Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459-489.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631-660.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87-103.
Kuwajima, K., Garvey, E. P., Finn, B. E., Matthews, C. B., & Sugai, S. (1991) *Biochemistry 30*, 7693-7703.
Manning, M. C., & Woody, R. W. (1989) *Biochemistry 28*, 8609-8613.
Oas, T. G., & Kim, P. S. (1988) *Nature 336*, 42-48.
Ohgushi, M., & Wada, A. (1983) *FEBS Lett. 164*, 21-24.
Ptitsyn, O. B. (1973) *Vestn. Akad. Nauk SSSR 5*, 57-68.
Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273-293.
Ptitsyn, O. B., Pain, R. H. Semisotnov, G. V., Zerovnik, E., & Razgulyaev, O. I. (1990) *FEBS Lett. 262*, 20-24.
Roder, H., Elöve, G. A., & Englander, S. W. (1988) *Nature 335*, 700-704.
Sears, D. W., & Beychok, S. (1979) in *Physical Principles and Techniques of Protein Chemistry, Part C* (Leach, S. J., Ed.) pp 445-593, Academic Press, New York.
Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., & Gilmanshin, R. I. (1991) *Biopolymers 31*, 119-128.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482-495.
Udgaoncar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694-699.

Antimicrobial Peptides from *Amaranthus caudatus* Seeds with Sequence Homology to the Cysteine/Glycine-Rich Domain of Chitin-Binding Proteins†

Willem F. Broekaert,*,‡ Wim Mariën,‡ Franky R. G. Terras,‡ Miguel F. C. De Bolle,‡ Paul Proost,§ Jozef Van Damme,§ Lieve Dillen,∥ Magda Claeys,∥ Sarah B. Rees,⊥ Jozef Vanderleyden,‡ and Bruno P. A. Cammue‡

F. A. Janssens Laboratory of Genetics, Catholic University of Leuven, W. De Croylaan 42, B-3001 Heverlee, Belgium, Rega Institute for Medical Research, Catholic University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium, Department of Pharmaceutical Sciences, Universitaire Instelling Antwerpen, Universiteitsplein 1, B-2610 Wilrijk, Belgium, and ICI Agrochemicals, Jealott's Hill Research Station, Bracknell, Berks RG12 6EY, U.K.

Received November 19, 1991; Revised Manuscript Received January 24, 1992

ABSTRACT: Two antimicrobial peptides (*Ac*-AMP1 and *Ac*-AMP2) were isolated from seeds of amaranth (*Amaranthus caudatus*), and their physicochemical and biological properties were characterized. On the basis of fast atom bombardment mass spectroscopy, *Ac*-AMP1 and *Ac*-AMP2 have monoisotopic molecular masses of 3025 and 3181, respectively. Both proteins have p*I* values above 10. The amino acid sequence of *Ac*-AMP1 (29 residues) is identical to that of *Ac*-AMP2 (30 residues), except that the latter has 1 additional residue at the carboxyl terminus. The sequences are highly homologous to the cysteine/glycine-rich domain occurring in many chitin-binding proteins. Both *Ac*-AMP1 and *Ac*-AMP2 bind to chitin in a reversible way. *Ac*-AMP1 and *Ac*-AMP2 inhibit the growth of different plant pathogenic fungi at much lower doses than other known antifungal chitin-binding proteins. In addition, they show some activity on Gram-positive bacteria. The antimicrobial effect of *Ac*-AMP1 and *Ac*-AMP2 is strongly antagonized by cations.

Chitin [poly(β-1,4-*N*-acetyl-D-glucosamine)] is a polysaccharide occurring in the cell wall of fungi and in the exoskeleton of invertebrates. Although plants have not been reported to contain chitin or chitin-like structures, proteins exhibiting strong affinity to this polysaccharide have been isolated from different plant sources [for a review, see Raikhel and Broekaert (1992)]. Examples of such chitin-binding proteins are basic chitinases from bean (Boller et al., 1983), wheat (Molano et al., 1979), and tobacco (Shinshi et al., 1987), chitin-binding lectins from wheat (Rice & Etzler, 1974), barley (Peumans et al., 1982), rice (Tsuda, 1979), and stinging nettle (Peumans et al., 1983), and a small protein from rubber tree latex, called hevein (Van Parijs et al., 1991). All these chitin-binding proteins share a homologous cysteine/glycine-rich domain of about 40-43 amino acids, which is repeated either 2-fold (in the nettle lectin) or 4-fold (in wheat, barley, and rice lectins) or fused to an unrelated domain (in basic chitinases).

Although the exact physiological role of these proteins remains uncertain, they all have been shown in in vitro experiments to exert antibiotic activities, suggesting a defense-related function. Indeed, antifungal properties have been ascribed to chitinases (Schlumbaum et al., 1986; Broekaert et al., 1988), nettle lectin (Broekaert et al., 1989), and hevein (Van Parijs et al., 1991). On the other hand, the wheat lectin causes deleterious effects on the development of insect larvae (Murdock et al., 1990; Czapla & Lang, 1990).

In the present paper, we describe the isolation and characterization of two antimicrobial peptides from the seeds of amaranth (*Amaranthus caudatus*) that are homologous to the cysteine/glycine-rich domain of chitin-binding proteins. The amaranth peptides inhibit the growth of fungi to a much higher

†This work was supported in part by the ECLAIR programme (AGRE-0005) of the Commission of the European Communities. W. F.B. is Research Associate of the National Fund for Scientific Research (Belgium). F.R.G.T. and B.P.A.C. acknowledge receipt of a predoctoral and postdoctoral fellowship, respectively, from the Belgian "Instituut ter Aanmoediging van het Wetenschappelijk Onderzoek in de Nijverheid en de Landbouw".

*To whom correspondence should be addressed.
‡F. A. Janssens Laboratory of Genetics, Catholic University of Leuven.
§Rega Institute for Medical Research, Catholic University of Leuven.
∥Universitaire Instelling Antwerpen.
⊥ICI Agrochemicals.

