Isolation and characterization of a 30 kDa protein with antifungal activity from leaves of *Engelmannia pinnatifida*

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During the course of screening plants for novel antifungal activity, we found that a high-molecular-mass fraction of an extract from leaves of *Engelmannia pinnatifida* exhibited potent and broad-spectrum antifungal activity. In this study a 30 kDa protein from *E. pinnatifida* leaves was purified to homogeneity by ammonium sulphate precipitation, gel filtration, Mono-Q and C₁₈ reverse-phase column chromatographies. The purified protein showed potent antifungal activity against various plant

pathogens with as little as 50 ng. The N-terminal amino acid sequence of the purified protein was determined as XXTKFDFFTLALQXPAXF, where X indicates an unidentified residue. This sequence showed 35–50 % sequence identity with purified style glycoproteins associated with self-incompatibility from wild tomato, tobacco and petunia, a phosphate-starvation-induced ribonuclease from cultured tomato cells and the SIR 63.4 kDa protein from yeast.

INTRODUCTION

Plants do not have an immune system directly comparable with that of animals [1,2]. Thus, to protect themselves from infection by a variety of pathogens, plants have evolved a host of defence mechanisms [3–5]. In fungal infection, these mechanisms include the synthesis of low-molecular-mass inhibitory compounds such as phenols, melanins, tannins or phytoalexins and the accumulation of proteins with the capability of acting directly on fungi to inhibit growth [3–5]. These proteins, called pathogenesis-related (PR) proteins, are divided into five classes [5]. PR proteins classes 2 and 3 contain chitinases and β -1,3-glucanases respectively [5]. Class 1 contains related proteins found in a wide variety of plants with molecular masses of approx. 15 kDa that are induced on infection by tobacco mosaic virus [5]. Class 4 includes acidic proteins with molecular masses of 13–14.5 kDa that have sequence similarity with wound-induced proteins of potato [5]. The mechanism of action of PR proteins classes 1 and 4 remains unknown [5]. Class 5 PR proteins include a number of proteins called thaumatin-like proteins [5], owing to their high degree of sequence similarity with the sweet protein thaumatin from *Thaumatococcus daniellii*. In addition to these PR proteins induced by pathogen attack or stress, other families of plant antifungal protein have been identified. These include cysteine-rich small proteins, thionins, ribosome inactivating proteins, lipid-transfer proteins and chitin binding proteins [5–10].

Although many purified PR proteins are reported to be antifungal *in vitro* [3–18], and tobacco and canola expressing a bean chitinase gene had an increased ability to survive in soils infected with *Rhizoctonia solani* [19], little is known for certain about the role of PR proteins in plants. We are interested in studying this role and the application of PR proteins to the development of fungus-resistant crops through gene transfer. With this in mind, we have isolated and characterized antifungal chitinases and thaumatin-like proteins from *Arabidopsis thaliana*,

corn and flax seeds and *Diospyros texana* fruits [12–18]. Details of results from the screening as well as relationship of the antifungal agents in these plants will be reported elsewhere. During the course of screening plants for novel antifungal activity, we found that a high-molecular-mass fraction of an extract from leaves of *Engelmannia pinnatifida* exhibited potent and broad-spectrum antifungal activity against many agronomically important pathogenic fungi. We describe here the isolation and characterization of a 30 kDa antifungal protein from *E. pinnatifida* leaves. Sequence analysis of the purified protein showed that it has significant sequence similarity with purified style glycoproteins associated with self-incompatibility from wild tomato, tobacco and petunia [20–22], a phosphate-starvation-induced ribonuclease from cultured tomato cells [23] and the SIR 63.4 kDa protein from yeast [24].

