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Isolation and Biochemical Characterization of an Antifungal Peptide from *Amaranthus hypochondriacus* Seeds

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An antifungal peptide, Ay-AMP, was isolated from *Amaranthus hypochondriacus* seeds by acidic extraction and then purified by reverse-phase high-pressure liquid chromatography. The molecular mass of this peptide, as determined by mass spectrometry, is 3184 Da. The peptide belongs to the superfamily of chitin-binding proteins, containing a single cysteine/glycine-rich chitin-binding domain, and it was found that Ay-AMP degrades chitin. Ay-AMP inhibits the growth, at very low doses, of different pathogenic fungi, such as *Candida albicans*, *Trichoderma* sp., *Fusarium solani*, *Penicillium chrysogenum*, *Geotrichum candidum*, *Aspergillus candidus*, *Aspergillus schraceus*, and *Alternaria alternata*. Ay-AMP is very resistant to the effect of proteases and heating; however, it showed an antagonistic effect with $CaCl_2$ and KCl.

KEYWORDS: Antifungal activity; antifungal peptide; peptide purification; *Amaranthus hypochondriacus*; chitin-binding peptide

INTRODUCTION

Plants use a variety of antimicrobial proteins and peptides to protect themselves from pathogen invasion, and they are mostly antifungal. Their mechanisms of action are as varied as their sources and include fungal cell wall polymer degradation, membrane channel, pore formation, damage to cellular ribosome, inhibition of DNA synthesis, and inhibition of the cell cycle (*1*). On the basis of homologies at the primary structure level, they can be classified into distinct families, including thionins (*2*), plant defensins (*3*), lipid-transfer proteins (*4*), chitinases (*5*), glucanases (*6*), hevein- and knottin-type antimicrobial peptides (*1*), and chitin-binding proteins (*7*).

The chitin-binding proteins have been isolated from diverse sources, and most of them have shown antifungal activity against phytopathogenic species, because chitin is the key component of the cell wall of these microorganisms. They have shown to affect fungal growth and development, disturbing the synthesis and/or deposition of chitin in the cell wall (5). The chitin-binding proteins from plants have been classified as PR-4 proteins. They are subdivided into two classes within this group. Class I has an N-terminal domain, which is similar to a domain present in hevein, a protein from rubber latex (*8*), whereas class II PR-4 proteins lack this domain. Examples of such chitin-binding proteins are chitinases from bean (*9*), wheat (*10*), and tobacco (*11*), chitin-binding lectins from wheat (*12*), barley (*13*), rice (*14*), breadfruit (*15*), *Pakia platycephala* (*16*), chitin-binding proteins and peptides from *Amaranthus retroflexus* and *Amaranthus caudatus* (*17*, *18*), sugar beet (*19*), *Pharbitis nil* L. (*20*), *Eucommia ulmoides* (*21*), and *Ginkgo biloba* (*22*). The size of chitin-binding proteins varies from 3.1 up to 20 kDa; they often have a basic pI and are highly resistant to extreme pH and protease treatment (*7*).

Since prehispanic time, every part of the *Amaranthus hypochondriacus* plant has been broadly used in Mexico, especially the seeds. Some proteins from *A. hypochondriacus* seeds have already been isolated in our laboratory, such as the nsLTP1 (nonspecific lipid-transfer protein) with 9.7 KDa (*23*), which plays a roll in the plant defense, and a globulin of 34.9 kDa, which is a storage protein very important in nutrient conservation (*24*).

Currently, there is a great interest in antimicrobial peptides, because these so-called "natural antibiotics" are promising to overcome the growing problem of antimicrobial resistance. There are already studies about antimicrobial peptides isolated from another amaranth seeds; however, there are not reports about these kind of peptides on the Mexican species *A. hypochondriacus*. Therefore, in this paper, we report the isolation, amino acid sequence determination, and biological properties of the antimicrobial peptide Ay-AMP from the seeds of *A. hypochondriacus*. According to its amino acid sequence and cysteine motif, Ay-AMP belongs to a superfamily of chitin-binding proteins, containing a single cysteine/glycine-rich chitin-binding domain.

