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Isolation of trichogin, an antifungal protein from fresh fruiting bodies of the edible mushroom *Tricholoma giganteum*

Yongxia Guo[a,b], Hexiang Wang[a,b], T.B. Ng[c,*]

[a] *Department of Microbiology, College of Biological Science, China Agricultural University, Beijing, China*
[b] *State Key Laboratory for Agrobiotechnology, Beijing, China*
[c] *Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China*

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Abstract

An antifungal protein was isolated from the mushroom *Tricholoma giganteum* var. golden blessings. The protocol included ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on CM-cellulose, and gel filtration by fast protein liquid chromatography on Superdex 75. The antifungal protein, designated trichogin, was unadsorbed on DEAE-cellulose but was adsorbed on Affi-gel blue gel and CM-cellulose. It exhibited antifungal activity against *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola*. Trichogin inhibited HIV-1 reverse transcriptase with an IC_{50} of 83 nM.

Keywords: Antifungal protein; *Fusarium oxysporum*; Reverse transcriptase; *Tricholoma giganteum*

1. Introduction

Antifungal proteins and peptides have been isolated from a vast number of animals [17,34], plants [1–7,9–11, 15,16,18–33,35] and fungi [8,13]. Despite the common role of defense against fungal pathogens, antifungal proteins are in fact an assembly of structurally diverse proteins. In the cohort are found thaumatin-like proteins [5,10,20,33,41,50], glucanases [8,27], protease inhibitors [3,4,11,18,43], ribosome inactivating proteins [16,21], chitinases and chitinase-like proteins [6,15,27,49], cyclophilin-like proteins [46,48], lectins [1,6], lipid transfer protein-like proteins [2], peroxidases [47], and others [19,42].

The literature about mushroom antifungal proteins is scanty and is confined to studies on *Lyophyllum shimeiji* [13], *Pleurotus eryngii* [38] and *Polyporus adusta* [39]. The purpose of the present investigation was to furnish evidence for the production of an antifungal protein from the common edible mushroom, *Tricholoma giganteum* var. golden blessing, and to compare its characteristics with other antifungal proteins, especially those of mushroom origin.

2. Materials and methods

2.1. Purification of trichogin

Fresh fruiting bodies (2 kg) of the mushroom *T. giganteum* var. golden blessings were extracted in distilled water (2 ml/g) for 60 min at 4 °C using a Waring blender. The extract was centrifuged at 12,000 rpm for 30 min. Tris–HCl buffer (pH 7.4) was added to the resulting superantant until the concentration of Tris attained 10 mM. The supernatant was first fractionated by ion exchange chromatography on a DEAE-cellulose (Sigma) column (5 cm × 20 cm) using 10 mM Tris–HCl buffer (pH 7.2) as the starting buffer. Unadsorbed proteins eluted with the starting buffer were collected as fraction D1 while adsorbed proteins desorbed with 0.8 M NaCl added to the starting buffer were collected as fraction

* Corresponding author. Tel.: +852 2 60 968 72; fax: +852 2 60 951 23.
E-mail address: s010042@cuhk.edu.hk (T.B. Ng).

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D2. Fraction D1 was subjected to affinity chromatography on an Affi-gel blue gel (Bio-Rad) column (2.5 cm × 20 cm) in 10 mM Tris–HCl buffer (pH 7.2). Following elution of unadsorbed proteins (fraction B1), adsorbed proteins (fraction B2) were eluted using 1 M NaCl added to the starting buffer. Fraction B2 was dialyzed before ion exchange chromatography on a 2.5 cm × 20 cm column of CM-cellulose (Sigma) in 10 mM Tris–HCl buffer (pH 7.2). Unadsorbed proteins were eluted with the buffer and collected as fraction CM1. Adsorbed proteins were separate into two peaks, CM2 and CM3. Fraction CM3 was further purified by gel filtration on a Superdex 75 HR 10/30 column by fast protein liquid chromatography using an AKTA Purifier System (Amersham Biosciences) in 0.2 M NH_4HCO_3 buffer (pH 8.5). The second peak eluted represented purified antifungal protein designated trichogin. The Superdex 75 column had been calibrated with molecular mass markers including bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (21 kDa).

