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Cordymin, an antifungal peptide from the medicinal fungus *Cordyceps militaris*

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ABSTRACT

Cordymin, an antifungal peptide with a molecular mass of 10,906 Da and an N-terminal amino acid sequence distinct from those of previously reported proteins, was purified from the medicinal mushroom *Cordyceps militaris*. The isolation protocol comprised ion exchange chromatography of the aqueous extract on SP-Sepharose and Mono S and gel filtration on Superdex 75 by a fast protein liquid chromatography system. Cordymin was adsorbed on both cation exchangers. The peptide inhibited mycelial growth in *Bipolaris maydis*, *Mycosphaerella arachidicola*, *Rhizoctonia solani* and *Candida albicans* with an IC_{50} of 50 μM, 10 μM, 80 μM, and 0.75 mM, respectively. However, there was no effect on *Aspergillus fumigatus*, *Fusarium oxysporum* and *Valsa mali* when tested up to 2 mM. The antifungal activity of the peptide was stable up to 100 °C and in the pH range 6–13, and unaffected by 10 mM Zn^{2+} and 10 mM Mg^{2+}. Cordymin inhibited HIV-1 reverse transcriptase with an IC_{50} of 55 μM. Cordymin displayed antiproliferative activity toward breast cancer cells (MCF-7) but there was no effect on colon cancer cells (HT-29). There was no mitogenic activity toward mouse spleen cells and no nitric oxide inducing activity toward mouse macrophages when tested up to 1 mM.

Introduction

'Dong Chong Xia Cao' which belongs to the genus *Cordyceps* forms a highly prized traditional medicine for health promotion and treatment for some ailments (Li et al. 2001, 2006). In traditional Chinese medicine, *Cordyceps sinensis* is used most often while *C. militaris* is a much cheaper substitute. A hemagglutinin has been isolated from *C. sinensis* (Hsu et al. 2009) and another hemagglutinins from *C. militaris* (Jung et al. 2007; Wong et al. 2009). An antibacterial protein devoid of antifungal activity has also been reported from *C. sinensis* (Ng and Wang 2005). In view of the relatively little amount of information available about the protein constituents of *C. militaris* and its much lower costliness in comparison with *C. sinensis*, we undertook the present study to ascertain if an antifungal protein could be isolated from *C. militaris*. This would reveal whether the medicinal fungus has one more potentially exploitable activity in addition to the previously reported attributes. Although antifungal proteins have been isolated from a diversity of organisms including animals (King et al. 2000; Morrison et al. 2002; Tsvetkova et al. 2006), plants (Leung et al. 2008; Lin et al. 2009; Wong et al. 2006), bacteria (Li et al. 2009; Park et al. 2008; Wong et al. 2008a) and fungi (Batta et al. 2009; Santos et al. 2009), very few in the literature are from medicinal fungi. The present report would furnish additional information.

Materials and methods

Dried fruiting bodies of *Cordyceps militaris* (100 g), collected from Guang Dong, Mainland China were homogenized in liquid nitrogen with a pestle, extracted in distilled water, and centrifuged. To the resulting supernatant, NH_4OAc (pH 4.5) buffer was added until a final concentration of 20 mM was attained. The sample was loaded on an SP-Sepharose column (5 cm × 16 cm) (GE, Healthcare). After removal of unadsorbed proteins, adsorbed proteins were eluted with 1 M NaCl in 20 mM NH_4OAc buffer (pH 4.5). The adsorbed fraction was dialyzed against distilled water and lyophilized. Then it was dissolved in 20 mM NH_4OAc buffer (pH 4.5) and applied on a Mono S column and eluted with the same buffer using an AKTA Purifier FPLC System (GE Healthcare). After the unadsorbed fraction had been eluted, the adsorbed protein was desorbed by using two consecution linear NaCl concentration gradients (0–0.2 M and 0.2–1 M) in 20 mM NH_4OAc buffer (pH 4.5). The fraction containing antifungal activity from the Mono S column was concentrated with an Amicon filter device before final purification

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on a Superdex 75 column in the same buffer. The single peak eluted constituted purified antifungal protein designated as cordymin.

Molecular mass determination by SDS-PAGE and mass spectrometry

SDS-PAGE was conducted as described by Nielsen and Reynolds (1978). After electrophoresis, the gel was stained with Coomassie Brilliant Blue. The molecular mass of the isolated peptide was estimated by comparison of its electrophoretic mobility with those of molecular mass marker proteins from GE Healthcare. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in an Applied Biosystems 4700 Proteomics Analyzer was also utilized for determining the molecular mass of the purified peptide (Wong et al. 2008b).

