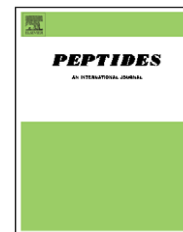


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Ganodermin, an antifungal protein from fruiting bodies of the medicinal mushroom *Ganoderma lucidum*

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ARTICLE INFO

Article history:

Received 23 May 2005

Received in revised form

10 June 2005

Accepted 10 June 2005

Published on line 21 July 2005

Keywords:

Antifungal protein

Ganoderma lucidum

Isolation

ABSTRACT

A 15-kDa antifungal protein, designated ganodermin, was isolated from the medicinal 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 *Phylospora piricola* with an IC₅₀ 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 NH₄OAc buffer, (pH 4.6) and then chromatographed on a column of CM-Sepharose (Amersham Biosciences) in 10 mM NH₄OAc buffer (pH 4.6). Following removal of unadsorbed proteins, a gradient (0–1 M) of NaCl in the NH₄OAc buffer was applied. The second and major adsorbed

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doi:10.1016/j.peptides.2005.06.009

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 IC₅₀ 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

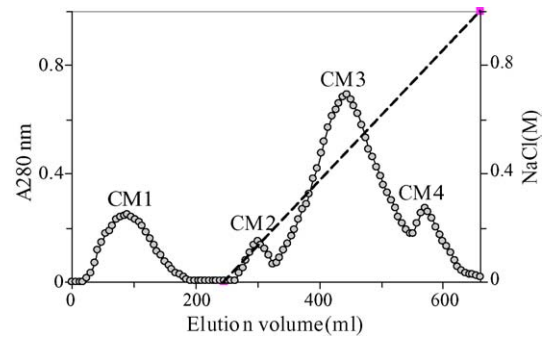


Fig. 1 – Ion exchange chromatography of peak B2 (derived from fraction of 1 kg fruiting body extract unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel) on a CM-Sepharose CL 6B column (2.5 cm × 15 cm). CM1 was eluted with 10 mM NH₄OAc buffer (pH 4.6). The dotted slanting line represents the linear concentration gradient of NaCl used to elute the peaks CM2, CM3 and CM4.

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 IC₅₀ 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). Ganodermin was devoid of protease inhibitory, deoxyribonuclease, ribonuclease or hemagglutinin (lectin) activities (data not shown).

4. Discussion

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

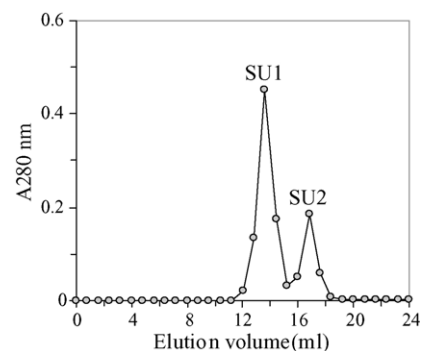


Fig. 2 – FPLC-gel filtration of 22 mg peak CM3 on a Superdex 75 HR 10/30 column. Buffer: 0.2 M NH₄HCO₃ buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. Peak SU1 represented purified antifungal protein (ganodermin).

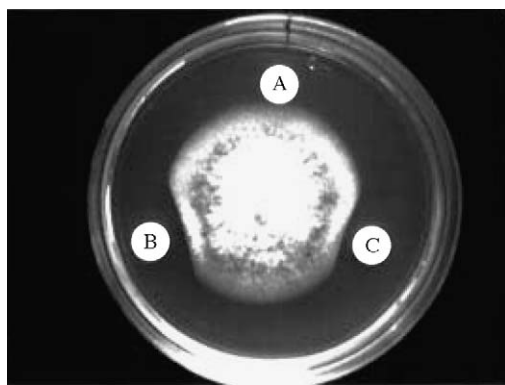


Fig. 3 – Antifungal activity of ganodermin toward *Botrytis cinerea*. (A) Negative control (0.1 M MES buffer, pH 6.5), (B) 180 μ g ganodermin and (C) 36 μ g ganodermin.

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.

Acknowledgments

We thank Miss Fion Yung for expert secretarial assistance and Medicine Panel of the CUHK Research Committee for a direct grant.