0006-2960/92/0431-4308$03.00/0

extent than other previously characterized antifungal chitin-binding proteins.

Materials and Methods

Materials. Seeds of *Amaranthus caudatus* L. were obtained from Gonthier (Wanze, Belgium). Growth of fungi, collection, and harvest of fungal spores were done as previously described (Broekaert et al., 1990a). The following fungal strains were used: *Alternaria brassicola* MUCL 20297, *Ascochyta pisi* MUCL 30164, *Botrytis cinerea* MUCL 30158, *Colletotrichum lindemuthianum* MUCL 9577, *Fusarium culmorum* IMI 180420, *Trichoderma hamatum* MUCL 29736, and *Verticillium dahliae* MUCL 19210. The bacterial strains were *Bacillus megaterium* ATCC 13632, *Escherichia coli* strain HB101, *Erwinia carotovora* strain 3912, and *Sarcina lutea* ATCC 9342.

Chitin was prepared by N-acetylation of chitosan (Sigma, St. Louis, MO) by the method of Molano et al. (1977). Nikkomycin Z was obtained from Calbiochem (San Diego, CA). The nettle lectin or *Urtica dioica* agglutinin (UDA)[1] was isolated from stinging nettle (*Urtica dioica*) rhizomes as previously described (Peumans et al., 1983). Chitinase and β-1,3-glucanase, isolated from pea pods by the method of Mauch et al. (1988), were kindly provided by Drs. A. Ludwig and T. Boller (University of Basel, Basel, Switzerland). Wheat thionin (the β-form of purothionin) was purified from wheat endosperm according to Redman and Fisher (1969).

Purification of A. caudatus Antimicrobial Peptides. A. caudatus seeds (1 kg) were ground in a coffee mill, and the resulting meal was extracted for 2 h at 4 °C with 3 L of an ice-cold extraction buffer containing 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 2 mM EDTA, 2 mM thiourea, 1 mM PMSF, and 1 mg/L leupeptin. The homogenate was squeezed through cheesecloth and clarified by centrifugation (5 min at 7000*g*). Solid ammonium sulfate was added to the supernatant to obtain 30% relative saturation, and the precipitate formed after standing for 1 h at room temperature was removed by centrifugation (10 min at 7000*g*). The supernatant was adjusted to 75% relative ammonium sulfate saturation and the precipitate formed overnight at room temperature collected by centrifugation (30 min at 7000*g*). After the pellet was redissolved in 300 mL of distilled water, the insoluble material was removed by further centrifugation (20 min at 7000*g*). The clear supernatant was dialyzed extensively against distilled water using benzoylated cellulose tubing (Sigma) with a molecular weight cutoff of 2000. After dialysis, the solution was adjusted to 50 mM Tris-HCl (pH 9) by addition of the 10-fold-concentrated buffer, and subsequently passed over a Q-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) column (12 × 5 cm) equilibrated with 50 mM Tris-HCl, pH 9. The proteins passed through the column were dialyzed extensively against 20 mM sodium phosphate buffer, pH 7. Further chromatographic purification was done as described in the legends to Figures 1 and 2.

Bioassays. Antifungal activity was measured by microspectrophotometry as previously described (Broekaert et al., 1990a). Routinely, tests were performed with 20 μL of a (filter-sterilized) test solution and 80 μL of a fungal spore suspension (2×10^4 spores/mL) in half-strength potato dextrose broth (Difco). For experiments on the antagonistic effect of cations, a synthetic growth medium was used instead of potato dextrose broth. This medium consisted of K_2HPO_4 (2.5 mM), $MgSO_4$ (50 μM), $CaCl_2$ (50 μM), $FeSO_4$ (5 μM), $CoCl_2$ (0.1 μM), $CuSO_4$ (0.1 μM), Na_2MoO_4 (2 μM), H_3BO_3 (0.5 μM), KI (0.1 μM), $ZnSO_4$ (0.5 μM), $MnSO_4$ (0.1 μM), glucose (10 g/L), asparagine (1 g/L), methionine (20 mg/L), myoinositol (2 mg/L), biotin (0.2 mg/L), thiamin hydrochloride (1 mg/L), and pyridoxine hydrochloride (0.2 mg/L). Control microcultures contained 20 μL of sterile distilled water and 80 μL of the fungal spore suspension. Unless otherwise stated, the test organism was *Fusarium culmorum*, and incubation was done at 25 °C for 48 h. Percent growth inhibition 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 595 nm of the control microculture. The corrected absorbance values equal the absorbance at 595 nm of the culture measured after 48 h minus the absorbance at 595 nm measured after 30 min. Values of growth inhibition lower than 15% are not indicated on the chromatograms.

Antibacterial activity was measured microspectrophotometrically as follows. A soft agarose medium (tryptone, 10 g/L; low melting point agarose, 5 g/L) was inoculated with bacteria to a cell density of 10^5 colony forming units/mL. Aliquots (80 μL) of the bacterial suspension were added to filter-sterilized samples (20 μL) in flat-bottom 96-well microplates. The absorbance at 595 nm of the culture was measured with the aid of a microplate reader after 30 min (blank values) and 24 h of incubation at 28 °C. The percent growth inhibition was calculated as in the antifungal activity assay.

Assays for human cell toxicity were performed either on umbilical vein endothelial cells (Alessi et al., 1988) or on skin-muscle fibroblasts (Van Damme et al., 1987) cultured in 96-well microplates. The growth medium was replaced by 80 μL of serum-free medium [Optimem 1 for endothelial cells or Eagle's minimal essential medium (EMEM) for fibroblasts, both from GIBCO] to which 20 μL of a filter-sterilized test solution was added. The cells were further incubated for 24 h at 37 °C under a 5% CO_2 atmosphere with 100% relative humidity. The viability of the cells was assessed microscopically after being stained with trypan blue (400 mg/L in phosphate-buffered saline, PBS) for 10 min.

