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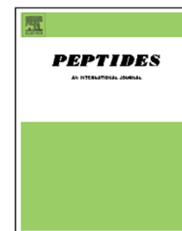

Ganodermin, an antifungal protein from fruiting bodies of the medicinal mushroom *Ganoderma lucidum*

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ABSTRACT

A 15-kDa antifungal protein, designated ganodermin, was isolated from the medical mushroom *Ganoderma lucidum*. The isolation procedure utilized chromatography on DEAE-cellulose, Affi-gel blue gel, CM-Sepharose and Superdex 75. Ganodermin was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and CM-Sepharose. Ganodermin inhibited the mycelial growth of *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola* with an IC_{50} value of 15.2 μM, 12.4 μM and 18.1 μM, respectively. It was devoid of hemagglutinating, deoxyribonuclease, ribonuclease and protease inhibitory activities.

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1. Introduction

Ganoderma lucidum is a medicinal mushroom that has antidiabetic, antioxidant, immunomodulatory, antitumor and antimetastatic activities [13]. However, very few studies on its protein components exist. Antifungal proteins have been isolated from a large number of plants [1–12,15–20,22–32,34,35] and animals [6,11,26], but only from a small number of mushrooms [9,15,18,19,29,30]. In view of the immense economic implications associated with antifungal proteins and the existence of many structurally different antifungal proteins, we undertook the present study to isolate an antifungal protein from *G. lucidum*, and to compare its characteristics with other mushroom and plant antifungal proteins, in order to ascertain whether it is a novel protein.

2. Materials and methods

2.1. *Isolation of antifungal protein*

Fresh fruiting bodies of *G. lucidum* (1 kg), collected on the campus of The Chinese University of Hong Kong (CUHK) and authenticated by Professor Shiuying Hu, Honorary Professor of Chinese Medicine (CUHK), were used. The fruiting bodies were homogenized in distilled water (2 ml/g). After centrifugation (10,000 × *g*, 30 min, 4 °C), the supernatant was passed through a column of DEAE-cellulose (Sigma) (5 cm × 20 cm) previously equilibrated and then eluted with 10 mM Tris–HCl buffer (pH 7.3). The unadsorbed fraction was separated on an Affi-gel blue gel (Bio-Rad) column (2.5 cm × 20 cm) in the same buffer. Adsorbed proteins were desorbed with 0.5 M NaCl, dialyzed against 10 mM NH_4OAc buffer, (pH 4.6) and then chromatographed on a column of CM-Sepharose (Amersham Biosciences) in 10 mM NH_4OAc buffer (pH 4.6). Following removal of unadsorbed proteins, a gradient (0–1 M) of NaCl in the NH_4OAc buffer was applied. The second and major adsorbed

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peak (CM3) was gel-filtered on a Superdex 75 column (Amersham Biosciences). The first peak obtained represented purified antifungal protein, which was designated ganodermin.

2.2. Electrophoresis and amino acid sequencing

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli and Favre [14]. The N-terminal amino acid sequence of ganodermin was determined using a Hewlett-Packard 1000A protein sequencer [26].

2.3. Assay for antifungal activity

The assay for antifungal activity toward the phytopathogenic fungi *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola* 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 (12 μl) containing 36 μg or 180 μg of ganodermin 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 crescent of inhibition around disks containing samples with antifungal activity [20,26]. The antifungal protein cicadin isolated from dried juvenile cicadas [26] was used as a positive control.

To determine the IC_{50} value for the antifungal activity (concentration producing 50% inhibition of mycelial growth), three doses (10 μM, 20 μM and 40 μM) of ganodermin and buffer (as negative control) were added separately to four aliquots of potato dextrose agar at 45 °C, mixed rapidly and poured into four separate small petri dishes. After the agar had cooled, a small amount of mycelia was added to each plate. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined. From a graph plotting inhibition of fungal growth against dose of ganodermin, the concentration required to produce 50% inhibition was calculated [26].

2.4. Assays for hemagglutinating (lectin), protease inhibitory, deoxyribonuclease and ribonuclease activities

The assays were performed as described in Refs. [35,34,25,23], respectively.

3. Results

Ion exchange chromatography of the *Ganoderma* extract on DEAE-cellulose yielded an unadsorbed peak (D1) with antifungal activity and an inactive adsorbed peak (D2). Affinity chromatography of D1 on Affi-gel blue gel resulted in an inactive unadsorbed peak B1 and an active adsorbed peak B2. When B2 was chromatographed on CM-Sepharose, a small unadsorbed peak CM1 and two very small adsorbed peaks, CM2 and CM4, were obtained (Fig. 1). Antifungal activity resided in the main adsorbed peak CM3 eluted between CM2 and CM4. CM3 was fractionated by gel filtration on Superdex 75 into a larger active peak SU1 and a smaller inactive peak SU2 (Fig. 2). Its molecular mass as estimated by gel filtration was 14 kDa. SU1 exhibited a single 15-kDa band in SDS-PAGE. Its N-terminal sequence was AGETHTVMINHAGRGAPKLVVGG-KKLS. A blast search revealed no significant homology to any protein. Ganodermin was obtained with a yield of 12 mg/kg fruiting bodies. It exerted antifungal action on *B. cinerea* (Fig. 3), *P. piricola* and *F. oxysporum*. The IC_{50} values of the antifungal activity of ganodermin toward *B. cinerea*, *F. oxysporum* and *P. piricola* were, respectively, 15.2 ± 0.7 μM, 12.4 ± 0.3 μM and 18.1 ± 0.5 μM (mean ± S.D., $n = 3$). Ganodemin was devoid of protease inhibitory, deoxyribonuclease, ribonuclease or hemagglutinin (lectin) activities (data not shown).

