microtitre plate assay. Hyphal suspensions of *F. moniliforme*, *M. phaseolina* and *T. vesiculosum* were suspended in 80 µL of potato dextrose broth containing 0·1 % Triton X-100 in microtitre plates (12 × 8 wells). Twelve micrograms (in 20 µL) of the purified protein were added to the hyphal suspensions. A control containing 80 µL of hyphal suspension and 20 µL of sterile water was used and the plate was incubated at 30 °C. Observations were made for inhibition in hyphal extension and changes in hyphal morphology in both untreated and treated wells after 24 and 48 h using a Nikon UFX-DX microscope.

Determination of molecular weight and pI of the protein

The inhibiting protein fraction was resolved in a 12 % SDS–PAGE along with standard molecular weight markers (Bangalore Genei Ltd, India), as described by Laemmli (1970), and stained with Ezee blue gel stainer (Bangalore Genei Ltd). The pI value of the inhibiting protein fraction was determined by isoelectric focusing in IEF 3–9 precast gel using the Phast automated electrophoretic system (GE Healthcare). Initially the gel was prefocused for 10 min followed by loading of the purified protein (40 ng μL^{-1}) along with broad range (pH 3–10) of IEF markers (GE Healthcare). The separation was done at 2000 V, 3·5 W, 15 °C, 75 Vh; 200V, 2·5 mA, 3·5 W, 15 °C, 15 Vh and 2000 V, 2·5 mA, 3·5 W, 15 °C, 410 Vh and run was for 500 Vh. After completion of a run, the gel was stained overnight in Ezee Blue gel stainer followed by destaining with distilled water.

In gel tryptic digestion and peptide sequencing

The purified protein was separated on a 12 % SDS–PAGE and stained with Ezee Blue. Gel plugs containing protein spots were transferred to microtitre plates for