Qualitative Assessment of Free Cysteine Thiol Groups. Analysis of thiol groups was performed by the 5,5′-dithiobis(nitrobenzoic acid) method (Creighton, 1989) using 10 nmol of reduced or unreduced peptides. Reduction was performed with dithiothreitol (Creighton, 1989) followed by reversed-phase chromatography to remove excess reagents (see legend to Figure 1).

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on precast commercial gels (PhastGel high density from Pharmacia) using a PhastSystem (Pharmacia) electrophoresis apparatus. The sample buffer contained 200 mM Tris-HCl (pH 8.3), 1% (w/v) SDS, 1 mM EDTA, 0.005% bromophenol blue, and, in case of analysis of reduced peptides, 1% (w/v) dithiothreitol (DTT). Peptides were fixed after electrophoresis in 6% glutaraldehyde and silver-stained according to Heukeshoven and Dernick (1985). Isoelectric focusing was done on precast Immobilin dry strips (Pharmacia) rehydrated in 8 M urea, using marker proteins in the p*I* range from 4.7 to 10.6 (Pharmacia). Antifungal activity detection after native cathodic gel electrophoresis was done as previously described (De Bolle et al., 1991) with the following modifications. Electrophoresis was performed on 10% acrylamide gels containing 240 mM Tris/277 mM MES (pH 7). The electro-

[1] Abbreviations: *Ac*-AMP, *Amaranthus caudatus* antimicrobial peptide; UDA, *Urtica dioica* agglutinin.

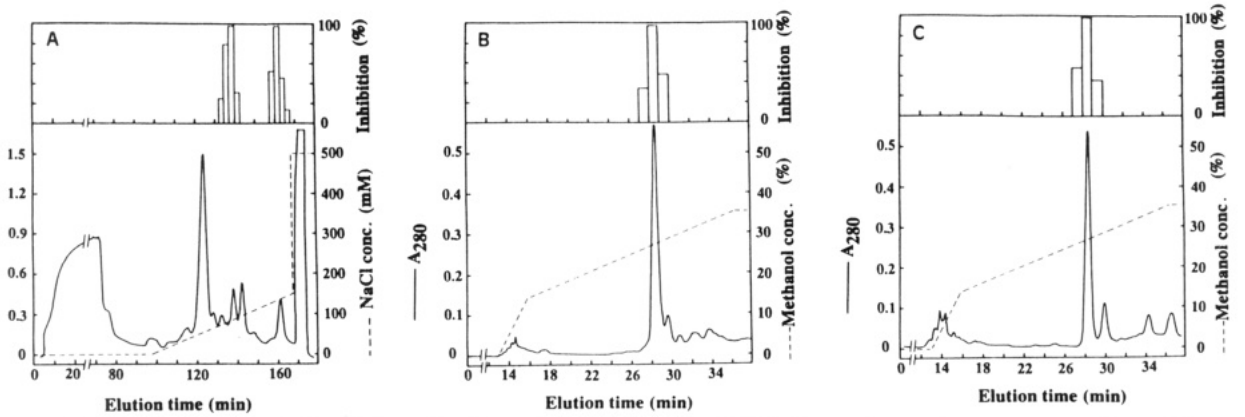

FIGURE 1: Purification of the antimicrobial peptides from *A. caudatus* seeds. (A) Cation-exchange chromatogram. About 100 mg of the basic protein fraction from *A. caudatus* seeds dissolved in 20 mM sodium phosphate buffer (pH 7) was applied on an S-Sepharose high-performance (Pharmacia) column (10 × 1.6 cm) previously equilibrated with the sodium phosphate buffer. The column was eluted at 3 mL/min with a linear gradient of 210 mL from 0 to 150 mM NaCl in 20 mM sodium phosphate buffer (pH 7). The eluate was monitored for protein by on-line measurement of the absorbance at 280 nm (lower panel) and collected in 7.5-mL fractions of which 20 μL was tested in the microspectrophotometric antifungal activity assay (upper panel). (B) Reversed-phase chromatogram of peak 2 material from cation-exchange chromatography. About 1 mg of the active protein fraction was loaded on a Pep-S (porous silica C_2/C_{18}, Pharmacia) column (25 × 0.93 cm) in equilibrium with 0.1% TFA. The column was eluted at 5 mL/min with the following gradients (solvent B is methanol containing 0.1% TFA): 0–13 min, 0% B; 13–16 min, 0–15% B; 16–36 min, 15–35% B. The eluate was monitored for protein by on-line measurement of the absorption at 280 nm (lower panel). Five-milliliter fractions of the eluate were collected, vacuum-dried, and finally dissolved in 0.5 mL of distilled water of which 10 μL was used in the microspectrophotometric antifungal activity assay (upper panel). (C) Reversed-phase chromatography of peak 4 material from cation-exchange chromatography. Chromatographic conditions were as described in (B).

phoresis buffer consisted of 100 mM L-histidine/41 mM MES (pH 6.5). The gels were kept cool (10 °C) during electrophoresis. The sample buffer contained 20% glycerol and 0.01% methylene blue.

Amino Acid Sequence Analysis. Cysteine residues of the peptides were modified by S-carboxamidomethylation (Creighton, 1989). Removal of excess reagents was done by reversed-phase chromatography. The resulting peptide fractions were subjected to amino acid sequence analysis in a 477A protein sequencer (Applied Biosystems) with on-line detection of phenylthiohydantoin amino acid derivatives in a 120A analyzer (Applied Biosystems).

Mass Spectrometry. Positive-ion fast atom bombardment mass spectra were obtained with a VG 70 SEQ double-focusing instrument equipped with an Ion Tech saddle-field fast atom gun using xenon atoms of 8 keV at a beam flux of 1 mA. The spectra were acquired in the multichannel analysis mode with an acquisition time of 1–2 min and a scan rate of 25 s/decade. The mass spectrometer was scanned exponentially from m/z 4900 to 800 with a resolution of approximately 3000. Cesium iodide cluster ions were used for calibration. The peptides (typically 1–2 μL; 4 μg) were applied to the probe tip which already contained 2 μL of the liquid matrix [1,4-dithiothreitol/1,4-dithioerythritol (5:1 v/v)] acidified with 1 μL of aqueous trichloroacetic acid (10% w/v).

