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Cicerin and arietin, novel chickpea peptides with different antifungal potencies

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Abstract

Two antifungal peptides with novel N-terminal sequences, designated cicerin and arietin were isolated from seeds of the chickpea (*Cicer arietinum*), respectively. Both peptides were adsorbed on Affi-gel blue gel and CM-Sepharose and exhibited a molecular weight of approximately 8.2 and 5.6 kDa, respectively. Arietin was more strongly adsorbed on CM-Sepharose than cicerin and manifested a higher translation-inhibiting activity in a rabbit reticulocyte lysate system and a higher antifungal potency toward *Mycosphaerella arachidicola*, *Fusarium oxysporum* and *Botrytis cinerea*. Both were devoid of mitogenic and anti-HIV-1 reverse transcriptase activities.

Keywords: Chickpea; Antifungal peptides

1. Introduction

Seeds of the cowpea (*Vigna unguiculata*) elaborate α- and β-antifungal proteins which have disparate molecular weights, N-terminal sequences and antifungal potencies [25]. Similarly antifungal chitinases, a thaumatin-like protein and a glucanase are produced by chickpea [7,16]. Antifungal lectin and chitinase have been isolated from potato [6], and antifungal protein and ribosome-inactivating protein with a lower antifungal activity have been reported from barley [14,15]. The results indicate that structurally different antifungal proteins may be synthesized by a single species.

A thaumatin-like protein, a β-1,3-glucanase and two chitinases have been described from the chickpea [7,16]. We report herein the isolation of two novel and structurally dissimilar antifungal peptides from the chickpea.

2. Materials and methods

Seeds of the chickpea (*Cicer arietinum*) obtained from a local market were homogenized in distilled water (0.3 g/ml). The homogenate was centrifuged and the supernatant was dialyzed against distilled water. Tris–HCl buffer (pH 7.2) was added to the dialyzed supernatant so that the final concentration of Tris was 10 mM before affinity chromatography on a column of Affi-gel blue gel (25 × 10 cm). The unadsorbed proteins were removed by washing the column with 10 mM Tris–HCl (pH 7.2). Adsorbed proteins eluted with a NaCl concentration gradient were dialyzed against 10 mM Tris–HCl (pH 7.2) prior to chromatography on a column of CM-Sepharose (1.5 × 18 cm). Unadsorbed proteins were removed by eluting with 10 mM Tris–HCl buffer (pH 7.2) and adsorbed proteins were removed by washing the column with a NaCl concentration gradient. The adsorbed proteins were dialyzed and then subjected to gel filtration on a Superdex 75 column in 100 mM NH_4HCO_3 (pH 9) by fast protein liquid chromatography.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli and Favre using 15% gel [11]. N-terminal sequencing of the purified antifungal peptides was conducted using a Hewlett-Packard G-1000A Edman degradation unit and an HP 1000 HPLC system. The purified antifungal peptides were assayed for cell-free translation-inhibiting activity in a rabbit reticulocyte lysate system as described by Lam et al. [13]. Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. The antifungal peptide (10 μl) was added to 10 μl

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of hot mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine and 1 μCi[4,5-^{3}H] leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl creatine kinase. Incubation proceeded at 37 °C for 30 min before addition of 330 μl 1 M NaOH and 1.2% H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casein hydrolyzate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed and dried with absolute alcohol passing through a cell harvester attached to vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckman liquid scintillation counter.

