Article

J. Mex. Chem. Soc. **2007**, *51(3)*, 136-140

ISSN 1870-249X

Antifungal Activity of a Protean Extract from *Amaranthus hypochondriacus* Seeds

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Recibido el 5 de marzo de 2007; aceptado el 15 de agosto de 2007

Abstract. Plants have developed a variety of protection mechanisms which include the synthesis of both, low molecular weight secondary metabolites and macromolecules as proteins and peptides with antimicrobial activity. There are previous reports of antifungal peptides found on the seeds of other *Amaranthus* species, so it is very probable that these kind of compounds are present in the *A. hypochondriacus* seeds. This was confirmed by the antifungal activity of a protean extract from *A. hypochondricus* against such pathogenic fungi as *Alternaria alternata, Fusarium solani, Candida albicans, Fusarium oxysporum, Trichoderma* sp. and *Aspergillus ochraceus*. The fungal growth inhibition was evaluated using the "poisoned" agar and the microspectrophotometry tests.
Key words: Antifungal activity, peptides, *Amaranthus hypochondriacus*, growth inhibition assay.

Resumen. Las plantas han desarrollado una variedad de mecanismos de protección contra patógenos entre los que se encuentra la síntesis de metabolitos secundarios y macromoléculas como proteínas y péptidos con actividad antimicrobiana. De las semillas de otras especies de *Amaranthus* se han aislado péptidos antifúngicos, así que es muy probable que también estén presentes en las semillas de *A. hypochondriacus.* Lo anterior se confirmó con la actividad antifúngica mostrada por un extracto proteínico de *A. hypochondricus* contra hongos como *Alternaria alternata, Fusarium solani, Candida albicans, Fusarium oxysporum, Trichoderma* sp. y *Aspergillus ochraceus.* La inhibición del crecimiento fúngico se evaluó por los métodos de agar "envenenado" y de análisis microespectrofotométrico.
Palabras clave: Actividad antifúngica, péptidos, *Amaranthus hypochondriacus*, bioensayos de inhibición de crecimiento.

Introduction

Over the past, decades numerous antimicrobial peptides (AMP) or membrane-lytic peptides have been isolated from insects [1], amphibians [2], plants [3] and mammals [4].

These peptides are present in most, if not all, plant species and they are mostly antifungal [5-7]. Based on homologies at the primary structure level, antifungal peptides can be classified into distinct families including thionins [8], plant defensins [9], lipid transfers proteins [10], pathogenesis related proteins and hevein- and knottin-type antimicrobial peptides. AMPs encompass a wide variety of structural motifs and so far contain even number of cysteines (4, 6 and 8), which are all pair wise connected by disulfide bridges, thus providing them high stability. The antifungal peptide mechanisms of action are as varied as their sources and include fungal cell wall polymer degradation, membrane channel and pore formation, damage to cellular ribosome, inhibition of DNA synthesis, and inhibition of the cell cycle [11]. Many of these peptides appear to act *via* a specific, but not receptor-mediated, permeabilization of microbial membranes, and this confers a considerable potential for their development as novel therapeutic agents that could overcome the resistance problem. The study of action mechanisms of these peptides is very important in order to develop them as new drugs, thus as express them in transgenic plants to enhance resistance against a particular microbial plant pathogen [12].

Amaranthus hypochondriacus belongs to Amaranthaceae family, and it is important in Mexican diet because of its proteins and essential amino acid concentrations. Since the prehispanic time, every part of the *A. hypochondriacus* plant has been broadly used in Mexico, specially the seeds, which are mostly consumed as a candy named "alegría". Some proteins from *A. hypochondriacus* seeds have already been isolated and studied, like the nsLTP1 (Non-specific lipid-transfer proteins) with 9.7 KDa, which plays a roll in the plant defense [13], a globulin of 34.9 kDa, which is a storage protein and it is very important in nutrient conservation [14], AmTI, a trypsin inhibitor [15], AAI, an α-amilase inhibitor peptide [16] and ALL, a dymeric glycoprotein specific to N-acetyl-D-galactosamine [17]. From the seeds of other amaranth species have been isolated peptides with antimicrobial activity like Ac-AMP1 and Ac-AMP2 from *A. caudatus* [18] and Ar-AMP from *A. retroflexus* [19], so it is probable that the *A. hypochondriacus* also has these kind of compounds and, on this paper, we are reporting the preliminary purification assays.

