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An antifungal peptide from red lentil seeds

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ABSTRACT

An antifungal peptide, with a molecular mass of 11 kDa, was isolated from dry seeds of the red lentil (*Lens culinaris*) using a procedure that involved four chromatographic steps. The antifungal peptide was unadsorbed on DEAE-cellulose, and adsorbed on Affi-gel blue gel and S-Sepharose. The final chromatographic step involved gel filtration by fast protein liquid chromatography on Superdex 75. The antifungal peptide inhibited mycelial growth in *Mycosphaerella arachidicola* with an IC_{50} of 36 μM. It also exhibited antifungal activity against *Fusarium oxysporum*, but there was no inhibitory activity toward tumor cell lines and human immunodeficiency virus type 1 reverse transcriptase (RT).

1. Introduction

The seeds of leguminous plants produce an array of proteins and peptides with inhibitory activity on fungal growth. These include glucanases [11,35], chitinases [35], chitinase-like proteins [19,60,63], chitin-binding proteins [13,32,33], allergen-like peptides [54], miraculin-like proteins [62], cyclophilin-like proteins [55], lipid transfer protein-like proteins [2,47], thaumatin-like proteins [5,36,37,52,61], peroxidases [56], defensins and defensin-like proteins [22,26,29,48–51], lectins/hemagglutinins [1,8,10,59], ribosome inactivating proteins [17,20,34], protease inhibitors [3,14,15,23,58], and novel proteins/peptides [6,7,12,21,27,30,31,38–40,43,44,46,53,57,63].

The aforementioned proteins/peptides have been isolated from a variety of leguminous seeds. However, to date no reports about the existence of an antifungal protein/peptide in lentil (*Lens culinaris*) are available in the literature. Lentil seeds form a common dish in many families. The intent of the present study was to isolate an antifungal peptide from lentil seeds. The peptide inhibits the fungi *Fusarium oxysporum* and *Mycosphaerella arachidicola*, which are pathogenic to cotton and peanut plants, respectively.

2. Materials and methods

Dry seeds of red lentil (*Lens culinaris*) (500 g) were made by homogenizing them in distilled water (3 ml/g). The homogenate was then centrifuged (14000 × *g* for 25 min). The supernatant was collected and loaded on a 5 cm × 20 cm column of DEAE-cellulose (Sigma) in 10 mM Tris–HCl buffer (pH 7.4). Following removal of unadsorbed proteins (fraction D1), the column was eluted with 1 M NaCl in the Tris–HCl buffer. Fraction D1 was dialyzed and then chromatographed on a 5 cm × 15 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.4). After the unadsorbed proteins (fraction B1) had been eluted, the column was eluted sequentially with 0.2 M NaCl and 1 M NaCl in the Tris–HCl buffer to yield fraction B2 and B3, respectively. Fraction B2 was dialyzed against 10 mM NH_4OAc buffer (pH 5) and chromatographed on a 2.5 cm × 20 cm column of SP-Sepharose (Amersham Biosciences). After elution of unadsorbed proteins (fraction S1) with 10 mM NH_4OAc buffer (pH 5), the column was eluted with a 0–1 M NaCl concentration gradient in the NH_4OAc buffer. The first adsorbed fraction (SP2) was then subjected to gel filtration on a Superdex 75 HR 10/30 column

