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An antimicrobial peptide Ar-AMP from amaranth (*Amaranthus retroflexus* L.) seeds

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Abstract

A 30-residue antimicrobial peptide Ar-AMP was isolated from the seeds of amaranth *Amaranthus retroflexus* L. essentially by a single step procedure using reversed-phase HPLC, and its in vitro biological activities were studied. The complete amino acid sequence of Ar-AMP was determined by Edman degradation in combination with mass spectrometric methods. In addition, the cDNA encoding Ar-AMP was obtained and sequenced. The cDNA encodes a precursor protein consisting of the N-terminal putative signal sequence of 25 amino acids, a mature peptide of 30 amino acids and a 34-residue long C-terminal region cleaved during post-translational processing. According to sequence similarity the Ar-AMP belongs to the hevein-like family of antimicrobial peptides with six cysteine residues. In spite of the fact that seeds were collected in 1967 and lost their germination capacity, Ar-AMP retained its biological activities. It effectively inhibited the growth of different fungi tested: *Fusarium culmorium* (Smith) Sacc., *Helminthosporium sativum* Pammel., King et Bakke, *Alternaria consortiale* Fr., and *Botrytis cinerea* Pers., caused morphological changes in *Rhizoctonia solani* Kühn at micromolar concentrations and protected barley seedlings from *H. sativum* infection.
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1. Introduction

In order to protect themselves against abundant phytopathogenic fungi, plants produce various organic compounds (Morrissey and Osbourn, 1999) and antimicrobial proteins [AMPs] (Selitrennikoff, 2001) and peptides (Broekaert et al., 1997). At present, several families of plant AMPs have been isolated from different organs (seeds, leaves, and flowers) (Broekaert et al., 1997; Garcia-Olmedo et al., 2001) and intercellular washing fluids (Kristensen et al., 2000). The characteristic feature of plant AMPs is high content of cysteine residues, which form three or more disulphide bridges. Only one family of linear cysteine-free peptides isolated from the roots of shepherd's purse *Capsella bursa-pastoris* has been described so far (Park et al., 2000). Most AMPs were isolated from cultivated higher plants and only few peptides with antimicrobial and antifungal properties were purified from weeds, which are highly resistant to pathogens including fungi.

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In this paper, we report the isolation, amino acid sequence determination, cloning, and biological properties of the antimicrobial peptide Ar-AMP from seeds of *Amaranthus retroflexus*, a weed that causes severe damage to crops in the south of Russia. According to its amino acid sequence and cysteine motif, Ar-AMP belongs to a superfamily of chitin-binding proteins, containing a single cysteine/glycine-rich chitin-binding domain.

2. Results and discussion

2.1. Isolation and sequence of peptide Ar-AMP

Acidic extract from 10 mg of *A. retroflexus* seeds showed antifungal activity toward *Helminthosporium sativum* by radial diffusion method (not shown). About 15 peptides in the molecular mass range of 3–6 kDa were detected in MALDI mass spectrum of this extract: 3155.88, 3602.31, 3910.25, 4107.94, 4234.05, 4343.73, 4375.08, 4419.27, 4528.06, 4534.38, 4545.00, 4650.00, 4755.00, 4869.78, 5101.99, 5388.20, 5549.65, and 5663.10 (the average masses are given and only the main peaks were calculated). The total extract was separated by RP-HPLC (Fig. 1). A 3155.88-Da peptide, named Ar-AMP, was active against *H. sativum* and was chosen for further analysis. By mass analysis of the intact, intact alkylated, and reduced and alkylated peptide, we showed that Ar-AMP contains 6 cysteine residues, all of which are involved in disulphide bridging. This follows from the observation that only after reduction and alkylation of the peptide with 4-vinylpyridine, its mass increased by 633 Da indicating *S*-pyridylethylation of 6 cysteine residues. The complete amino acid sequence of Ar-AMP was unambiguously established using Edman degradation in combination with mass spectrometry: AGECVQGRCPSGMCCSQFGYCGR-GPKYCGR.