MATERIALS AND METHODS

Biological Materials. Amaranth seeds were used as plant material for peptide extraction and analysis. Amaranth (*A. hypochondriacus*)

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dry seeds were bought from local producers from the state of Tlaxcala in México. The seeds were stored at room temperature. The fungi *Candida albicans*, *Aspergillus flavus*, *Aspergillus candidus*, *Aspergillus schraceus*, *Trichoderma* sp., *Fusarium solani*, *Fusarium oxysporum*, *Penicillium chrysogenum*, *Rhizopus arrhizus*, *Alternaria alternata*, *Trichophytum mentagrophytes*, and *Geotrichum candidum* were supplied by the UNAM mycology laboratory, UNIGRAS. Their spores were collected as described by Broekaert and collaborators (*25*) and stored at −70 °C.

Chemicals. All chemicals used in this study were of the highest grade available from Sigma-Aldrich (Saint Louis, MO). Milli-Q water (Millipore, Bedford, MA) with a resistance greater than 18 MΩ was used throughout. The samples and solutions were sterilized by filtration through 0.22 μm sterile filters (Millipore, Bedford, MA) prior to use.

Peptide Extraction and Purification. Ground seeds were extracted with 10% (v/v) acetic acid for over 1 h. The extract was centrifuged at 13 000 rpm. The soluble part was precipitated with acetone overnight. The precipitated fraction was collected by centrifugation at 3000 rpm, redissolved in 0.1% trifluoroacetic acid (TFA) in water (v/v) and injected onto a C18 RP-column (*17*).

The peptides were eluted with a linear acetonitrile gradient (0–40% of solvent B in solvent A for 60 min). Solvent A is 0.1% TFA in water (v/v), and solvent B is acetonitrile (*17*). The fractions were tested against *C. albicans* and *P. chrysogenum*.

Antifungal Activity. The microbiological tests were performed on microplates using phytopathogenic and dermopathogenic fungi, *P. chrysogenum*, and *C. albicans*, following the methodology developed by Broekaert and collaborators in 1990 (*25*), with some variations. Routinely, tests were performed with 20 μL of a filter-sterilized test solution, 10 μL of a fungal spore suspension (2×10^4 spores/mL), and 70 μL of potato dextrose broth (PDB). Control microcultures contained 20 μL of sterile distilled water instead of the test solution. It was incubated at room temperature for 48 h. The growth inhibition percentage is defined as 100 times the ratio of the corrected absorbance of the control microculture minus the corrected absorbance of the test microculture over the corrected absorbance at 570 nm of the 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. A 1 mg/mL dose of nistatine (Alpharma, México D.F.) was used as a positive control. The absorbance was measured in an Elx-808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

Minimal Inhibitory Concentration (MIC). A total of 11 concentrations of Ay-AMP (40−0.04 μg/mL) were used to test the fungal growth inhibition against *C. albicans*, *A. flavus*, *A. candidus*, *A. schraceus*, *Trichoderma* sp., *F. solani*, *F. oxysporum*, *P. chrysogenum*, *Rhizopus arrhizus*, *A. alternata*, *T. mentagrophytes*, and *G. candidum*. A total of 30 μL from a 200 μg/mL stock solution of Ay-AMP was taken and put on the first column of flat-bottom microplates. Then, double serial dilutions were made with distilled sterile water for the remaining columns (*26*). The microbial test was made as explained above, and the MIC was assumed as the minimal concentration that inhibits 80% fungal growth. Triplicate assays were conducted.

IC_{50}. The dose–response relation of many agonists yields sigmoidal curves when the response is plotted against the logarithm of the dose. There is no generally accepted theory that explains the shape of such curves; however, it was found that such curves are often approximately linear between 20 and 80%. Therefore, the data in the 20–80% region may be subjected to linear regression, in which the ordinate is the inhibition percent and abscissa is the log dose (*27*). To perform the test, logarithmic dilutions were made of the peptide and then the activity was measured as explained above. The doses tested were 31.62, 10.0, 3.16, 1.0, 0.32, and 0.1 μg/mL. Triplicate assays were conducted.

Protein Quantification. Protein concentration in the extract and the peptide concentration were 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. Triplicate assays were conducted.