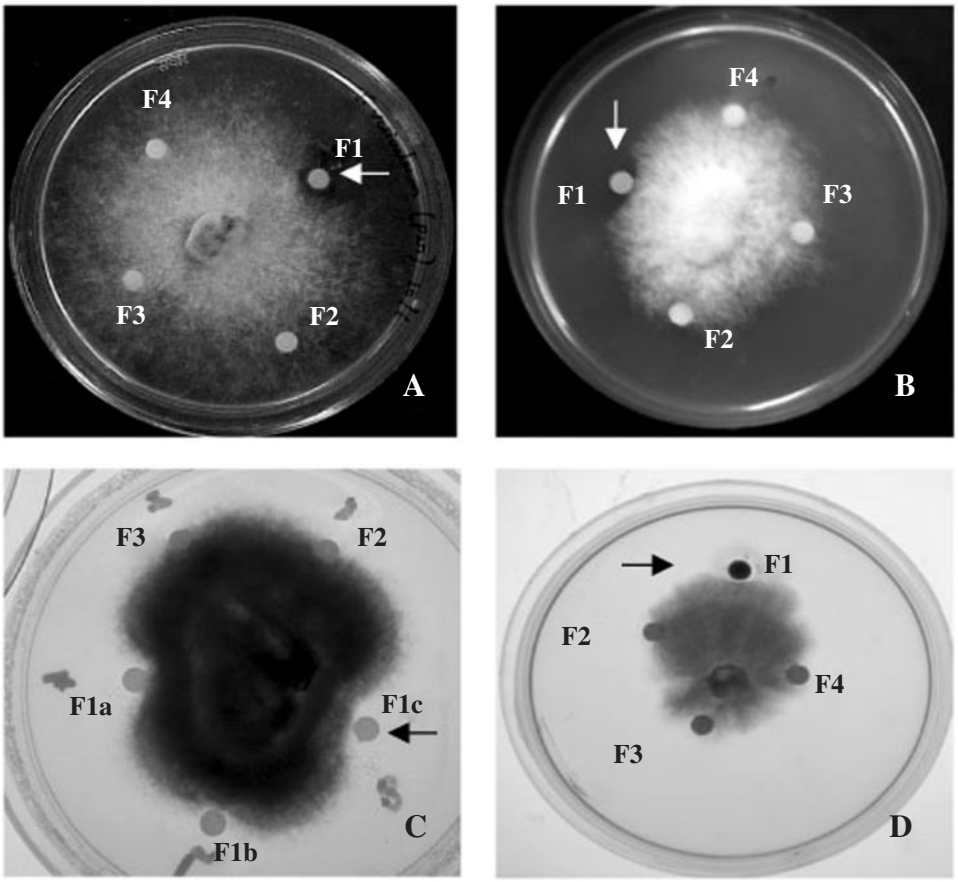

FIG. 2. *In vitro* hyphal extension inhibition assay against phytopathogens. (A) Hyphal extension inhibition assay in *Trichosporium vesiculosum* (Pan isolate). F1, F2, F3 and F4: purified protein from fractions 1, 2, 3 and 4, respectively, assayed against the pathogen. The arrow indicates the inhibition zone. (B) Hyphal extension inhibition assay in *Trichosporium vesiculosum* (Sal isolate). F1, F2, F3 and F4: purified protein from fractions 1, 2, 3 and 4, respectively, assayed against the pathogen. The arrow indicates the inhibition zone. (C) Hyphal extension inhibition assay in *Macrophomina phaseolina*. F1a, F1b and F1c: 4 µg, 6 µg and 10 µg of purified protein from fraction 1 assayed against the pathogen; F2 and F3: purified protein from fractions 2 and 3 assayed against the pathogen. The arrow indicates the inhibition zone. (D) Hyphal extension inhibition assay in *Fusarium moniliforme*. F1, F2, F3 and F4: purified protein from fractions 1, 2, 3 and 4 assayed against the pathogen. The arrow indicates the inhibition zone.

automated tryptic digestion and peptide extraction on a ProGest Workstation (Genomic Solutions, Ann Arbor, MI, USA) using the standard ProGest long trypsin protocol. Following digestion, the peptide extracts were lyophilized in a vacuum concentrator, re-suspended in 10 mL of 0·1 % formic acid and used for MS–MS analyses.

Mass spectroscopic peptide separation and sequencing was carried out on Applied Biosystems (CA, USA)QSTAR PULSARi™ quadrupole time of flight mass spectrometer coupled to an Amersham Ettan™ MDLC nano HPLC workstation (Applied Biosystems). Following digestion, concentrated peptide digest was loaded onto an LC Packings C18, 5 mm PepMap™ nano-precolumn, washed of salts with 400 mL of 0·5 % acetonitrile, 0·05 % TFA and eluted with linear gradient of 5 % acetonitrile, 0·05 % TFA to 65 % acetonitrile, 0·05 % TFA through a flow splitter onto an LC Packings C18, 3 mm PepMap™ nano-column for direct infusion at 200 nl min^{-1} through a nano-spray tip into the mass spectrometer.

TOFms spectra were collected between the mass range 100 and 2000 amu throughout the gradient elution, and precursor ion selection and product ion spectra were generated using Applied Biosystems BioAnalyst™ software's fully automated switching and acquisition procedures. Only multiple charged precursor ion species were selected for fragmentation and peptide sequencing.

Database searches and protein identification

For protein identification, all MS–MS product ion spectra generated from each sample were used in a MASCOT (www.matrixscience.com) database search of the NCNInr database of all available Viridiplantae sequences (Perkins *et al*., 1999). The similarity of the peptide sequence with existing sequences in the database was also determined using blastp at http://www.ncbi.nlm.nih.gov/BLAST (Altschul *et al.*, 1997).