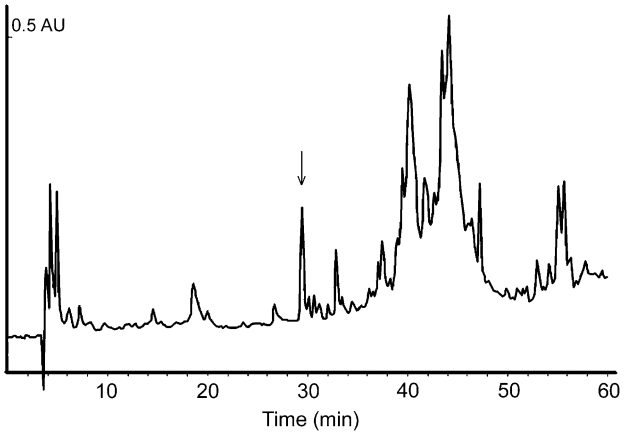

Fig. 1. RP-HPLC of the total acidic extract (about 5 mg) from *A. retroflexus* seeds. The antimicrobial peptide Ar-AMP eluted at approximately 30 min is shown by vertical arrow. For details see Section 4.

The calculated monoisotopic molecular mass of Ar-AMP of 3153.2 Da was in good agreement with the measured monoisotopic value (3153.6 Da), suggesting that there are no other post-translational modifications of the peptide, except for the formation of disulphide bridges.

2.2. Isolation and sequencing of cDNA encoding Ar-AMP

To isolate the cDNA encoding Ar-AMP, a specific primer AMP-3′RACE was designed on the basis of the experimentally determined first 8 N-terminal amino acids of Ar-AMP. First strand cDNA for PCR amplification was synthesized from total RNA using the RLdT oligonucleotide designed to act as a reverse transcription primer and also to provide a unique 3′-sequence to act as a recognition site during subsequent PCR-mediated amplification. Selective RACE amplification of the 3′-cDNA ends was then undertaken using the RLdT-specific oligonucleotide primer and the degenerate oligonucleotide primer AMP-3′RACE. The data on the nucleotide sequence of the cDNA coding for the 3′-terminal fragment of the Ar-AMP gene formed the basis for the construction of primers used in amplification of the 5′-terminal region of the cDNA Ar-AMP. Using "SMART™ RACE 5′ cDNA Amplification" (CLONTECH Laboratories, Inc.) technology and primers AMP-5′RACE1 and AMP-5′RACE2, the 5′-terminal cDNA fragment of the Ar-AMP gene was cloned. Translation of cDNA sequences obtained with the AMP-5′RACE2 primer resulted in the amino acid sequence of Ar-AMP. The deduced amino acid sequence of the cDNA clone encodes a predicted polypeptide of 89 amino acids (GenBank Accession No. AY861660). This polypeptide consists of three domains: N-terminal putative signal peptide (25 amino acids), a domain corresponding to the mature peptide (30 amino acids), and a C-terminal propeptide (34 amino acids). The nucleotide sequence analysis suggests that Ar-AMP is synthesized as part of at least an 89 amino acid residue precursor, which loses 25 N-terminal amino acids (a signal peptide) and 34 carboxy-terminal amino acids during post-translational processing. Similar processing of the N- and C-termini has been observed for other precursor proteins from *Amaranthus caudatus* (De Bolle et al., 1993).

Searches with the BLAST program, literature data on some recently isolated plant antimicrobial peptides and sequence alignments demonstrated the homology of Ar-AMP to a super family of chitin-binding proteins, containing a single cysteine/glycine-rich chitin-binding domain. It demonstrates the highest similarity to Ac-AMP2 isolated from seeds of the cultivated amaranth

A. caudatus (Broekaert et al., 1992) and differs from the *A. caudatus* peptide in three positions: Val-3, Arg-6 and Lys-23 in Ac-AMP2 are replaced by Ala-3, Gln-6 and Arg-23 in Ar-AMP (Fig. 2). By sequence homology, Ar-AMP, as well as Ac-AMP2, belongs to the family of 6-Cys hevein-like AMPs. They both differ from hevein and 8-Cys hevein-like peptides by the lack of the seventh and eighth cysteine residues (Broekaert et al., 1997). A comparison of the nucleotide sequence of the Ar-AMP cDNA with that of the gene encoding AMP2 from *A. caudatus* (De Bolle et al., 1993) revealed complete identity in the coding regions of both genes except for the codons for the variant amino acid residues (16 substitutions were found) and low homology in the 5′- and 3′-noncoding regions (not shown). High homology in the coding region of the genes suggests stable conservation of the translated propeptide.

