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An antifungal defensin from *Phaseolus vulgaris* cv. ‘Cloud Bean’

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**A R T I C L E  I N F O**

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**A B S T R A C T**

An antifungal peptide with a defensin-like sequence and exhibiting a molecular mass of 7.3 kDa was purified from dried seeds of *Phaseolus vulgaris* ‘Cloud Bean’. The isolation procedure entailed anion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, cation exchange chromatography on SP-Sepharose, and gel filtration by fast protein liquid chromatography on Superdex 75. Although the antifungal peptide was unadsorbed on DEAE-cellulose, it was adsorbed on both Affi-gel blue gel and SP-Sepharose. The antifungal peptide exerted antifungal activity against *Mycosphaerella arachidicola* with an IC\(_{50}\) value of 1.8 μM. It was also active against *Fusarium oxysporum* with an IC\(_{50}\) value of 2.2 μM. It had no inhibitory effect on HIV-1 reverse transcriptase when tested up to 100 μM. Proliferation of L1210 mouse leukemia cells and MBL2 lymphoma cells was inhibited by the antifungal peptide with an IC\(_{50}\) of 10 μM and 40 μM, respectively.

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**Introduction**

Fungi inflict tremendous damage to humans, other animals, and plants. Some fungal diseases are devastating to plants leading to crop destruction and enormous economic losses. Antifungal proteins have arrested the attention of investigators because of the tremendous economic implications.

To date, many different types of plant antifungal proteins are known. Among them are thaumatin-like proteins (Chu and Ng 2003; Pressey 1997; Wang and Ng 2002; Ye et al. 1999), glucanases (Vogelsang and Barz 1993), chitinases and chitinases-like proteins (Lam et al. 2000; Vogelsang and Barz 1993), ribosome inactivating proteins (Roberts and Selitrennikoff 1986), defensins (Thevissen et al. 2003; Wong and Ng 2003), peroxidases (Ye and Ng 2002), allergen-like proteins (Ye et al. 2001a), protease inhibitors (Chilosi et al. 2000; Ye et al. 2001a,b), lectins (Ye et al. 2001b), lipid transfer proteins (Cammue et al. 1995; Wang et al. 2004), embryo abundant protein-like proteins (Wang and Ng 2000), cyclophilin-like proteins (Ye and Ng 2000), and others (Wang et al. 2001; Wang and Ng 2005; Ye et al. 2002). Many of the aforementioned antifungal proteins are also referred to as pathogenesis related proteins (Van Loon 1990). Antifungal proteins have also been purified from animals including insects (Iijima et al. 1993).

The aim of the present investigation was to isolate an antifungal protein from the seeds of the cloud bean cultivar of *Phaseolus vulgaris* and to ascertain which type of antifungal protein it belongs to. Its characteristics and activities were compared with those of other antifungal proteins.

**Materials and methods**

**Isolation of antifungal protein**

Dried seeds of *Phaseolus vulgaris* ‘Cloud Bean’ (250 g) from Mainland China were purchased from a vendor in Hong Kong. They were authenticated by Prof. Shiu Ying Hu, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong and then deposited in Laboratory 302, School of Biomedical Sciences, under the voucher number PVCB 195. They were homogenized in distilled water (6 ml/g) and the homogenate was centrifuged at 12 000 × g for 20 min at 4°C. The supernatant was collected and loaded on a column (5 cm × 20 cm) of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.2). Following removal of unadsorbed proteins in fraction D1, adsorbed proteins were eluted from the column with 0.2 M NaCl and then with 1 M NaCl added to the 10 mM Tris–HCl buffer, to yield fractions D2 and D3, respectively. Fraction D1 was applied to a column (5 cm × 18 cm) of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.2). Following elution of unadsorbed proteins in fraction D1, adsorbed proteins were eluted from the column with 0.2 M NaCl and then with 1 M NaCl added to the 10 mM Tris–HCl buffer, to yield fractions D2 and D3, respectively. Fraction D1 was applied to a column (5 cm × 18 cm) of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.2). Following elution of unadsorbed proteins in fraction D1, adsorbed proteins were desorbed initially with 0.2 M NaCl in the Tris–HCl buffer to yield fraction B1, and subsequently with 1 M NaCl in the Tris–HCl buffer to yield fraction B2. Fraction B1 was further purified by ion exchange chromatography on a column (2.5 cm × 20 cm) of SP-Sepharose (GE Healthcare) in 10 mM NH\(_4\)OAc buffer (pH 5.0). After elution of unadsorbed proteins (fraction S1), adsorbed proteins were desorbed with a linear
concentration (0–1 M) gradient of NaCl. The fraction with antifungal activity (S2) was then further fractionated by fast protein liquid chromatography on a Superdex 75 HR 10/30 column (GE Healthcare) in 0.2 M NH₄HCO₃ buffer (pH 8.5). The last absorbance peak (SU3) represented purified antifungal peptide.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