Peroxidase activity staining

The protein sample was dissolved in loading buffer without SDS and thiol-reducing agents and separated in a 12 % SDS–PAGE without prior boiling as described by Christensen *et al.* (1998). Subsequent to electrophoresis, the gel was equilibrated in 20 mM sodium acetate

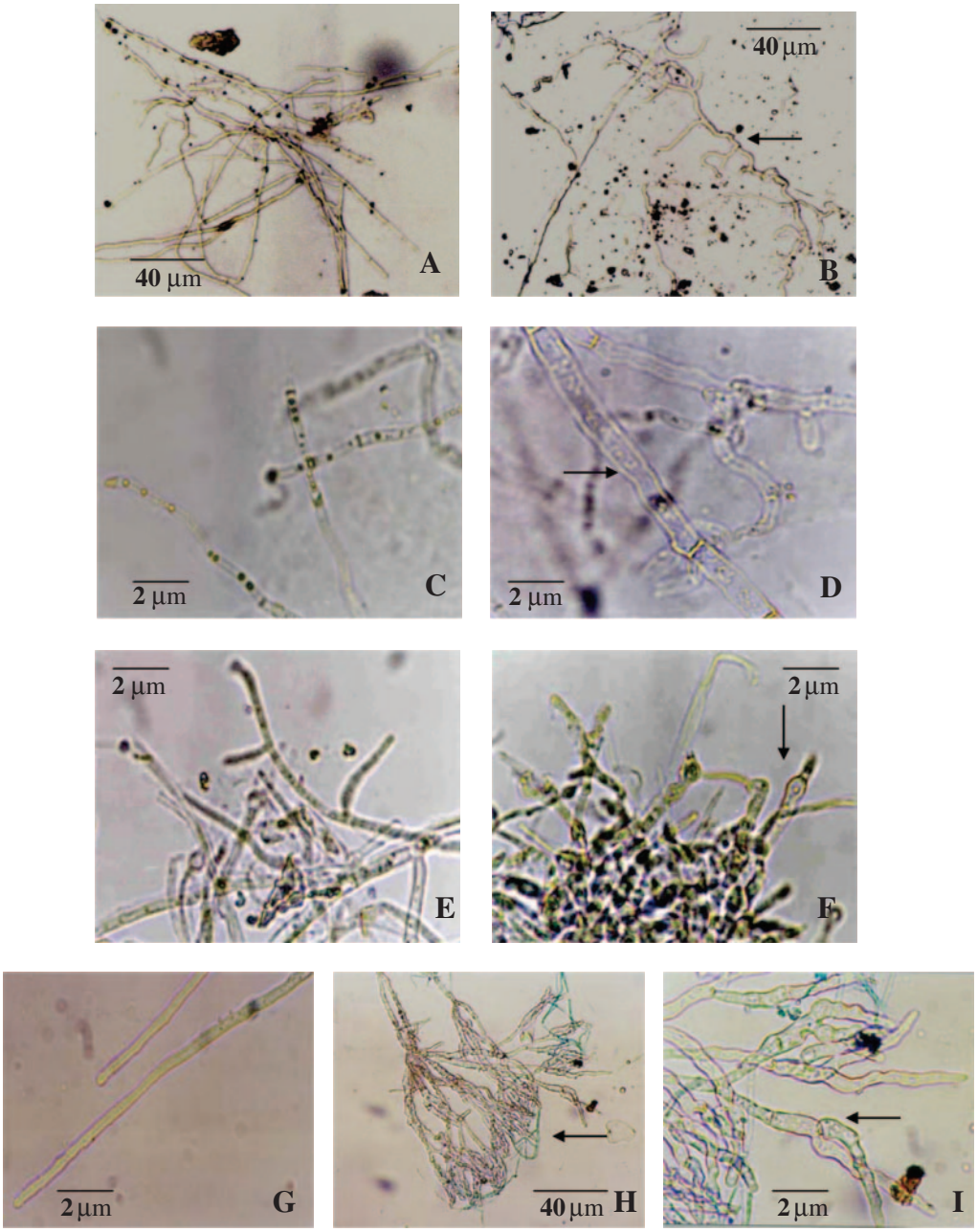

FIG. 3. *In vitro* hyphal morphology studies. (A) Hyphal morphology of *Trichosporium vesiculosum* (Pan isolate) in a control well. (B) Hyphal morphology of *Trichosporium vesiculosum* (Pan isolate) in a protein-treated well. The arrow indicates a distorted hypha. (C) Hyphal morphology of *Trichosporium vesiculosum* (Sal isolate) in a control well. (D) Hyphal morphology of *Trichosporium vesiculosum* (Sal isolate) in a protein-treated well. The arrow indicates a hypha with increased volume. (E) Hyphal morphology of *Macrophomina phaseolina* in a control well. (F) Hyphal morphology of *Macrophomina phaseolina* in a protein-treated well. The arrow indicates hyphal tip distortion. (G) Hyphal morphology of *Fusarium moniliforme* in control well. (H and I) Hyphal morphology of *Fusarium moniliforme* in a protein-treated well. The arrow indicates increased hyphal branching.

buffer, pH 5·0, followed by 30-min incubation in sodium acetate buffer containing 0·03 % H_2O_2 and 0·6 % (w/v) diaminobenzidine (DAB). The reaction was terminated by washing the gel in distilled water four or five times.

Determination of pH optima for peroxidase activity

