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Purification of Angularin, A Novel Antifungal Peptide from Adzuki Beans

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Abstract: An antifungal peptide was isolated from the adzuki bean with a procedure involving affinity chromatography on Affi-gel blue gel and ion exchange chromatography on CM-Sepharose. The protein designated angularin was adsorbed on both types of chromatographic media and possessed a molecular weight of 8 kDa. Angularin exhibited antifungal activity against a variety of fungal species including *Mycospharella arachidiocola* and *Botrytis cinerea*. It inhibited mycelial growth in *B. cinerea* with an IC_{50} of 14.3 μM. *Fusarium oxysporum* and *Rhizoctonia solani* were not inhibited. Angularin demonstrated inhibitory activity on translation in the rabbit reticulocyte lysate system ($IC_{50} = 8.0$ μM) but did not affect proliferation of splenocytes. The activity of HIV-1 reverse transcriptase was inhibited in the presence of angularin. Its *N*-terminal sequence was GEPGQKE.

Keywords: angularin; antifungal peptide; adzuki beans

INTRODUCTION

Leguminous plants represent a rich source of antifungal proteins which fall into different categories. To name some of the categories, there are thaumatin-like proteins [1], chitinases [2–7], glucanases [2], miraculin-like proteins [8], cyclophilin-like proteins [9], protease inhibitor [10] and allergen Ara H1-like peptides [11].

In view of the variety of structurally disparate antifungal proteins elaborated by leguminous plants, we undertook the present investigation to look for novel antifungal protein(s) and/or peptide(s) in the adzuki bean (red bean). The results disclosed the presence of an antifungal peptide with an *N*-terminal sequence manifesting no homology to known proteins. The isolated antifungal peptide also inhibited the activity of human immunodeficiency virus type 1 reverse transcriptase and curtailed translation in a cell-free rabbit reticulocyte lysate system. The present report constitutes one of the few regarding leguminous antifungal peptides. Most of the previous articles pertain to antifungal proteins elaborated by legumes.

MATERIALS AND METHODS

Isolation Procedure

Seeds of adzuki beans (*Vigna angularis*) from a local dealer were immersed in distilled water for several hours before homogenization. The homogenate was centrifuged and the resulting supernatant was dialysed against distilled water. Tris-HCl buffer (pH 7.2) was added until the final concentration was 10 mM. The supernatant was applied on a column of Affi-gel blue gel previously equilibrated with and then eluted with 10 mM Tris-HCl buffer (pH 7.2). After elution of unadsorbed proteins, the adsorbed proteins were desorbed by using a linear concentration gradient

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(0–0.5 M) of NaCl in the aforementioned buffer. The first adsorbed fraction (fraction B1) was dialysed against 10 mM Tris-HCl buffer (pH 7.2) and then chromatographed on a column of CM-Sepharose in the same buffer. Following removal of unadsorbed proteins, the adsorbed proteins (AB1, AB2 and AB3) were eluted by inclusion of a linear concentration gradient (0–0.5 M) of NaCl in the Tris-HCl buffer.

Determination of Molecular Weight

The homogeneity of the purified antifungal peptide designated angularin represented by fraction AB1 and its molecular weight were estimated by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [12]. Molecular weight determination was also carried out by fast protein liquid chromatography on a Superdex 75 column (Amersham Pharmacia Biotech).

Analysis of *N*-terminal Sequence

N-terminal sequencing of the proteins was conducted by employing a Hewlett-Packard HP G1000A Edman degradation unit and an HP1000 HPLC system [13].

Assay for Antifungal Activity

The antifungal activity of angularin was assessed by using sterile petri plates (100 × 15 cm) which contained 10 ml potato dextrose agar. After the mycelial colony had developed, sterile paper disks with a diameter of 0.625 cm were laid at a distance of 1 cm from the rim of the mycelial colony. An aliquot of the peptide in 10 mM Tris-HCl buffer (pH 7.2) was applied to a disk. The petri plate was incubated at 23 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control and had produced a crescent of inhibition around disks with antifungal samples [14]. The fungal species used in the assay included *Mycosphaerella arachidicola*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Botrytis cinerea*.

To determine the IC_{50} value for the antifungal activity of angularin three doses of the 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 [14].

