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An antifungal protein from ginger rhizomes

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Abstract

There are very few reports on antifungal proteins from rhizomes and there is none from the family of Zingiberaceae. An antifungal protein with a novel N-terminal sequence was isolated from ginger rhizomes utilizing a protocol that involved ion exchange chromatography on DEAE–cellulose, affinity chromatography on Affi-gel blue gel, and fast protein liquid chromatography on Superdex 75. The protein was unadsorbed on DEAE–cellulose and adsorbed on Affi-gel blue gel. It exhibited an apparent molecular mass of 32 kDa and exerted antifungal activity toward various fungi including *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora piricola*.

Keywords: Ginger; Rhizome; Antifungal protein; Purification

Animals and plants produce an array of defensive molecules including antimicrobial proteins and peptides [2,5–7,9,13,24,28–30,32,33]. One class of antimicrobial proteins and peptides is comprised of antifungal proteins and peptides, which is an assembly of structurally diverse molecules such as thaumatin-like proteins [7–10], chitinases and chitinase-like proteins [1–6], ribonucleases [12–14], protease inhibitors [11], ribosome inactivating proteins [15,16], glucanases [17], embryo-abundant proteins [18], cyclophilin-like proteins [19], allergen-like proteins [20], deoxyribonucleases [21], defensin-like peptides [22], and miraculin-like proteins [23]. Antifungal proteins and peptides play a putative protective role against the devastating effects of fungal attacks.

Ginger is widely used in cooking. Zingiberol, zingiberene, bisabolene, α-curcumene, linalool, cineole, gingerol, and gingerone are organic compounds present in ginger [24], but little is known about its protein constituents. The purpose of this study was to isolate an antifungal protein from ginger and compare its characteristics with those of previously described antifungal proteins in order to ascertain if it is a novel antifungal compound.

Materials and methods

Isolation of antifungal protein. Fresh ginger (*Zingiber officinalis*) rhizomes (2 kg) were obtained from a local market. They were first extracted with distilled water (3.5 ml/grhizome) in a Waring blender. After centrifugation (13,000*g*, 20 min), the supernatant was removed. Tris–HCl buffer (pH 7.2) was added to the supernatant until the concentration of Tris attained 10 mM. The supernatant was then applied to a DEAE–cellulose (Sigma) column (5 × 20 cm) in 10 mM Tris–HCl buffer (pH 7.0). The column was first eluted with the buffer to obtain unadsorbed proteins (fraction D1) and subsequently with 0.8 M NaCl in Tris–HCl buffer to desorb adsorbed proteins (fraction D2). Fraction D1 was subjected to affinity chromatography on an Affi-gel blue gel (Bio-Rad) column (2.5 × 20 cm). The column was eluted with 10 mM Tris–HCl buffer (pH 7.0) to obtain the unadsorbed fraction B1 and then with a linear concentration gradient (0–1.5 M) of NaCl to obtain adsorbed fractions B2 and B3. Fraction B3 was subsequently chromatographed on a Superdex 75 HR 10/30 column

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(Amersham Biosciences) in 0.2 M NH_4HCO_3 (pH 8.5) using an AKTA Purifier (Amersham Biosciences) to yield three peaks SU1, SU2, and SU3. SU2 represented the purified antifungal protein.

Electrophoresis, molecular mass determination, and N-terminal sequence analysis. The purified ginger antifungal protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [25]. Gel filtration on an FPLC-Superose 12 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass of the protein. The N-terminal sequence of the protein was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [26].

Assay of antifungal activity. The assay of the purified protein for antifungal activity toward *Botrytis cinerea*, *Physalospora piricola*, *Mycosphaerella arachidicola*, and *Fusarium oxysporum* was carried out in 100 × 15 mm petri plates containing 10 ml potato dextrose agar (Sigma). After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot of a solution of ginger antifungal protein was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [3].