The effect of pH on peroxidase activity was determined by estimating its activity in buffers ranging from pH 3·0 to 9·0. The enzyme activity was measured with 1 mM DAB as substrate and 0·03 % (w/v) H_2O_2 and the rate of change in A452 was monitored for 2 min as described by Christensen *et al.* (1998) with slight modifications. The buffers used in the assay included 200 mM acetate (pH 3·0–5·0) and 200 mM phosphate (pH 6·0–9·0).

Further, the above study was corroborated with the effect of pH on the antifungal nature of the enzyme. Hyphal mass of *Trichosporium vesiculosum* was incubated in potato

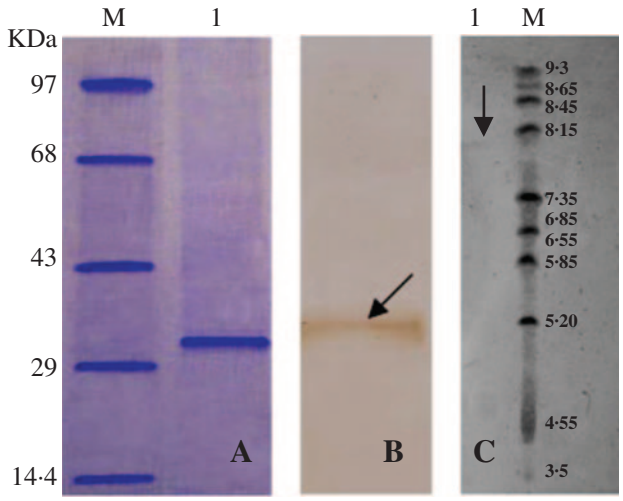

FIG. 4. (A) 12 % SDS–PAGE of purified protein from fraction 1. Lane M, molecular weight marker; lane 1, purified protein from fraction 1. (B) Peroxidase activity staining in 12 % SDS–PAGE. (C) Determination of the pI of the purified protein fraction. Lane M, IEF markers (pI 3·50, amyloglucosidase; pI 4·55, soybean trypsin inhibitor; pI 5·20, β-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; pI 9·30, trypsinogen). Lane 1, purified protein from fraction 1.

dextrose broth with a pH ranging from 4·0 to 9·0 in microtitre plates in triplicate. The fresh weight of the hyphae was determined at 0 h followed by addition of 10 µg of protein. Sterile water was added in control wells. The plate was incubated at 30 °C and the fresh weights of all the control and treated hyphae were recorded after 24 and 48 h.

Determination of temperature optima for peroxidase activity

The thermal stability of the enzyme was determined by incubating the protein sample for 30 min in 200 mM phosphate buffer, pH 6·0, at temperatures ranging from 5 °C to 80 °C. The peroxidase activity was determined spectrophotometrically at 452 nm using 1 mM DAB and 0·03 % (w/v) H_2O_2.