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

SDS-PAGE was conducted according to the method of Laemmli and Favre [12]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of trichogin 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 trichogin (about 500 pmol) was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system. The initial and repetitive yields of the sequencing experiments exceeded 95% and 90%, respectively [14].

2.4. Assay of antifungal activity

The assay of trichogin 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 of a solution of trichogin 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 [30].

For quantitative assays, three doses of antifungal protein (1, 5, and 25 μM) were added to potato dextrose agar (4 ml) at 45 °C, these being mixed rapidly and poured into three separate 6 cm Petri dishes. After the agar had cooled down to room temperature a small amount (1 mm × 1 mm) of mycelia, the same amount to each plate, was inoculated. Buffer only was employed for a negative control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth and hence the IC_{50} was determined. IC_{50} is defined as the concentration of antifungal protein required to reduce the area of mycelial colony to 50%.

2.5. Assay for ribonuclease activity

The activity of trichogin toward yeast tRNA was assayed by determining the generation of acid-soluble, UV-absorbing material with the method of Ng and Wang [19]. Trichogin was incubated with 200 μg tRNA in 150 μl of 100 mM MES buffer (pH 6.0) at 37 °C for 1 h. The reaction was terminated by introduction of 25 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 minute in the acid-soluble fraction per milliliter of reaction mixture under the specific conditions.

2.6. Assay of lectin activity

In the assay for lectin (hemagglutinating) activity, a serial two-fold dilution of the sample solution 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 read after about 1 h when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutinating activity, was reckoned as one hemagglutination unit or one titer.

2.7. Assay for HIV reverse transcriptase inhibitory activity

The assay for ability to inhibit HIV-1 reverse transcriptase was carried out by using an enzyme-linked immunosorbent assay kit from Boehringer Mannheim (Germany) as previously described [32,37,44,50]. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(a) oligo (dT) 15. Instead of radio-labeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich enzyme-linked immunosorbent assay protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxi-

dase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 490 nm can be read using a microtiter plate (ELISA) reader and is directly proportional to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of trichogin was calculated as percent inhibition as compared to a control without the protein.

3. Results

3.1. Isolation of antifungal protein

The extract of *T. giganteum* fruiting bodies was fractionated on DEAE-cellulose into an unadsorbed fraction D1 with antifungal activity and an inactive adsorbed fraction D2. Fraction D1 was separated on Affi-gel blue gel into an unadsorbed fraction B1 devoid of antifungal activity and an adsorbed fraction B2 in which antifungal activity resided. Ion exchange chromatography of fraction B2 on CM-cellulose yielded an unadsorbed fraction CM1 and an adsorbed fraction CM2, both devoid of antifungal activity. Antifungal activity was concentrated in the second adsorbed fraction CM3. Upon gel filtration on Superdex 75, CM3 was resolved into two peaks, SU1 and SU2, of similar size (Fig. 1). Antifungal activity resided in the second peak SU2. SU2 exhibited a molecular mass of 27 kDa both in gel filtration (Fig. 1) and in SDS-PAGE (Fig. 2). The yields of the various chromatographic fractions are presented in Table 1. The N-terminal sequence of trichogin, QVHWPMF, is not found in any reported antifungal protein. It is dissimilar to those of previously isolated mushroom antifungal proteins (Table 2).

3.2. Antifungal activity and other activities of antifungal protein

The purified antifungal protein, designated trichogin, exerted antifungal activity toward *F. oxysporum* (Fig. 3). *M. arachidicola* (Fig. 4) and *Physalospora piricola* (Fig. 5). The IC_{50} of the antifungal activity of trichogin to-

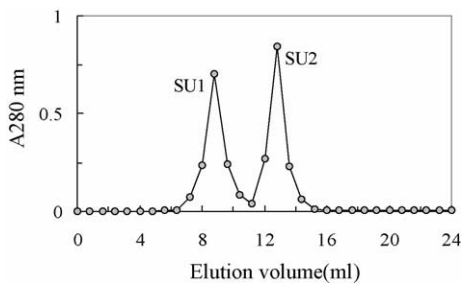

Fig. 1. Gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column, buffer: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml.