Electrophoresis. Sodium dodecyl sulfate−polyacrilamide gel electrophoresis (SDS−PAGE, 16%) was performed on a Bio-Rad electrophoresis apparatus. The sample buffer contained 1 M Tris-HCl at pH 6.8, 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.01% bromophenol blue, and 20% (v/v) glycerol. Peptides were fixed after electrophoresis in 10% (v/v) trichloroacetic acid and 40% (v/v) methanol and then Coomassie-stained. Isoelectric focusing was performed on precast IEF gels in the pI range from 3 to 9, and a marker protein was used in the same pI range (Amersham, Piscataway, NJ).

In-Gel Digestion. In-gel digestion of samples was carried out using the method described by Wilm et al. (*28*). Briefly, excised gel spots were treated with 50 mM ammonium bicarbonate in 50% acetonitrile to remove Coomassie Brilliant Blue. The disulfide bonds were reduced with 10 mM dithiothreitol for 45 min at 56 °C, and SH groups were subsequently alkylated with 55 mM iodoacetamide. The gel pieces were washed, then dehydrated with acetonitrile, reswelled on ice in a digestion buffer containing 12.5 ng/μL trypsin and 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. Peptides were extracted with 50% acetonitrile and desalted by ZipTip C18.

Liquid Chromatography−Tandem Mass Spectrometry (LC−MS/MS) Analysis. The mixture of tryptic gel-digested peptides was condensed to a concentration of approximately 500 pmol/5 μL of 1% acetic acid and directly loaded into a Finnigan LCQ ion-trap mass spectrometer (San Jose, CA) using a Surveyor MS syringe pump delivery system. The eluate at 10 μL/min was split to allow only 5% of the sample to enter the nanospray source (0.5 μL/min). Tryptic peptides were separated online using a capillary/needle PicoFrit (ProteoPep) RP18 from New Objective (Woburn, MA) with a 5–75% linear gradient of solvent B (0.1% acetic acid in 90% acetonitrile) for 30 min. The spray voltage and the capillary temperature were set at 1.7 kV and 130 °C, respectively. For MS experiments, the fragmentation source was operated with 25–35 V of collision energy and 35–45% (arbitrary units) of normalized collision energy and then scan with an activated wide band. All spectra were obtained in the positive-ion mode. The data acquisition was performed on an Xcalibur Windows NT PC data system. The MS/MS spectra from enzymatically produced peptides were analyzed manually and using Sequest (Thermo Electron Co.), Mascot (Matrix Science Ltd.), and Protein Prospector (UCSF).

Alignment. The amino acid sequences were aligned using the ANTHEPROT 2000 (version 6.0) program. The alignment was performed with selected sequences extracted from the following identified proteins: Ay-AMP from *A. hypochondriacus* (this work), hevein from *Hevea brasiliensis* (*8*), Pn-AMP1 from *Pharbitis nil* L. (*20*), EAFP2 from *Eucommia ulmoides* (*21*), Ar-AMP from *Amaranthus retroflexus* (*17*), IWF4 from *Beta vulgaris* (*19*), and GAFP from *Ginkgo biloba* (*22*).

Chitin-Binding Assay. Because of the sequence similarity between Ay-AMP and chitin-binding proteins, we tested the Ay-AMP ability to bind on a chitin substrate. Microcolumns packed with chitin (New England BioLabs) were loaded with Ay-AMP and eluted with phosphate-buffered saline (PBS) at pH 7.4 and 100 mM acetic acid at pH 2.8. The fractions were analyzed by SDS−PAGE.

Chitinase Activity Assay. Chitinase activity was determined in 96-well microplates and a 100 μL assay mixture containing protein sample (10 μL), 1 mM 4-nitrophenyl-*N*,*N*′-diacetyl-β-D-chitobioside (*p*-NP-$(GlcNAc)_2$) (25 μL), and 100 mM sodium acetate buffer at pH 5.0 (65 μL). The reaction was incubated at 37 °C for 10 min, with constant agitation, and then the enzymatic reaction was terminated by the addition of 1.0 M sodium carbonate (50 μL). The amount of *p*-nitrophenol (*p*-NP) released was determined spectrophotometrically at 405 nm in an Elx-808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). The molar concentration of *p*-NP was calculated by a calibration curve constructed with *p*-NP concentrations varying from 0 to 30 nmol (*29*).