The effect of temperature on the antifungal nature of the enzyme was conducted as explained earlier. Hyphal mass of *T. vesiculosum* was incubated in antifungal assay media with or without the enzyme and incubated at 5, 10, 20, 30, 40 and 50 °C. The fresh weights of the hyphae at 0, 24 and 48 h were recorded. All experiments were conducted in triplicate.

Determination of peroxidase activity in leaf tissues of A. calamus

Leaf tissues of 30-d-old sprouting were sectioned manually (10–15 µm) and incubated in the dark for 20 min in 20 mM phosphate buffer, pH 6·0, containing 0·05 mM DAB and 0·01 % H_2O_2, as described by Dehon *et al*. (2002). Controls were maintained by incubating the sections in buffer or buffer with DAB or H_2O_2 and observations were made using a Nikon UFX-DX microscope.

RESULTS

Extraction and purification of antifungal protein

The total leaf protein extracted from *A. calamus* was purified through cation exchange chromatography and the peak-giving fractions were individually tested for their antifungal activity as described above (data not shown). The fraction showing the antifungal activity was further purified by gel filtration using a Superose 12 10/300 GL column. Four peak-giving fractions (Fig. 1) were obtained which were desalted and concentrated for further use.

Antifungal activity

All the four fractions purified were subjected to antifungal assay against the fungal pathogens, *T. vesiculosum* (both isolates), *M. phaseolina* and *F. moniliforme*. In the hyphal extension inhibition assay, an inhibition zone was observed in all the pathogens tested at varying concentrations, 8 µg for *T. vesiculosum* (Panampally isolate), 10 µg for *T. vesiculosum* (Salem isolate) and *M. phaseolina*, and 12 µg for *F. moniliforme* when tested with fraction 1, while the other three fractions did not inhibit the pathogens (Fig. 2).

The microtitre plate assay indicated that the purified protein affected the hyphal morphology causing stunted growth with abnormal swelling in both isolates of *T. vesiculosum* (Fig. 3A–D) and *M. phaseolina* (Fig. 3E, F). In *F. moniliforme*, an increased hyphal branching was observed along with stunted growth after 48 h of protein treatment (Fig. 3G–I).

Determination of molecular weight and pI of the antifungal protein

The purified protein from fraction 1 was separated on a 12 % SDS–PAGE and presence of a single band was observed at approx. 32 kDa (Fig. 4A). The pI of the purified protein was determined to be approx. 7·93 (Fig. 4C).

Peptide sequencing and identification of the protein

The purified protein was subjected to tryptic digestion followed by LC–MS–MS. The digest generated ten peptide fragments of which six were unique fragments with sequence coverage of 37 % with putative bacterial-induced peroxidase from *Oryza sativa* (japonica cultivar group) (accession number BAD61674). The peptides matched the full length of the sequence including N-terminal and C-terminal regions of the *Oryza* bacterial-induced peroxidase sequences. The blastp search also revealed the similarity of the peptide sequence with putative bacterial-induced peroxidases from rice with accession numbers BAD61674, BAD61677 and BAD61671, with similarity percentages of 49 %, 42 % and 41 %, respectively (Fig. 5).

Enzyme activity staining and determination of pH and temperature optima

The purified protein separated on PAGE was stained for peroxidase using DAB and H_2O_2, and a single band was

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BAD61674      1   MPAPPPLAAVSARRCSGPHRPPR 23
BAD61677      1   ----------------------- 23
BAD61671      1   ----------------------- 23
AcPOX             MPAPPPLAAVSARRCSGPHRPPR

BAD61674       80 RSCPATVSCADVLALAARDAVAMLSGPSWGVLLGRKDSLTAS IDMANKDLPNPKDSLAEL  139
BAD61677      123 RSCPATVSCADVLALAARDAVAMLGGPSWGVLLGRKDSLTAS IDMAKEDLPNPKDSLAEL  182
BAD61671      123 RSCPATVSCADVLALAARDAVAMLGGPSWGVLLGRKDSLAARMDMANKDLPRPTDSLAEL   182
AcPOX             -SCPATVSCADVLALAAR----------------------------------------