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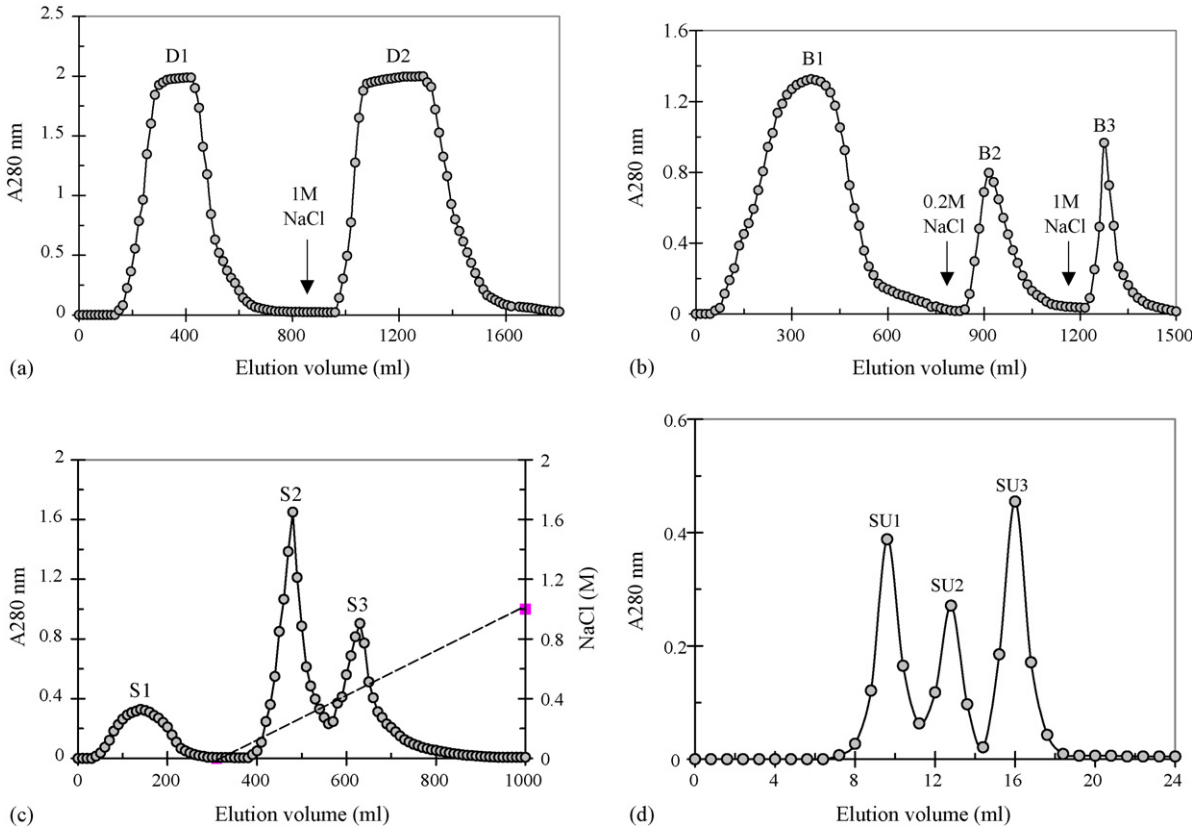

Fig. 1 – (a) Ion exchange chromatography on a DEAE-cellulose column (5 cm × 20 cm). Sample: Supernatant obtained by centrifugation of red lentil seed homogenate. Starting buffer: 10 mM Tris–HCl buffer (pH 7.4). Arrow indicates application of 1 M NaCl in starting buffer to yield fraction D2. Antifungal activity was detected in fraction D1. (b) Affinity chromatography on an Affi-gel blue gel column (5 cm × 15 cm). Sample: unadsorbed fraction (D1) from DEAE-cellulose column. Starting buffer: 10 mM Tris–HCl buffer (pH 7.4). Arrows indicate application of 0.2 M NaCl and 1 M NaCl in starting buffer to elute fractions B2 and B3, respectively. Antifungal activity was detected in fraction B2. (c) Ion exchange chromatography on a SP-Sepharose column (2.5 cm × 20 cm). Sample: fraction of bean extract that was unadsorbed on DEAE-cellulose and subsequently adsorbed on Affi-gel blue gel and eluted with 0.2 M NaCl. Starting buffer: 10 mM NH_4OAc buffer (pH 5). Dotted line indicates 0–1 M NaCl gradient in 10 mM NH_4OAc buffer (pH 5) used to elute adsorbed proteins. Antifungal activity was detected only in fraction S2. (d) Gel filtration by fast protein liquid chromatography on a Superdex 75 HR10/30 column using an AKTA Purifier (Amersham Biosciences). Sample: fraction S2 from SP-Sepharose column. Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. Antifungal activity was found exclusively in fraction SU3.

(Amersham Biosciences) in 0.2 M NH_4HCO_3 buffer (pH 8.5) by FPLC using an AKTA Purifier (Amersham Biosciences). The third absorbance peak SU3 represented purified antifungal peptide.

2.1. *Electrophoresis, molecular mass determination, and N-terminal sequence analysis*

The purified peptide was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [16]. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass of the peptide. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also employed to determine the molecular mass of the purified peptide using an Applied Biosystems 4700 Proteomics Analyzer. The N-terminal sequence of the peptide was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [18].

2.2. *Assay of antifungal activity*

The assay for antifungal activity toward *Mycosphaerella arachidicola* 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 (15 μl) of lentil antifungal peptide 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 crescents of inhibition around disks containing samples with antifungal activity.

