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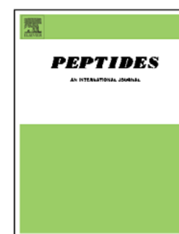

An antifungal protein from the pea *Pisum sativum* var. arvense Poir

H.X. Wang [a], *T.B. Ng* [b,*]

[a] *State Key Laboratory for Agrobiotechnology and Department of Microbiology, China Agricultural University, Beijing, China*
[b] *Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China*

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ABSTRACT

An antifungal protein with a molecular mass of 11 kDa and a lysine-rich N-terminal sequence was isolated from the seeds of the pea *Pisum sativum* var. arvense Poir. The antifungal protein was unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel and CM-cellulose. It exerted antifungal activity against *Physalospora piricola* with an IC_{50} of 0.62 μM, and also antifungal activity against *Fusarium oxysporum* and *Mycosphaerella arachidicola*. It inhibited human immunodeficiency virus type 1 reverse transcriptase with an IC_{50} of 4.7 μM.

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1. Introduction

Leguminous plants produce a number of proteins including lectins [51,57], trypsin inhibitors [56], ribosome inactivating proteins [17] and antifungal proteins [9,32,44,47,50,52–61]. These proteins serve to protect the plants from pathogens and predators.

Antifungal proteins help plants to combat phytopathogenic fungi and thus protect plants from the devastating damage caused by fungal infections and prevent massive economic losses. Transgenic plants expressing antifungal proteins are expected to be resistant to fungal infections. Consequently, research on antifungal proteins has attracted the attention of many investigators. Antifungal proteins have been found in or isolated from mammals [26], insects [20,36], plants [2,27,31,35] and fungi [10,11,16,24].

The pea *Pisum sativium* is a common vegetable. From it a lectin [51] and a ribosome inactivating protein [17] have been isolated. No antifungal activity of these proteins has been reported although lectins [1,8,57] and ribosome inactivating proteins [16] with antifungal activity have been isolated from other sources.

The isolation and characterization of an antifungal protein from its seeds are described herein. The isolation of leguminous antifungal proteins is considered to be a worthwhile task. This is because plants produce a host of antifungal proteins comprising thaumatin-like proteins [5,9,13,25,33,34,48,50], chitinases [28,30], chitinase-like proteins [18,58], chitin binding proteins [12,29], defensins, defensin-like proteins [23,45–47], miraculin-like proteins [60], embryo-abundant proteins [38], ribosome inactivating proteins [19], lipid transfer protein-like proteins [2,44], protease inhibitors [3,4,14,21] and new proteins/peptides [7,22,49].

* *Corresponding author*. Tel.: +852 2609 6872; fax: +852 2603 5123.
E-mail address: biochemistry@cuhk.edu.hk (T.B. Ng).
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2. Materials and methods

2.1. Purification of antifungal protein

Dried seeds of *P. sativum* var. arvense Poir (500 g) were obtained from a local Indian shop. After homogenization in distilled water (3 ml/g) and centrifugation (12,000 × *g* for 20 min), the resulting supernatant was loaded on a 5 cm × 20 cm column of DEAE-cellulose (Sigma) in 10 mM Tris–HCl buffer (pH 7.2). After the flowthrough material had been collected as fraction D1, adsorbed proteins were desorbed with 10 mM Tris–HCl buffer containing 0.8 M NaCl to yield fraction D2. Fraction D1 was subjected to affinity chromatography on a 5 cm × 10 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.2). After removal of the flowthrough fraction B1, adsorbed proteins were desorbed stepwise with 0.2 M NaCl and 1 M NaCl in the Tris–HCl buffer to yield fractions B2 and B3, respectively. Fraction B2 was then chromatographed on a 2.5 cm × 20 cm column of CM-cellulose (Sigma) in 10 mM NH_4OAc buffer (pH 4.5). After unadsorbed proteins had been eluted as fraction CM1, adsorbed proteins were desorbed with a linear concentration (0–1 M) gradient of NaCl. The second adsorbed fraction CM3 was subjected to gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (Amersham Biosciences). The last fraction (SU3) constituted purified antifungal protein.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

It was conducted according to the method of Laemmli and Favre using 18% gel [15]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of the isolated peptide was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

2.3. Amino acid sequence analysis

The N-terminal amino acid sequence of the isolated peptide was analyzed by means of automated Edman degradation using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system [17].