The assay for ability to inhibit human immunodeficiency virus (HIV) reverse transcriptase activity was carried out as detailed by Collins et al. [4] using a non-radioactive ELISA kit. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hydrid poly(A) oligo (dT) 15. In place 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 ELISA protocol: biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next

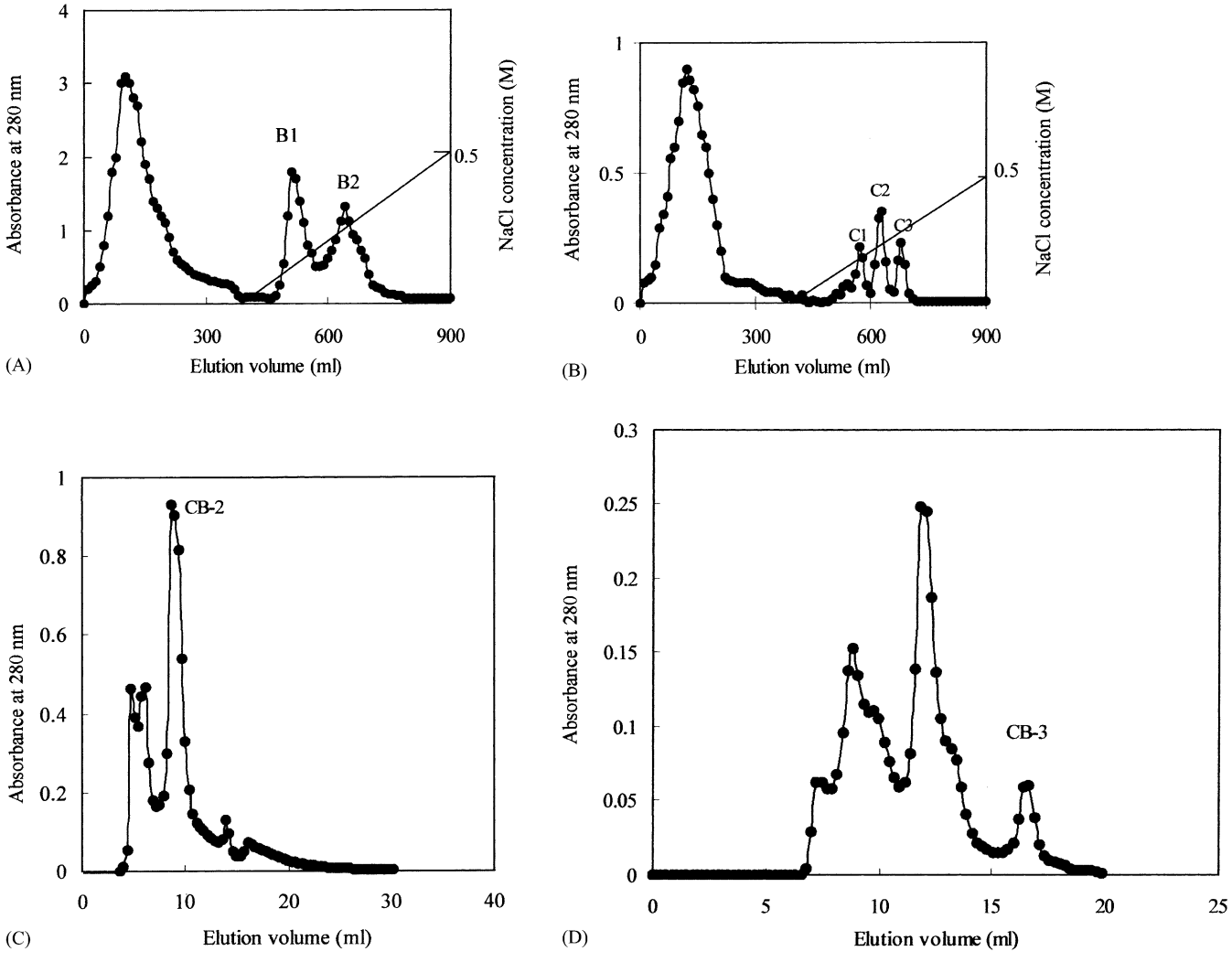


Fig. 1. (A) Affinity chromatography of a crude extract of chickpeas on a column of Affi-gel blue gel which had been equilibrated with and was eluted with 10 mM Tris–HCl buffer (pH 7.2). The linear NaCl concentration gradient (0–0.5 M) used to elute the column after the large unadsorbed peak had been eluted is indicated by the slanting line across the chromatogram. Peak B2 was the only peak with antifungal activity. (B) Ion-exchange chromatography of peak B2 on a column of CM-Sepharose which had been equilibrated with and eluted with 10 mM Tris–HCl buffer (pH 7.2). The linear NaCl concentration gradient (0–0.5 M) used to elute the column after the large unadsorbed peak had been eluted is indicated by the slanting line across the chromatogram. Peaks C2 and C3 contained antifungal activity. (C) Gel filtration of peak C2 on an FPLC-Superdex 75 column in 100 mM NH_4HCO_3 (pH 9). Peak CB-2 represents the purified antifungal protein cicerin. (D) Gel filtration of peak C3 on an FPLC-Superdex 75 column in 100 mM NH_4HCO_3 (pH 9). Peak CB-3 represents the purified antifungal protein arietin.

step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of antifungal peptide was calculated as percent inhibition as compared to a control without the antifungal peptide. The antifungal peptides were assayed for mitogenic activity in mouse splenocytes as detailed by Wang et al. [18].

