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A thaumatin-like antifungal protein from the emperor banana

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ABSTRACT

A 20-kDa protein with substantial N-terminal sequence homology to thaumatin-like proteins was isolated from ripe fruits of the emperor banana, *Musa basjoo* cv. 'emperor banana'. The isolation procedure entailed $(NH_4)_2SO_4$ precipitation, ion exchange chromatography on DEAE-cellulose, and affinity chromatography on Affi-gel blue gel. The thaumatin-like protein inhibited mycelial growth in *Fusarium oxysporum* and *Mycosphaerella arachidicola*. However, it did not affect the mitogenic response of murine splenocytes or [methyl-^{3}H] thymidine incorporation by tumor cells. The activity of HIV-1 reverse transcriptase was slightly inhibited.

1. Introduction

Antifungal proteins and peptides have drawn the attention of a large number of researchers by virtue of their potential value in protecting economically important crops from fungal attack. It is well documented that there are many types of antifungal proteins and peptides with different amino acid sequences and antifungal potencies. They include thaumatin-like proteins [4,8,9,21–23,30,39,45], protease inhibitors [1,2,12,19], glucanases [27], chitinases [7,27], chitinase-like proteins [16,46], cyclophilin-like proteins [41], allergen-like proteins [40], miraculin-like proteins [47], lectins [7,44], peroxidases [42], ribosome inactivating proteins [17], defensins [34–38], lipid transfer proteins [33], and novel proteins and peptides [18,24].

Thaumatin-like proteins (TLPs) are antifungal proteins with an amino acid sequence highly homologous to that of the sweet protein thaumatin from the West African plant *Thaumatococcus danielli* [25]. Interestingly, despite the structural similarity between TLPs and thaumatin, TLPs are not sweet in taste whereas thaumatin does not exhibit antifungal activity [25,45].

TLPs are produced by a range of plants including the dicots grape [23] and tomato [21,22], the monocots barley, wheat, oat and sorghum [9,26], and fungi [14]. Their molecular masses lie within the range of 21–25.8 kDa.

The emperor banana is a special cultivar of banana, characterized by a smaller and less bright yellow cylindrical fruit with a smooth peel compared with much larger fruits with ridged skin from the regular banana (*Musa acceminata*). Recently, a lectin has been purified from emperor banana [38]. It would thus be interesting to isolate another protein from the emperor banana.

The purification and characterization of a TLP from emperor banana are described herein.

2. Materials and methods

2.1. Isolation of antifungal peptide

Emperor bananas (2 kg) were extracted with 2000 ml distilled water and then centrifuged. Ammonium sulfate was added to the supernatant to 20% saturation. After centrifugation,

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$(NH_4)_2SO_4$ was added to the resulting supernatant to 80% saturation. Tris–HCl buffer (pH 7.3) was added to the supernatant obtained after centrifugation of the extract until the concentration of Tris attained 20 mM. It was subjected to affinity chromatography on a 1.5 cm × 10 cm column of Affi-gel blue gel in 10 mM Tris–HCL buffer (pH 7.3). After unadsorbed proteins (fraction B1) had been eluted, adsorbed proteins (fraction B2) were desorbed with 1.5 M NaCl in 10 mM Tris–HCl buffer (pH 7.3). The adsorbed peak was then dialyzed against 20 mM NH_4OAc buffer (pH 4.6) prior to FPLC-ion exchange chromatography on a 1-ml Mono S column (Amersham Biosciences) in the same buffer. Following removal of unbound proteins, the bound proteins were desorbed using a linear NaCl concentration gradient (0–1 M) in 20 mM NH_4OAc (pH 4.6). Bound proteins eluted with 1 M NaCl were then subjected to gel filtration on a Superdex 75 HR10/30 column (Amersham Biosciences) in 0.15 M NH_4HCO_3 (pH 8.0).

2.2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

It was conducted according to the method of Laemmli and Favre [13]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of the antifungal protein was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

2.3. *Amino acid sequence analysis*

The N-terminal amino acid sequence of the antifungal protein was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with a high performance liquid chromatography system as previously described [15].

2.4. *Assay of antifungal activity*

The assay for antifungal activity toward *Mycosphaerella arachidicola* and *Fusarium oxysporum* was carried out in 100 mm × 15 mm petri plates containing 10 ml of potato dextrose agar. 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 (15 μl) of the antifungal protein was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped the disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [34].

To determine the IC_{50} value for the antifungal activity, five doses of the antifungal protein were added separately to five aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into five 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 the 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 determined. IC_{50} value is the concentration of the antifungal protein required to reduce the area of the mycelial colony to 50% [34].