It was conducted as described by Laemmli and Favre (1973) using 18% gel. Following electrophoresis under non-reducing conditions, the gel was stained with Coomassie Brilliant Blue. The molecular mass of the antifungal peptide was estimated by comparison of its electrophoretic mobility with those of molecular mass marker proteins from GE Healthcare including horse myoglobin peptides of different molecular mass: 16 949, 14 404, 10 700, 8 159 and 6 214 Da. The molecular mass was also determined using gel filtration on a Superdex Peptide column (GE Healthcare).

Amino acid sequence analysis

The amino acid sequence of the antifungal peptide was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer by means of automated Edman degradation. Microsequencing was conducted using 18% gel. Following electrophoresis under non-reducing conditions, the gel was stained with Coomassie Brilliant Blue. The molecular mass of the antifungal peptide was estimated by comparison of its electrophoretic mobility with those of molecular mass marker proteins from GE Healthcare including horse myoglobin peptides of different molecular mass: 16 949, 14 404, 10 700, 8 159 and 6 214 Da. The molecular mass was also determined using gel filtration on a Superdex Peptide column (GE Healthcare).

Determination of protein concentration

It was carried out by using the dye binding reagent (Bio-Rad).

Assay of antifungal activity

The assay for antifungal activity toward Mycosphaerella arachidicola, Physalospora piricola and Fusarium oxysporum was conducted 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 laid at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (15 μl) of the antifungal peptide was added to a disk. The plates were then left at 23 °C for 72 h until mycelial growth had surrounded the disks containing the control and had produced crescents of inhibition around disks containing samples with antifungal activity (Wang and Ng 2001).

To determine the IC₅₀ value for the antifungal activity, three doses of the antifungal peptide were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into three separate small Petri dishes. After the agar had cooled down, the same amount of mycelia was added to each plate. Buffer without the antifungal peptide was used 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 was calculated. From a graph plotting % reduction in area of mycelial colony (IC₅₀) compared with the control was determined (Wong and Ng 2003).

Test of ability to permeabilize fungal membranes

The assay was conducted by the uptake of SYTOX Green, a high-affinity affinity stain that penetrates cells with compromised membranes as detailed by Thevissen et al. (2003). Briefly, fungi were cultured in the presence or in the absence of cloud bean defensin. SYTOX Green (Invitrogen) was added to the fungal cultures (0.5 mM final concentration). After incubation for 10 min, the fungal cells were examined for the presence of the dye by using a fluorescence microscope (Nikon TE2000). An excitation wavelength of 500–540 nm was used.

Assay of inhibitory activity toward human immunodeficiency virus reverse transcriptase (HIV-1 RT)

The assay was performed according to instructions provided with the assay kit from Boehringer-Mannheim (Germany). The assay makes use of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of 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 ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. Next, an antibody to digoxigenin conjugated to peroxidase binds to the digoxigenin-labeled DNA. Finally, the peroxidase substrate is added. The peroxidase enzyme catalyzes cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm, which is directly correlated to the level of RT activity, can be measured with a microtiter plate (ELISA) reader. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the isolated peptide was calculated as percent inhibition as compared to a control without the peptide (Ng et al. 2002; Ng and Wang 2001). The defensin gynmin (Wong and Ng 2003) was used as a positive control.