The antifungal activity of the purified peptides was assayed using sterile petri plates (100 × 15 mm) containing 10 ml potato dextrose agar. After the mycelial colony had developed, at a distance of 1 cm from the rim of the mycelial colony were placed sterile paper disks (0.625 cm in diameter). The test sample was added to a disk, and the plate was incubated at 23 °C until mycelial growth from the central plate had enveloped disks containing the control (buffer) and had formed crescents of inhibition around disks with samples expressing antifungal activity. The fungi studied comprised *Mycosphaerella arachidicola*, *Fusarium oxysporum* and *Botrytis cinerea* [1]. The purified peptides are stable at the incubation temperature.

3. Results

Affinity chromatography of a crude extract of chickpeas on Affi-gel blue gel yielded a large unadsorbed peak and two smaller adsorbed peaks B1 and B2 (Fig. 1A). B2 was subjected to ion-exchange chromatography on CM-Sepharose to give an unadsorbed peak and three adsorbed peaks C1, C2 and C3 (Fig. 1B). C2 and C3 were then separately fractionated by gel filtration on Superdex 75 to give two and three peaks, respectively. The last peak in each chromatogram represents the purified antifungal peptide. They (CB-2 and CB-3) are designated cicerin and arietin, respectively (Fig. 1C and D). From 100 g chickpeas, 21.8 g crude extract and 256 mg B2 were obtained. Subsequently, 13.6 mg C2 and 2.8 mg CB2 (cicerin), and 8.1 mg C3 and 0.8 mg CB3 (arietin) were derived from B2. Their N-terminal sequences are presented in Table 1. A BLAST search did not reveal related sequences. Both arietin and cicerin demonstrated very low cell-free translation-inhibitory activity, with an IC_{50} of approximately 40 and 4.5 μM, respectively (Tables 1 and 2). Both cicerin and arietin demonstrated a low molecular weight of 8.2 and 5.6 kDa in gel filtration (Fig. 1C and D) and SDS–PAGE (Fig. 2, lanes d and b, respectively). The antifungal activity of cicerin and arietin towards various fungal species is illustrated in Fig. 3. Arietin appeared to have a stronger antifungal activity than cicerin. The IC_{50} values for antifungal activity of cicerin and arietin toward *B. cinerea* were 12 μM (Fig. 4a) and 2 μM (Fig. 4b), respectively. Cicerin and arietin devoid of mitogenic activity in mouse splenocytes when tested at 45 μm and inhibitory activity toward HIV-1 reverse transcriptase when tested at 200 μm.