2.4. Assay for cell-free translation-inhibitory activity

Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. An assay based on the rabbit reticulocyte lysate system was used. The isolate peptide (10 μl) was added to 10 μl of radioactive 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 a vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckman liquid scintillation counter [17].

2.5. Assay of antifungal activity

The assay for antifungal activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Coprinus comatus* was carried out in 100 mm × 15 mm Petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, at a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile blank paper disks (0.625 cm in diameter). An aliquot of a solution of the isolated peptide was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [45–47].

To determine the IC_{50} value for the antifungal activity, three doses of the isolated 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, a small amount of mycelia, the same amount to each plate, was added. Buffer only without 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 [45,47].

2.6. Assay for HIV reverse transcriptase inhibitory activity

The ability of the peptide to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described in ref. [46].

2.7. Assays for DNase, RNase, peroxidase, trypsin inhibitory and hemagglutinating (lectin) activities

The assays were conducted as described in refs. [39,42,53,56,57], respectively.

3. Results

The yields of the various chromatographic fractions are presented in Table 1. Antifungal activity was detected in the fraction of pea extract unadsorbed on DEAE-cellulose (D1) and subsequently adsorbed on Affi-gel blue gel (B2) (data not

Table 1 – Yields of different chromatographic fractions (from 500 g dry seed)

Fraction	Yield (mg)	Fraction	Yield (mg)
D1	930.3	CM1	29.6
D2	1157.0	CM2	65.4
B1	179.5	CM3	94.1
B2	272.1	SU1	15.3
B3	208.9	SU2	18.8
		SU3	30.7

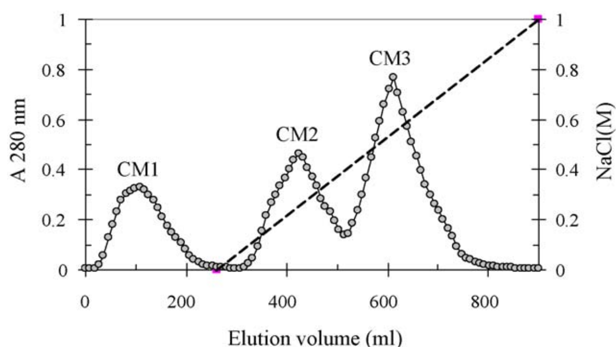

Fig. 1 – Ion exchange chromatography on CM-cellulose. Column dimensions: 2.5 cm × 20 cm. Sample: fraction B2 from Affi-gel blue gel column. Buffer: 10 mM NH_4OAc buffer (pH 4.5). Dotted line across the right half of the chromatogram represents 0–1 M NaCl concentration gradient used to desorb adsorbed proteins.

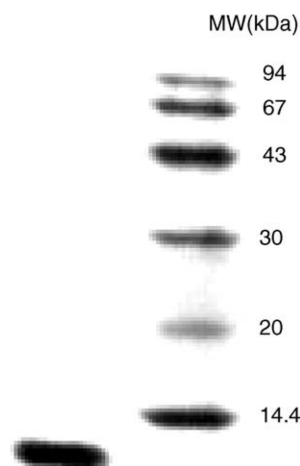

Fig. 3 – SDS-PAGE results. Left lane: *Pisum sativum* antifungal protein (fraction SU3). Right lane: molecular mass standard from Amersham Biosciences.

shown). Fraction B2 was resolved on CM-cellulose into an unadsorbed fraction CM1 and two adsorbed fractions (CM2 and CM3) of increasing size (Fig. 1). Fraction CM3 was fractionated on Superdex 75 into three fractions. Antifungal activity resided in the last fraction SU3 (Fig. 2). Fraction SU3 appeared as a single band with a molecular mass of 11 kDa in SDS-PAGE (Fig. 3) and a peak with the same molecular mass in gel filtration (Fig. 2). It represented purified antifungal protein. Its N-terminal sequence is shown in Table 2. It showed some similarities to defensins. Its antifungal activity toward the fungi *M. arachidicola*, *F. oxysporum* and *Physalospora piricola* is illustrated in Figs. 4–6, respectively. Its antifungal activity toward *P. piricola* was determined to be 0.62 μM (Fig. 6). It caused 21.2, 60.7 and 91.5% inhibition of HIV-1 reverse transcriptase at 0.8, 8 and 80 μM, respectively. The IC_{50} was 4.7 μM. It had no detectable DNase, RNase, peroxidase, trypsin inhibitory or hemagglutinating activity (data not shown). A comparison of the isolated antifungal protein with other antifungal proteins is presented in Table 3.