Amino acid sequence analysis

The N-terminal amino acid sequence of cordymin was analyzed by means of automated Edman degradation using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system (Wong et al. 2008b).

Assay of antifungal activity in filamentous fungi

The assay of activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola*, *Valsa mali*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Aspergillus fumigatus* (all from China Agricultural University) was performed in petri dishes containing potato dextrose agar (PDA). Each of the fungal species used is pathogenic to a different agriculturally important crop. The crops comprise apple, orange, cotton, and maize, etc. After the mycelial colony has developed, sterile blank paper disks were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot of a solution of cordymin was added to individual disks. The dish was incubated at 25 °C for 20–48 h until mycelial growth had enveloped disks containing the positive control (haricot bean defensin) and had formed crescents of inhibition around disks containing samples with antifungal activity.

To determine the IC_{50} value for the antifungal activity, five doses of the protein were added separately to five aliquots each containing PDA, mixed and poured into five separate small dishes. Buffer only served as a control. After the agar had cooled down, a known amount of mycelia will be added. After incubation at 25 °C for 20–72 h, the concentration of antifungal protein leading to 50% decrease in the area of mycelial colony, defined as the IC_{50}, was determined (Wong and Ng 2006b).

Assay of antifungal activity in Candida albicans

The fungal strain tested was *Candida albicans* ATCC 820. Cells from early logarithmic-phase cultures were washed twice in 50 mM sodium phosphate buffer (pH 7.4), and resuspended in culture medium RPMI 1640, and adjusted to 2.0×10^6 CFU/ml. Cordymin solutions at different concentrations were then added to the cell suspensions and incubated in a shaker for 12 h. Then, the mixtures were serially diluted with RPMI 1640, and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted. The number of fungi for each dilution was determined from the average colony counts for three plates (Wong and Ng 2005). Defensin was employed as a positive control.

Assay for HIV-reverse transcriptase inhibitory activity

The ability of cordymin to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described by Collins et al. (1997). Defensin was employed as a positive control.

Assay of antiproliferative activity

Breast cancer (MCF-7) and colon cancer (HT-29) cells were suspended in medium. An aliquot of this cell suspension was seeded to a plate, followed by incubation overnight at 37 °C in an atmosphere of 5% CO_2 in air. The antifungal protein was then added and incubated for 24 h or 48 h. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was spiked into each well and the plates incubated for 4 h. The supernatant was removed and dimethyl sulfoxide was added to dissolve the MTT-formazan formed at the bottom of the wells. After 10 min, OD595 nm was measured. Haricot bean defensin was used as a positive control (Wong and Ng 2006a).

Assay of mitogenic activity

BALB/c mice (25–30 g) were sacrificed by cervical dislocation and the spleens aseptically dissected out. Spleen cells were isolated by pressing the tissue through a sterilized sieve, and resuspended in culture medium. The cells were seeded into a plate and cordymin was added. After incubation at 37 °C, [methyl-^{3}H]-thymidine was added, and the cells were incubated for 6 h. The cells were harvested onto a glass fiber filter using an automated cell harvester. The radioactivity was measured in a liquid scintillation counter. Haricot bean defensin with mitogenic activity, and Con A which is a lectin with highly potent mitogenic activity, were used as a sample control and a positive control, respectively (Wong and Ng 2005).

Assay of nitric oxide production by macrophages

The assay was executed as detailed by Wong and Ng (2006a). Macrophages collected from the peritoneal cavity of mice following an intraperitoneal injection of thioglycolate were washed, resuspended in RPMI 1640, and seeded in a plate for 1 h before incubation with cordymin for 24 h. Culture medium from each culture well was allowed to react with Griess reagent for 10 min before OD540 nm was read. Lipopolysaccharide was used as a positive control.