Table 1
N-terminal sequences of the chickpea antifungal proteins arietin and cicerin

Arietin	GVGYKVVVTTTAAADDDDVV
Cicerin	ARCENFADSYRQPPISSSQT

Table 2
Inhibition of cell-free translation in rabbit reticulocyte lysate by the chickpea antifungal proteins arietin and cicerin

	Concentration (μM)	% Inhibition (mean ± S.D., $N = 3$)
Arietin	200.0	83.4 ± 2.9
	40.0	55.6 ± 3.1
	8.0	27.3 ± 1.8
	1.6	14.1 ± 0.9
Cicerin	72.0	90.5 ± 3.2
	18.0	67.9 ± 1.6
	4.5	53.5 ± 2.7
	1.2	31.6 ± 1.2

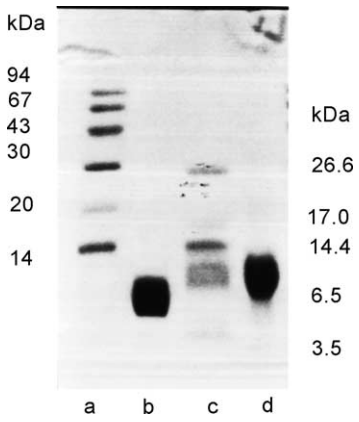


Fig. 2. SDS–PAGE of cicerin and arietin. Lane a: pharmacia molecular weight standards (from top downward, phosphorylase b, 94 kDa; bovine serum albumin (BSA), 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor 20 kDa and α-lactalbumin, 14 kDa). Lane b: arietin. Lane c: low molecular weight standards (from top downward, triose phosphate isomerase, 26.6 kDa; myoglobin, 17 kDa; α-lactalbumin, 14.4 kDa; aprotinin, 6.5 kDa; oxidized insulin B chain, 3.5 kDa). Lane d: cicerin.

4. Discussion

Antifungal proteins of different structures have been isolated. They include chitinases [16,22,23], ribosome inactivating proteins [15], cyclophilin-like proteins [19], miraculin-like proteins [24], lectins [2,6] and cysteine