EXPERIMENTAL

Materials

The leaves of *Engelmannia pinnatifida* Nutt. were collected from Texas through the Missouri Botanical Garden. *E. pinnatifida* (also called cut-leaved daisy or Engelmann's daisy) is a common plant (perennial) of the plains and prairies, closely resembles the sunflower, but has the daisy characteristic of closing the flower heads at night and opening them in bright sunlight. The rough, hairy plants grow 30–90 cm tall and are topped by broad clusters of showy yellow flower heads approx. 4 cm across. The 8–10 ray flowers are approx. 1.3 cm long and are indented at the tip. The erect to spreading stems form a round crown. Leaves are alternate and deeply cut, 8–15 cm long. The upper leaves often have coarse teeth [25]. The Mono-Q column (HR 5/5) and PD-10 columns were purchased from Pharmacia LKB Biotechnology and the Vydac C₁₈ reverse-phase column (4.6 mm $\times$ 250 mm) was from the Separation Group through P. J. Cobert Associates. Centriprep-10 concentrators with a molecular mass-cut-off of

Abbreviations used: PR proteins, pathogenesis-related proteins; TFA, trifluoroacetic acid.

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10 kDa were obtained from Amicon. Molecular-mass standards for SDS/PAGE were from Bio-Rad Laboratories. All plant pathogens, including *Fusarium oxysporum*, *Alternaria solani*, *Sclerotinia sclerotiorum*, *Gaeumannomyces graminis*, *Phytophthora megasperma* and *Phytophthora infestans*, were provided by the Ceregen Technology Group of Monsanto Company. The other reagents were of the highest grade commercially available.

Preparation of plant extracts for screening

Approx. 15 g of plant material was homogenized in 50 ml of 10 mM sodium acetate buffer, pH 5.0. After centrifugation the supernatant was collected, filtered through a 0.2 µm membrane and concentrated to approx. 2.5 ml with an Amicon Centriprep-10 concentrator. The concentrated solution was then passed over a PD-10 column equilibrated with the above buffer to remove any remaining low-molecular-mass molecules. The high-molecular-mass molecule fraction (3.5 ml) was used to screen for antifungal activities against various plant pathogens.

Purification of a 30 kDa antifungal protein

All purification procedures were performed at 4 °C unless otherwise noted. Briefly, 300 g of the leaves of *E. pinatifida* were homogenized in 500 ml of 10 mM sodium acetate buffer, pH 5.0. The precipitate from the extract was discarded after centrifugation, and solid ammonium sulphate was added to the supernatant to 80 % of saturation. After stirring overnight, the precipitate was collected by centrifugation, resuspended in 10 mM sodium acetate buffer, pH 5.0, and dialysed extensively against 10 mM Tris/HCl buffer, pH 8.0, with a 12 kDa dialysis membrane. The dialysed solution was then concentrated to approx. 25 ml with an Amicon Centriprep concentrator with 10 kDa molecular-mass cut-off. The concentrated solution was passed through a PD-10 gel filtration column equilibrated with 10 mM Tris/HCl buffer, pH 8.0, to remove any remaining small molecules. The high-molecular-mass fraction was then applied on to a Mono-Q column that had been equilibrated with 10 mM Tris/HCl buffer, pH 8.0. Proteins were eluted from the column with a gradient of NaCl (0–1.0 M) in 10 mM Tris/HCl buffer, pH 8.0, at a flow rate of 1 ml/min with a Waters Associates HPLC system, model 510. The active fractions were pooled and purified further on a Vydac C₁₈ reverse-phase column equilibrated with 0.1 % trifluoroacetic acid (TFA) in water. After a 5 min washing phase, proteins were eluted by a linear gradient of acetonitrile [0–80 % (v/v)] in 0.1 % TFA at a flow rate of 1 ml/min with a Waters Associates HPLC system, model 510. All fractions were collected, concentrated to dryness with a Savant concentrator system, redissolved in 0.5 ml of 10 mM sodium acetate buffer, pH 5.0, and assayed for antifungal activity. A fraction that was eluted at about 60 % acetonitrile showed strong activity. SDS/PAGE showed that this fraction contained a homogeneous protein with a molecular mass of approx. 30 kDa.