To determine the IC_{50} value for the antifungal activity of ginger antifungal protein, three doses of the protein were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly, and poured into three separate small petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was added. Buffer only without ginger antifungal protein served as a control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth was determined by calculating the % reduction in area of mycelial colony [27].

Assay of hemagglutinating activity. In the assay for lectin (hemagglutinating) activity, a serial twofold dilution of a solution of ginger antifungal protein in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of rabbit red blood cells in phosphate-buffered saline (pH 7.2) at 20 °C. The results were observed after about 1 h when the blank had fully sedimented, to see if the red blood cells had agglutinated. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg protein [28].

Assay of ribonuclease activity. Ginger antifungal protein was assayed for RNase activity toward tRNA by determining the generation of acid-soluble, UV-absorbing species with the method of Wang and Ng [29]. The protein was incubated with 200 μg yeast tRNA in 150 μl of 100 mM Mes (pH 6) at 37 °C for 1 h. The reaction was terminated by introduction of 350 μl of ice-cold 3.4% (v/v) perchloric acid. After leaving on ice for 15 min, the sample was centrifuged (15,000*g*, 15 min) at 4 °C. The OD_{260} of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in OD_{260} of one per min in the acid-soluble fraction per ml of reaction mixture under the specified condition.

Assay of protease activity. In the assay for protease activity, a solution of casein (Sigma), which was used as substrate, was freshly prepared as follows. To 2 g casein, 10 ml distilled water, and 10 ml of 0.2 M NaOH were added. After addition of another 60 ml distilled water, the mixture was stirred to make a solution. The pH of the solution was adjusted to pH 7.5 with HCl and the solution was heated at 90 °C for 15 min before cooling down and dilution with 100 ml of 100 mM Tris–HCl (pH 8) buffer containing 40 mM $CaCl_2$. The precipitate was removed and the resulting solution was used. The tested sample (ginger antifungal protein) or trypsin solution (positive control) (50 μl) was mixed with 350 μl of the above mentioned casein solution. After 25 min, 1 ml of 4% (w/v) trichloroacetic acid was added. The reaction mixture was allowed to stand at room temperature for 30 min before centrifugation for 15 min at 15,000*g*. The absorbance of the supernatant, which reflects the amount of casein fragments produced by proteolytic action, was read at 280 nm against water as blank.

Assay of protease inhibitory activity. The assay was conducted, as described above, for the protease assay using trypsin as enzyme. The purified ginger antifungal protein was tested for its ability to inhibit trypsin in 50 mM Tris–HCl buffer (pH 8) after incubation at 25 °C for 30 min.

Results

Ion exchange chromatography of ginger rhizome extract on DEAE–cellulose produced an unadsorbed fraction D1 with antifungal activity and an adsorbed fraction devoid of antifungal activity (data not shown). Affinity chromatography of fraction D1 on Affi-gel blue gel yielded an unadsorbed fraction and two adsorbed fractions B2 and B3. Antifungal activity was located only in fraction B2 (Fig. 1). Fraction B2 was resolved on Superdex 75 into three peaks, SU1, SU2, and SU3 (Fig. 2). SU2 appeared as a single band with a molecular mass of 32 kDa in SDS–PAGE (Fig. 3) and demonstrated the same molecular mass in gel filtration. The N-terminal sequence of ginger antifungal protein is presented in Table 1. It shows some similarity to a partial sequence of a homeobox protein. The purification of the protein is given in Table 2.