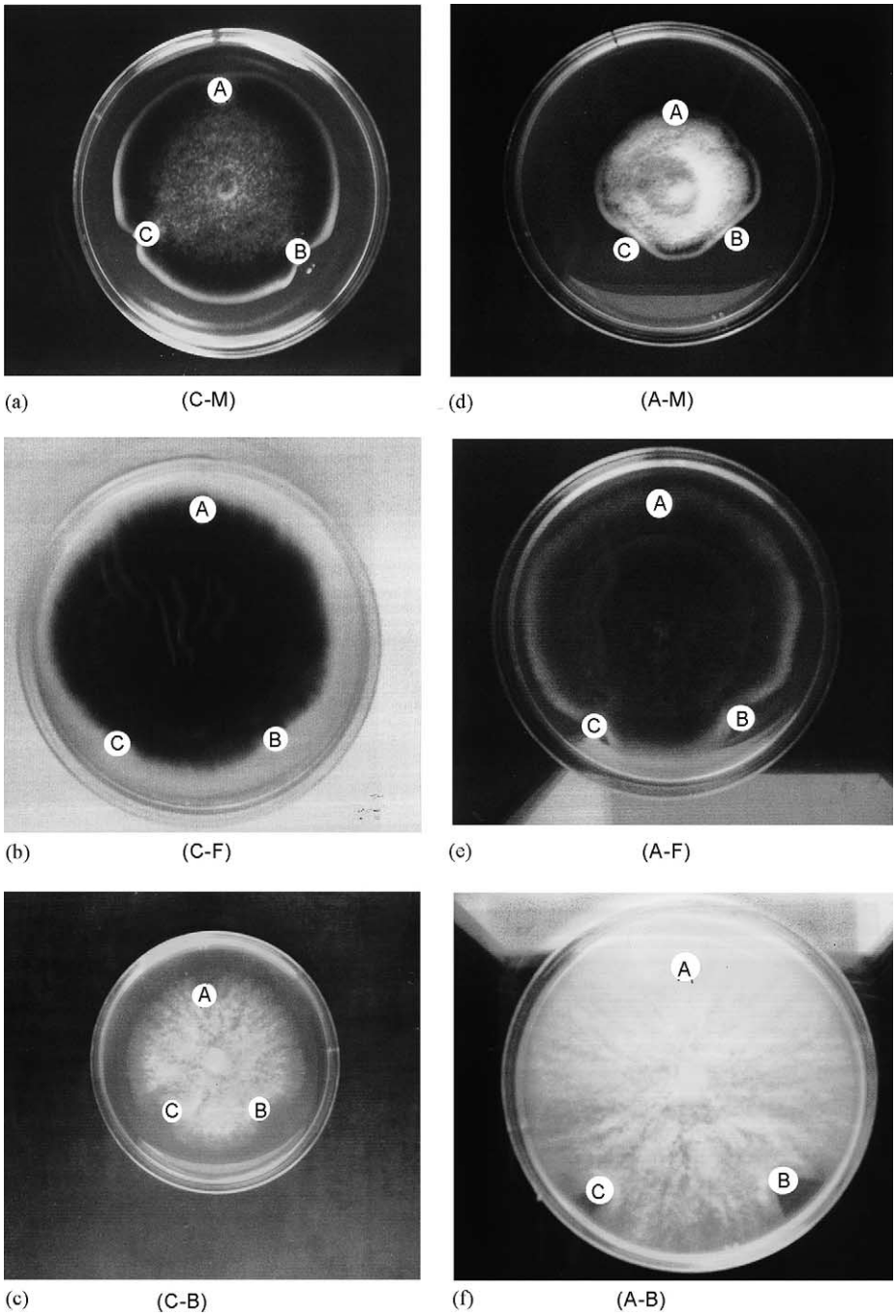
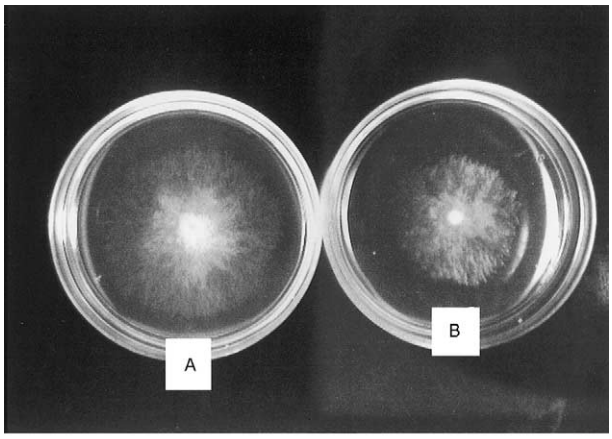
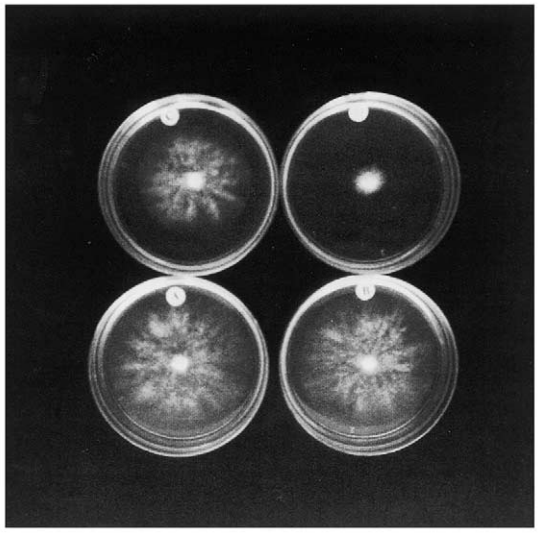