Assay of antifungal activity

Antifungal activity was determined under sterile conditions by using a hyphal extension-inhibition assay as described by Roberts and Selitrennikoff [26]. Fungal mycelia were harvested from actively growing fungal plates and placed in the centre of Petri dishes containing the nutrient agar used for maintenance of the fungus under test. After incubation of these dishes for 20–24 h at room temperature to allow for mycelial growth, sterile filter paper discs were placed on the agar surface in front of the advancing fungal mycelium and then 35 µl of the protein solution in 10 mM sodium acetate buffer, pH 5.0, was applied to each

disc. The plates were incubated at room temperature for 20 h and then photographed. In this manner, if the material being tested was antifungal, a crescent-shaped zone of inhibition was observed around the disc.

Other methods

Protein concentration was determined by the method of Bradford [27], with BSA as the standard. The purity and molecular mass of the purified protein were determined by SDS/PAGE [28] with silver staining. Protein samples were hydrolysed in 6 M HCl at 110 °C for 24 h under vacuum and analysed on a Beckman 6300 High Performance Analyzer. Automated Edman degradation was carried out on an Applied Biosystems 470A Protein Sequencer as described [29]. The databases searched for similarity were PIR Protein Version 45, SwissProt Version 31 and GenPept Version 91 [30–33].

RESULTS AND DISCUSSION

To obtain novel antifungal proteins, we recently initiated the screening of plants for antifungal activity. We found 14 extracts from various plant tissues, including seeds, stems, leaves, flowers and fruits, that exhibited antifungal activity against the agronomically important pathogens of wheat take-all (*G. graminis*), potato late blight (*P. infestans*), soybean root rot (*P. megasperma*), canola stem rot (*S. sclerotiorum*), potato wilt (*F. oxysporum*) and tomato early blight (*A. solani*). Details of results from the screening as well as relationship of the antifungal agents in these plants will be reported elsewhere. Although some plant extracts showed a broad spectrum of activity against many pathogens, few extracts displayed a very narrow spectrum of activity (results not shown). Because the plant extract might contain more than one antifungal protein, and each might show different activities against different pathogens, it is difficult to predict which extract contains the protein of interest. In this study, an extract from leaves of *E. pinatifida* was selected for further purification because it showed potent and broad-spectrum antifungal activity against many important pathogens under the assay conditions. This antifungal activity was observed in extracts from the leaves but not from other tissues. Furthermore the activity was found only from leaves of young but not mature plants. This was not unexpected, because there have been reports indicating that some PR proteins are expressed only in certain tissues of plants during development [3–6].

Figure 1 Fractionation of *E. pinatifida* extract by Mono-Q column

Plant extract was applied on to a Mono-Q column equilibrated with 10 mM Tris/HCl buffer, pH 8.0. Proteins were eluted with a gradient of NaCl (0–1 M) in 10 mM Tris/HCl buffer, pH 8.0, at a flow rate of 1 ml/min with a Waters Associates HPLC system, model 510. Proteins were detected by monitoring changes in the absorbance at 280 nm. The horizontal bar shows the position of active fractions.

Figure 2 Fractionation of active fractions from the Mono-Q column by C_{18} reverse-phase HPLC

Active fractions from the Mono-Q column were pooled and purified further on a Vydac C_{18} reverse-phase column equilibrated with 0.1% TFA in water. After a 5 min washing phase, proteins were eluted by a linear gradient of acetonitrile (0–80%) in 0.1% TFA at a flow rate of 1 ml/min with a Waters Associate HPLC system, model 510. Proteins were detected by monitoring changes in the absorbance at 280 nm.

Figure 3 SDS/PAGE of the purified protein

Lanes 1 and 3, molecular-mass standards; lane 2, active fraction after C_{18} reverse-phase HPLC.