The antifungal activity of SU2 (designated ginger antifungal protein) toward *B. cinerea*, *F. oxysporum*, *M. arachidicola*, and *P. piricola* was observed. Its activity was the weakest toward *M. arachidicola* and the strongest toward *P. piricola* (Figs. 4–7). The IC_{50} values of the antifungal activity of ginger antifungal protein toward *B. cinerea*, *F. oxysporum*, *M. arachidicola*, and *P. piricola*

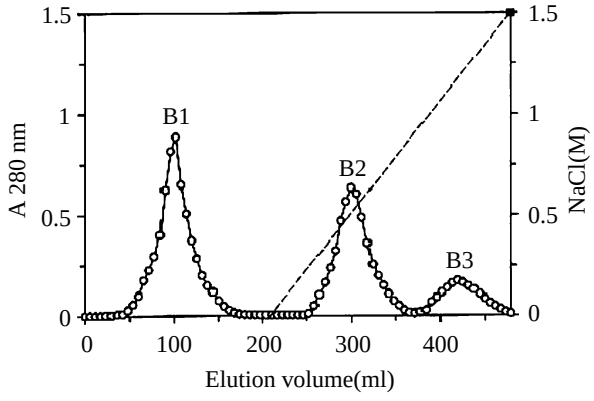

Fig. 1. Affinity chromatography on an Affi-gel blue gel column (2.5 × 20 cm). Sample, fraction D1 unadsorbed on DEAE–cellulose. Buffer, 10 mM Tris–HCl buffer (pH 7.0) for eluting fraction B1 and a linear concentration gradient (0–1.5 M) of NaCl in 10 mM Tris–HCl buffer (pH 7.0) for eluting fractions B2 and B3.

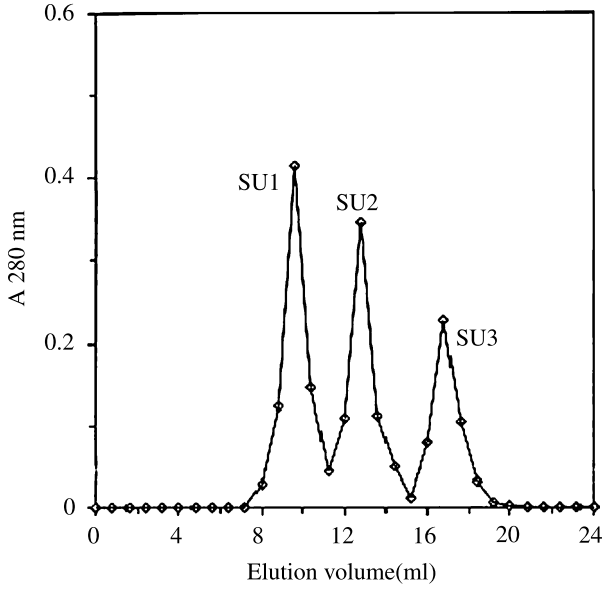

Fig. 2. Fast protein liquid chromatography-gel filtration of fraction B2 on a Superdex 75 HR 10/30 column using an AKTA Purifier (Amersham Biosciences) system. Buffer, 02 M NH_4HCO_3 (pH 8.5). Flow rate, 0.4 ml/min. Fraction size, 0.8 ml.

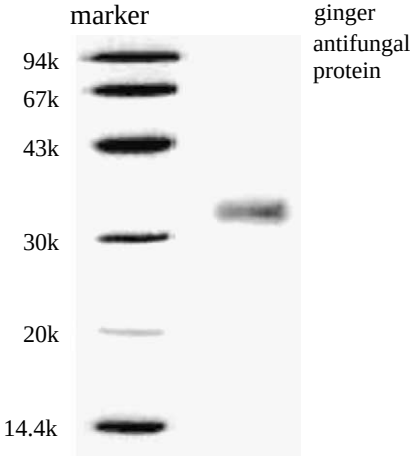

Fig. 3. SDS–PAGE of ginger antifungal protein.

were, respectively, 27.4, 24.7, 36.3, and 13.1 μM. Ginger antifungal protein did not exhibit protease, protease inhibitory, lectin or ribonuclease activity at a dose of 100, 50, and 10 μg, respectively (data not shown).