Assay of ability to inhibit HIV-1 integrase

Expression and purification of recombinant HIV-1 integrase

The plasmid that expressed His-tagged wild-type HIV-1 integrase, pT7–7–His (YTIX–HIV-1-IN, was a generous gift from Professor S.A. Chow (School of Medicine, UCLA). To express the protein, a 1–1 culture of E. coli BL21 (DE3) cells containing the expression plasmid was grown at 37 °C until OD600 reached 0.7–0.8. Cells were induced by addition of 0.8 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and harvested, after 4 h of incubation, by centrifugation at 6000 × g for 10 min at 4 °C. Cells were suspended at a concentration of 10 ml/g wet cell paste in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM EDTA, 2 mM ZnCl₂, 50 mM NaCl and 5 mM imidazole. Lysozyme was added to a concentration of 0.2 mg/ml. After incubation at 4 °C for 1 h, the lysate was sonicated and centrifuged at 40 000 × g at 4 °C for 20 min. The pellet was homogenized in 50 ml buffer A (20 mM Tris–HCl, pH 8.0, 2 M NaCl, 2 mM β-mercaptoethanol) containing 5 mM imidazole. The suspension was rotated at 4 °C for 1 h, and cleared by centrifugation at 40 000 × g at 4 °C for 20 min. The supernatant was loaded onto a 1–ml chelating Sepharose (GE Healthcare) column charged with 50 mM imidazole. The column was washed with five column volumes of buffer A containing 5 mM imidazole, and the protein was eluted with three column volumes of buffer A containing 200 mM and 400 mM imidazole, respectively. Protein-containing fractions were pooled, and EDTA was added to a final concentration of 5 mM. The protein was dia lyzed against buffer B (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 M NaCl, 20% glycerol) containing 2 mM β-mercaptoethanol, and then against buffer B containing 1 mM dithiothreitol. Aliquots of the protein were stored at −70 °C (Loizidou et al. 2009).

HIV-1 integrase assay

A non-radioactive ELISA-based HIV-1 integrase assay was performed according to the DNA-coated plate method. In this study, 1 μg of 5-methyl reduced plasmid 5kS was coated onto each well in the presence of 2 M NaCl as target DNA. The donor DNA was
prepared by annealing VU5BR (5′-biotin-GTGTGAAAAATCTCTAGCAGT-3′) and VU5 (5′-ACTGCTAGAGATTTTCCACAGT-3′) in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 0.1 M NaCl at 80 °C followed by 30 min at room temperature. Integrase reaction was performed in 20 mM HEPES (pH 7.5) containing 10 mM MnCl2, 30 mM NaCl, 10 mM dithiothreitol and 0.05% Nonidet-P40 (Sigma). After the integrase reaction, the biotinylated DNA immobilized on the wells was detected by incubation with streptavidin-conjugated alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany), followed by colorimetric detection with 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl2. The absorbance due to the alkaline phosphatase reaction was measured at 415 nm. The ribosome inactivating protein trichosanthin was used as a positive control (Loizidou et al. 2009).

Assay of antiproliferative activity

The antiproliferative activity of the purified peptide was determined as follows. The cell lines L1210 (mouse leukemia) and MBL2 (lymphoma) from American Type Culture Collection were maintained in Dulbecco Modified Eagles’ Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 mg/l streptomycin and 100 IU/ml penicillin, at 37 °C in a humidified atmosphere of 5% CO2. Cells (1 × 104) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 3 h before addition of the peptide. Incubation was carried out for another 48 h. Radioactive precursor, 1/H9262 Ci, [methyl-3H] thymidine (GE Healthcare) was added to each well and incubated for 6 h. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting (Wong and Ng 2003).

Screening for inhibitory effect on SARS coronavirus protease

The activity of SARS coronavirus (CoV) protease was indicated by a cleavage of designed substrate which was composed of two proteins linked by a cleavage site for SARS CoV protease. The reaction was performed in a mixture containing 5 μM SARS CoV protease, 5 μM sample, 20 μM substrate and buffer [20 mM Tris–HCl (pH 7.5), 20 mM NaCl and 10 mM beta-mercaptoethanol] for 40 min at 37 °C. After 40 min, the reaction was stopped by heating at 100 °C for 2 min. Then the reaction mixture was analysed by SDS-PAGE. If SARS CoV protease is inhibited by the test sample, there is only one band, which is the intact substrate, shown in SDS-PAGE (Leung et al. 2008).