RESULTS AND DISCUSSION

Isolation and Peptide Purification. Acidic extract from 10 mg of *A. hypochondriacus* seeds showed antifungal activity toward *C. albicans* and *P. chrysogenum* using the microculture method (results not shown). The total extract was fractionated by RP-HPLC (**Figure 1**). Every peak was tested against *C. albicans* and *P. chrysogenum*. One peak was active against both fungi

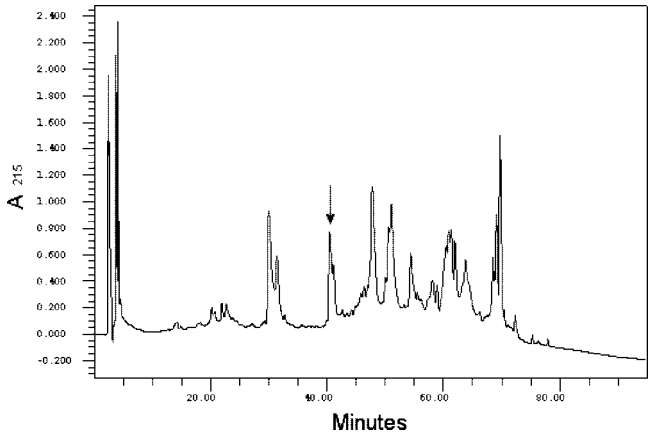

Figure 1. RP-HPLC chromatogram of the acidic extract from *A. hypochondriacus*. The antimicrobial peptide Ay-AMP eluted at approximately 40 min is shown by a vertical arrow.

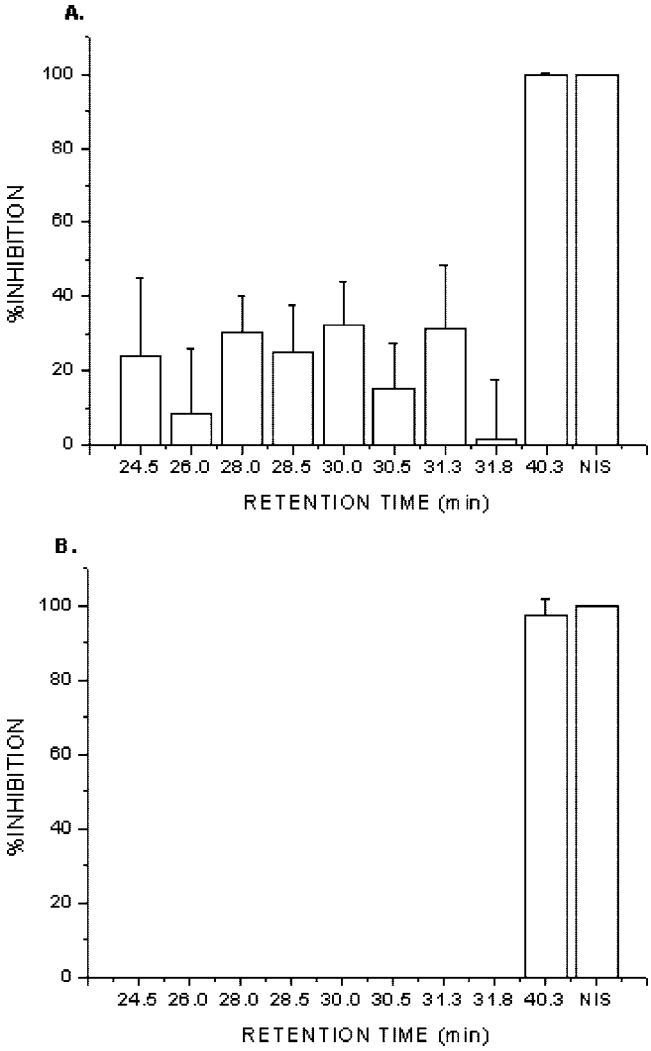

Figure 2. Antifungal activity plot. Chromatogram peaks tested against (**A**) *C. albicans* and (**B**) *P. chrysogenum*. In the plot, NIS is the activity of the positive control, 1 mg/mL nistatine.