BAD61674      140 IRMFEKNGLDERDLTALSGAHTVGMAHDCKNYDDRIYSRVGQGGDSIDPSFAAQRRQECE  199
BAD61677      183 IRMFKEHDLDERDLTALSGAHTVGMAHDCKNYDDRIYSRVGQGGDSIDPSFAALRRQECE  242
BAD61671      183 IRMFKENNLDERDLTALSGAHTVGRTH SCEHYEERIYSLVGQGGDSIDPSFAAQRRQECE 242
AcPOX             ------NGLDERDLTALSGAHTVGMAHDCK---------VGQGGDSIDPSFAAQR ----

BAD61674      200 QKHGNATAPFDERTPAKFDNAYYIDLLARRGLLTSDQELYTQGCETGDLVKTYAMNGDVF 259
BAD61677      243 QKHDKATAPFDERTPAKFDNAYYVDLLARRGLLTSDQELYTQGCQTGDLVKTYAMNGDVF 302
BAD61671      243 QKHGNATAPFDERTPAKFDNAYYVDLLARRGLLTSDQELYTQGCETGDLVKTYAMNGDVF 302
AcPOX             ------------------ FDNAYYI DL L A R----------------------------

BAD61674      260 FADFVRAMVKMGNIRPKHWWTPAEVR  285
BAD61677      303 FADFTRAMVKMGNIRPKHWWTPAEVR  328
BAD61671      303 FADFARAMVKMGNIRPKHWWTPTEVR  328
AcPOX             ------AMVKMGNIRPKHWWTPAEVR
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FIG. 5. Similarity of peroxidase sequence from *A. calamus* (AcPOX) with putative bacterial-induced peroxidases from *Oryza sativa*.

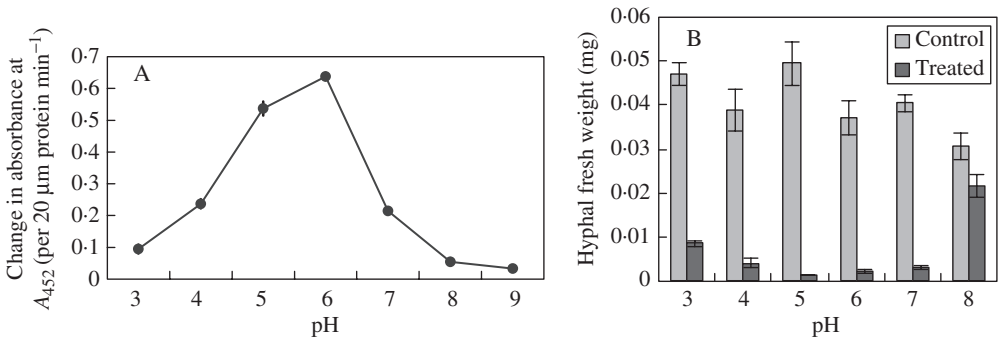

FIG. 6. Effect of pH on peroxidase and antifungal activity. (A) Effect of pH on peroxidase activity. (B) Effect of pH on antifungal activity of peroxidase.

observed (Fig. 4B). The pH and temperature sensitivity of the peroxidase was determined, wherein a continuous increase in activity was observed from pH 3 to pH 6, with a steep decrease in activity with a further increase in pH. The optimum pH for maximum enzyme activity was observed at 5·6 (Fig. 6A). The effect of pH on antifungal activity of the enzyme was determined by recording the change in fresh weight of hyphal mass after 48 h of incubation. Minimum growth of hyphal mass was observed at pH 5 and pH 6 corroborating with enzyme activity (Fig. 6B). Similarly, the temperature stability of the enzyme was determined and a continuous increase in activity was observed from 5 to 40 °C with maximum activity at 36 °C. A drastic decrease with almost 70 % loss in activity was observed at 70 °C. The antifungal activity of the enzyme was at its maximum from 10 °C to 40 °C with minimal hyphal growth at 30 °C (Fig. 7A, B).