2.3. Biological activity of Ar-AMP

We assayed the ability of Ar-AMP to inhibit the growth of different phytopathogenic fungi. Fig. 3 shows the inhibition zones in the presence of Ar-AMP manifested by active sporulation in *H. sativum, F. culmorum* and by the formation of microsclerocia in *Rhizoctonia solani*. The experiments were conducted with various Ar-AMP concentrations (Table 1). The highest inhibitory activity of Ar-AMP was observed against *F. culmorum*, the lowest inhibition was noted against *Alternaria consortiale*. The Ar-AMP did not inhibit the growth of *R. solani*, but affected active development of sclerotia around the well (Fig. 3). Regarding fungus morphology, *R. solani* displayed the highest sensitivity to Ar-AMP, while *Botrytis cinerea* and *A. consortiale* were the most tolerant.

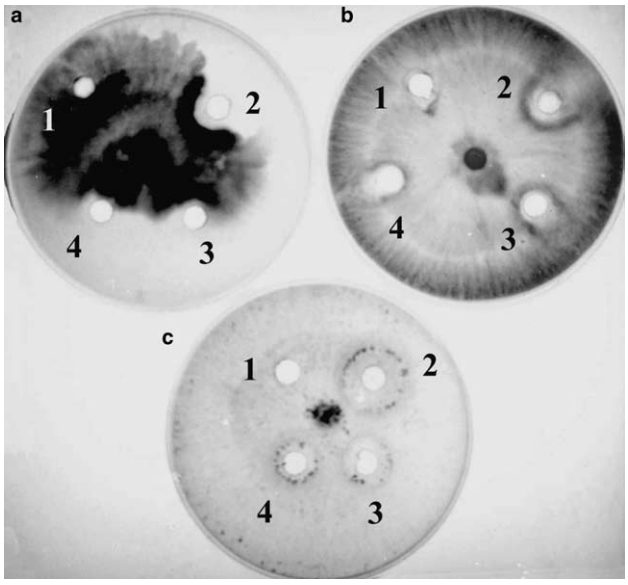

Fig. 3. Antifungal activity of peptide Ar-AMP toward (a) *H. sativum*, (b) *F. culmorum* and (c) *R. solani*. 1, Control (water); 2, 3 and 4, 290, 95 and 32 mmol of peptide Ar-AMP, respectively.

We also tested the effect of Ar-AMP on plant seedlings upon fungal infection. Seedlings of the barley variety Pirkka (sensitive to common root rot) were grown in the presence of *H. sativum* at different

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Ar-AMP     AGECVQGR----CPSGM----CCSQFGYCGRGPK----YCG-R--------- 30
Ac-AMP2    VGECVRGR----CPSGM----CCSQFGYCGKGPK----YCG-R--------- 30
Pa-AMP1    -AGCIKNGGR--CNASAGPPYCCSSY--CFQIAGQSYGVCKNR--------- 38
Mj-AMP1    -AQCIGNGGR--CNENVGPPYCCSGF--CLRQPGQGYGYCKNR--------- 38
Fa-AMP1    -AQCGAQGGGATCPGGL----CCSQ--WCGSTPK----YCG--AGCQSNCK- 38
Hevein     -EQCGRQAGGKLCPNNL----CCSQWGWCGSTDE----YCSPDHNCQSNCKD 43
             *        *  .     *** :           **
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Fig. 2. Alignment of AMP selected sequences. The sequences were aligned using the CLUSTAL W (1.82) program. The symbols denoting the degree of conservation are as follows: (“*”) identical residues; (“:”) conserved substitutions; (“.”) semi-conserved substitutions. Cysteine residues are shadowed. In the amino acid sequence of hevein, the amino acid residues involved in saccharide binding are shown in bold (SMART ChtBD1). The selected sequences were extracted from the following identified proteins: Ar-AMP, from *A. retroflexus* (this work); Ac-AMP2 (P27275) from *A. caudatus*; Pa-AMP (P81418) from *Phytolacca americana*; Mj-AMP1 (P25403) from *Mirabilis jalapa*; AAI (P80403), hevein (P02877) from *Hevea brasiliensis*.