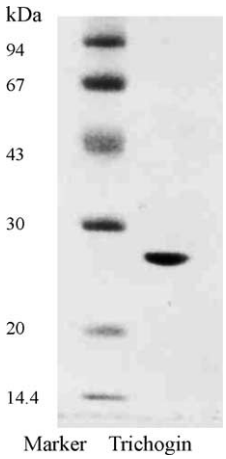

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Left lane: molecular mass markers from Amersham Biosciences, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). Right lane: fraction SU2 from Superdex 75 column representing purified antifungal protein trichogin.

Table 1
Protein yields of various chromatographic fractions

Fraction	Yield (mg)
Extract	4820
D1	1526
D2	1804
B1	618
B2	380
CM1	88
CM2	166
CM3	93
S1	30
S2	35

Table 2
Comparison of N-terminal sequence of trichogin with those of other mushroom antifungal proteins

Trichogin	QVHWPMF
Alveolarin	GVCDMADLA
Eryngin	ATRVVYCNRRSGSVVGGDDTVYYEG
Lyophyllum antifungal protein	AGTEIVTCYNAGTKVPRGPSAXGGAIDFFN

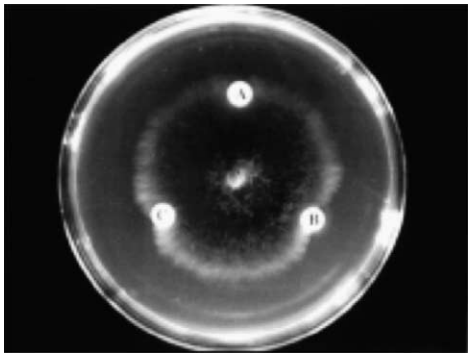

Fig. 3. Antifungal activity of trichogin toward *Fusarium oxysporum*: (A) control (15 μl 0.1 M MES buffer, pH 6); (B) 80 μg trichogin in 0.1 M MES buffer, pH 6; (C) 16 μg trichogin in 0.1 M MES buffer, pH 6.

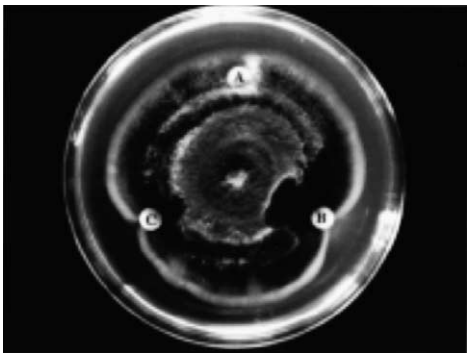

Fig. 4. Antifungal activity of trichogin toward *Mycosphaerella arachidicola*: (A) control (15 μl 0.1 M MES buffer, pH 6); (B) 80 μg trichogin in 0.1 M MES buffer, pH 6; (C) 16 μg trichogin in 0.1 M MES buffer, pH 6.

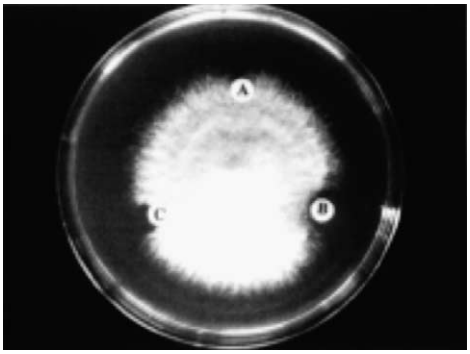

Fig. 5. Antifungal activity of trichogin toward *Physalospora piricola*: (A) control (15 μl 0.1 M MES buffer, pH 6); (B) 80 μg trichogin in 0.1 M MES buffer, pH 6; (C) 16 μg trichogin in 0.1 M MES buffer, pH 6.

ward *M. arachidicola* was estimated to be 3.8 ± 0.28 μM (mean ± SEM, $n = 3$) (Fig. 6). Trichogin inhibited HIV-1 reverse transcriptase with an IC_{50} of 83 ± 2.7 nM (mean ± SEM, $n = 3$). However, it was devoid of ribonuclease and hemagglutinating activities (data not shown).