2.5. *Assay of mitogenic activity*

The isolated antifungal protein was tested in this assay since some antifungal proteins, such as chrysancorin [32] demonstrate activity in this assay. Four C57BL/6 mice (20–25 g) were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml, and 100 μg streptomycin/ml. The cells (7×10^5 cells/100 μl/well) were seeded into a 96-well culture plate and serial concentrations of the antifungal protein in 100 μl medium were added. After incubation of the cells at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, 10 μl [methyl-^{3}H]-thymidine (0.25 μCi, Amersham, Biosciences) was added, and the cells were incubated for a further 6 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass fiber filter, and the radioactivity was measured with a Beckman model LS 6000SC scintillation counter. All reported values are the means of triplicate samples [32,34–38].

2.6. *Assay for HIV reverse transcriptase inhibitory activity*

The isolated antifungal protein was tested for this activity since some antifungal proteins possess an inhibitory activity toward human immunodeficiency virus type 1 (HIV-1) reverse transcriptase [20,29,43,44,46]. The assay showing inhibition of HIV-1 reverse transcriptase was assessed by using an enzyme-linked immunosorbent assay kit from Boehringer Mannheim (Germany) as previously described [3,34–38]. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the antifungal protein was calculated as percent inhibition as compared to a control without the protein.

3. Results

When the $(NH_4)_2SO_4$ precipitate of the crude banana extract was chromatographed on Affi-gel blue gel, a broad unad-

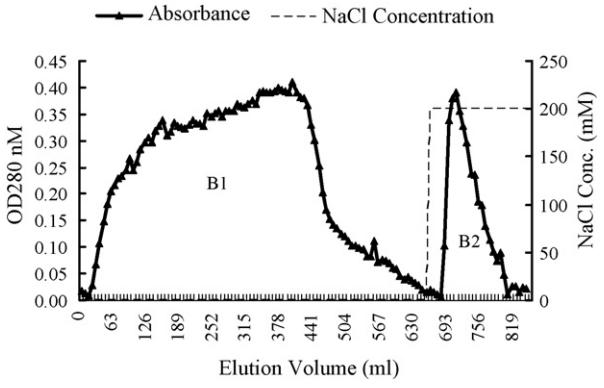

Fig. 1 – Elution profile of dissolved $(NH_4)_2SO_4$ precipitate of banana extract on Affi-gel blue gel. Absorbance curve indicated by line with triangles; NaCl concentration indicated by broken straight lines.

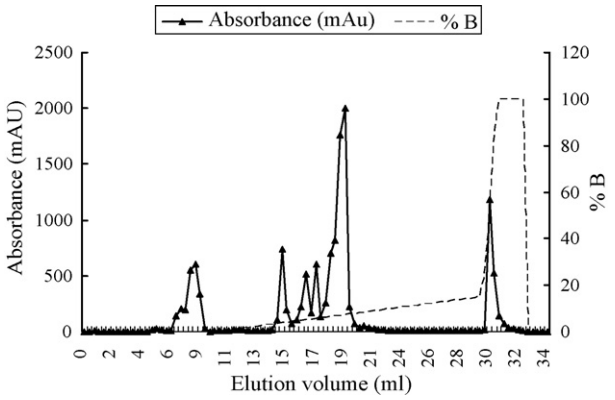

Fig. 2 – Elution profile of fraction B2 on Mono S. 60% B means elution with 20 mM NH_4OA_C buffer (pH 4.6) containing 600 mM NaCl. Absorbance curve indicated by line with triangles; NaCl concentration indicated by broken straight lines.

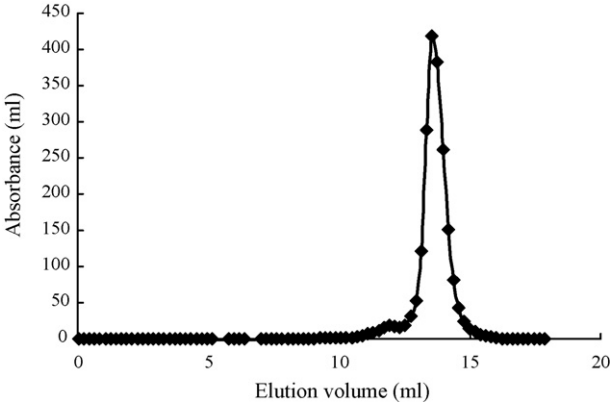

Fig. 3 – FPLC-gel filtration of most strongly adsorbed fraction from Mono S column on Superdex 75.