Assay for HIV Reverse Transcriptase Inhibiting Activity

Angularin was tested for this activity since some antifungal proteins possess an inhibitory activity toward HIV-1 reverse transcriptase. The assay for ability to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described by Collins *et al.* [15]. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(a) · oligo (dT)15. In place of radio-labelled nucleotides, digoxigenin- and biotin-labelled 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-labelled DNA binds to the surface of microtitre plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labelled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyses the cleavage of the substrate, producing a coloured reaction product. The absorbance of the samples at 405 nm can be determined using a microtitre 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 angularin was calculated as the percent inhibition compared with a control without the protein.

Assay for Mitogenic Activity

Four C57BL/6 mice (20–25 g) were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml and 100 µg streptomycin/ml. The cells (7×10^5 cells/100 µl/well) were seeded into a 96-well culture plate and serial dilutions of a solution (containing 100 µg in the first well) in 100 µl medium were added. After incubation of the cells at 37 °C in a humidified atmosphere of 5% CO_2 for

24 h, 10 µl methyl [^{3}H]-thymidine (0.25 µCi, Amersham Pharmacia Biotech) was added, and the cells were incubated for a further 6 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Beckman model LS 6500 scintillation counter. All reported values are the means of triplicate samples [16].

Assay for Translation-inhibiting Activity

The assay of angularin for the ability to inhibit synthesis from ^{3}H-leucine in a cell-free rabbit reticulocyte lysate system was carried out as previously described. Many antifungal proteins have been reported to have cell-free translation-inhibitory activity. Angularin was thus tested for this activity. Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anaemic by phenylhydrazine injections. An assay based on the rabbit reticulocyte lysate system [14] was used. Angularin (10 µl) was added to 10 µl 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 haemin and 5 µl creatine kinase. Incubation proceeded at 37 °C for 30 minx 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 hydrolysate in a 96-well plate to precipitate radioactively labelled protein. The precipitate was collected on a glass fibre 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.

RESULTS

Purification and N-terminal Sequence

The adzuki bean extract yielded a large unadsorbed peak and two adsorbed peaks B1 and B2 upon affinity chromatography on Affi-gel blue gel (Figure 1). The adsorbed peak B1 was fractionated on CM-Sepharose into a large unadsorbed peak and three adsorbed peaks AB1, AB2 and AB3 (Figure 2). The adsorbed peaks AB1 contained a purified antifungal protein designated angularin. The yields throughout the various stages of purification from 125 g seeds were crude extract, 9.15 g; fraction B1 absorbed on Affi-gel blue gel, 147 mg; fraction AB1 (purified angularin) adsorbed on CM-Sepharose, 3.8 mg. The *N*-terminal sequence of angularin is GEPGQKE.

The molecular weight of angularin was 8 kDa in SDS-PAGE (Figure 3) and in gel filtration (data not shown).

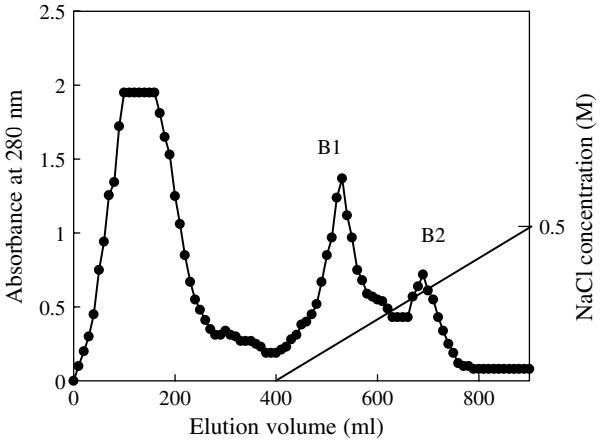

Figure 1 Fractionation of the crude extract of adzuki bean seeds on an Affi-gel blue gel column equilibrated with the binding buffer (10 mM Tris-HCl, pH 7.2). The gel was washed with the binding buffer and eluted with a linear gradient of 0–500 mM NaCl in the same buffer. Antifungal activity was detected in CM-Sepharose fraction B1.

