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Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase

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Abstract

An antifungal peptide with a molecular mass around 7 kDa and an N-terminal sequence highly homologous to defensin was isolated from ground beans (*Vigna sesquipedalis* cv. 'Ground Bean'). The peptide was adsorbed on Affi-gel blue gel and on Mono S. It exerted an antifungal action on *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola*; and an antibacterial action on *Escherichia coli* B, *Proteus vulgaris*, *Mycobacterium phlei* and *Bacillus megaterium*. The antimicrobial activity was inhibited in presence of the 5 mM $CaCl_2$ and $MgCl_2$, but no inhibition was observed in 5 mM NaCl. The peptide exerted antiproliferative activity toward breast cancer (MCF-7) cells and leukemia M1 cells, this activity could not be inhibited by the ions mentioned above. It also exhibited some inhibitory activity toward human immunodeficiency virus-type 1 reverse transcriptase.
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1. Introduction

Plants are under pressure as they are a source of food for a wide range of microorganisms, including viruses, bacteria, fungi, nematodes and insects. In order to survive, plants must possess a defense system to block the entry of these invaders [14].

Several molecules in plants have been reported to contribute to plant defense activity: (1) proteins or peptides, such as lectins [3,6], ribosomal inactivating proteins [11], chitinases [6,17], proteases [25], defensins [31], peroxidases, ubiquitin-like peptides [15], ribonucleases [16], arginine- and glutamate-rich peptides [27,18] and some unclassified proteins [34], (2) organic compounds, classified into phytoanticipins and phytoalexins, which include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins and dienes [21], and (3) active nitrogen and oxygen species, such as reactive nitrogen oxide species and hydrogen peroxide [1].

In this study, an antifungal peptide was purified from ground beans (*Vigna sesquipedalis* cv. 'Ground Bean'), which is a common vegetable in the Chinese diet. Its characteristics were compared with those of previously isolated antifungal peptides.

2. Materials and methods

2.1. Materials

Dried ground beans were purchased from Nanjing, Mainland China. Affi-gel blue gel was purchased from Bio-Rad, and Mono S and Superdex 75 columns were from Amersham Biosciences. Chemicals for sequence analysis were obtained

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from Hewlett-Packard (Palo Alto, CA, USA). All other chemicals used were of reagent grade.

2.2. Purification of antifungal peptide

The beans (200 g) were soaked overnight in distilled water (1 l) and then blended in a Waring blender. Tris–HCl buffer (pH 7.6) was added to the supernatant obtained after centrifuging the slurry until a concentration of 10 mM was attained. The supernatant was then chromatographed on a column (2.5 cm × 10 cm) of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.8). After removal of the unadsorbed fraction, adsorbed proteins were eluted with 1 M NaCl in the same buffer. The adsorbed fraction was dialyzed, lyophilized, and then applied on an FPLC Mono S column (1 ml, Amersham Biosciences) in 20 mM NH_4OAc buffer (pH 4.5). Unadsorbed proteins were washed off the column with the starting buffer. Adsorbed proteins were eluted with two linear NaCl concentration gradients (0–0.3 and 0.3–1 M) in the starting buffer. Fraction S4, in which antifungal activity resided, was then subjected to gel filtration by FPLC on a Superdex 75 HR 10/30 column (Amersham Biosciences) in 10 mM NH_4HCO_3 buffer (pH 9.4). The last peak represented purified antifungal peptide which was designated as sesquin.

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

It was conducted according to the method of Laemmli and Favre [9] using 18% gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of sesquin was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

2.4. Amino acid sequence analysis

The N-terminal amino acid sequence of sesquin was analyzed by means of automated Edman degradation using a Hewlett-Packard 1000A protein sequencer equipped with an HPLC system [13].

2.5. Assay of antifungal activity

The assay of sesquin for antifungal activity toward *Mycosphaerella arachidicola* and *Fusarium oxysporum* was carried out in 90 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 of a solution of sesquin was added to a disk. The plates were incubated at 25 °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 [33].

To determine the IC_{50} value for the antifungal activity of sesquin, four doses of the peptide were added separately to four aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into four 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 [28].

To investigate thermal stability, pH stability and effects of ions, sesquin was pretreated accordingly and the assay of antifungal activity was then conducted as mentioned above.

2.6. Assay of antibacterial activity

Bacteria were incubated in 10 ml of nutrient broth in a thermal shaker for 12 h at 37 °C, and then 5 ml of this bacterial suspension was transferred to 50 ml of nutrient broth and incubated for another 3–5 h (the exact duration depends on the bacterial species) in order to shift bacterial growth to the mid-logarithmic phase. The bacterial suspension was then centrifuged at 2000 × *g* for 10 min, and the bacterial pellet was washed and resuspended in normal saline. A total of 10^5 or 10^7 of bacteria per ml was obtained by dilution guided by the optical density at 595 nm. In the experiment, every condition was prepared in triplicate; one aliquot of bacteria was mixed with sesquin at 0.50.25 and 0.125 mg/ml, respectively; one aliquot was mixed with different concentrations of sesquin in 5 mM $CaCl_2$; one aliquot was mixed with different concentrations of sesquin in 5 mM $MgCl_2$; and one aliquot was mixed with only bacteria in saline as a control. The samples were then incubated in a shaker and aliquots were obtained at four time points (0, 3, 6 and 12 h), serially diluted with nutrient broth, and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted. The number of bacteria for each condition and dilution was determined from the average colony counts for three plates [29].

2.7. Assay for HIV reverse transcriptase inhibitory activity

The ability of sesquin to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described by Collins et al. [4].

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 molecules, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity allows sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the

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