(**Figure 2**) and was chosen for further analysis. It was named Ay-AMP. The electrophoresis showed a clear band for the peak with antifungal activity (**Figure 3**), and apparently, it is pure.

Mass Spectrometry. The spectrum showed that Ay-AMP was homogeneous and pure (**Figure 4**). The molecular mass was estimated to be 3184.0 Da in the reduced state.

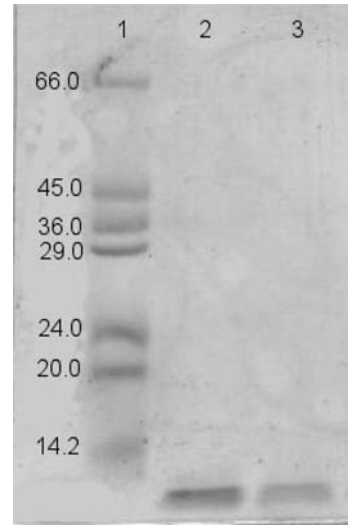

Figure 3. SDS−PAGE analysis of purified Ay-AMP. Both lines 2 and 3 are reduced Ay-AMP. Line 1 is the molecular-weight markers.

The calculated monoisotopic molecular mass of Ay-AMP of 3187.35 Da was in good agreement with the measured monoisotopic molecular mass, 3184.0 Da, suggesting that there are no other post-translational modifications of the peptide, except for the formation of disulphide bridges.

Amino Acid Sequence. The amino acid sequence of Ay-AMP was partially determined by LC−MS/MS analysis. From this analysis, we obtained 15 residues, CPSGMCCSQFGYCGK. This sequence has 100% homology with a fragment of the Ac-AMP2 sequence, which was isolated from *A. caudatus* seeds (*18*), and it covered more than 50% of the total sequence. Both peptides Ay-AMP and Ac-AMP2 have the same molecular mass. The undetermined sequence of Ay-AMP has just one isobaric amino acid in position 26, lysine with molecular mass 128 Da, which could be replaced by glutamine, which has the same molecular mass, 128 Da. However, the basic residues, such as lysine, are very conserved in these antimicrobial peptides because they are extremely important to the peptide activity. Considering all of the evidence discussed, we can say that the sequence of Ay-AMP is the same as that of the Ac-AMP2 (**Figure 5A**), and because of that, it also has the same three-dimensional structure (*30*) (**Figure 6**). On the basis of the hypothesis of a monophyletic origin of grain amaranths, it is conceivable that the Ay-AMP and Ac-AMP2 sequences might be conserved through domestication of *Amaranthus hybridus*, the putative common ancestor (*31*). There is a previous antecedent of this fact; the nsLTP isolated from *A. hypochondriacus* seeds has exactly the same sequence as that of one isolated from *A. caudatus* (*23*).

Ay-AMP is 30 amino acids in length, and its theoretical isoelectric point, calculated in expasy (*32*), from the sequence data is 8.92. The experimental pI value of Ay-AMP is higher than 9 (results not shown).

The alignment of the Ay-AMP amino acid sequence with some chitin-binding proteins demonstrated the similarity of Ay-AMP to a superfamily of chitin-binding proteins, containing a single cysteine/glycine-rich domain, such as hevein (**Figure 5B**). The hevein domain is highly conserved, containing six to eight cysteine residues, six in Ay-AMP, which are all involved in intramolecular disulfide bridges. Additionally, the hevein domain contains one serine and three aromatic residues at a conserved position (*7*).

It is obvious the high similarity between Ay-AMP and Ar-AMP (**Figure 5**), an antifungal peptide isolated from *A.*