Chitin-Binding Assay. Peptide samples (50 μg) dissolved in 1 mL of phosphate-buffered saline (pH 7) were applied on a chitin microcolumn (2.5 × 6 mm) and recycled 3 times over the column. The column was eluted 5 times with 1 mL of phosphate-buffered saline and once with 1 mL of 100 mM acetic acid (pH 2.8). Fractions (1 mL) of the eluate were desalted and concentrated by reversed-phase chromatography and finally redissolved in 50 μL of sample buffer for SDS–PAGE analysis.

RESULTS

Purification Procedure. The antimicrobial peptides of *A. caudatus* seeds were purified by a four-step procedure. The first step involved ammonium sulfate fractionation of proteins precipitating in the interval of 30–75% relative saturation. In the second step, the basic protein fraction (pI > 9) was isolated by passage over a Q-Sepharose (Pharmacia) anion-exchange column equilibated at pH 9. The proteins not retained by the column contained all antifungal activity and were further separated in a third step by cation-exchange chromatography at pH 7 on an S-Sepharose high-performance (Pharmacia) column. Elution of the column with a linear gradient from 0 to 150 mM NaCl yielded four distinct peaks (Figure 1A). The antifungal activity coeluted with the material from peaks 2 and 4, respectively. The active fractions were purified in the final step by reversed-phase chromatography on a C_2/C_{18} silica column (Figure 1B,C). Both materials from peaks 2 and 4 yielded well-resolved major peaks that coeluted with the antifungal activity. The active factor purified from peak 2 material is further referred to as *Ac*-AMP1 (*Amaranthus caudatus* antimicrobial peptide 1) and that from peak 4 as *Ac*-AMP2. The purified factors were analyzed by native gel electrophoresis, whereby *Ac*-AMP1 and *Ac*-AMP2 both showed single protein bands (Figure 2). In addition, by using a zymographic technique, it was demonstrated that antifungal activity comigrated exactly with the protein bands in the gel

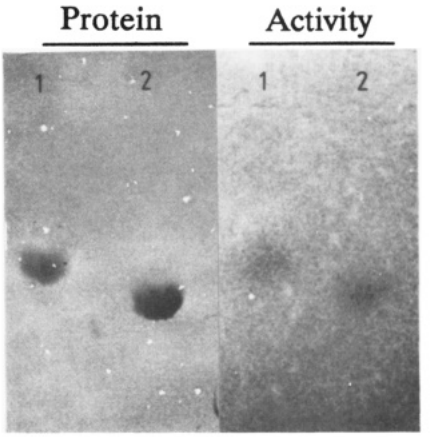

FIGURE 2: Native cathodic gel electrophoresis and zymography of purified *A. caudatus* antimicrobial peptides. Peptides (20 μg per lane) were detected by silver-staining of a diffusion blot prepared from the gel. The gel was overlaid with a soft agar gel containing viable *T. hamatum* spores and incubated at 25 °C for 3 days. Lanes 1, *Ac*-AMP1; lanes 2, *Ac*-AMP2.

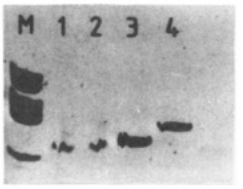

FIGURE 3: SDS–PAGE analysis of purified *A. caudatus* antimicrobial peptides. Lane 1, reduced *Ac*-AMP1; lane 2, reduced *Ac*-AMP2; lane 3, unreduced *Ac*-AMP1; lane 4, unreduced *Ac*-AMP2. The peptides (200 ng) were separated on precast PhastGel high-density (Pharmacia) gels. Molecular weight markers (lane M) had the following sizes: 17K, 14.5K, 8K, 6K, and 2.5K.

(Figure 2), confirming the proteinaceous nature and the purity of the isolated factors.

Molecular Structure. The amaranth antimicrobial peptides were analyzed by SDS–PAGE before and after reduction with dithiothreitol (Figure 3). Reduced *Ac*-AMP1 and *Ac*-AMP2 both migrated as single bands with an apparent molecular weight of about 3K. The wavy aspect of the bands was consistent in multiple independent SDS–PAGE analyses. In their unreduced state, *Ac*-AMP1 and *Ac*-AMP2 yielded a 4- and a 5.5-kDa band, respectively. Estimation of the molecular weights of the native *Ac*-AMPs by gel filtration on a Superdex-75 column (30 × 1.6 cm) (Pharmacia) yielded values of about 2K (results not shown). The unreduced *Ac*-AMPs did not react with the thiol reagent 5,5′-dithiobis(nitrobenzoic acid), whereas the reduced peptides did, indicating that all cysteine residues participate in disulfide bonds (results not shown). The p*I* values of *Ac*-AMP1 and *Ac*-AMP2 are 10.3 and higher than 10.6, respectively, as evidenced by isoelectric focusing (results not shown).

Amino Acid Sequence. The amino acid sequence of the reduced and carboxamidomethylated antimicrobial peptides was determined by direct N-terminal sequencing (Figure 4A). *Ac*-AMP1 is 29 amino acids in length, whereas *Ac*-AMP2 has 30 residues. The sequence of *Ac*-AMP2 is identical to that of *Ac*-AMP1 except that it has one additional amino acid at its carboxyl terminus. The *Ac*-AMPs are particularly rich in cysteine (six residues), glycine (seven residues), and basic amino acids (four and five residues for *Ac*-AMP1 and *Ac*-AMP2, respectively). The theoretical isoelectric points calculated from the sequence data are 10.1 and 11.0 for *Ac*-AMP1 and *Ac*-AMP2, respectively, assuming that all cysteine residues participate in disulfide linkages.