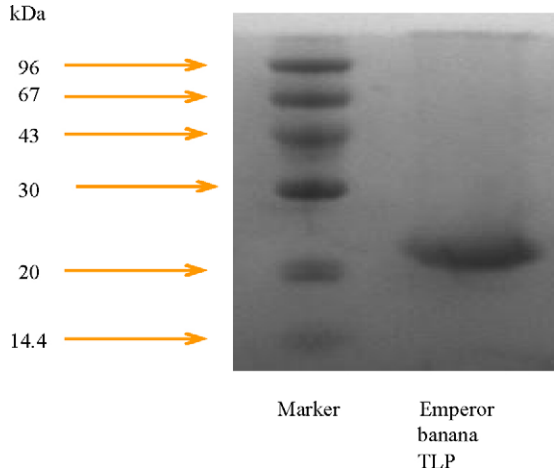

Fig. 4 – SDS-PAGE of emperor banana antifungal protein.

sorbed fraction (B1) devoid of antifungal activity and a much smaller adsorbed fraction (B2) with antifungal activity were obtained (Fig. 1). B2 was subsequently resolved on Mono S into a number of fractions, the most strongly adsorbed fraction of which exhibited antifungal activity (Fig. 2). This fraction appeared as a sharp absorbance peak upon gel filtration on Superdex 75 (Fig. 3) and a single band in SDS-PAGE (Fig. 4). The estimated molecular mass was 22 kDa based on gel filtration and SDS-PAGE. The N-terminal sequence of the purified protein manifested extensive homology to thaumatin-like proteins (Table 1). The protein inhibited mycelial growth in *M. arachidicola* and *F. oxysporum* (Fig. 5), with an IC_{50} of about 20 μM for the latter (Fig. 6A and B). The antifungal activity was stable between pH 1 and pH 11 (Fig. 7), and up to 70 °C (Fig. 8). Its inhibitory activity on HIV-1 reverse transcriptase was weak. At 16 μM and 64 μM there was only 13.6 ± 0.1% and 16.7 ± 0.1% inhibition. The

Table 1 – N-terminal sequence and comparison with other thaumatin-like (TLPs) (Results of a BLAST search)

Musa acuminata TLP	Residue number	N-terminal sequence	Residue number	% identity
Musa basjoo TLP	1	ATAFFEFVNRCCYTVAAAAV	20	100
Thaumatin	2	AT--FEIVNRCSYTVWAAA	18	75
Musa acuminat TLP	27	AT--FXIVNRCSYTVWAAAV	44	70
Sambucus nigra TLP	25	AT--FDIVNRCSYTVWAAA	41	68
Nicotiana tabacum TLP	26	AT--FDIVNKCTYTVWAAA	42	63

Underscored amino acid residues are identical to corresponding residues in emperor banana (*M. basjoo*) TLP.

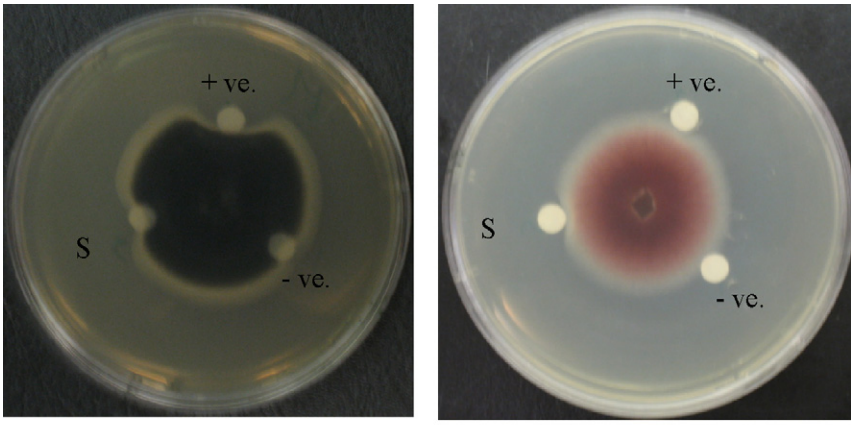

Fig. 5 – Antifungal activity of emperor banana thaumatin-like protein. Left disk: TLP (mg). Right disk: buffer, pH uppermost disk: μg nystatin (positive control).

protein (10 μg) retained its antifungal activity after exposure to 10 μg trypsin in 67 mM phosphate buffer (pH 7.6) for 1, 2, and 4 h at room temperature 25 °C (data not shown) (Table 2).