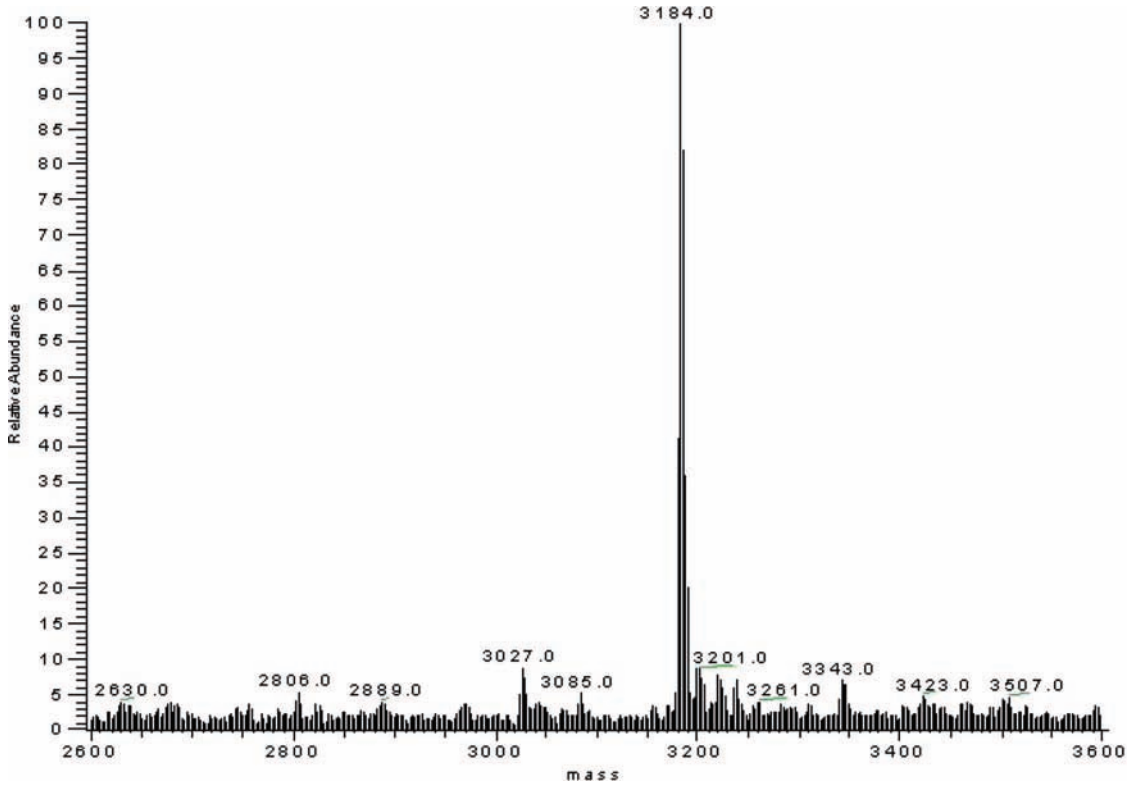

Figure 4. Electrospray mass spectrum of Ay-AMP. The major peak corresponds to the singly protonated molecule at *m*/*z* 3184.0.

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A.
Ay-AMP      VGECVR---GR-CPSGMCCSQFGYCGKGPKYCGR

B.
HEVEIN      -EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQS-NCKD
Pn-AMP1     -QQCGRQASGRLCGNRLCCSQWGYCGSTASYCGA--GCQS-QCRS
EAFP2       -QTCAS-RCPRPCNAGLCCSIYGYCGSGAAYCGAGN-CRC-QCRG
Ar-AMP      AGECVQ---GR-CPSGMCCSQFGYCGRGPKYCGR
IWF4        SGECNMY--GR-CPPGYCCSKFGYCGVGRAYCG
GAFP        DPTCSVLGDFK-CNPGRCCSKINYCGAC—YQWRFGLTPAR
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Figure 5. Comparison of the N-terminal amino acid sequence of Ay-AMP with chitin-binding proteins. (**A**) Amino acid sequence of Ay-AMP. (**B**) Sequences were aligned using the ANTHEPROT 2000 (version 6.0) program. Chitin-binding domains are shadowed. The alignment was performed with selected sequences extracted from the following identified proteins: Ay-AMP from *A. hypochondriacus*, hevein from *H. brasiliensis*, Pn-AMP1 from *P. nil* L., EAFP2 from *E. ulmoides*, Ar-AMP from *A. retroflexus*, IWF4 from potato, and GAFP from *G. biloba*.