Experimental

Biological material

Amaranth (*Amaranthus hypochondriacus*) dry seeds were obtained from Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, the seeds were stored at room temperature and humidity. The fungi *Candida albicans, Aspergillus ochraceus, Trichoderma* sp., *Fusarium solani, F.*

oxysporum, and *Alternaria alternata* were supplied by the UNAM mycology laboratory UNIGRAS.

Obtention of protean extract

300 g of *A. hypochondriacus* seeds were ground in a coffee mill and the resulting flour was extracted at 4 °C for 2 h with 600 mL of water. The homogenate was squeezed through a cheesecloth and clarified by centrifugation (30 min at 7000 × g). Enough solid ammonium sulfate was then added to the supernatant so as to obtain a 30 % relative saturation. The supernatant was allowed to stand for an hour at room temperature and the precipitate formed was removed by centrifugation (30 min at 15000 × g); the supernatant was further adjusted to 75 % relative ammonium sulfate saturation and allowed to stand overnight at 4 °C. The precipitate formed was collected by centrifugation (30 min at 15000 × g) and the resulting pellet (about 10 g) redissolved in 100 mL of water, the solution was dialyzed extensively against distilled water using regenerated cellulose membrane tubing (Spectrum Laboratories, Inc. Rancho Dominguez, CA) with a molecular cut off of 2000 Da. After dialysis the solution was adjusted to 50 mM Tris-HCl (pH 9) by adding ten-fold concentrated buffer [18]. This is the test solution that was used for the fungal growth inhibition test.

Fractionation of the protean extract

5 mL of the protean extract was passed through a Sephadex G-50 (Pharmacia) column (143.5 × 2 cm) in equilibrium with 50 mM Tris-HCl, pH 9. The fractions were eluted with the same equilibrating buffer and then were tested against *A. alternata, F. solani, C. albicans, F. oxysporum, T.* sp. and *A. ochraceus.*

Analysis of fractions

A number of G-50 column fractions were analyzed by electrophoresis in a 16 % Tris-Tricine SDS-PAGE using Kaleidoscope Polypeptide Standards (Bio-Rad, Hercules, CA).

Protein quantification

Protein concentration, in the extract and the G-50 fractions, was determined using the BCA protein assay kit (Pierce, Rockford, IL). Bovine serum albumin was employed as a standard. The protein concentration was expressed as micrograms per milliliter. Three replicates were conducted.

Antifungal activity

"Poisoned" agar method

Plates were prepared by adding 2.5 mg/mL of sterile test solution to 5 mL of Potato Dextrose Agar (PDA) (Sigma-Aldrich, Saint Louis, Mo); a 5 mm mycelial plug of each fungus was cultivated in the center of the plate, and incubated at 25 °C. The negative control plate was prepared in the same way using the buffer 50 mM Tris-HCl pH 9 instead of the test solution. Fungal growth inhibition was determinated after several days, depending on the fungus, until the negative control plates were completely full. Results are reported as an average with its respective standard deviation [20]. All tests were run in triplicate.

Microspectrophotometry method

This semi-quantitative method was modified from Broekaert [21]. Assays were performed with 20 μL (0.22 mm filter-sterilized) of test solution (200 μg/mL), 10 μL fungal spore suspension (2×10^4 spores/mL) and 70 μL half strength Potato Dextrose Broth (PDB) (Sigma-Aldrich Saint Louis, Mo). Negative control microcultures contained exactly the same but 20 μL of sterile distilled water instead of test solution. The absorbance of the cultures was measured at 570 nm in an Ultra Microplate Reader (ELx 808, Bio-Tek Instruments) after 30 min, then the cultures were incubated at 25 °C for 48 h and the absorbance was measured again at 570 nm. Percent growth inhibition is defined as 100 times the ratio of the corrected absorbance of the negative control microculture minus the corrected absorbance of the test microculture over the corrected absorbance at 570 nm of the negative control microculture. The corrected absorbance value is the absorbance at 570 nm of the culture measured after 48 h minus the absorbance at 570 nm of the culture measured after 30 min. Test solutions showing a growth inhibition under 20 % were considered inactive. A 1 mg/mL dose of Nistatine (NIS) (Alpharma, México, D.F.) was used as a positive control. Three replicates were conducted.