The amino acid sequence of *Ac*-AMP2 shows striking similarity to the cysteine/glycine-rich domains of chitin-binding proteins, such as chitinases, chitin-binding lectins, and hevein (Figure 4B). Sequence alignment of *Ac*-AMP2 and the N-terminus of a basic chitinase from tobacco (Shinshi et al., 1987) showed 14 identical amino acids and 5 conserved changes in the first 30 residues. A single gap of four amino acids had to be introduced in the N-terminal portion of *Ac*-AMP2 to allow optimal alignment with the chitin-binding proteins. With this alignment, all of the cysteine residues appeared at invariant positions.

Mass Spectrometry. Fast atom bombardment mass spectrometry of *Ac*-AMP1 and *Ac*-AMP2 showed abundant protonated molecules (isotope mixtures) forming peaks at m/z 3028.4 and 3184.2, respectively, as well as doubly protonated molecules. Detection of doubly protonated molecules could be expected in view of the very basic nature of the peptides. In order to determine the monoisotopic molecular masses, the molecular ion regions were analyzed in detail and the isotopic patterns compared with the calculated ones. These data indicate that the singly charged molecular ions $(M + H)^+$ of *Ac*-AMP1 and *Ac*-AMP2 are at m/z 3026.4 and 3182.2, implying that the corresponding monoisotopic relative molecular masses are 3025.4 and 3181.2. The mass spectrum obtained from *Ac*-AMP2 and the isotopic pattern of its $(M + H)^+$ ion region are illustrated in Figure 5.

Chitin-Binding Activity. Because of the similarity in amino acid sequence between the *Ac*-AMPs and chitin-binding proteins, we investigated the ability of the former to bind on a chitin substrate. Microcolumns packed with chitin were loaded with either *Ac*-AMP1 or *Ac*-AMP2, and eluted subsequently at neutral pH and low pH (pH 2.8). As shown in Figure 6, the *Ac*-AMPs were absent from the fraction passed through the column and from the neutral pH washings, but instead were recovered in the low-pH desorption buffer. These results indicate that both *Ac*-AMP1 and *Ac*-AMP2 exhibit binding affinity toward chitin.

Antifungal Activity. The antifungal activity of the *Ac*-AMPs was not abolished after treatment with proteinase K, Pronase E, chymotrypsin, or trypsin. Moreover, the *Ac*-AMPs were not affected by heat treatments at up to 100 °C for 10 min nor by exposure to pH conditions as extreme as pH 2 or pH 11. The remarkable stability of the *Ac*-AMPs may be explained by their small size and the presence of intramolecular disulfide bridges. Reduction of the cystine residues by dithiothreitol, however, completely abolished the antifungal activity (results not shown).

The *Ac*-AMPs were also assessed for their antifungal potency on six different plant pathogenic fungi (*Alternaria brassicola*, *Ascochyta pisi*, *Botrytis cinerea*, *Colletotrichum*

A

Ac-AMP1	V G E C V R G R C P S G M C C S Q F G Y C G K G P K Y C G
Ac-AMP2	V G E C V R G R C P S G M C C S Q F G Y C G K G P K Y C G R

B

To chit	* E Q C G S Q A G G A R C A S G L C C S K F G W C G N T N D Y C G P G * N C Q S * Q C P G G
Be chit	* E Q C G r Q A G G A l C *P* g G n C C S q F G W C G s T t D Y C G P G * * C Q S * Q C * G G
Hevein	* E Q C G r Q A G G k l C *P* n n L C C S q *W* G W C G s T d *E* Y C s P d h N C Q S * *N* C k d s
Wh lect	* q r C G e Q G s n n e C *P* n n L C C S q *Y* G *Y* C G m g g D Y C G k G * * C Q d g a C w t s
UDA	* q r C G S Q G G G G t C *P* a l r C C S i *W* G W C G a *S* s p Y C
Ac-AMP2	v g e C v r * * * * G R C *P* S G *M* C C S q F G *Y* C G k g p k Y C G r

FIGURE 4: Amino acid sequences of the *Ac*-AMPs and related proteins. (A) Amino acid sequences of *Ac*-AMP1 and *Ac*-AMP2. (B) Alignment of N-terminal amino acid sequences from tobacco chitinase (Shinshi et al., 1987), bean chitinase (Broglie et al., 1986), hevein (Broekaert et al., 1990b), wheat lectin (Raikhel & Wilkins, 1987), and nettle lectin (Chapot et al., 1986) and the sequence of *Ac*-AMP2. Sequence identities with the tobacco chitinase are indicated in capitals; conserved changes are marked in italic and nonconserved changes in lower case. Conserved changes are considered as substitutions within the amino acid homology groups FWY, MILV, RKH, ED, NQ, ST, and PAG. Gaps introduced for optimal alignment are represented by asterisks.

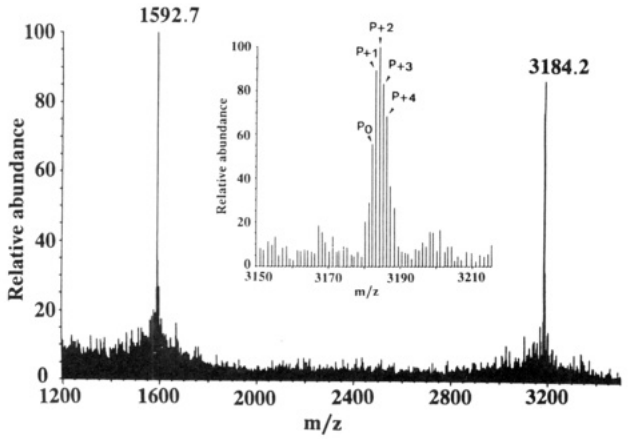

FIGURE 5: Fast atom bombardment mass spectrum of *Ac*-AMP2. The two major peaks correspond to the singly protonated molecules at m/z 3184.2 and the corresponding doubly charged protonated molecules at m/z 1592.7. (Inset) Isotopic pattern of *Ac*-AMP2 obtained by using a resolution of 3000. The expected isotopic pattern is as follows: P_0, 52%; P_{+1}, 92%; P_{+2}, 100%; P_{+3}, 80%; P_{+4}, 52%. Comparison of the measured isotopic pattern with the calculated pattern allows the identification of the monoisotopic $(M + H)^+$ peak P_0 at m/z 3182.2.