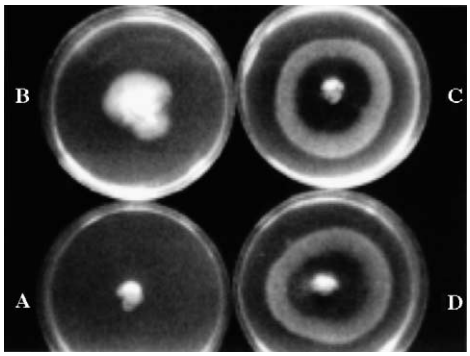

Fig. 6. Determination of IC_{50} value for antifungal activity of trichogin. Doses used: plate A (25 μM) (lower left), plate B (5 μM) (upper left), plate C (1 μM) (upper right) and plate D (control i.e. 0.1 M MES buffer, pH 6) (lower right). IC_{50} value was determined to be 3.8 μM.

4. Discussion

A variety of molecular masses have been reported for antifungal proteins of fungal origin [8,13,38,39], as is the case of angiosperm antifungal proteins [5,19,29–33,42–44]. The molecular mass of trichogin isolated in the present study lies in the middle of the range. It is much smaller than the value of 52 kDa for panaxagin and quinqueginsin from ginseng [19,31] and larger than the value of 14 kDa for antifungal protein from *L. shimeiji* [13]. Like the majority of antifungal proteins, trichogin is monomeric. Only very few antifungal proteins like those from ginseng [19,31] are dimeric.

Trichogin can be isolated with the use of a purification protocol proven to be effective for isolating antifungal proteins of diverse origins [13,15,29–40]. The behavior of trichogin on the various ion exchange and affinity chromatographic media employed in the isolation procedure is similar to that reported for other antifungal proteins [29–38,40,42–52]. It is unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and CM-cellulose. The use of 10 mM Tris–HCl buffer (pH 7.3) as the starting buffer for both DEAE-cellulose and Affi-gel blue gel chromatography obviates the need for dialysis between the two steps and thus simplifies the procedure.

The fungal species sensitive to the growth-inhibitory action of *L. shimeiji* antifungal protein, including *F. oxysporum*, *M. arachidicola* and *P. piricola*, are also inhibited by trichogin. It probably deserves mention that antifungal proteins from shallot bulbs [35] and asparagus seeds [32] demonstrate antifungal action against only *Botrytis cinerea* out of the several fungal species tested. However, many angiosperm antifungal proteins exert antifungal action on more than one fungal species. This was seen in antifungal proteins from chestnut [5], chive [15], *Ginkgo biloba* [29], garlic [30], cicada [34], lily bulb [36], bamboo shoot [37], garland chrysanthemum seeds [40], chickpea [42,46], peanut [45], French bean [47,50], mungbean [48], field bean [49] and cowpea [52].

The antifungal activity of trichogin is high compared with those of other antifungal proteins including chestnut [5], garlic [30], lily bulb [36], chickpea [42,46], mungbean [48] and cowpea [52].

Trichogin inhibits HIV-1 reverse transcriptase with a potency ($IC_{50} = 83$ nM) higher than those of *L. shimeiji* antifungal protein [13], cowpea antifungal proteins [52], chickpea cyclophilin-like antifungal protein [46], red kidney bean lectin [44], shallot antifungal peptide [35], and antifungal proteins from American ginseng [31] and Chinese ginseng [19]. Many angiosperm antifungal proteins are also endowed with the ability to inhibit the retroviral enzyme [15,19,31,35,44,46].

Trichogin is devoid of ribonuclease and lectin activities. This is important in view of the reports that some ribonucleases [19,31] and lectins [1,6,44] exhibit antifungal activity.

The N-terminal sequence of trichogin is novel and has not been reported for antifungal proteins in the literature. It is also

substantially different from those in the mushroom antifungal proteins *L. shimeiji* antifungal protein [13], eryngin [38] and alveolarin [39].

The present report on trichogin adds to the rather meager literature on mushroom antifungal proteins.

Acknowledgment

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