Assay of protease activity

This assay was conducted in view of the observation that a protease exhibits antifungal activity (Park et al. 2009). In the assay, a solution of casein (Sigma) used as substrate was freshly prepared as described by Wong et al. (2008a). Briefly, to 2 g of casein, 10 ml of distilled water and 10 ml of 0.2 mol l−1 NaOH were introduced. Subsequent to addition of 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 exposed to 90 °C for 15 min before cooling down and dilution with 100 ml of 100 mM of Tris–HCl buffer (pH 8) containing 40 mM $CaCl_2$. The precipitate was discarded, and the resulting solution was used. The test sample (cordymin) or trypsin solution (positive control) (50 μl) was mixed with 350 μl of the aforementioned casein solution. After incubation for 25 min, 1 ml of 4% (w/v) trichloroacetic acid was added. The reaction mixture was left at room temperature for 30 min prior to centrifugation at 15,000 × g for 15 min. The absorbance of the supernatant, which indicates the amount of casein fragments formed by proteolytic action of the test sample, was read at 280 nm against water as blank.

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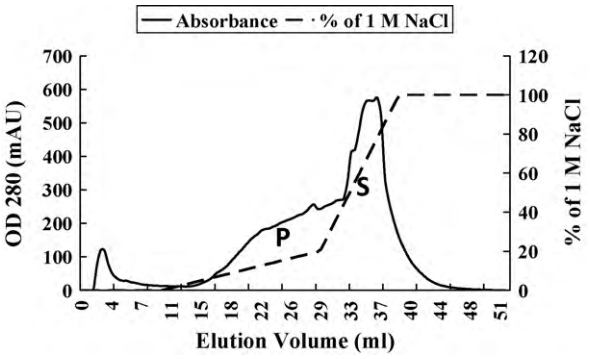

Fig. 1. FPLC on a 1-ml Mono S™ column previously equilibrated with and then eluted with 20 mM NH_4OAc buffer (pH 4.6) at a flow rate of 2 ml/min. The adsorbed fraction was eluted with one breakpoint linear gradient of 0–0.2 M, followed by 0.2–1 M NaCl in the same buffer as shown by the broken line across the chromatogram. Only the fraction labeled S showed antifungal activity.

Results

Isolation and physicochemical characteristics

Cation exchange chromatography of the extract of *Cordyceps militaris* on SP-Sepharose in 10 mM NH_4OAc buffer (pH 4.5) yielded an unadsorbed fraction devoid of antifungal activity and an adsorbed fraction with antifungal activity which was eluted with 1 M NaCl in 10 mM NH_4OAc buffer (pH 4.5) (data not shown). FPLC of the adsorbed fraction on Mono S resulted in a small unadsorbed fraction without antifungal activity. Elution of the adsorbed proteins with the two linear NaCl concentration gradients (0–0.2 M and 0.2–1 M) in 10 mM NH_4OAc buffer (pH 4.5) produced two major adsorbed fractions, S and P (Fig. 1). Antifungal activity was detected only in fraction S. Fraction S was subsequently resolved on Superdex 75 into a main fraction C with antifungal activity and some tiny fractions without activity (Fig. 2). Fraction C displayed a single band with a molecular mass below 14 kDa in SDS-PAGE (Fig. 3). The purity and molecular mass of fraction C were also examined by mass spectrometry. The spectrum shows only one peak with a molecular mass of 10906.62 Da (Fig. 4). The peptide in fraction C was named cordymin.

The N-terminal sequence of cordymin was AMAPPYGYRTPDAAQ. It did not show any significant similarity with other known proteins/peptides in Pubmed database.

Biological characteristics

Cordymin inhibited mycelial growth in a number of fungal species including *Bipolaris maydis*, *Mycosphaerella arachidicola*

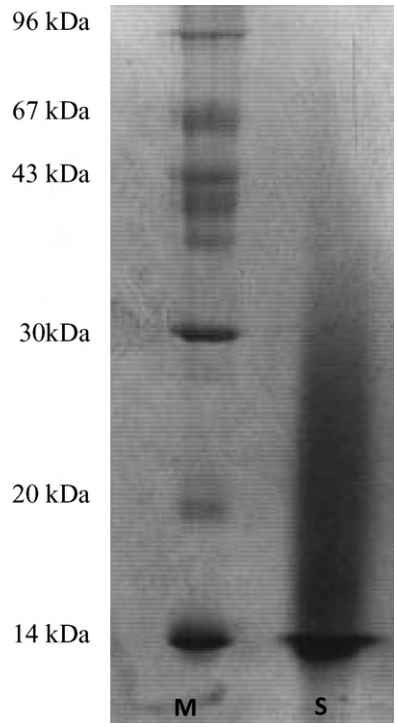

Fig. 3. SDS-PAGE results. Right lane S: peak C from Superdex 75 column representing purified cordymin from *Cordyceps militaris*. Left lane M: molecular mass standards from GE Healthcare.