Results and discussion

The concentration of total protein on the protean extract was approximately 15 mg/mL in every extraction. The protean extract of *A. hypochondriacus* seeds showed good growth inhibition activity against *A. alternata, T. sp., F. oxysporum* and *F. solani* when tested using the "poisoned" agar method (Figure 1). The most susceptible fungus was *F. solani* with a mycelial growth of just 6.0 mm; on the contrary, *F. oxysporum* was the most resistant with the most largest mycelial growth, about 30.8 mm (Table 1). The fungi *A. ochraceus*, and *C. albicans* were not tested with the "poisoned" agar method because these fungi do not grow in a radial way. A concentration of 2 mg/mL of protean extract was tested using the microspectrophotometry method against *A. schraceus, C. albicans, F. oxysporum* and *Trichoderma* sp. The fungi *F. solani* and *A. alternata* did not produce spores, so they could not be tested with this methodology. The obtained results show that *F. oxysporum* is the less sensitive to the antifungal effect of the protean extract with just a 35.15 % of growth inhibition, while *C. albicans* is very sensitive, showing a 100 % of growth inhibi-

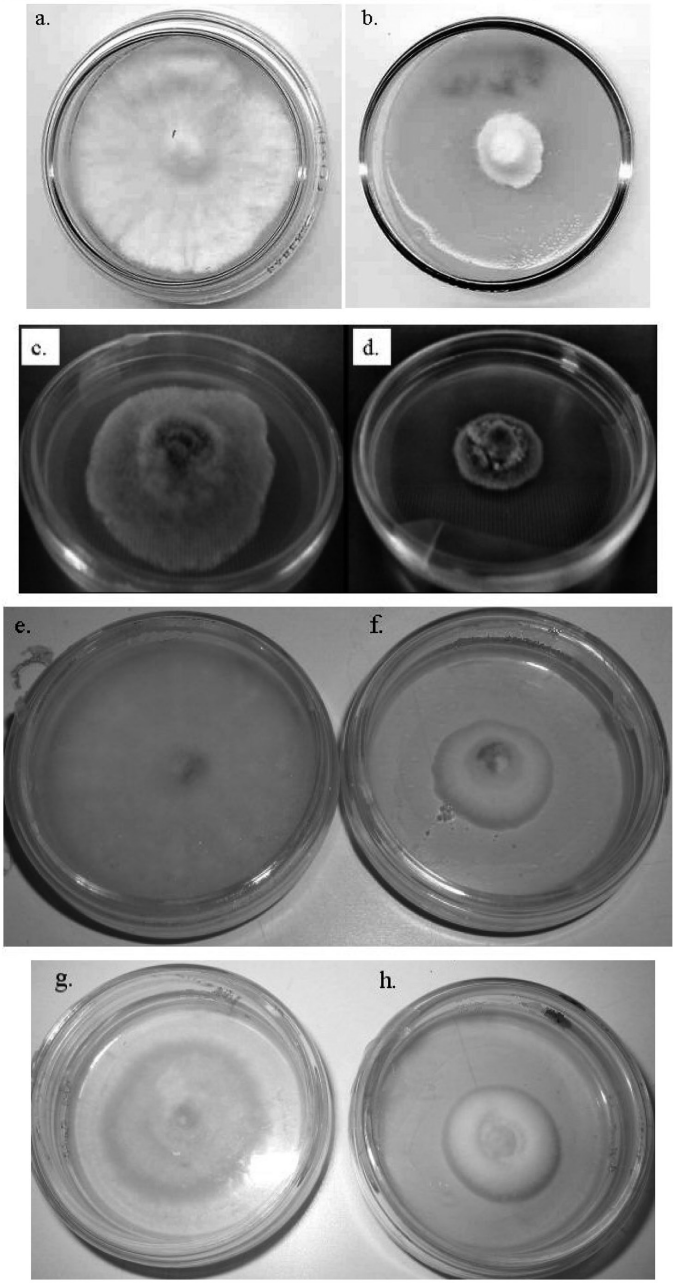

Fig. 1. Antifungal activity of the negative control (buffer 50 mM Tris-HCl pH 9) and the protean extract of *A. hypochondriacus* against *F. solani* (**a** and **b**, respectively), against *A. alternata* (**c** and **d**, respectively), against *Trichoderma* sp. (**e** and **f**, respectively) and *F. oxysporum* (**g** and **h**, respectively).

tion (Figure 2). The antifungal activity against *F. oxysporum* using both methodologies, "poisoned" agar and microspectrophotometry, are in good correlation because in both cases the protean extract showed a low antifungal activity.