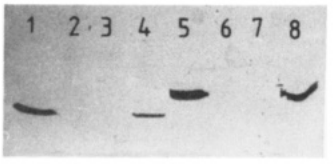

FIGURE 6: Chitin binding of the *Ac*-AMPs. *Ac*-AMP1 (lanes 1–4) and *Ac*-AMP2 (lanes 5–8) were subjected to affinity chromatography on a chitin column, and the eluate fractions were analyzed by SDS–PAGE. Lanes 1 and 5, antimicrobial peptides at equivalent amounts as those loaded on the columns; lanes 2 and 6, fractions passed through the column; lanes 3 and 7, fractions eluted with PBS (pH 7); lanes 4 and 8, fractions eluted with 100 mM acetic acid (pH 2.8).

Table I: Antifungal Activity of *Ac*-AMP1, *Ac*-AMP2, UDA, and Pea Chitinase

fungus	IC_{50} (μg/mL)[a]			
	Ac-AMP1	*Ac*-AMP2	UDA	chitinase
A. brassicola	7	4	200	400
A. pisi	8	8	1000	>500
B. cinera	10	8	>1000	>500
C. lindemuthianum	8	8	20	>500
F. culmorum	2	2	>1000	>500
T. hamatum	7	3	90	1.5
V. dahliae	6	8	80	500

[a] The concentration required for 50% growth inhibition after 48 h of incubation.

lindemuthianum, *Fusarium culmorum*, and *Verticillium dahliae*) and one saprophytic fungus (*Trichoderma hamatum*) (Table I). The concentration required for 50% growth inhibition after 48 h of incubation (IC_{50}) varied from 2 to 10 μg/mL, depending on the test organism. The antifungal potency of *Ac*-AMP1 was almost identical to that of *Ac*-AMP2.

To assess possible synergistic effects between the *Ac*-AMPs and other antifungal agents, the following combinations were tested on *B. cinerea* and *C. lindemuthianum* (the ratios between parentheses indicate mass ratios used in the tests with *B. cinerea* and *C. lindemuthianum*, respectively): *Ac*-AMP2 and UDA (1:100, 1:2.5), *Ac*-AMP2 and pea chitinase (1:100, 1:100), *Ac*-AMP2 and pea glucanase (1:100, 1:100), *Ac*-AMP2 and wheat thionin (1:2, 1:2.5), and *Ac*-AMP2 and Nikkomycin Z (3:1, 20:1). The given mass ratios were chosen as to reflect equal amounts of activity units of each antifungal agent, taking 1:100 as an upper limit. However, none of the tested combinations showed increased antifungal activity relative to the individual agents (results not shown).

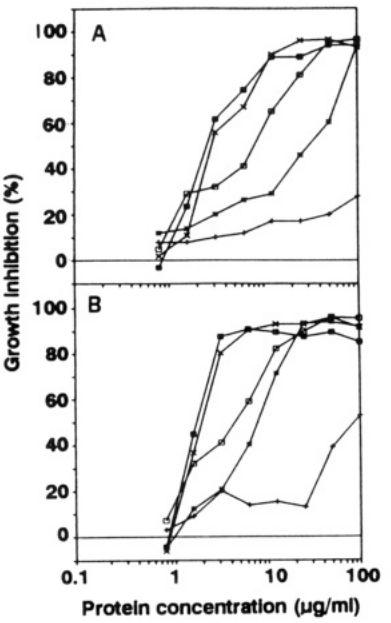

FIGURE 7: Antagonistic effect of K^+ and Ca^{2+} on growth inhibition of *B. cinerea* caused by *Ac*-AMP1 (panel A) and *Ac*-AMP2 (panel B). The dose–response curves were determined using a low ionic strength synthetic growth medium (■), supplemented with 10 mM KCl (×), 50 mM KCl (□), 1 mM $CaCl_2$ (*), and 5 mM $CaCl_2$ (+).

The specific activity of the *Ac*-AMPs was found to be strongly dependent on the ionic constitution of the growth medium. Figure 7 shows the dose–response curves of *Ac*-AMP1 and *Ac*-AMP2 on *B. cinerea* in a low ionic strength synthetic growth medium with and without different additions of KCl or $CaCl_2$. In the reference medium, containing 2.5 mM monovalent cations and 0.1 mM divalent cations, *Ac*-AMP1 and *Ac*-AMP2 had IC_{50} values of 2.2 and 1.6 μg/mL, respectively. Administration of KCl at 10 mM to this medium did not significantly affect the dose–response curves, whereas KCl at concentrations of 50 mM increased the IC_{50} values by about 3-fold. $CaCl_2$ had a much more dramatic antagonistic effect. When supplemented at 1 mM to the reference medium, $CaCl_2$ caused a 5–6-fold increase of the IC_{50} values. At 5 mM $CaCl_2$, the drops in specific activity were more than 50-fold.

Specificity of Antibiotic Effects. To address the question whether or not the activity of the *Ac*-AMPs is restricted to fungi, we have tested their effect in two other bioassays using either bacteria or human cultured cells as indicator cells. *Ac*-AMP1 and *Ac*-AMP2 were inhibitory to the Gram-positive bacteria *Bacillus megaterium* (IC_{50} values of 40 and 10 μg/mL, respectively) and *Sarcina lutea* (IC_{50} values of 250 and 40 μg/mL, respectively). As for the antifungal effects, the antibacterial activity of the *Ac*-AMPs was dependent on the ionic composition of the growth medium: addition of KCl (50 mM) and $CaCl_2$ (1 mM) to the bacterial medium (1% tryptone) decreased the antibacterial potency by about 12-fold. *Ac*-AMP1 and *Ac*-AMP2 did not influence the growth of the Gram-negative bacteria *Escherichia coli* and *Erwinia carotovora* when included in the culture medium at up to 500 μg/mL. Likewise, *Ac*-AMP1 and *Ac*-AMP2 did not affect the viability of either human umbilical endothelial cells or human skin-muscle fibroblasts at concentrations up to 500 μg/mL (results not shown).