REFERENCES

- [1] Broekaert WF, Van Parijs J, Leyns F, Joos H, Peumans WJ. A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. *Science* 1989;245:1100–2.
- [2] Cammue BPA, Thevissen K, Hendriks M, Eggermont K, Goderis IJ, Proost P, Van Damme J, Osborn RW, Guerbet F, Kader JC, Broekaert WF. A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer protein. *Plant Physiol* 1995;109:445–55.
- [3] Chilosi G, Caruso C, Caporale C, Leonardi L, Bertini L, Buzi A, Nobile M. Antifungal activity of a Bowman-Birk type trypsin inhibitor from wheat kernel. *J Phytopathol* 2000;148:477–81.
- [4] Chu KT, Liu KH, Ng TB. Cicerarin, a novel antifungal peptide from the green chickpea. *Peptides* 2003;24:659–63.
- [5] Chu KT, Xia LX, Ng TB. Pleurostrin, an antifungal peptide from the oyster mushroom. *Peptides* 2005;26:2098–103.
- [6] Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hertu C, Hoffmann JA. Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence similarity to plant antifungal peptides. *J Biol Chem* 1995;269:33159–67.
- [7] Gozia O, Ciopraga J, Bentia T, Lungu M, Zamfirescu I, Tudor R, Roseanu A, Nitu F. Antifungal properties of lectin and new chitinases from potato tuber. *FEBS Lett* 1995;370:245–9.
- [8] Graham JS, Burkhart W, Xiong J, Gillikin JW. Complete amino acid sequence of soybean leaf P21-similarity to the thaumatin-like polypeptides. *Plant Physiol* 1992;98:163–5.
- [9] Grenier J, Potvin C, Asselin A. Some fungi express β -1,3-glucanases similar to thaumatin-like proteins. *Mycologia* 2001;92:841–8.
- [10] Huang X, Xie WJ, Gong ZZ. Characteristics and antifungal activity of a chitin binding protein from *Ginkgo biloba*. *FEBS Lett* 2000;478:123–6.
- [11] Iijima R, Kurata S, Natori S. Purification, characterization and cDNA cloning of an antifungal protein from the hemolymph of *Sarcophaga peregrina* (flash fly). *J Biol Chem* 1993;268:12055–62.
- [12] Joshi BN, Sainani MN, Bastawade KB, Gupta VS, Ranjekar PK. Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. *Biochem Biophys Res Commun* 1998;246:382–7.
- [13] Kimura X, Taniguchi M, Baba K. Antitumor and antimetastatic effects on liver of triterpenoid fractions of *Ganoderma lucidum*: mechanism of action and isolation of an active substance. *Anticancer Res* 2002;22:3309–18.
- [14] Laemmli UK, Favre M. Gel electrophoresis of proteins. *J Mol Biol* 1973;80:575–99.
- [15] Lam SK, Ng TB. First simultaneous isolation of a ribosome inactivating protein and an antifungal protein from mushroom (*Lyophyllum shimeiji*) together with evidence for synergism of their antifungal effects. *Arch Biochem Biophys* 2001;393:271–80.
- [16] Ng TB. Antifungal proteins and peptides of leguminous and non-leguminous origins. *Peptides* 2004;25:1215–22.
- [17] Ng TB, Wang HX. Panaxagin, a new protein from Chinese ginseng possesses antifungal, antiviral, translation-inhibiting and ribosome-inactivating activities. *Life Sci* 2000;68:739–49.
- [18] Ngai PH, Ng TB. Lectin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency virus-1 reverse transcriptase and proliferation of leukemia cells. *Life Sci* 2003;73:63–74.

- [19] Ngai PH, Zhao Z, Ng TB. Agrocybin, an antifungal peptide from the edible mushroom *Agrocybe cylindracea*. *Peptides* 2005;26:191-6.
- [20] Roberts WK, Selitrennikoff CP. Isolation and partial characterization of two antifungal proteins from barley. *Biochim Biophys Acta* 1986;880:161-70.
- [21] Tanaka S, Ko K, Kino K, Tsuchiya K, Yamashita A, Murasugi A, Sakuam S, Tsuno H. An immunomodulatory protein from a fungus *Ganoderma lucidum* having similarity to immunoglobulin variable region. *J Biol Chem* 1989;264:16372-7.
- [22] Tattersall DB, Van Heeswijck R, Hoj PB. Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. *Plant Physiol* 1997;114:759-69.
- [23] Wang H, Ng TB. Quinqueginsin, a novel protein with anti-human immunodeficiency virus, antifungal, ribonuclease and cell-free translation-inhibitory activities from American ginseng roots. *Biochem Biophys Res Commun* 2000;269:203-8.
- [24] Wang H, Ng TB. Ginkbilobin, a novel antifungal protein from *Ginkgo biloba* seeds with sequence similarity to embryo-abundant protein. *Biochem Biophys Res Commun* 2000;279:407-11.
- [25] Wang H, Ng TB. Isolation of a novel deoxyribonuclease with antifungal activity from *Asparagus officinalis* seeds. *Biochem Biophys Res Commun* 2001;289:102-4.
- [26] Wang H, Ng TB. Isolation of cicadin, a novel and potent antifungal peptide from juvenile cicadas. *Peptides* 2002;23:7-11.
- [27] Wang H, Ng TB. Ascalin, a new antifungal peptide with human immunodeficiency virus type 1 reverse transcriptase inhibitory activity from shallot bulbs. *Peptides* 2002;23:1025-9.
- [28] Wang H, Ng TB. Isolation of an antifungal thaumatin-like protein from kiwi fruits. *Phytochemistry* 2002;61:1-6.
- [29] Wang H, Ng TB. Eryngin, a novel antifungal peptide from fruiting bodies of the edible mushroom *Pleurotus eryngii*. *Peptides* 2004;25:1-5.
- [30] Wang H, Ng TB. Alveolarin, a novel antifungal polypeptide from the wild mushroom, *Polyorus alveolaris*. *Peptides* 2004;25:693-6.
- [31] Wang H, Ye XY, Ng TB. Purification of chrysancorin, a novel antifungal protein with mitogenic activity from garland chrysanthemum seeds. *Biol Chem* 2001;382:947-51.
- [32] Wong JH, Ng TB. Gymnin, a potent defensin-like antifungal peptide from the Yunnan bean *Gymnocladus chinensis* Baill. *Peptides* 2003;24:963-8.
- [33] Wang HX, Ng TB, Chiu SW. A distinctive ribonuclease from fresh fruiting bodies of the medicinal mushroom *Ganoderma lucidum*. *Biochem Biophys Res Commun* 2004;314:519-22.
- [34] Ye XY, Ng TB, Rao PF. A Bowman-Birk-type trypsin-chymotrypsin inhibitor from broad beans. *Biochem Biophys Res Commun* 2001;289:91-6.
- [35] Ye XY, Ng TB, Tsang PWK, Wang J. Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*) activities. *J Protein Chem* 2001;20:367-75.