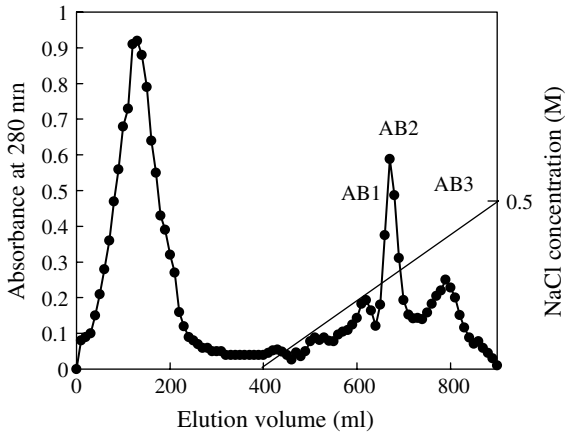

Figure 2 Elution profile of fraction B1 from the SP-Toyopearl column. After chromatography on Affi-gel blue gel, adsorbed fraction B1 was dialysed and then applied to CM-Sepharose column in 10 mM Tris-HCl buffer (pH 7.2). The column was then washed with the binding buffer. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 500 mM in the same Tris-HCl buffer (pH 7.2) to form peaks AB1, AB2 and AB3.

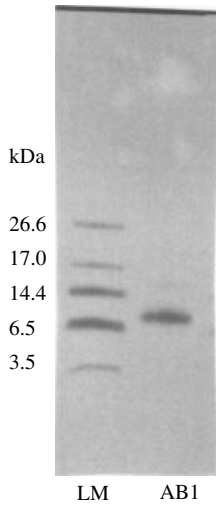

Figure 3 SDS-polyacrylamide gel electrophoresis of angularin (AB1). Lane a: low molecular weight standards (from top downward triose phosphate isomerase, 26.6 kDa; myoglobin, 17 kDa; α-lactalbumin, 14.4 kDa; aprotinin, 6.5 kDa and oxidized insulin B chain, 3.5 kDa); Lane b: angularin.

Antifungal Activity

The antifungal activity of angularin is illustrated in Figure 4(I–II). The activity was strong toward *Mycosphaerella arachidicola*. Angularin inhibited *Botrytis cinerea* with an IC_{50} of 14.3 μM (Figure 4III).

Translation-inhibitory and HIV-1 reverse Transcriptase-inhibiting Activities

Angularin exerted an inhibitory action on translation in rabbit reticulocyte lysate with an IC_{50} of 8 μM (Table 1). Angularin reduced the activity of HIV-1 reverse transcriptase. No inhibition was detected at 7 μM. An inhibition of 27.5% ± 2.1% (mean ± SD, $n = 3$) was brought about at 70 μM.

Effect on Splenocyte Proliferation

Uptake of methyl [^{3}H] thymidine was not affected by doses of angularin up to 2 μM.

DISCUSSION

Angularin is similar to previously reported antifungal proteins in its low cell-free translation-inhibitory potency compared with ribosome inactivating proteins [13, 17]. Ribosome inactivating proteins inhibit translation with an IC_{50} in the picomolar and nanomolar ranges, whereas antifungal proteins do so with an IC_{50} in the micromolar range. This translation-inhibiting activity may be related to its antifungal activity. However, it has been reported that ribosome inactivating proteins exert a lower antifungal potency than antifungal proteins [18, 19]. Angularin also inhibits the activity of HIV-1 reverse transcriptase, in accordance with observations on other antifungal proteins [1, 6–9, 11]. The potency with which it inhibits the HIV enzyme is within the lower end of the range of potencies reported for anti-HIV natural products [20].

Some antifungal proteins such as mungin, a cyclophilin-like from mung beans, exert a suppressive action on proliferation of murine splenocytes [9]. Other antifungal proteins like hypogin, the allergen-like protein from peanuts [11], and chrysancorin from garland chrysanthemum seeds [21] on the other hand, stimulate splenocyte proliferation. Angularin is devoid of an effect in this regard.

Angularin retards mycelial growth in *Mycosphaerella arachidicola* and *Botrytis cinerea*. Its antifungal potency is similar to that of broad bean trypsin inhibitor [22].

Angularin resembles previously reported antifungal proteins [1, 6–9, 11, 21] in its chromatographic behaviour on Affi-gel blue gel and CM-Sepharose. It is perhaps noteworthy that ribosome inactivating proteins have similar chromatographic behaviour on these media [13, 23, 24]. Ribosome inactivating proteins exhibit antifungal activity which, however, may be weaker than that of antifungal proteins [18, 19].

A wide range of molecular weights has been reported for antifungal proteins. Some non-leguminous antifungal proteins are homodimeric, e.g. ribonucleases from American ginseng and Chinese ginseng [25, 26] or heterodimeric, e.g. sanchi ginseng [27]. The molecular weights of these proteins are around 55 kDa. Pisavin, a miraculin-like antifungal protein from *Pisum sativum*, has a molecular weight of 36 kDa [8]. Dolichin, a chitinase from the field bean, possesses a molecular weight of 28 kDa [6]. Mungin, a cyclophilin-like antifungal protein from mung beans, is smaller, about 18 kDa in molecular weight [9]. Some leguminous antifungal compounds are peptides, e.g. hyopgin from peanuts [11], and arietin and cicerin from chickpeas [28], with a molecular weight of a few kilodaltons. Agularin has a molecular weight of 8 kDa. Its *N*-terminal sequence does not exhibit similarity to known proteins.