For purification, an extract from 300 g of *E. pinnatifida* leaves was made and fractionated on a Mono-Q column (Figure 1). The active partly purified fraction from the Mono-Q column was then further purified on a Vydac C_{18} reverse-phase column (Figure 2). A fraction that eluted at approx. 60% acetonitrile showed strong antifungal activity. SDS/PAGE and subsequent N-terminal amino acid sequence analysis showed that this fraction contained a homogeneous protein with a molecular mass of approx. 30 kDa (Figure 3). From 300 g of *E. pinnatifida*

Table 1 Purification of a 30 kDa antifungal protein from *E. pinnatifida* leaves (300 g)

Because the hyphal extension-inhibition assay was used [26], it is difficult to report the antifungal activity in numerical terms. The activity of the protein in this work was determined by the distance from the centre of the disc to the growth of the fungi. Active (+), moderately active (++), strongly active (+++) and very strongly active (++++) denote distances less than 1 mm, between 1 and 3 mm, between 3 and 6 mm and more than 6 mm respectively. See the Experimental section for details of the assay.

	Volume (ml)	Protein (mg)*	Antifungal activity against <i>G. graminis</i>
Extract	35.0	336.0	+
Mono-Q column	52.0	19.4	++
C_{18} HPLC	1.0	0.035†	++++

* Protein concentration was determined by the Bradford method [27] with BSA as the standard.

† Protein concentration was determined by amino acid analysis.

leaves, approx. 35 μ g of the purified protein was obtained as calculated from amino acid analysis (Table 1). The purified protein showed potent antifungal activity against the agronomically important pathogens potato late blight (*P. infestans*) and wheat take-all (*G. graminis*) with as little as 50 ng of protein per disc (Figure 4 and Table 2). It also strongly inhibited the growth of other important plant pathogens such as *P. megasperma* and *S. sclerotiorum* (Table 2). The purified protein, however, had no effect on the growth of the human pathogen *Candida albicans* (Table 2). Similar to many purified PR proteins [9], the purified 30 kDa antifungal protein is relatively soluble at low pH and resistant to heat treatment and protease digestion (results not shown). It also showed characteristics typical of a hydrophobic protein, as judged from its elution pattern on a C_{18} reverse-phase column (Figure 2) and its high content of hydro-

Figure 4 Antifungal activities *in vitro* of the purified protein against *P. infestans* (A) and *G. graminis* (B)

Approx. 50 ng of the purified protein in 35 μ l of 10 mM sodium acetate buffer, pH 5.0, was applied on to the discs numbered 1. For controls, 35 μ l of the above buffer and 35 μ l of water were tested on discs numbered 2 and 3 respectively. See the Experimental section for details of the assay.

Table 2 Antifungal activity of the purified protein against various fungal pathogens

About 50 ng of purified protein in 35 μ l of 10 mM sodium acetate buffer, pH 5.0, was used for determination of antifungal activity. See Table 1 for a description of the symbols used.

Tested fungal pathogens	Antifungal activity
<i>Fusarium oxysporum</i>	+++
<i>Alternaria solani</i>	+++
<i>Sclerotinia sclerotiorum</i>	+++
<i>Gaeumannomyces graminis</i>	+++
<i>Phytophthora megasperma</i>	+++
<i>Phytophthora infestans</i>	+++
<i>Candida albicans</i>	Not active

phobic amino acid residues such as tyrosine, valine, phenylalanine, isoleucine and leucine in its amino acid composition (Table 3).

To gain more insight into the structure–function relationship, the purified protein was subjected to sequence analysis and its N-terminal amino acid sequence was determined (Figure 5). Of the first 18 residues, 6 were either phenylalanine or leucine, which agreed with the hydrophobic characteristic of the protein as described. Results from a computer search [30–33], based on an overlap of 14 residues, for similarity with other protein sequences showed that the above sequence had no significant similarity with any antifungal proteins published so far. Thus the purified 30 kDa antifungal protein might be a representative of a novel

Table 3 Amino acid composition of 30 kDa antifungal protein from *E. pinnatifida* leaves

The results are ratios of amino acids recovered after hydrolysis *in vacuo* with 6 M HCl at 110 °C for 24 h. Numbers in parentheses represent the closest integer to the analytical values shown. Values are not corrected for the destruction of threonine and serine. Abbreviation: N.D., not determined.