Fig. 3. Antifungal activity of cicerin towards: (a) *M. arachidicola* (A) 10 mM Tris–HCl buffer (pH 7.2), (B) 36 nmol cicerin (C) 7.2 nmol cicerin; (b) *F. oxysporium* (A) 10 mM Tris–HCl buffer (pH 7.2), (B) 36 nmol cicerin, (C) 7.2 nmol cicerin; (c) *B. cinerea* (A) 10 mM Tris–HCl buffer (pH 7.2), (B) 36 nmol cicerin, (C) 7.2 nmol cicerin. Antifungal activity of arietin towards: (d) *M. arachidicola* (A) 10 mM Tris–HCl buffer (pH 7.2), (B) 18 nmol arietin, (C) 3.6 nmol arietin; (e) *F. oxysporium* (A) 10 mM Tris–HCl buffer (pH 7.2), (B) 18 nmol arietin, (C) 3.6 nmol arietin; (f) *B. cinerea* (A) 10 mM Tris–HCl buffer (pH 7.2), (B) 18 nmol arietin, (C) 3.6 nmol arietin.

protease inhibitor [10]. Antifungal peptides have also been purified, such as chitinase-binding peptides from *Ginkgo biloba* leaves [8], a peptide with N-terminal sequence similarity to peanut allergen Ara H1 [21], the antimicrobial peptide Ace-AMP1 from onion seeds [3], and antifungal peptides with different sequences from insects [5,9]. The antifungal peptides isolated from chickpea in the present study were characterized by novel N-terminal sequences. Although the two chickpea peptides cicerin and arietin were similar in molecular weight (5–8 kDa), they differed somewhat in antifungal activity. Arietin was more potent against *B. cinereas*, *M. arachidicola* and *F. oxysporum*. Cicerin also



(a) (E-1)



(b) (E-2)

Fig. 4. (a) Inhibitory activity of 9.14 μM cicerin on growth of *B. cinerea*. Plate A represents control (no cicerin) and plate B, 9.14 μM cicerin. The other concentrations tested but not shown included 0.61, 3.05 and 15.24 μM. The IC_{50} was estimated to be 12 μM. (b) Inhibitory activity of 0.13, 0.67, and 3.33 μM arietin on *B. cinerea*. Plate A represents control; plate B, 0.13 μM; plate C, 0.67 μM; and plate D, 3.33 μM. The IC_{50} was determined to be 2 μM.

exhibited a higher cell-free translation-inhibiting activity than arietin.

In general, the chickpea antifungal peptides, cicerin and arietin were stronger in antifungal activity than mungin, a cyclophilin-like antifungal protein [19]; French bean thaumatin-like protein [22]; sativin, a miraculin-like antifungal protein [24]; and dolichin, a chitinase-like antifungal protein [23]. Cicerin and arietin were similar to cowpea chitinases [25] and *Panax notoginseng* chitinase [12] in their antifungal potency. However, the chickpea peptides were weaker than ginkbilobin, an embryo-abundant protein [17] and antifungal peptides from red and pinto bean [20]. Although the antifungal potency of cicerin and arietin may be similar to some of the chitinases, their N-terminal amino acid sequences are distinctly different, indicating that they are indeed new peptides with a fungistatic action.

Some of the leguminous antifungal proteins previously investigated were capable of inhibiting HIV-1 reverse transcriptase [23] or possessed immunomodulatory activity [19]. However, cicerin and arietin lacked this ability.

It is interesting to note that cicerin and arietin could be isolated with an isolation procedure used successfully for the purification of antifungal proteins from leguminous, as well as non-leguminous species [1,19,23–25]. The various antifungal proteins and peptides behaved similarly in that they were adsorbed on Affi-gel blue gel and CM-Sepharose.

The finding of two chickpea antifungal peptides in the present investigation, together with the previous report of thaumatin-like protein, glucanase and chitinases from the chickpea [7,16], point out the existence of multiple proteins/peptides with antifungal activity in the chickpea.