Peroxidase activity in leaf tissues of A. calamus

Enzyme activity was demonstrated in the leaf tissues of *A. calamus*. The electron-dense brown stain of DAB was observed in the epidermal layers of the leaves and in the lumen of the xylem vessels. The control without H_2O_2 and DAB and with H_2O_2 alone did not develop any stain (Fig. 8A, B).

DISCUSSION

Acorus calamus (commonly known as 'sweet flag'), belonging to the family Araceae, is a perennial plant which grows in the wild in swamps and lakes and along rivers throughout the world. It is cultivated in many countries to satisfy the demand for its essential oil which is used for flavouring and in the perfumery and pharmaceutical industries. The present study describes the purification of peroxidase from leaves of *A. calamus* and its inhibitory effect on growth of fungal hyphae *in vitro.*

Peroxidases (EC 1·11·1·7) are a superfamily of enzymes that are ubiquitous in plants, fungi and vertebrates. Within the superfamily, three distantly related structural classes have been identified (Welinder, 1992). The structurally diverse secretary plant peroxidases (class III) are

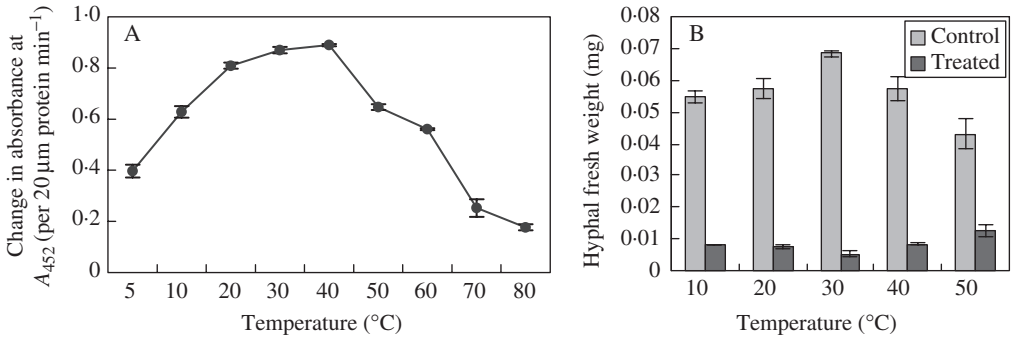

FIG. 7. Effect of temperature on peroxidase and antifungal activity. (A) Effect of temperature on peroxidase activity. (B) Effect of temperature on antifungal activity of peroxidase.

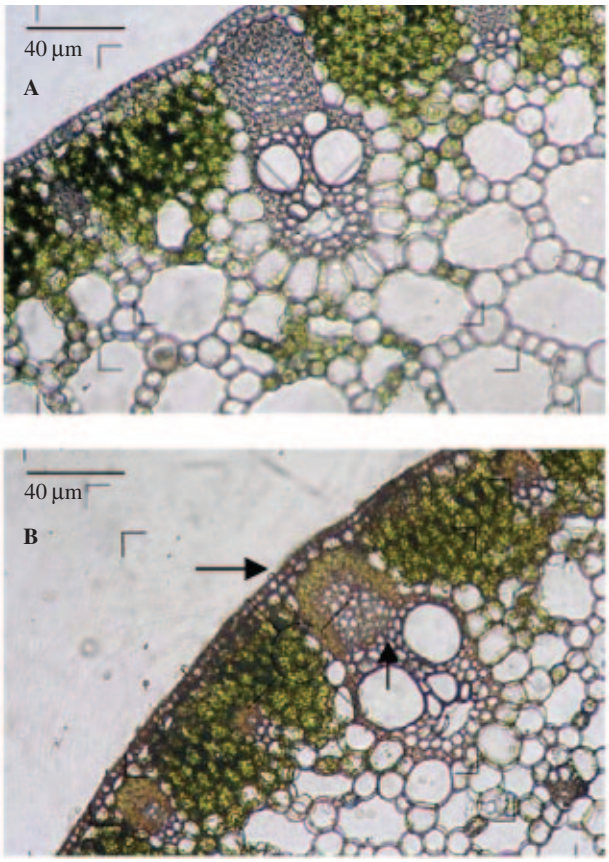

FIG. 8. Peroxidase activity in leaves of *A. calamus*. (A) Section of leaf tissue treated with H_2O_2. (B) Section of leaf tissue treated with DAB and H_2O_2. Arrows indicate the peroxidase activity in the epidermal cells and xylem lumen.