Amino acid	Number of residues
Asx	22.59 (23)
Thr	16.24 (16)
Ser	23.94 (24)
Glx	30.80 (31)
Pro	14.56 (15)
Gly	34.97 (35)
Ala	15.23 (15)
Cys	7.53 (8)
Val	15.56 (16)
Met	0.00 (0)
Ile	12.37 (12)
Leu	20.91 (21)
Tyr	15.23 (15)
Phe	16.24 (16)
Trp	N.D.
His	7.70 (8)
Lys	16.74 (17)
Arg	9.80 (10)
Total residues	282

30 kDa protein	X-X-T-K-F-D-F-F-T-L-A-L-Q-X-P-A-X-F
Yeast SIR 63.4 kDa protein	K-F-D-F-F-G-F-A-L-C-S-A-G-L-V 50%
Tomato S ₃	D-F-D-Y-L-Q-L-V-L-Q-X-P-R-S-F 50%
Tomato S ₁	Y-F-E-Y-L-Q-L-V-L-Q-X-P-T-T-F 50%
Petunia S ₂	A-N-F-D-Y-F-Q-L-V-L-T-W-P-S-S-F 50%
Petunia S ₃	A-N-F-D-Y-F-Q-L-V-L-T-W-P-A-S-F 50%
Tobacco S ₃	A-F-E-Y-M-Q-L-V-L-Q-W-P-A-A-F 50%
Tobacco S ₂	A-F-E-Y-M-Q-L-V-L-T-W-P-I-T-F 43%
Tobacco S ₆	A-F-E-Y-M-Q-L-V-L-Q-W-P-T-A-F 43%
Tobacco S ₂	D-F-D-Y-M-Q-L-V-L-T-X-P-A-S-F 43%
Tobacco S ₁₁	D-F-E-Y-L-Q-L-V-L-T-W-P-A-S-F 43%
Tomato ribonuclease	A-K-D-F-D-F-F-Y-F-V-Q-W-P-G-S-Y 35%
Consensus	. . . F

Figure 5 Amino acid sequence of the purified 30 kDa protein in comparison with those of style glycoproteins from wild tomato [20], tobacco [20,21], petunia [22], a ribonuclease from cultured tomato cells [23] and the SIR 63.4 kDa protein from yeast [24]

X denotes an unidentified amino acid residue. The complete proteins from the Protein/GenBank were scanned. The databases searched were PIR Protein Version 45, SwissProt Version 31 and GenPept Version 91 [30–33]. The percentage identity in the sequence comparison changed depending on the number of residues of overlap during comparison. The results shown here are based on 14 amino acids overlap.

class of antifungal proteins. The above sequence, however, exhibited 43–50% sequence identity with the N-terminal sequences of a family of style proteins associated with self-incompatibility from wild tomato, tobacco and petunia [20–22]. The purified protein also showed 50% sequence identity with the yeast SIR 63.4 kDa protein [24] (Figure 5). In addition, although the purified 30 kDa antifungal protein showed no ribonuclease activity, it has 35% sequence identity with a phosphate-starvation-induced ribonuclease from cultured tomato cells [23]. It is worth noting that tobacco style glycoproteins S₂, S₃ and S₆ have been shown to have ribonuclease activity [34]. However, no antifungal activity of the style glycoproteins, plant ribonucleases or other ribonucleases has been reported. It is also worth noting

that, during preparation of this manuscript, Moiseyev et al. [35] reported the isolation of an 18 kDa ribonuclease from a callus cell culture of *Panax ginseng* C. A. Mey strain R1 that has 60–70% sequence identity with two intracellular PR proteins from parsley [36,37].

In summary, the results in this study provide evidence for the existence of a novel class of antifungal proteins that show sequence similarity with plant ribonucleases, plant style proteins associated with self-incompatibility and the yeast SIR 63.4 kDa protein. Since the purified protein exhibited potent and broad spectrum of antifungal activity, it may play a role in protecting plants against attack from pathogens.

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