Partial purification of the antifungal extract using a Sephadex G-50 column yielded ten fractions (Figure 3), which were analyzed by electrophoresis and were evaluated for antifungal activity, using the microspectrophotometry method,

Table 1. Micellium growth inhibition of the protean extract from *A. hypochondriacus* seeds.

	Micellium diameter (mm)	
Fungi	Protean extract	Negative control
A. alternata	7.0 ± 0.0	41.0 ± 2.1
F. solani	6.0 ± 0.0	48.0 ± 1.0
Trichoderma sp.	19.3 ± 2.5	50.0 ± 0.0
F. oxysporum	30.8 ± 3.1	40.7 ± 1.6

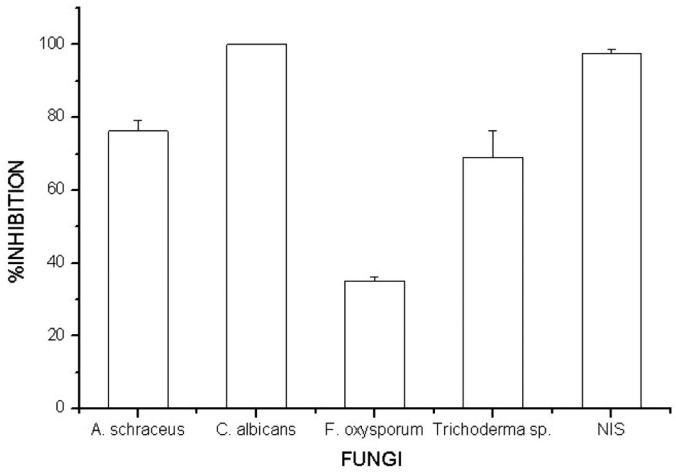

Fig. 2. Antifungal activity of the protean extract against *Aspergills ochracues*, *C. albicans*, *F. oxysporum* and *Trichoderma* sp. by the microspectrophotometry assay. The test solution had a concentration of 2 mg/mL. NIS is the positive control.

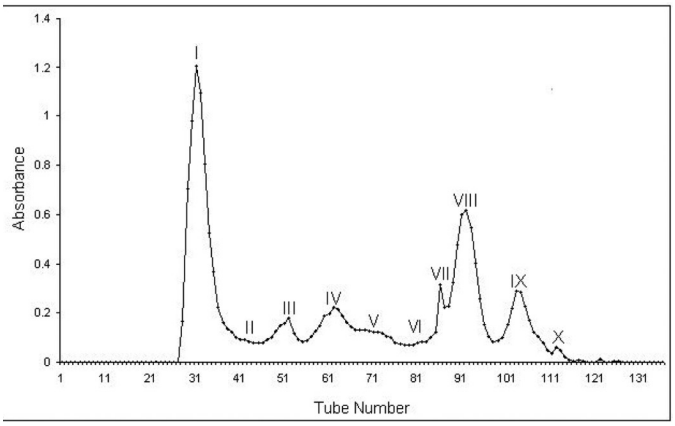

Fig. 3. Chromatographic profile of the fractions obtained by purification of the protean extract from *A. hypochondriacus* using Sephadex G-50. I to X are the fractions collected.

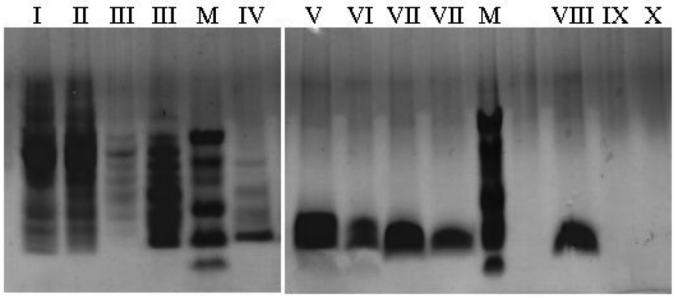

Fig. 4. Electrophoretic protein analysis of Sephadex G-50 fractions on a Tris-Tricine 16 % SDS-PAGE. The numbers over the gel (I to X) represent the peaks on the Sephadex G-50 column and the M correspond to the molecular weight marker (Bio-Rad, Hercules, CA).