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References

[1] Benhamou N, Broglie K, Broglie R, Chet I. Antifungal effect of bean endochitinase on *Rhizoctonia solani*: ultrastructural changes and cytochemical aspect of chitin breakdown. Can J Microbiol 1993;39:318–28.
[2] 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.
[3] Cammue BPA, Thevissen K, Hendriks M, Eggermont K, Goderis IJ, Proost P, et al. A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer protein. Plant Physiol 1995;109:445–55.
[4] Collins RA, Ng TB, Fong WP, Wan CC, Yeung HW. A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs. Life Sci 1997;60 PL:345–51.
[5] Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hertu C, et al. 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.
[6] Gozia O, Ciopraga J, Bentia T, Lungu M, Zamfirescu I, Tudor R, et al. Antifungal properties of lectin and new chitinases from potato tuber. FEBS Lett 1995;370:245–9.
[7] Hanselle, T. Thesis West faelisehe Wilhelms-Universitaet Muenster, Institute for Biochemistry and Biotechnology of Plants, 1988.
[8] Huang X, Xie WJ, Gong ZZ. Characteristics and antifungal activity of a chitin binding protein from *Ginkgo biloba*. FEBS Lett 2000;478:123–6.
[9] Iijina 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.
[10] 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.
[11] Laemmli UK, Favre M. Gel electrophoresis of proteins. J Mol Biol 1973;80:575–99.

[12] Lam SK, Ng TB. Isolation of a small chitinase-like antifungal protein from *Panax notoginseng* (sanchi ginseng) roots. Int J Biochem Cell Biol 2001;33:287–92.
[13] Lam SSL, Wang HX, Ng TB. Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea *Pisum sativum*. Biochem Biophys Res Commun 1998;253:135–42.
[14] Leah R, Tommerup H, Svendsen I, Mundy J. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J Biol Chem 1991;246:1564–73.
[15] Roberts WK, Selitrennikoff CP. Isolation and partial characterization of two antifungal proteins from barley. Biochim Biophys Acta 1986;880:161–70.
[16] Vogelsang R, Barz W. Purification, characterization and differential hormonal regulation of a β-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.). Planta 1993;189:60–9.
[17] 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.
[18] Wang HX, Ng TB, Ooi VEC, Liu WK, Chang ST. A polysaccharide–peptide complex from cultured mycelia of the mushroom *Tricholoma mongolicum* with immunoenhancing and antitumor activities. Biochem Cell Biol 1996;74:95–100.
[19] Ye XY, Ng TB. Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. J Peptide Res 2001;57:330–6.
[20] Ye XY, Ng TB. Mungin, a novel cyclophilin-like antifungal protein from the mung bean. Biochem Biophys Res Commun 2000;273:1111–5.
[21] Ye XY, Ng TB. Peptides from pinto bean and red bean with sequence homology to cowpea 10-kDa protein precursor exhibit antifungal, mitogenic, and HIV-1 reverse transcriptase-inhibitory activities. Biochem Biophys Res Commun 2001;285:424–9.
[22] Ye XY, Wang HX, Ng TB. Dolichin, a new chitinase-like antifungal protein isolated from field beans (*Dolichos lablab*). Biochem Biophys Res Commun 2000;269:155–9.
[23] Ye XY, Wang HX, Ng TB. First chromatographic isolation of an antifungal thaumatin-like protein from French bean legumes and demonstration of its antifungal activity. Biochem Biophys Res Commun 1999;263:130–4.
[24] Ye XY, Wang HX, Ng TB. Structurally dissimilar proteins with antiviral and antifungal potency from cowpea (*Vigna unguiculata*) seeds. Life Sci 2000;67:3199–207.
[25] Ye XY, Wang HX, Ng TB. Sativin, a novel antifungal miraculin-like protein isolated from legumes of the sugar snap *Pisum sativum* var. *macrocarpon*. Life Sci 2000;67:775–81.