Table 2
Antifungal activities and yields at various stages of purification of ginger antifungal protein from 2 kg ginger rhizomes

Chromatographic fraction	Antifungal activity toward *P. piricola* (IC_{50})	Yield (mg)	Purification
Crude extract	8.53 mg/ml	2510	—
D1	3.75 mg/ml	527	2.3
B2	1.34 mg/ml	134.6	2.8
SU2 (ginger antifungal protein)	13.1 μM	26.4	3.2

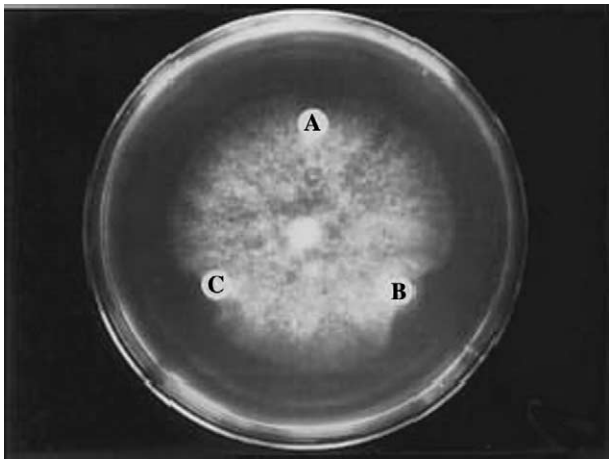

Fig. 4. Antifungal activity of ginger antifungal protein toward *B. cinerea*. (A) Control (20 μl of 50 mM Mes buffer; pH 6.0); (B) 160 μg ginger antifungal protein in 20 μl Mes buffer, and (C) 32 μg ginger antifungal protein in 20 μl Mes buffer.

Discussion

The present report constitutes the first concerning the isolation of an antifungal protein from family Zingiberaceae. The antifungal protein purified from ginger rhizomes is capable of exerting an inhibitory effect on mycelial growth in several fungal species. Its antifungal potency is similar to those reported for other antifungal proteins. Some antifungal proteins, such as ascalin from shallot bulbs and deoxyribonuclease from asparagus seeds, are able to inhibit only one fungal species, namely *B. cinerea*, out of the several species tested [21,30].

Ginger antifungal protein is devoid of hemagglutinating activity indicating that it is not a lectin. This is noteworthy since some lectins, such as potato lectin [2] and

Table 1
N-terminal sequence of ginger antifungal protein (results of BLAST search)

Protein	Residue No.	Amino acid sequence	Residue No.	Protein length (No. of amino acids)
Ginger antifungal protein	1	NGPAAQAA-------ENNLA	13	
Homebox protein CHX10	289	GPDAQAAISQEELRENSIA	307	361

-, space created to maximize sequence similarity.
Identical corresponding amino acid residues are underlined.
Residue No. 1 and residue No. 13 refer to N and A being the 1st and 13th amino acids in ginger antifungal protein.

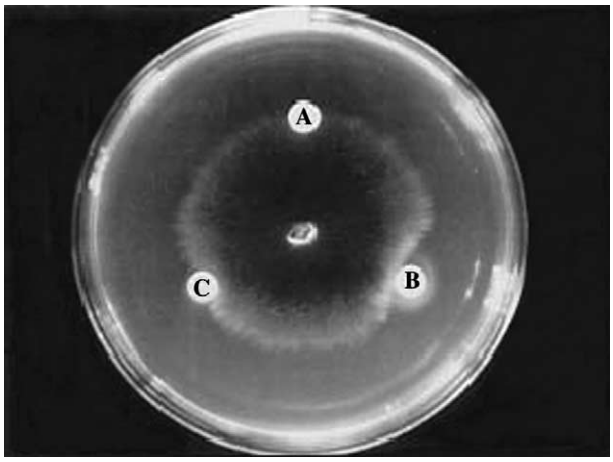

Fig. 5. Antifungal activity of ginger antifungal protein toward *F. oxysporum*. (A) Control (20 μl of 50 mM Mes buffer, pH 6.0), (B) 160 μg ginger antifungal protein in 20 μl Mes buffer, and (C) 32 μg ginger antifungal protein in 20 μl Mes buffer.