Results

Isolation of defensin

The extract of cloud beans was fractionated into three fractions of approximately equal size: an unadsorbed fraction D1 with antifungal activity and two adsorbed fractions D2 and D3 without activity (Table 1). Fraction D1 was resolved on Affi-gel blue gel into a larger unadsorbed fraction B1 devoid of antifungal activity, an adsorbed fraction B2 with antifungal activity eluted with 0.2 M NaCl in the Tris–HCl buffer, and an adsorbed fraction B3 eluted with 1.0 M NaCl in the Tris–HCl buffer but without antifungal activity (Table 1). Fraction B2 was separated on SP-Sepharose into a broad unadsorbed fraction S1 and three sharp adsorbed fractions S2, S3 and S4 (Fig. 1). Antifungal activity was detected only in the largest fraction S2 (Table 1). Fraction S2 was separated by gel filtration on Superdex 75 into three fractions SU1, SU2 and SU3 (Fig. 2). SU3, which displayed a molecular mass of 7.3 kDa, was the only fraction with antifungal activity (Table 1). Its amino acid sequence, which

| Column   | Chromatographic fraction | Yield (mg) | IC50 (μg/ml) |
|----------|--------------------------|------------|--------------|
| DEAE-cellulose | D1 unadsorbed*          | 1561       | 302.7        |
|           | D2 adsorbed              | 1092       | –            |
|           | D3 adsorbed              | 1224       | –            |
| Affi-gel blue gel | B1 unadsorbed          | 600        | –            |
| S-Sepharose   | S1 unadsorbed           | 23         | –            |
|           | S2 adsorbed              | 102        | 36.5         |
|           | S3 adsorbed              | 49         | –            |
|           | S4 adsorbed              | 58         | –            |
| Superdex 75 | SU1                     | 23         | –            |
|           | SU2                     | 19         | –            |
|           | SU3                     | 30         | 13.1         |

* Defensin-enriched fractions are highlighted in boldface.
manifests pronounced homology to plant defensins, is recorded in Table 2. Fraction SU3 appeared in SDS-PAGE as a single band with a molecular mass of 7.3 kDa (Fig. 3).

Biological activities of isolated defensin

The inhibitory action of the purified antifungal peptide represented by fraction SU3 on the fungi *Mycosphaerella arachidicola* and *Fusarium oxysporum* is shown in Figs. 4 and 5, respectively. There was a dose-dependent inhibition of mycelial growth. The antifungal peptide suppressed mycelial growth in *M. arachidicola* with an IC₅₀ value of 1.8 μM (Fig. 6) and *Fusarium oxysporum* with an IC₅₀ value of 2.2 μM. The antifungal peptide did not reduce the activity of HIV-1 reverse transcriptase. Neither did it affect the activities of HIV-1 integrase and SARS coronavirus proteinase (data not shown). But it inhibited the proliferation of L1210 and MBL2 tumor cells with an IC₅₀ of 1.0 μM and 40 μM, respectively (Table 3). In the assay of SYTOX Green uptake, cloud bean defensin (10 μM) could induce membrane permeabilization in *M. arachidicola* and *F. oxysporum* as

### Table 2

N-terminal amino acid sequence (1–40) of *Phaseolus vulgaris* ‘Cloud Bean’ defensin in comparison with related legume defensins.

| Species                                | N-terminal amino acid sequence (1–40) |
|----------------------------------------|---------------------------------------|
| *Phaseolus vulgaris* ‘Cloud Bean’      | KTYENLADYKGVYFTTGSHDDTHKNEHKRLSRGRYRDFF |
| *Clitora ternatea*                     | NLCERASLTWGNCGNTGHCICDTCQCNWESAHGCHKRGN |
| *Phaseolus vulgaris* cv. ‘White Cloud Bean’ | KTCNADTFTRGCFCSTNCDHCKNKEHLSLCRGRDFR  |
| *Pisum sativum*                        | NTCENLDSYKCVCFGGCDGRHCRCTQCAISGRCRDFFRCW |
| *Vicia faba*                           | LLGRCKVKNSRFNGPCLTDTYHCTVTCRGEGYKGDCCHGLR |
| *Vigna radiata* D1                     | ANCKTESMTFSFCITKPKACCFACSFSGCNKCKILEK  |
| *Vigna radiata* Ph1                    | ATCKAECFTWDEVIKNFKVCACEAEDSGDNCSKILEK  |
| *Vigna unguiculata*                    | KTCNVLDTYRGCPFHTGSCDDHCKNKEHLSLGCRCDDFR |

### Table 3

Antiproliferative activity (inhibition rate) of ‘Cloud Bean’ defensin toward L1210 and MBL2 tumor cells. Results are presented as mean ± SD (*n* = 3).