(Fig. 5), *Rhizoctonia solani*, and planktonic form of the yeast *Candida albicans* (Fig. 6) with an IC_{50} of 50 μM, 10 μM, 80 μM, and 0.75 mM, respectively. No inhibitory activity was demonstrated toward *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Valsa mali* when tested up to 2 mM. The antifungal activity of cordymin was stable throughout the temperature range 0–100 °C and at pH 4.5, 7.4 and 9.4 (data not shown). The activity was unaffected in the presence of 10 mM $ZnCl_2$ and 10 mM $MgCl_2$ (data not shown). Cordymin did not show any protease activity when tested up to 1 mM (data not shown). Its antifungal activity was not eliminated by trypsin digestion at 37 °C for 30 min at a cordymin: trypsin ratio of 1:1 (w:w).

Cordymin exerted an antiproliferative action on MCF 7 breast tumor cells (Fig. 7), but there was no effect on HT 29 colon cancer cells when tested up to 1 mM. It was devoid of mitogenic activity on mouse splenocytes (Table 1) and nitric oxide inducing activity toward mouse macrophages when tested up to 1 mM (data not

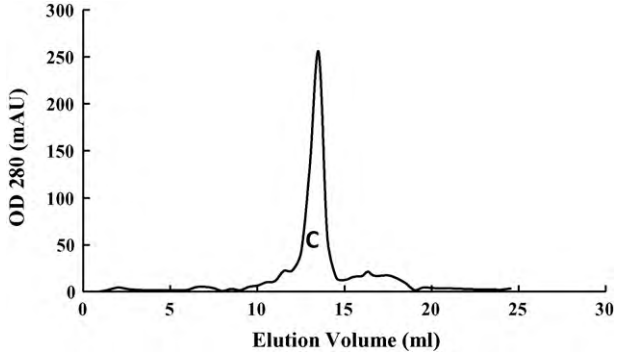

Fig. 2. Gel filtration of peak S (from Mono S™ column) on a Superdex 75 column in 20 mM NH_4HCO_3 buffer (pH 9.4) at a flow rate 0.5 ml/min.

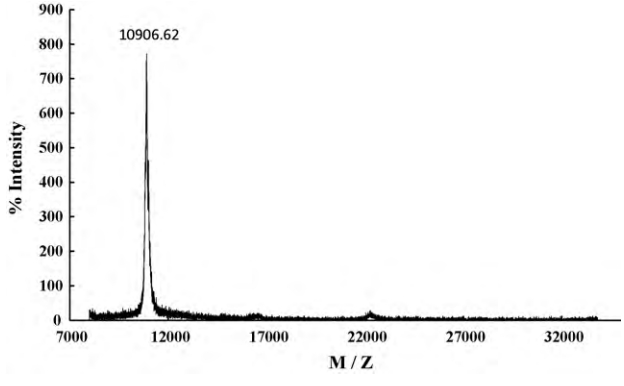

Fig. 4. Molecular mass determination by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

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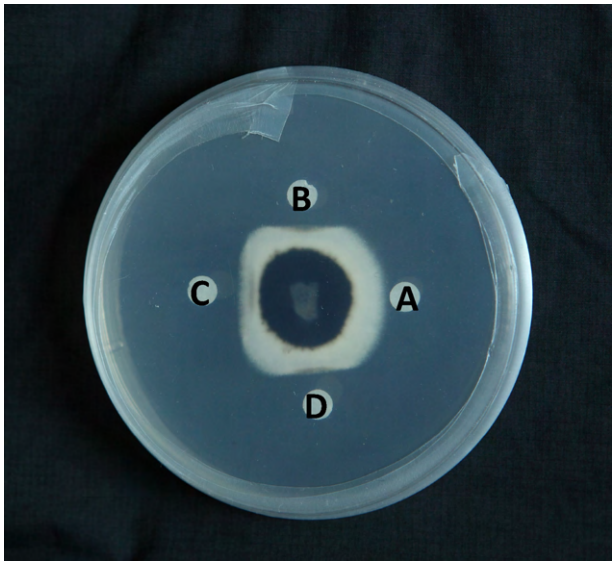

Fig. 5. Determination of antifungal activity of cordymin toward *Mycosphaerella arachidicola* (A: phosphate buffer saline (PBS, pH 7.6); B: 20 μM cordymin in PBS; C: 10 U nystatin in PBS; D: 5 μM defensin (haricot bean) in PBS).

shown). Cordymin also exerted an inhibitory effect toward HIV-1 reverse transcriptase with IC_{50} 55 μM.