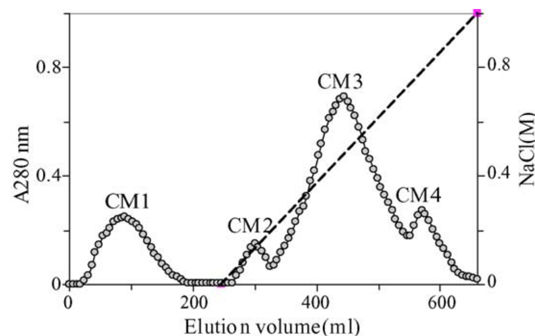

Fig. 1 – Ion exchange chromatography of peak B2 (derived from fraction of 1 kg fruiting body extract unadsrobed on DEAE-cellulose and adsorbed on Affi-gel blue gel) on a CM-Sepharose CL 6B column (2.5 cm × 15 cm). CM1 was eluted with 10 mM NH_4OAc buffer (pH 4.6). The dotted slanting line represents the linear concentration gradient of NaCl used to elute the peaks CM2, CM3 and CM4.

4. Discussion

The chromatographic behavior of ganodermin on DEAE-cellulose, Affi-gel blue gel and CM-Sepharose resembles that

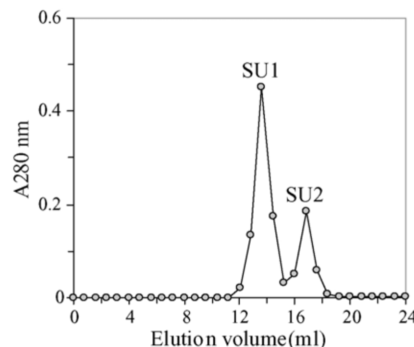

Fig. 2 – FPLC-gel filtration of 22 mg peak CM3 on a Superdex 75 HR 10/30 column. Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. Peak SU1 represented purified antifungal protein (ganodermin).

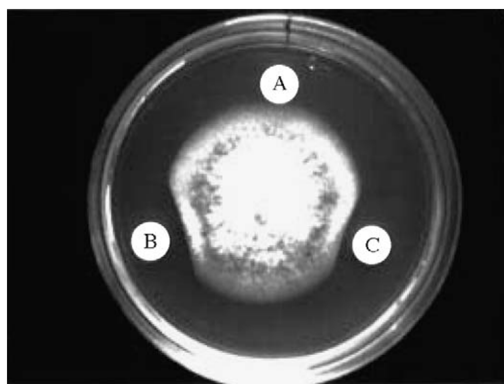

Fig. 3 – Antifungal activity of ganodermin toward *Botrytis cinerea*. (A) Negative control (0.1 M MES buffer, pH 6.5), (B) 180 μg ganodermin and (C) 36 μg ganodermin.

of angiosperm and animal antifungal proteins [4,5,15–19,23–32,34,35] and *Lyophyllum* antifungal protein from the mushroom *Lyophyllum shimeiji* [15]. It is unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel and CM-Sepharose. Molecular masses ranging from several kilodaltons to about 67 kDa have been reported for antifungal proteins [4,5,10–12,15–20,23–32,34,35]. The molecular mass of ganodermin is close to that of *Lyophyllum* antifungal protein [15]. Pleurostrin, an antifungal peptide with about half the size of ganodermin, has been isolated from the oyster mushroom *Pleurotus ostreatus* [5]. Ganodermin resembles two mushroom antifungal proteins, *Lyophyllum* antifungal protein and eryngin to a small extent in N-terminal sequence, and angiosperm thaumatin-like proteins and thaumatin only slightly. However, there is no similarity to other mushroom antifungal proteins. Ganodermin inhibits mycelial growth in the phytopathogenic fungi *B. cinerea*, *F. oxysporum* and *P. piricola*. *Lyophyllum* antifungal protein is inhibitory to *P. piricola* and *M. arachidicola* [15]. Its antifungal potency is similar to those of previously reported antifungal proteins [4,5,15,18,19,24,26,28–32]. *B. cinerea*, *F. oxysporum* and *P. piricola* are the pathogens of cucumber, cotton and apple, respectively. Toxins produced by these fungi render food consumed by human jeopardous to health. Thus, the isolation of a protein with inhibitory activity toward these fungi has important implications for human health.

Some antifungal proteins have hemagglutinating (lectin), deoxyribonuclease, ribonuclease and protease inhibitory activities [1,3,7,12,17,23,25,34,35]. Ganodermin does not have any of these activities.

Very few bioactive proteins, such as a lectin [21] and a ribonuclease [33], have been isolated from *G. lucidum*. The isolation of an antifungal protein adds to the scanty literature on this medicinal mushroom.

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