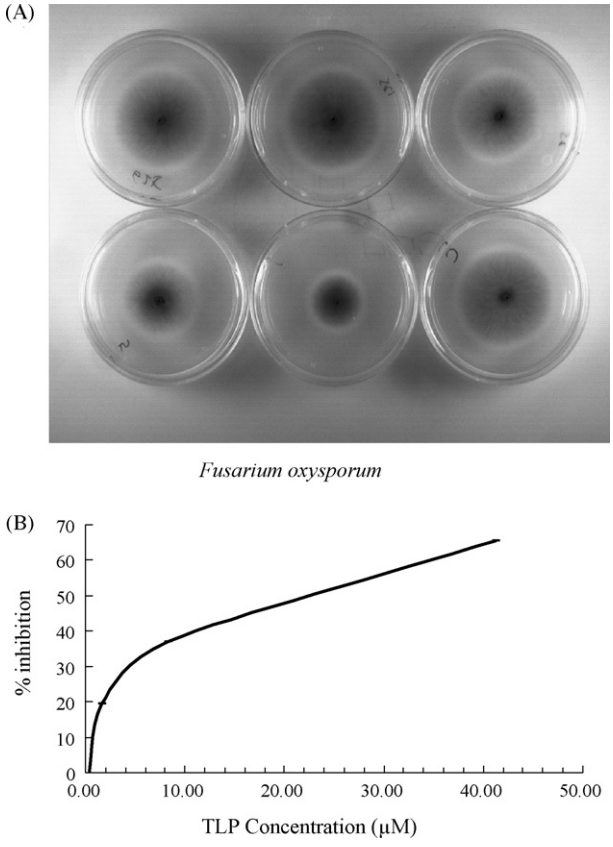

Fig. 6 – (A) IC_{50} determination of antifungal activity of emperor banana thaumatin-like protein toward *F. oxysporum* (TLP). Upper row: left (0.07 μM TLP, middle (0.35 μM), and right (1.7 μM). Lower row: left (8.6 μM), middle (43 μM), and right (0 μM). (B) Graph plotting data from (A).

4. Discussion

TLPs have been isolated from many different plant tissues including kiwi fruits [30,39], *Diospyros kaki* fruits [28], and French beans [45]. Emperor banana TLP isolated in the present investigation resembles those TLPs in possessing a molecular mass of about 20 kDa. Its N-terminal sequence demonstrates marked homology to previously reported TLPs. The chromatographic behavior of emperor banana TLP on Affi-gel blue gel and ion exchangers is also similar to that of other TLPs [30,45] and other types of antifungal proteins [16,20,29,31,32,47].

F. oxysporum is one of the fungi attacking bananas. It causes Panama disease in bananas and in addition infects a wide spectrum of plant species. *M. arachidicola* is the major fungus attacking peanuts. Thus, the inhibitory action of emperor banana TLP on these two fungal species may be exploited in the protection of bananas and peanuts from fungal invasions.

The antifungal protein isolated in the present investigation displays an N-terminal sequence with resemblance to those of thaumatin-like proteins. The molecular mass of emperor banana TLP (20 kDa) is similar to those of other TLPs reported in the literature. French bean TLP has a molecular mass of 20 kDa, kiwi fruit TLP a molecular mass of 21 kDa, and maize TLP a molecular mass of 22 kDa. The molecular masses of tomato TLP [21], grape TLP [23], and *Diospyros texana* TLP [28] are 23, 24, and 27 kDa, respectively.

Table 2 – Protein yields and antifungal potencies at different stages of purification of emperor banana thaumatin-like protein

Fraction	Protein yield from 5 kg bananas (mg)	IC_{50} toward *F. oxysporum* (mg/mL)
Crude extract	1500	9.2
Adsorbed peak (B2) from Affi-gel blue gel	142	5.5
Most strongly adsorbed peak from Mono S	25	1.1
Major peak from Superdex 75	5	0.4

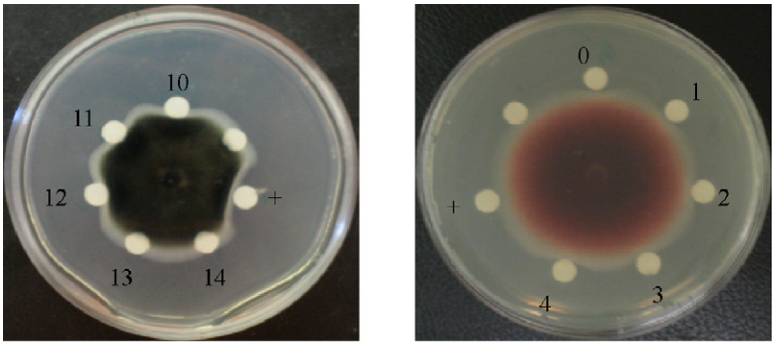

Fig. 7 – pH stability of antifungal activity of emperor banana TLP toward *M. arachidicola*, Left plate: starting at 12 noon position in clockwise direction pH 10, distilled water as negative control, mystatin (0.5 μg), as positive control pH 14, 13, 12, and 11. Right plate: arrangement similar to left plate, pH 2, 3, and 4, nystatin (0.5 μg) and distilled water.