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To determine the IC_{50} value for the anti-fungal activity, three concentrations of lentil antifungal peptide (0.6 μM, 3 μM, and 15 μM) 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, a small amount of mycelia, the same amount to each plate was added. Buffer only without lentil antifungal peptide served 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 determined. IC_{50} is defined as the concentration of the antifungal peptide that produces 50% reduction in the area of the mycelial colony.

2.3. *Assay for antiproliferative activity toward leukemia cells*

The antiproliferative activity of the purified peptide was determined as follows. The cell line L1210 (leukemia) and MBL2 (lymphoma) were purchased from American Type Tissue Culture. The cell lines 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% CO_2. Cells (1×10^4) 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 μCi ([^{3}H-methyl]-thymidine, from Amersham Biosciences) was then added to each well and incubated for 6 h. The cultures were then harvested using a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting [48–51].

2.4. *Assay for HIV-1 reverse transcriptase inhibitory activity*

The assay for HIV reverse transcriptase (RT) inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannhein (Germany). The assay takes advantage 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. The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. 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) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the peptide was calculated as percent inhibition as compared to a control without the protein [45].

3. Results

Ion exchange chromatography of red lentil seed extract on DEAE-cellulose yielded two fractions, an unadsorbed fraction D1 and an adsorbed fraction D2 (Fig. 1a). Antifungal activity resided in fraction D1 (Table 1). Affinity chromatography of fraction D1 on Affi-gel blue gel yielded large unadsorbed fraction B1 devoid of antifungal activity and two adsorbed fractions B2 and B3 both smaller than fraction B1 (Fig. 1b). Fraction B2 with antifungal activity was then further purified on S-Sepharose to yield a small inactive unadsorbed fraction S1, a large adsorbed fraction S2, and a smaller adsorbed fraction S3 (Fig. 1c). Antifungal activity was detected only in fraction S2 (Table 1), which was then subjected to FPLC–gel filtration on Superdex 75 to give three fractions: SU1, SU2, and SU3 (Fig. 1d). Antifungal activity resided in fraction SU3. This fraction demonstrated a molecular mass of 11 kDa in gel filtration on Superdex 75 (Fig. 1d) and smaller than 14.4 kDa in SDS-PAGE (Fig. 2). The molecular mass determined by MALDI-TOF MS was 11067 Da (data not shown). It inhibited mycelial growth in the fungi *Mycosphaerella arachidicola* (Fig. 3a) and *Fusarium oxysporum* (Fig. 3b). The IC_{50} value for the antifungal activity on *M. arachidicola* was 36 μM (0.4 mg/ml). Table 1 presents the yields and antifungal activities of the various chromatographic fractions. Table 2 presents the N-terminal sequences of the purified antifungal peptide. It exhibits marked homology to lentil lectin. The purified antifungal peptide did not inhibit the activity of HIV-1 reverse transcriptase and [methyl-^{3}H] thymidine incorporation by L1210 and MBL2 cells when tested up to 150 μM (data not shown).

Table 1 – Yields and antfiungal activities of chromatographic fractions (from 500 g seeds)

Fraction	Yield (mg)	IC_{50} (mg/ml)[a]	Purification fold
D1	2429	24.9	1
D2	2766	–	–
B1	955	–	–
B2	480	4.6	5.4
B3	365	–	–
S1	41	–	–
S2	165	1.5	16.6
S3	138	–	–
SU1	40	–	–
SU2	28	–	–
SU3	47	0.4	62.3

[a] IC_{50} against *Mycosphaerella arachidicola*.

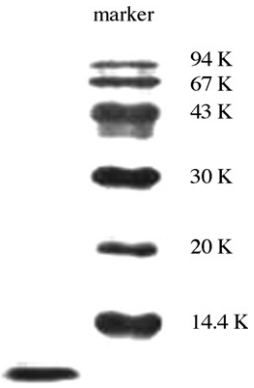

Fig. 2 – SDS–PAGE results of purified antifungal peptide (left lane) and molecular mass marker (right lane).