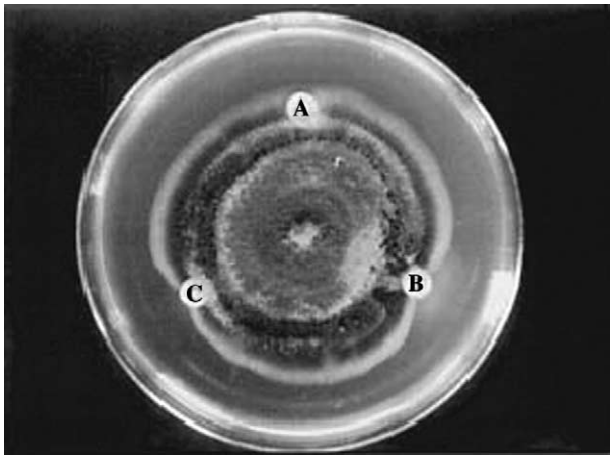

Fig. 6. Antifungal activity of ginger antifungal protein toward *M. arachidicola*. (A) Control (20 μl of 50 mM Mes buffer, pH 6.0), (B) 160 μg ginger antifungal protein in 20 μl Mes buffer, and (C) 32 μg ginger antifungal protein in 20 μl Mes buffer.

red kidney bean lectin [31], exhibit antifungal activity. Antifungal proteins from American ginseng [14], Chinese ginseng [13], and sanchi ginseng [12] exhibit ribonuclease activity. Ginger antifungal protein is also without proteolytic activity, or protease inhibitory activity although some protease inhibitors display antifungal activity [11].

Ginger antifungal protein is similar in molecular mass to a number of previously isolated antifungal proteins including chitinases from different leguminous species [5,6], thaumatin-like proteins from kiwi fruits [9,10], and French beans [32].

The chromatographic behavior of ginger antifungal protein on DEAE–cellulose and Affi-gel blue gel resembles those of many other antifungal proteins [13,14,18,19,27]. In the case of ginger antifungal protein,

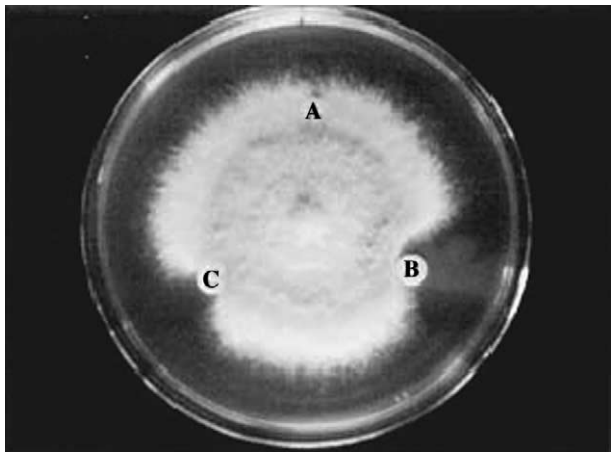

Fig. 7. Antifungal activity of ginger antifungal protein toward *P. piricola*. (A) Control (20 μl of 50 mM Mes buffer, pH 6.0), (B) 160 μg ginger antifungal protein in 20 μl Mes buffer, and (C) 32 μg ginger antifungal protein in 20 μl Mes buffer.

gel filtration on Superdex 75, following the step of affinity chromatography on Affi-gel blue gel is sufficient to obtain the antifungal protein in a purified state. As regards some of the other antifungal proteins, an additional chromatographic step on a CM-ion exchanger prior to the gel filtration step is required for complete purification [14,18].

The N-terminal sequence of ginger antifungal protein is not found in the sequence of any known antifungal protein. Ginger antifungal protein shows sequence similarity to a portion of a homeobox protein [33]. In fungi, homeobox genes are involved in hyphal formation [34,35]. This structural feature may be related to the suppressive action of ginger antifungal protein on hyphal growth.

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