Emperor banana antifungal protein was isolated with a procedure applicable to other antifungal proteins [16,20,29–32,44–47]. It was adsorbed on Affi-gel blue gel, and Mono S. Ammonium sulfate precipitation was included as an early step in the purification potocol for maize TLP, *D. texana* TLP, and kiwi fruit TLP [10,28,30]. This step was also utilized in the purification of emperor banana TLP. Ion exchangers were also used for isolation of TLPs from maize, tobacco, tomato, *D. texana,* grape, French bean, and kiwi fruit. However, hydrophobic interaction chromatography employed for purification of tobacco TLP [36] and soybean TLP [8] was not used for emperor banana TLP. The affinity chromatographic resin Affi-gel blue gel used in the purification of TLPs from French bean [25], kiwi fruits [31] and emperor banana was not employed for the isolation of other TLPs. The Mono S and Affi-gel blue gel steps were very effective because a large amount of inactive proteins were removed. The antifungal protein obtained was already highly purified after chromatography on Affi-gel blue gel and Mono S.

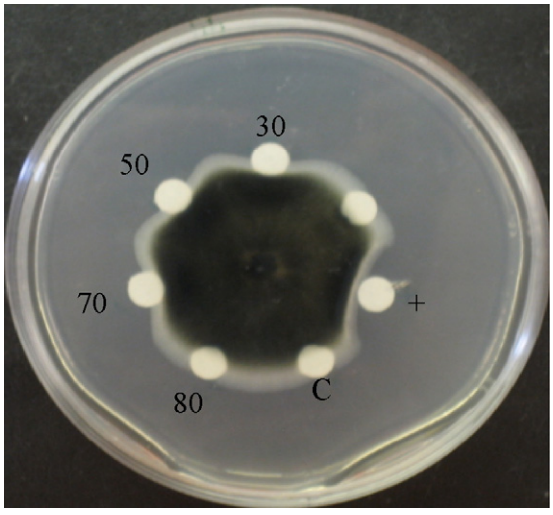

Fig. 8 – Thermal stability of antifungal activity of emperor banana thaumatin-like protein toward *M. arachidicola*. Starting at 12 noon position in a clockwise direction: 30 °C, distilled water as negative control, mystatin (0.5 mg as positive control), 0.15 M NH_4HCO_3 buffer (pH 8.0), 80, 70, and 50 °C.

Emperor banana antifungal protein demonstrates antifungal activity against *F. oxysporum* and *M. arachidicola*. Its antifungal activity is more potent than the thaumatin-like proteins isolated from French bean legumes [45] and kiwi fruits [30] in our laboratory. *F. oxysporum* is insensitive to kiwi fruit TLP and weakly sensitive to French bean TLP, but it is inhibited by emperor banana TLP. Emperor banana TLP is also more potent than kiwi fruit TLP on *M. arachidicola*.

Emperor banana TLP lacks mitogenic activity toward mouse splenocytes. In this regard it is noteworthy that some antifungal proteins such as chrysancorin from garland chrysanthemum seeds [32] and trypsin inhibitor from broad beans [43] exhibit mitogenic activity while others like mungin from mungbeans [41] may demonstrate anti-mitogenic activity.

Chestnut (*Castanopsis*) TLP shows a considerably more potent inhibitory effect on HIV-1 reverse transcriptase (IC_{50} = 1.6 μM) than kiwi fruit TLP ($IC_{50} \geq 27$ μM) and French bean TLP (IC_{50} = 200 μM) [30,45]. Low activity is exhibited by emperor banana TLP toward the retroviral enzyme.

A variety of antifungal proteins such as chive chitinase-like protein and bean defensin-like peptides inhibit proliferation of tumor cells [16,34–38]. It is somewhat unexpected that emperor banana TLP is devoid of these activities.

Kiwi fruit and cherry thaumatin-like proteins and European chestnut chitinase are allergens. The peanut antifungal peptide exhibits an allergen-like N-terminal amino acid sequence [5,6,11]. Whether emperor banana TLP possesses allergenic characteristic awaits elucidation.

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