Cordymin did not reveal protease activity toward casein.

Discussion

Antifungal proteins have aroused the attention of many researchers because of their ability to deter pathogenic fungi from invading agricultural crops and from causing diseases in animals. Plants expressing the genes encoding antifungal proteins have augmented resistance against fungal pathogens and huge economic losses due to deleterious fungal infections can be averted. Antifungal proteins may also protect animals including human from debilitating infections.

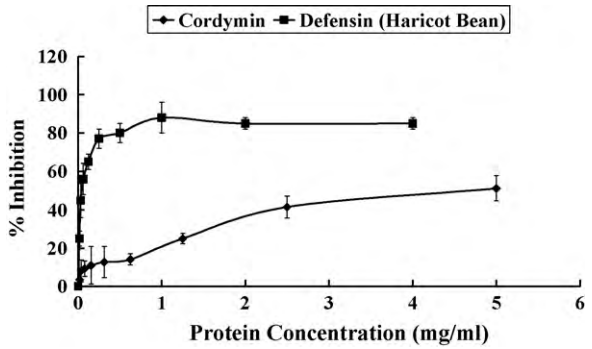

Fig. 7. Antiproliferative activity of cordymin and defensin (Haricot bean) towards breast cancer (MCF-7) cells after treatment for 24 h as indicated by MTT assay results (data represent means ± SD, $n = 3$).

Antifungal proteins with different amino acid sequences are produced by different organisms. For instance, defensins with distinct structures are produced by humans, other animals and plants (Wong et al. 2007). In addition to defensins, plants produce a repertoire of structurally diverse antifungal proteins (Selitrennikoff 2001). Fungi including medicinal fungi and others also produce a variety of antifungal proteins (Nig 2004). Ganodermin is an antifungal protein isolated from the renounced medicinal fungus *Ganoderma lucidum* (Wang and Ng 2006). The antifungal protein cordymin isolated from *C. militaris* in this study differs in N-terminal sequence from ganodermin and also antifungal proteins/peptides from other edible/non-medicinal fungi (Chu et al. 2005; Wang and Ng 2004a), indicating that it is a protein distinct from previously reported antifungal proteins.

Cordymin is adsorbed on cation exchangers. This chromatographic characteristic is also encountered in other antifungal proteins such as juncin (Ye and Ng 2009), protein from caper seeds (Lam and Ng 2009), and cicadin (Wang and Ng 2002a). The feature is also consistent with the observation that some antifungal proteins are unadsorbed on anion exchangers like DEAE-cellulose (Wong and Ng 2006b). The molecular mass of cordymin is within the range reported for antifungal proteins/peptides.

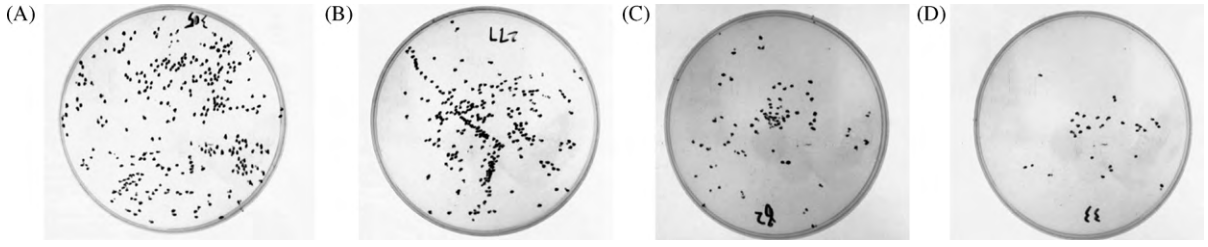

Fig. 6. Determination of IC_{50} value of antifungal activity of cordymin toward *Candida albicans* (A: 0 M cordymin; B: 0.5 mM cordymin; C: 1 mM cordymin, D: 2 mM cordymin).

Table 1
Test of cordymin for mitogenic activity toward murine splenocytes as reflected in uptake of [methyl-^{3}H]-thymidine (data represent means ± SD, $n = 3$).