haem-containing enzymes which oxidize several substrates in the presence of H_2O_2 (Penel *et al*., 1992; Vianello *et al.*, 1997). The majority of them are *N*-glycosylated and are believed to be localized in the cell wall or vacuoles. They show a broad range of substrate specificity and high thermal stability (Welinder, 1992; Bernards *et al.*, 1999; Rodríguez *et al.*, 2002; Mika and Luthje, 2003; Carvalho *et al*., 2003). A dedicated database called PeroxiBase has been developed for class III peroxidases (Bakalovic *et al*., 2006). The thermal stability of peroxidase was also observed in the present study, wherein enzyme stability was demonstrated from 5 °C to 60 °C with an optimum temperature of 36 °C. The enzyme activity was observed in the epidermal tissues and xylem lumen of leaf tissues of *A. calamus.* This is characteristic of pathogen-induced peroxidases, which have been shown to localize in the cell walls and extracellular spaces in leaf mesophyll tissues and in vessel lumen and walls of xylem elements in rice, after infection with *X. oryzae* pv. *oryzae* (Young *et al*., 1995). Similarly, accumulation of a cationic peroxidase PO-C1 was observed in the xylem parenchyma and in vessel walls and lumen of rice (Hilaire *et al*., 2001) and *Betula pubescens* subsp. *czerepanovii* (Ruuhola and Yang, 2006).

Peroxidases have been shown to accumulate specifically during infection. Several roles have been attributed to the plant haem peroxidases in host–pathogen interactions (Moerschbacher, 1992; Chittoor *et al.*, 1999; Kristensen *et al.*, 1999). The possible defence-related functions are formation of structural barriers by polymerization of lignin and suberin (Hammerschmidt and Kuc, 1982; Espelie *et al*., 1986), cross-linking of wall protein (Bradley *et al*., 1992; Iiyama *et al*., 1994) and dimerization of antimicrobial phenols by peroxidase oxidative activity that are toxic to pathogens (Martinez *et al*., 2000). Extracellular peroxidases catalyse the generation of reactive oxygen species by oxidation of the plant hormone, indole-3-acetic acid, and defence-related compounds ,like salicylic acid, aromatic monoamines and chitooligosaccharides, in the presence of H_2O_2, causing oxidative burst, thereby limiting the pathogen invasion (Blee *et al*., 2001; Bolwell *et al*., 2002; Kawano, 2003).

In vitro studies have indicated that peroxidases in the presence of H_2O_2 inhibit the growth of fungi (Joseph *et al.*, 1998; Yang and Anderson, 1999). However, few reports have suggested the antifungal nature of specific peroxidases in the absence of H_2O_2. In wheat, a basic non-glycosylated haem peroxidase (WP1) was purified from kernel tissues that inhibited the germ tube elongation of pathogens (Caruso *et al*., 2001). Similarly, Ye and Ng (2002) isolated a novel peroxidase from French bean (a legume) which exhibited antifungal activity against fungal species like *Coprinus comatus*, *Mycospaerella*

arachidicola, *Fusarium oxysporum* and *Botrytis cinerea*. Joseph *et al.* (1998) reported a similar inhibitory role for peroxidases in *Hibiscus esculentus* and *Vigna sinensis* ssp. *sesquipedalis* against its pathogens, *Pseudocercospora abelmoschi* and *P. cruenta*. In the present study, the inhibitory effect of haem peroxidase, in the absence of H_2O_2, on the hyphal elongation of pathogens like *T. vesiculosum*, *F. moniliforme* and *M. phaseolina* was also demonstrated. This study indicates the role of peroxidase in first line defence against invading pathogens.

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