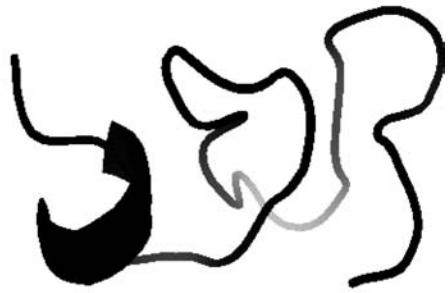

Figure 6. Three-dimensional structure of Ay-AMP. Because Ay-AMP has the same amino acid sequence as Ac-AMP2, the three-dimensional structure of Ay-AMP is exactly the same as the structure solved for Ac-AMP2 by ^{1}H nuclear magnetic resonance (NMR) (*30*).

retroflexus, a weed that causes damage to crops in the south of Russia (*17*). The difference is just in three positions V1, R6, and K23 in Ay-AMP, which are replaced by A1, R6, and R23 in Ar-AMP.

Table 1. Antifungal Activity of Ay-AMP

fungus	IC_{50} (μg/mL)	MIC (μg/mL)
Trichoderma sp.	0.24	6.41
Candida albicans	0.33	2.74
Geotrichum candidum	0.61	1.84
Aspergillus candidus	1.27	3.90
Alternaria alternata	2.26	11.67
Penicillium chrysogenum	2.66	6.22
Aspergillus schraceus	7.87	27.43
Fusarium solani	11.88	39.90

It is also noteworthy that several proteins that have been shown to possess antimicrobial properties share some key structural features with Ay-AMP, namely, a highly basic nature, a small-sized polypeptide chain, and a high content of cysteines. Examples of that are the thionins occurring in seeds and leaves of different plants, with about 45−47 residues and 4 disulfide bridges (*33*), and plant defensin, with around 45−54 amino acids and 4 disulfide bridges (*34*). Thionins and defensin have a broad spectrum of antibiotic properties because they are toxic to fungi, bacteria, and mammalian cells (*35*, *36*).

Antifungal Activity. Ay-AMP was assessed for its antifungal potency on 12 different pathogenic fungi and active against eight of them (**Table 1**). On the fungi where there was found antifungal activity, the IC_{50} varied from 0.24 to 11.88 μg/mL, depending upon the test organism. The MIC, to inhibit 80% of fungal growth, took values from 2.74 to 39.90 μg/mL. Ay-AMP did not have growth inhibitory activity against *A. flavus*, *R. arrhizus*, *T. mentagrophytes*, and *F. oxysporum* at any tested concentration. The highest inhibitory activity of Ay-AMP was observed against *Trichoderma* sp., and the lowest inhibition was noted against *F. solani*.

To assess possible synergistic effects between Ay-AMP and another antifungal agent, thaumatin from *Taumatococcus daniellii* (Sigma-Aldrich, St. Louis, MO), was used. The mixture with an Ay-AMP/thaumatin ratio of 1:1 (wt/wt) was tested against

P. chrysogenum, and a high synergistic effect was observed. The IC_{50} fell from 2.66 to 0.66 μg/mL.

The specific activity of Ay-AMP was found to be dependent upon the ionic constitution of the growth medium. The addition of 10 mM KCl or 1 mM $CaCl_2$ to the PDB medium did not affect the dose–response curves against *P. chrysogenum*, whereas 50 mM KCl and 5 mM $CaCl_2$ increase the IC_{50} values by about 12-fold. This is in concordance with other antifungal peptides that are antagonized by mono- and divalent ions, such as plant defensins, in which antifungal activity is reduced by an increase in ionic strength, especially by divalent cations (*1*), and thionins, which are inhibited by Ca^{2+} at a concentration above 5 mM but not by Mg^{2+} of Ba^{2+} at levels up to 10 mM or monovalent cations at concentrations up to 50 mM (*37*), and in the case of EAFP1 and EAFP2 from *Eucommia ulmoides*, which exhibit chitin-binding properties similar to other hevein-like peptides, when 5 mM calcium ions are added to the medium, IC_{50} values rise more than 100-fold (*38*). There are very few antifungal peptides that are not affected by monovalent cations, such as K^+ up to 50 mM; this is the case of Rs-AFP1 and Rs-AFP2, polypeptides rich in cysteine isolated from radish seeds, in which the $CaCl_2$ concentration of 5 mM is necessary to cause activity reduction (*36*).