Table 2 – N-terminal sequence of lentil antifungal peptide

		Length	
Lentil antifungal peptide (1–30)	TETNSFSITKFSPDGNKLIFQGDGYTTKGK		
Lentil lectin (31–60)	TETTSFSITKFSPDQKNLIFQGDGYTTKGK	275	amino acids
Chickpea lectin (24–53)	TETTSFSITKFSPDQKNLIFQGDGYTTKGK	268	
Lens ervoides lectin (31–60)	TETTSFSITKFSPDQQNLIFQGDGYTTKEK	275	
Lens lamottei (31–60)	TETTSFSITKFSPDQQNLIFQGDGYTTKEK	275	
Lens nigricans (31–60)	TETTSFSITKFSPDQQNLIFQGDGYTTKEK	275	
Lathyrus sativus (22–51)	TETTSFLITITKFSPDQQNLIFQGDGYTTKEK	251	

4. Discussion

The red lentil antifungal peptide isolated in the present study possesses an N-terminal sequence similar to lentil lectin and yet it is much smaller in molecular mass than lentil lectin [9]. Some lectins demonstrate an antifungal action, e.g. red kidney bean hemagglutinin [59], wheat germ agglutinin [4], and lectin from stinging nettle rhizomes [8]. However, other lectins are devoid of antifungal activity. The amino acid sequences of lentil lectin, broad bean lectin, and Con A are highly homologous [9]. Tetrameric Con A (*Canavalia ensiformis*) is also similar to dimeric *Canavalia gladiata* lectin in sequence [51]. However, all these lectins are devoid of antifungal activity (Wang and Ng, unpublished data). The N-terminal sequence of the 11-kDa lentil antifungal peptide is similar to that of the 17-kDa subunit of lentil lectin. Taken together, the evidence suggests that lentil antifungal peptide is an entity distinct from lentil lectin. The present findings are reminiscent of the report that proteolytic fragments of ovalbumin but not ovalbumin display antimicrobial activity [28].

Lentil antifungal peptide is unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel, like many other antifungal proteins [37–47]. It also resembles antifungal proteins of other origins in its spectrum of antifungal activity [37–39,41,43–51,53–63]. It is worth mentioning that antifungal proteins from the shallot bulbs [40] and asparagus seeds [42] inhibit mycelial growth in only one out of the several fungal species tested.

An array of antifungal proteins inhibit HIV-1 reverse transcriptase, protease and integrase [25]. Lentil antifungal peptide also curtails the activity of HIV-1 reverse transcriptase, probably by protein–protein interaction, which is how HIV-1 protease inhibits the homologous reverse transcriptase. Its inhibitory potency is within the range of inhibitory potencies reported for anti-HIV natural products [24]. Some antifungal proteins exert an antiproliferative activity toward tumor cells [19,48,51]. In contrast, lentil antifungal peptide is inactive in this regard, probably due to structural differences.

In summary, lentil antifungal peptide is a lectin-like peptide present in red lentil seeds. The peptide could be used in transgenic plants against the banana pathogen *M. arachidicol* and the cotton pathogen *F. oxysporum*.

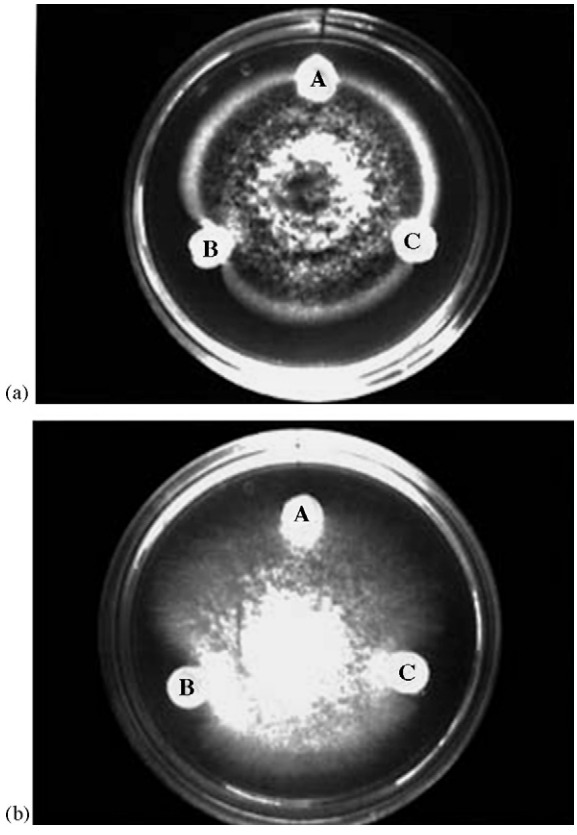

Fig. 3 – Inhibitory activity of purified antifungal peptide on mycelial growth in (a) *Mycosphaerella arachidicola*; (b) *Fusarium oxysporum*. (A) 15 μl 50 mM 15 μl MES buffer. (B) 60 μg antifungal peptide in 15 μl MES buffer (C) 12 μg antifungal peptide in 15 μl MES buffer.

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