| Dose (μM) | %Inhibition of [methyl-³H] thymidine incorporation L1210 cells MBL2 cells |
|-----------|-------------------------------------------------------------------------|
| 40        | 98.2 ± 3.6                                                              |
| 20        | 97.8 ± 3.8                                                              |
| 10        | 94.6 ± 3.5                                                              |
| 5         | 88.5 ± 4.1                                                              |
| 2.5       | 77.3 ± 2.8                                                              |
| 1.3       | 63.5 ± 3.2                                                              |
| 0.7       | 28.9 ± 1.9                                                              |
| IC₅₀ (μM) | 1.0                                                                     |
|           | 40                                                                      |
Table 4

Comparison of biochemical characteristics and activities of defensins from Phaseolus vulgaris cv. ‘Cloud Bean’ and Phaseolus vulgaris cv. ‘White Cloud Bean’.

|                     | Cloud bean defensin | White cloud bean defensin |
|---------------------|---------------------|---------------------------|
| Molecular mass (kDa) | 7.3                 | 7.4                       |
| Chromatographic behavior on | Adsorbed | Adsorbed |
| Affi-gel blue gel |                          |                           |
| DEAE-cellulose | Unadsorbed | –                         |
| SP-Sepharose | Adsorbed | –                         |
| Antifungal activity (IC50) | 2.2 μM | 2.3 μM                |
| Fusarium oxysporum | 1.8 μM | 0.7 μM                |
| Mycosphaerella arachidicola | –   | 2.8 μM                |
| Botrytis cinerea |                           |                           |
| Antiproliferative activity (IC50) |                           |                           |
| L1210 | 10 μM | –                        |
| MBL2 | 40 μM | –                        |
| MCF7 | No activity at 100 μM | 5.7 μM                |
| HIV-1 reverse transcriptase inhibitory activity (IC50) | 120 μM |                           |

Cloud bean defensin potently inhibits proliferation of tumor cells. Antiproliferative activity toward tumor cells is also an attribute of defensins (Wong and Ng 2003), defense-like peptides and also other antifungal proteins including ribosome inactivating proteins (Lam et al. 1998) and chitinase-like proteins (Lam et al. 2000). This activity of cloud bean defensin may be a consequence of the protein synthesis inhibitory activity which is a characteristic of antifungal proteins (Ng and Ye 2003) including ribosome inactivating proteins (Ng and Parkash 2002). It is noteworthy that cloud bean defensin is inhibitory to both L1210 and MBL2 tumor cells. The antifungal protein passiflavin (Lam and Ng 2009) inhibits proliferation in breast cancer MCF7 cells, but not in hepatoma HepG2 cells.

Some antifungal proteins including defense-like peptides (Wong and Ng 2003), protease inhibitors (Ye et al. 2001a), thauatin-like proteins (Wang and Ng 2002), chitinase-like proteins (Lam et al. 2000) and ribosome inactivating proteins (Wong et al. 2008a,b) demonstrate HIV-1 reverse transcriptase inhibitory activity. It is somewhat surprising that cloud bean defensin is devoid of inhibitory activity toward the retroviral enzyme. Its lack of inhibition on HIV-1 integrase and SARS CoV protease is in concord with previous reports on other defensins (Leung et al. 2008).

The present findings on cloud bean defensin is reminiscent of the observation that mungin, an antifungal protein from mung beans, is without HIV-1 reverse transcriptase inhibitory activity (Ye and Ng 2000). The isolated defense-like antifungal peptide potently inhibits fungal growth in both fungal species examined. Some antifungal proteins are able to inhibit only one out of the several fungi tested (Wang and Ng 2001). Other antifungal proteins have a lower antifungal potency than cloud bean defensin (Ye et al. 1999).

To recapitulate, a defensin with potent antifungal, and antiproliferative activities was isolated from cloud beans. Like French bean defensin (Leung et al. 2008), it is devoid of inhibitory activity toward HIV-1 integrase and SARS coronavirus proteinase.

White cloud bean and cloud bean are two cultivars of Phaseolus vulgaris. Their defensins are similar in molecular mass, N-terminal sequence and antifungal potency. However, white cloud bean defensin, but not cloud bean defensin, has inhibitory activity toward HIV-1 reverse transcriptase. The former appears to be more potent in inhibitory activity toward tumor cells. Hence similar, but not identical proteins are produced by different cultivars.

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