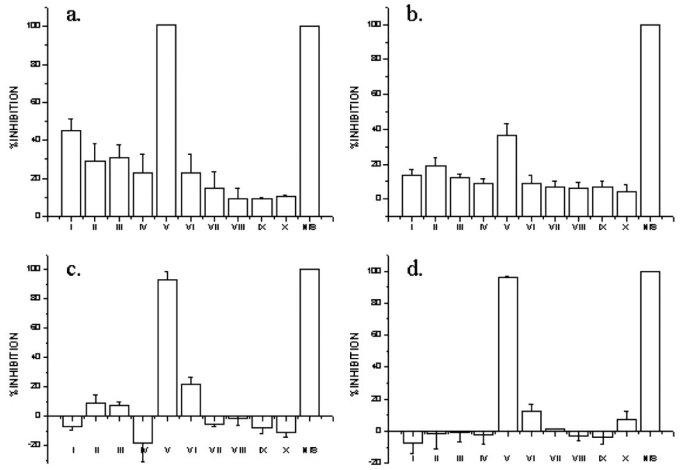

Fig. 5. Antifungal activity of fractions I-VIII against *C. albicans* (**a**), *F. oxysporum* (**b**), *Aspergillus ochraceus* (**c**), *Trichoderma* sp. (**d**). The abscissa corresponds to the Sephadex G-50 fractions; the ordinate corresponds to the fungal growth inhibition percentage.

against *C. albicans*, *T.* sp., *A. ochraceus* and *F. oxysporum.* The "poisoned" agar method was not used with the Sephadex G-50 fractions because, in this methodology, a large volume of test solution is needed, about 4 mL for each fungus, while in the microspectrophotometry method just 60 μL, for each fungus, are employed.

The electrophoretic analysis of the Sephadex G-50 fractions, shows that fractions IX and X do not contain proteins or peptides (Figure 4), so they may contain small molecules like secondary metabolites, these fractions were not active against any of the fungi (Figure 5). Fractions I to VIII contain proteins and peptides, with the proteins in the first fractions and the peptides in the last ones (Figure 4). Fractions I to VI showed fungal growth inhibition higher than 20 % against *C. albicans*, with fraction V being the most active (100 % of inhibition, Figure 5a), but only fraction V showed fungal growth inhibition, higher than 20 %, against *F. oxysporum*, *A. ochraceus* and *Trichoderma* sp (Figures 5b, 5c and 5d) and, according to its electrophoretic pattern (Figure 4), fraction V is rich in peptides which was expected to be the responsible for the antifungal activity. Fractions VI to VIII contain peptides as well, but their antifungal activity were higher than 20 % only against *C. albicans*, with an growth inhibition percentage of 23.15; 14.93 and 9.57 respectively, so these fractions contain peptides without antifungal activity.

To be sure that the antifungal activity was due to peptides instead of secondary metabolites, besides the partial purification through the Sephadex G-50 column, which separate proteins from peptides and peptides from small molecules, the protean extract was over heating, 100 °C for 15 min, and then tested against the same fungi and it was found that the protean extract antifungal activity was completely lost against the tested fungi. The extract was over heating because these kind of peptides are very resistant to the effect of proteases and heating [18].

Conclusions

Proteins extracted from *A. hypochondriacus* seeds resulted in having a high antifungal activity against dermo and phytopathogenic fungi. Peptide fraction V showed the highest antifungal activity against most of the fungi tested. We will perform further studies to identify the fungi *T.* sp., to purify and to characterize the biochemistry and spectroscopy features of the antifungal peptide, in order to understand its role in plant defense mechanism and to provide a valuable tool for engineering resistance against phytopathogenic fungi in plants.

Acknowledgements

We acknowledge support from DGAPA, UNAM grant (IN211605), DGEP for a doctoral fellowship, received during two years and a half, and M. Sc. Cristina Pérez, from UNIGRAS, for supplying and identifying the fungi.

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