Table 2 – NH_2-terminal sequence of *P. sativum* antifungal protein

Pisum sativum antifungal protein	AKKK KTEEN
Pachyrhizus erosus defensin (1–5)	KTCEN
Vigna radiata PDF1 (29–33)	KTCEN
Cajanus cajan antifungal protein (28–32)	KTCEN

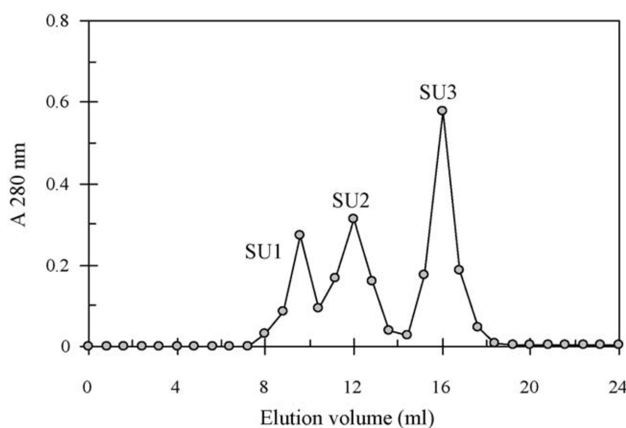

Fig. 2 – Gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column using an AKTA Purifier. Sample: fraction CM3 from CM-sepharose column. Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml.

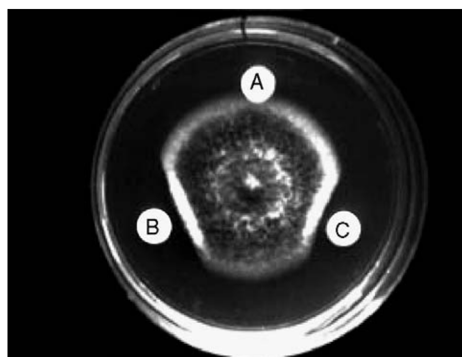

Fig. 4 – Inhibitory activity of *P. sativum* antifungal protein on mycelial growth in *Mycospaerella arachidicola*. (A) Fifteen microliters 50 mM MES buffer. (B) Seventy-five micrograms antifungal protein in MES buffer. (C) Fifteen micrograms antifungal protein in MES buffer.

4. Discussion

The antifungal protein isolated in this study has a lysine-rich N-terminal sequence. Its antifungal potency as indicated by the IC_{50} value (below 1 μM) is higher than the potencies (IC_{50} values well above 1 μM) of most of the previously reported antifungal proteins [34,35]. It inhibits mycelial growth in each of three fungal species tested: *F. oxysporum*, *M. arachidicola* and

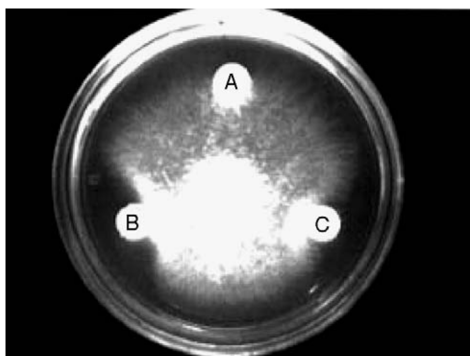

Fig. 5 – Inhibitory activity of *P. sativum* antifungal protein on mycelial growth in *Fusarium oxysporum*. (A) Fifteen microliters 50 mM MES buffer. (B) Seventy-five micrograms antifungal protein in MES buffer. (C) Fifteen micrograms antifungal protein in MES buffer.