In sum, an antifungal peptide with a novel *N*-terminal sequence was isolated from adzuki beans.

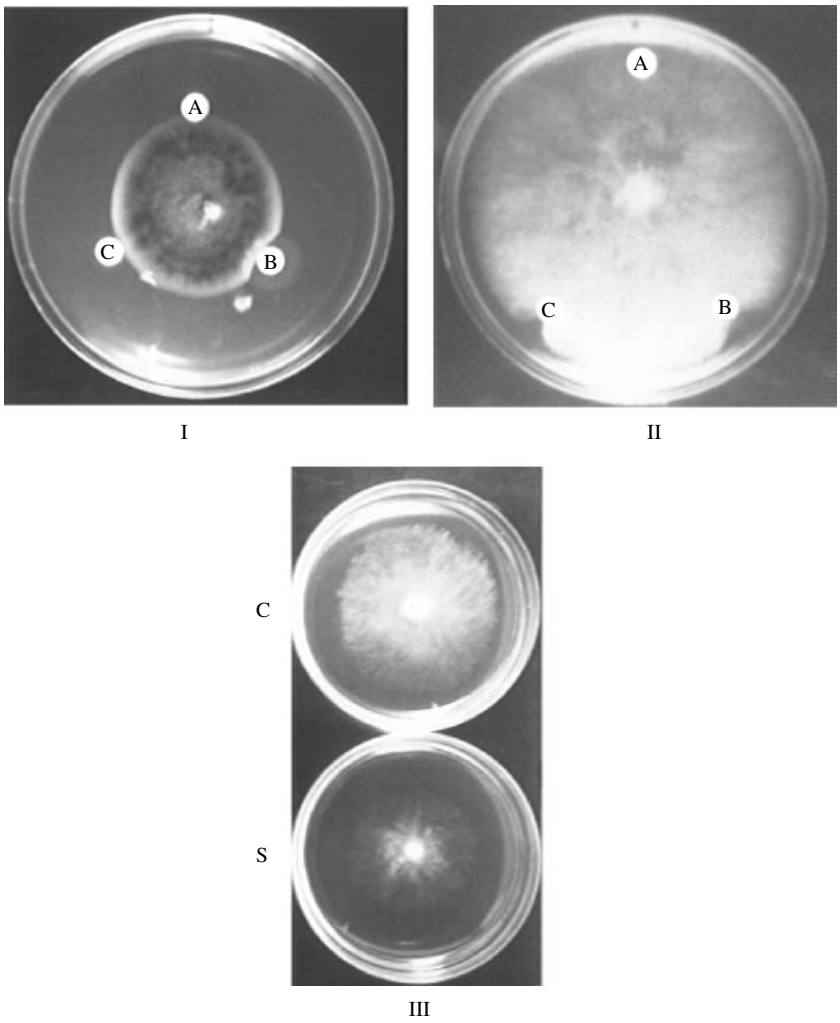

Figure 4 (I) Inhibitory activity of angularin toward *Mycosphaerella arachidicola* A, 10 mM Tris-HCl buffer, pH 7.2; B, 300 μg angularin; C, 60 μg angularin. (II) Inhibitory activity of angularin toward *Botrytis cinerea* A, 10 mM Tris-HCl buffer, pH 7.2; B, 300 μg angularin; C, 60 μg angularin (III) Determination of IC_{50} value of antifungal activity of angularin toward *Botrytis cinerea*. Plate C represents the control and plate S represents treatment with 12.6 μM angularin. Treatment with 0.16 μM and 3.1 μM angularin not shown. IC_{50} calculated to be 14.3 μM.

Table 1 Inhibition of Cell-Free Translation in Rabbit Reticulocyte Lysate by Angularin

	Concentration (μM)	Inhibition (%)
Angularin	200.0	85.5 ± 1.5
	40.0	67.2 ± 1.6
	8.0	51.1 ± 2.1
	1.6	29.2 ± 0.8

The IC_{50} value for angularin is around 8.0 μM. Results are mean ± SD($N = 3$).

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