Concentration of cordymin/defensin/Con A (μM)	[Methyl-^{3}H]-thymidine uptake (CPM)		
	Cordymin	Defensin	Con A
1000	323 ± 11	234 ± 23	348 ± 23
500	476 ± 12	289 ± 14	209 ± 29
250	435 ± 21	370 ± 25	222 ± 22
125	332 ± 28	351 ± 10	243 ± 12
62	453 ± 16	480 ± 20	233 ± 12
31	419 ± 18	442 ± 14	245 ± 28
16	440 ± 23	362 ± 13	1236 ± 128
8	348 ± 10	287 ± 32	12330 ± 831
4	493 ± 22	1287 ± 89	8736 ± 526
0	228 ± 18	332 ± 36	250 ± 23

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Cordymin exerts antifungal activity against several fungal species including *B. maydis*, *M. arachidicola and R. solani*, unlike the antifungal proteins from shallot bulbs (Wang and Ng 2002b) and asparagus seeds that inhibit only one out of the several fungal species tested. The antifungal potency of cordymin is higher than that of many antifungal proteins. Its antifungal activity is unaffected by zinc and magnesium ions, unlike defensins which are influenced by the ambient ionic strength (Wong et al. 2006). Cordymin is also dissimilar from antifungal proteins such as leguminous defensin (Wong et al. 2006) in that it is devoid of mitogenic activity toward splenocytes and nitric oxide inducing activity toward macrophages. However, it is known that not all antifungal proteins/peptides have these two attributes (Ye and Ng 2009).

Cordymin elicits a reduction in the proliferation of MCF-7 breast cancer cells but not that of HT-29 colon cancer cells. This specificity of antiproliferative action is reminiscent of the vastly different inhibitory potencies of ribosome inactivating proteins toward different cancer cells (Tsao et al. 1990).

The observation that cordymin inhibits activity of HIV-reverse transcriptase reinforces the contention that it is an antipathogenic or a defense protein. Other antifungal proteins (Samaranayake et al. 2001; Wang and Ng 2002a), lectins/hemagglutinins (Li et al. 2008; Ye et al. 2001), ribosome inactivating proteins (Jiratchariyakul et al. 2001; Lee-Huang et al. 1995), ribonucleases (Wang and Ng 2004b; Xia et al. 2005), and protease inhibitors (Ye and Ng 2002; Zhang et al. 2008), which are also defense proteins, display similar activity. The mechanism of inhibition probably involves protein–protein interaction, as in inhibition of the retroviral reverse transcriptase by the homologous protease (Bottcher and Grosse 1997).

Cordymin is a distinct from protein from *C. sinensis* antibacterial protein. This is apparent from a comparison of their N-terminal sequences, molecular masses and biological activities. The latter lacks antifungal activity (Ng and Wang 2005).

Cordymin is distinct in several aspects from the antifungal protein (CMP) isolated by Park et al. (2009) from *C. militaris*. The spectra of antifungal activities of the two peptides are not identical. CMP exerts a strong antifungal effect against *Fusarium oxysporum*, but cordymin does not show antifungal effect on *F. oxysporum* when tested up to 2 mM. CMP is adsorbed on anion exchanger DEAE-Sepharose, but cordymin is not. CMP shows a molecular mass about 12 kDa, but cordymin is only 10 kDa in molecular mass. The hemagglutinin reported by the Korean group of investigators also differs from the hemagglutinin purified by us from the same medicinal fungus (Jung et al. 2007; Wong et al. 2009). The discrepancies between the data on *Cordyceps militaris* antifungal peptide and hemagglutinin from the Korean group and our group suggest the possibility that different strains of *C. militaris* were used in the two studies.

The antifungal protein isolated by Park et al. (2009), has not been assayed for stability in presence of Zn^{2+} and Mg^{2+} ions, HIV-reverse transcriptase inhibitory activity, mitogenic activity toward spleen cells, and nitric oxide inducing activity toward macrophages.

Cordymin is an exploitable peptide in view of its pronounced thermostability (0–100 °C) and relative pH stability (pH 4.5–9.4), trypsin stability, relatively wide spectrum of antifungal activity (activity in 4 out of 7 fungal species tested), specific antiproliferative action on MCF-7 breast cancer cells, and inhibitory effect on HIV-1 reverse transcriptase. Cordymin manifests many of the attributes typical of defense proteins encompassing antifungal, anti-HIV-1 reverse transcriptase, and antiproliferative activities.

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