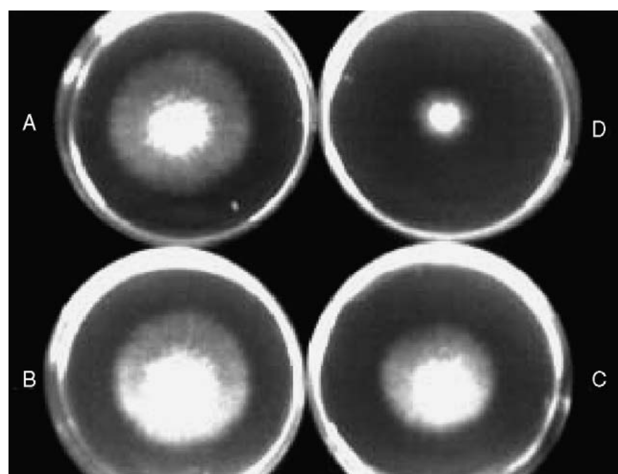

Fig. 6 – Determination of IC_{50} value of antifungal activity of *P. sativum* antifungal protein toward *Fusarium oxysporum*. (A) Control, (B) 0.16 μM antifungal protein, (C) 0.8 μM antifungal protein and (D) 4 μM antifungal protein. IC_{50} was determined to be 1.4 μM.

P. piricola. This is in contrast to some of the antifungal proteins which inhibit only one out of the several fungal species examined [37,39], and similar to the majority of antifungal proteins which are inhibitory to a number of fungi [34–36,38,40–47,50,52–61].

The pea antifungal protein obtained in this study was purified by using a procedure found to be efficient for other antifungal proteins [34–43,45–47,50–61]. Generally speaking, they are unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and CM-sepharose/CM-cellulose. The final purification step is gel filtration on Superdex 75.

Some antifungal proteins, e.g. mungin, a cyclophilin-like antifungal protein from mung beans, are devoid of HIV-1 reverse transcriptase activity [54]. The isolated pea antifungal protein demonstrated an HIV-1 reverse transcriptase inhibitory activity with an IC_{50} of about 5 μM, which represents a fairly potent activity.

The entire N-terminal sequence of pea antifungal peptide is not found in the sequence of any known protein, indicating that the antifungal peptide may be a novel peptide. Nevertheless, it exhibits some sequence similarity to leguminous defensins.

Antifungal proteins show a wide range of molecular masses, from 6 to 7 [45–47,52] to tens of kilodaltons [33,34,41,42,52,58,60]. Pea antifungal peptide manifests a molecular mass of 11 kDa, which is at the lower end of the range. Pea antifungal peptide is devoid of DNase, RNase, peroxidase, lectin and trypsin inhibitory activities, indicating that its antifungal activity is not due to any of these proteins. It is known that some DNases [39], RNases [42], peroxidases [53], lectins [1,8,57] and trypsin inhibitors [6,56] have an antifungal action.

In summary, a small antifungal protein with high potency was isolated from a variety of the pea *P. sativum*.

Table 3 – Comparison of *Pisum sativum* antifungal protein with other antifungal proteins

	P. sativum antifungal proteins	Other antifungal proteins
Molecular mass (kDa)	11	6–67
Chromatographic behavior		
(i) DEAE-cellulose	Unadsorbed	Unadsorbed
(ii) Affi-gel blue gel	Adsorbed	Adsorbed
(iii) CM-cellulose (Mono S)	Adsorbed	Adsorbed
Antifungal activity		
(i) *F. oxysporum*	Present	Less than 1 μM to over 10 μM
(ii) *M. arachidicola*	Present	Less than 1 μM to over 10 μM
(iii) *P. piricola*	IC_{50} = 0.62 μM	Less than 1 μM to over 10 μM
HIV-1 reverse transcriptase inhibitory activity (IC_{50})	4.7 μM	Less than 1 μM to over 30 μM
DNase activity	Undetectable	Some DNases have antifungal activity
RNase activity	Undetectable	Some RNases have antifungal activity
Peroxidase activity	Undetectable	Some peroxidases have antifungal activity
Lectin activity	Undetectable	Some lectins have antifungal activity
Trypsin inhibitory activity	Undetectable	Some trypsin inhibitors have antifungal activity

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