The treatment of Ay-AMP with different proteases, such as pepsin, papain, chymotrypsin and trypsin, in an enzyme/peptide ratio of 1:60 (wt/wt) incubated for 5 h at 37 °C, did not affect its antifungal activity, except for trypsin, which increased the IC_{50} values by about 10-fold. The antifungal activity was not affected by boiling Ay-AMP for over 10 min. The high resistance of this peptide to the proteolysis and heating may be due to its small size and the presence of intramolecular disulfide bridges.

Even when the antifungal activity of Ay-AMP and Ac-AMP2 were tested against different fungi, their antifungal properties and potency are similar, because both of them show values of IC_{50} under 10 μg/mL and they were antagonized by mono- and divalent ions. Additionally, both peptides Ay-AMP and Ac-AMP2 were very resistant to the heating and protease effect (*18*).

The Ay-AMP was not cytotoxic against human T lymphocytes at 50 μg/mL, which is a higher concentration than the MIC to inhibit the fungal growth against any of the eight sensitive fungi.

The mechanism of action of this peptide, such as the chitin-binding proteins, is mainly due to their affinity to chitin. Because nascent chitin of the hyphal apex, where hyphal growth and, therefore, cell-wall assembly take place, is the most accessible, chitin-binding proteins localize along the fungal cell walls and accumulated at septa and hyphal tips by the union to the fungus cell-wall chitin, as its name indicates.

Chitin-Binding Assay. The amino acid sequence of Ay-AMP indicates that it belongs to a superfamily of chitin-binding proteins, and it was confirmed by the reversible binding of this peptide to a mini-chitin column. **Figure 7** shows that Ay-AMP is almost missing in the fraction of PBS washings but was recovered in the low pH desorption buffer. This result indicates that Ay-AMP exhibits binding affinity toward chitin.

Chitinase Activity. The Ay-AMP released *p*-NP from the substrate at peptide concentrations as low as 2−8 μg/mL. This could be a reflection of the exochitinase mechanism of action of these peptides. The exochitinolytic splitting of diacetylchitobiose from the nonreducing end of this substrate liberates *p*-nitrophenol as the product. It should be noticed that *p*-NP-$(GlcNAc)_2$ is not specific for chitinases, however. Roberts and Selitrennikoff in 1988 found that bacterial chitinases had over 200-fold more specific activity than the β-*N*-acetylglucosaminidase for the hydrolysis of the substrate, suggesting that hydrolysis of the chromogenic substrate by nonchitinases may not be a significant problem (*39*).

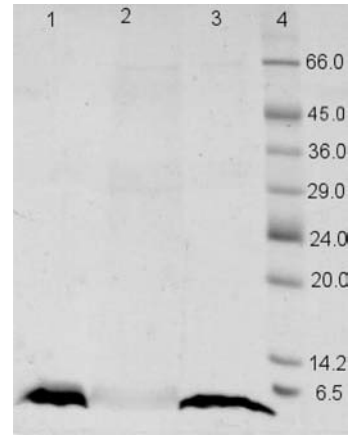

Figure 7. Chitin-binding assay of the Ay-AMP. Line 1 corresponds to Ay-AMP loaded on the column; line 2 is the fraction eluted with 100 mM PBS at pH 7.4; line 3 is the fraction eluted with 100 mM acetic acid at pH 2.8; and line 4 corresponds to the molecular-weight markers.

In conclusion, Ay-AMP is a very potent antifungal peptide isolated from the *A. hypochondriacus* seeds, which, besides exhibiting binding affinity toward chitin, shows chitinase activity. Ay-AMP may be a component of active defense, providing a valuable tool for engineering resistance against phytopathogenic fungi in plants.

ABBREVIATIONS USED

PR, pathogenesis related; TFA, trifluoroacetic acid; RP-HPLC, reverse-phase high-pressure liquid chromatography; pI, isoelectric point; MIC, minimal inhibitory concentration; IC_{50}, inhibitory concentration to inhibit 50% fungal growth; LC−MS/MS, liquid chromatography−tandem mass spectrometry; *p*-NP-$(GlcNAc)_2$, 4-nitrophenyl-*N*,*N*′-diacetyl-β-D-chitobioside; *p*-NP, *p*-nitrophenol; PDB, potato dextrose broth; PBS, phosphate-buffered saline.

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