Discussion

We have isolated from amaranth seeds two potent antimicrobial peptides, designated *Ac*-AMP1 and *Ac*-AMP2.

Ac-AMP1 and *Ac*-AMP2 were identified, by direct amino-terminal sequencing, as 29- and 30-residue peptides, respectively. According to the sequence data, *Ac*-AMP2 differs from *Ac*-AMP1 only by the occurrence of a single additional carboxyl-terminal residue. As expected by the fact that this residue is a basic amino acid (Arg-30), *Ac*-AMP2 has a higher p*I* value. It also explains why *Ac*-AMP2 is retained more strongly than *Ac*-AMP1 by the cation-exchange resin. Since *Ac*-AMP1 appears to be a truncated form of *Ac*-AMP2, it is possible that it is derived from the same precursor molecule by differential posttranslational processing. Alternatively, *Ac*-AMP1 may result from carboxypeptidase degradation of *Ac*-AMP2 during the isolation procedure.

Fast atom bombardment mass spectrometry at high resolution indicated that *Ac*-AMP1 and *Ac*-AMP2 have monoisotopic molecular masses of 3025.4 and 3181.2, respectively. These values are within 0.2 Da of the theoretical masses calculated from the amino acid sequence data, assuming that all six cysteines form three disulfide bridges and that all other amino acids are unmodified. The absence of free thiol groups in the *Ac*-AMPs was confirmed by chemical thiol dosage. The molecular weight estimation of the reduced *Ac*-AMPs by SDS–PAGE (3K) is in good agreement with the data obtained by mass spectrometry and amino acid sequencing. However, the unreduced peptides seem to migrate at higher apparent molecular masses (4 and 5.5 kDa for *Ac*-AMP1 and *Ac*-AMP2, respectively). These results may indicate that the *Ac*-AMPs are dimeric proteins, whose conformation is stabilized by disulfide linkages. Alternatively, the lower mobility of the unreduced peptides may be explained by assuming that such highly compact structures have a relatively low binding capacity for SDS. Also, the relatively large differences in motility between unreduced *Ac*-AFP1 and *Ac*-AFP2, despite their near-identity in primary structure, suggest that molecular weight estimation of small unreduced proteins with high cystine contents is not very reliable.

The sequence of the amaranth antimicrobial peptides shows striking homology to the cysteine/glycine-rich domain that occurs in different chitin-binding proteins, including basic chitinases, *N*-acetylglucosamine-specific lectins, and hevein (Raikhel & Broekaert, 1992). Moreover, *Ac*-AMP1 and *Ac*-AMP2 bind to chitin and can be desorbed from this polysaccharide at low pH, a property shared by other chitin-binding proteins (Molano et al., 1979; Peumans et al., 1983; Van Parijs et al., 1991). It is therefore clear that the *Ac*-AMPs can be considered as new members of the family of chitin-binding proteins. However, when compared to the regular 40–43 amino acid cysteine/glycine-rich domains found in the chitin-binding proteins characterized so far, the *Ac*-AMPs distinguish themselves by several features, including a higher abundance of basic amino acids, the presence of an additional amino-terminal residue, the occurrence of a gap of 4 amino acids at positions 6–9, and the lack of a carboxyl-terminal portion of 10–12 residues.

Apart from the sequence homology with chitin-binding proteins, structural homology is also evident when comparing the *Ac*-AMPs to two antimicrobial peptides isolated from *Mirabilis jalapa* seeds (Cammue et al., 1992). The *M. jalapa* antimicrobial peptides are also homodimers composed of highly basic, small-sized (3.5–4 kDa) cysteine-rich polypeptides. Although there is no significant sequence homology between the *Ac*-AMPs and the antimicrobial peptides from *M. jalapa*, both types of proteins contain three disulfide bridges formed by six cysteine residues, of which the third and fourth residues are at adjacent positions, flanked by a serine residue.

It is also noteworthy that several proteins that have been shown to possess antimicrobial properties share some key structural features with the *Ac*-AMPs, namely, a highly basic nature, a small-sized polypeptide chain, and a high content of cysteine. Proteins that fit into this superfamily, next to the *Ac*-AMPs and the *M. jalapa* antimicrobial peptides (Cammue et al., 1992), are the thionins occurring in seeds and leaves of different plants (45–47 amino acids, 4 disulfide bridges) (Bohlmann & Apel, 1991), the defensins, found in mammals (Lehrer et al., 1991) and insects (Lambert et al., 1989) (29–40 amino acids, 3 disulfide bridges), and an antifungal protein secreted by the fungus *Aspergillus giganteus* (51 amino acids, 4 disulfide bridges) (Nakaya et al., 1990). Thionins and defensins exhibit a broad array of antibiotic properties since they are toxic to fungi, bacteria, and mammalian cells [see Bohlman and Apel (1991) and Lehrer et al. (1991) and references cited therein]. In our tests, however, the *Ac*-AMPs were devoid of activity against cultured mammalian cells and only inhibited two Gram-positive bacteria but not two Gram-negative bacteria. A similar degree of specificity was also observed for the antimicrobial peptides from *Mirabilis jalapa* (Cammue et al., 1992).

Ac-AMP1 and *Ac*-AMP2 appear to be potent inhibitors of all seven fungi tested in this study. Their specific activity is comparable to that of wheat thionin, which also typically inhibits fungal growth with IC_{50} values between 1 and 10 μg/mL (Cammue et al., 1992). Relative to other chitin-binding proteins, such as UDA or chitinase, the *Ac*-AMPs have much higher specific activities. UDA only inhibits three out of the seven fungi tested at concentrations below 100 μg/mL, whereas at this concentration the pea chitinase is only inhibitory to one out of seven tested fungi (Table I).

A striking observation is that the antifungal activity of the *Ac*-AMPs is strongly reduced by the presence of inorganic salts. Since addition of $CaCl_2$ at 1 mM caused a stronger reduction in antifungal activity than KCl at 50 mM, it is clear that the antagonistic effect of salts is primarily due to the cations, with divalent cations being more potent antagonists than monovalent cations. It has previously been observed that the toxic activity of thionins on yeast is strongly antagonized by different divalent cations (Okada et al., 1970). Also, Roberts and Selitrennikoff (1990) found that zeamatin, a 22-kDa antifungal protein from maize seeds, shows a 40-fold reduction in specific activity upon addition of NaCl to a concentration of 100 mM. The characteristic ability of zeamatin of acting in synergism with the antifungal drug nikkomycin Z is not shared by the *Ac*-AMPs (W. F. Broekaert and F. R. G. Terras, unpublished results).

The unique properties of the *Ac*-AMPs as potent inhibitors of fungal growth in vitro suggest that they may play a role in the defense of seeds or seedlings against invasion by fungal organisms. However, it remains to be shown that the *Ac*-AMPs can exert antifungal effects in their natural microenvironment, considering the ionic strength-dependent activity of these proteins.

ACKNOWLEDGMENTS

We are grateful to A. Ludwig and T. Boller for providing samples of pea chitinase and pea glucanase.

Registry No. *Ac*-AMP1, 139632-17-0; *Ac*-AMP2, 139632-18-1; chitin, 1398-61-4.

REFERENCES

Alessi, M., Declerck, P. J., De Mol, M., Nelles, L., & Collen, D. (1988) *Eur. J. Biochem. 175*, 531–540.

Bohlmann, H., & Apel, K. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42*, 227–240.

Boller, T., Gehri, A., Mauch, F., & Vogeli, U. (1983) *Planta 157*, 22–31.

Broekaert, W. F., Van Parijs, J., Allen, A. K., & Peumans, W. J. (1988) *Physiol. Mol. Plant Pathol. 33*, 319–331.

Broekaert, W. F., Van Parijs, J., Leyns, F., Joos, H., & Peumans, W. J. (1989) *Science 245*, 1100–1102.

Broekaert, W. F., Terras, F. R. G., Cammue, B. P. A., & Vanderleyden, J. (1990a) *FEMS Microbiol. Lett. 69*, 55–60.

Broekaert, W. F., Lee, H. I., Kush, A., Chua, N. H., & Raikhel, N. V. (1990b) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7633–7637.

Broglie, K. E., Gaynor, J. J., & Broglie, R. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6820–6824.

Cammue, B. P. A., De Bolle, M. F. C., Terras, F. R. G., Proost, P., Van Damme, J., Rees, S. B., Vanderleyden, J., & Broekaert, W. F. (1992) *J. Biol. Chem. 267*, 2228–2233.

Chapot, M. P., Peumans, W. J., & Strosberg, A. D. (1986) *FEBS Lett. 195*, 231–234.

Creighton, T. E. (1989) in *Protein structure, a practical approach* (Creighton, T. E., Ed.) pp 155–167, IRL Press, Oxford.

Czapla, T. H., & Lang, B. A. (1990) *J. Econ. Entomol. 83*, 2480–2485.

De Bolle, M. F. C., Goderis, I. J., Terras, F. R. G., Cammue, B. P. A., & Broekaert, W. F. (1991) *Electrophoresis 12*, 442–444.

Heukeshoven, J., & Dernick, R. (1985) *Electrophoresis 6*, 103–112.

Lambert, J., Keppi, E., Dimarcq, J.-L., Wicker, C., Reichhart, J. M., Dunbar, B., Lepage, P., Van Dorsselaer, A., Hoffmann, J., Fothergill, J., & Hoffmann, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 262–266.

Lehrer, R. I., Ganz, T., & Selsted, M. E. (1991) *Cell 64*, 229–230.

Mauch, F., Hadwiger, L. A., & Boller, T. (1988) *Plant Physiol. 87*, 325–333.

Molano, J., Duran, A., & Cabib, E. (1977) *Anal. Biochem. 83*, 648–656.

Molano, J., Polasheck, I., Duran, A., & Cabib, E. (1979) *J. Biol. Chem. 254*, 4901–4907.

Murdock, L. L., Huesing, J. E., Nielsen, S. S., Pratt, R. C., & Shade, R. E. (1990) *Phytochemistry 29*, 85–89.

Nakaya, K., Omata, K., Okahashi, I., Nakamura, Y., Kolkenbrock, H., & Ulbrich, N. (1990) *Eur. J. Biochem. 193*, 31–38.

Okada, F., Yoshizumi, H., & Terashima, Y. (1970) *Agric. Biol. Chem. 34*, 1084–1088.

Peumans, W. J., Stinissen, H. M., & Carlier, A. R. (1982) *Biochem. J. 203*, 239–243.

Peumans, W. J., De Ley, M., & Broekaert, W. F. (1983) *FEBS Lett. 177*, 99–103.

Raikhel, N. V., & Wilkins, T. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6745–6749.

Raikhel, N. V., & Broekaert, W. F. (1992) in *Control of plant gene expression* (Verma, D. P., Ed.) Telford Press, London (in press).

Redman, D. G., & Fisher, N. (1969) *J. Sci. Food Agric. 20*, 427–432.

Rice, R. H., & Etzler, M. E. (1974) *Biochem. Biophys. Res. Commun. 59*, 414–419.

Roberts, W. K., & Selitrennikoff, C. P. (1990) *J. Gen. Microbiol. 136*, 1771–1778.

Schlumbaum, A., Mauch, F., Vögeli, U., & Boller, T. (1986) *Nature 324*, 365–367.

Shinshi, H., Mohnen, D., & Meins, F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 89–93.

Tsuda, M. (1979) *J. Biochem. (Tokyo) 86*, 1451–1461.

Van Damme, J., Cayphas, S., Opdenakker, G., Billiau, A., & Van Snick, J. (1987) *Eur. J. Immunol. 17*, 1–7.

Van Parijs, J., Broekaert, W. F., Goldstein, I. J., & Peumans, W. J. (1991) *Planta 183*, 258–264.