Table 1
Antifungal activity of peptide Ar-AMP

Ar-AMP (μM)	*H. sativum*		*F. culmorum*		*B. cinerea*		*A. consortiale*		*R. solani*	
	a	b	a	b	a	b	a	b	a	b
286.0	+++	Yes	+++	Yes	++	No	+++	No	–	Yes
95.3	++	Yes	++	Yes	+	No	++	No	–	Yes
31.8	+	No	+	Yes	+	No	+	No	–	Yes
10.6	+	No	+	Yes	+	No	–	No	–	Yes
3.5	–	No	+	No	–	No	–	No	–	Yes
1.2	–	No	–	No	–	No	–	No	–	No

[a] Inhibition of fungal growth; the symbols denoting the diameter of the inhibition zone are as follows: (“+++”) 8–10 mm; (“++”) 3–7 mm; (“+“) less than 3 mm; (“–”) no inhibition.
[b] Morphological changes of fungi.

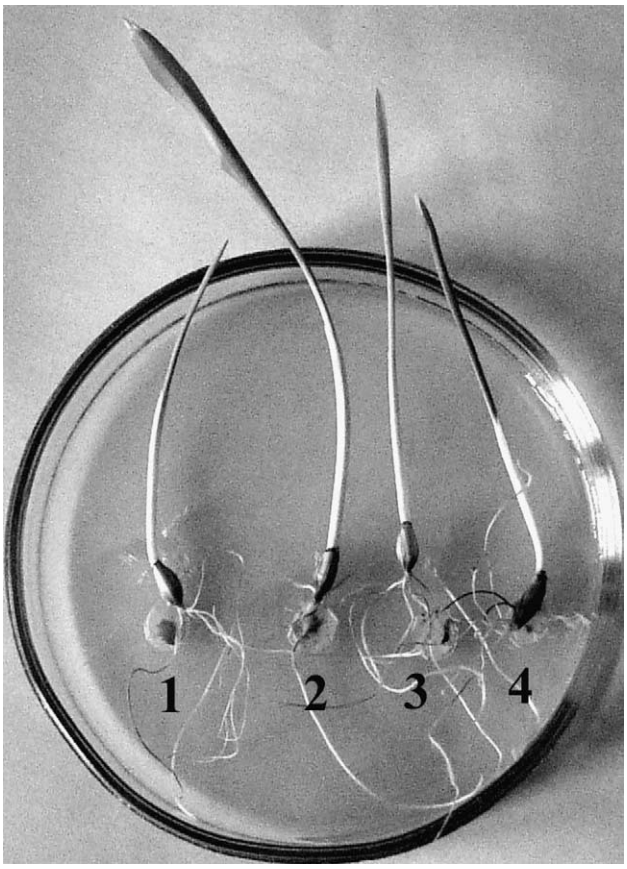

Fig. 4. The protective effect of peptide Ar-AMP against common root rot of barley seedlings (variety Pirkka). 1, Control (10^6 conidia/ml); 2, 3 and 4, 290, 95 and 32 mmol of Ar-AMP, respectively. For details see Section 4.

Ar-AMP concentrations (Fig. 4). Barley seeds inoculated only with *H. sativum* conidia were used as control. We showed that Ar-AMP decreased root rot infection of barley at concentrations from 4 to 32 μM, thus increasing the mass of seedlings. An observed decrease in the infection level is associated not only with inoculation of roots with Ar-AMP, but likely with the protective effect of their own AMPs excreted by seeds during germination and accumulated in the developing seedlings (Hejgaard et al., 1992).

3. Conclusion

We have developed a procedure for the isolation of an antifungal peptide from *Amaranthus* species essentially by a single step RP-HPLC. Although seeds were collected in 1967 and lost their germinating capacity, Ar-AMP retained its antifungal activity. The complete amino acid sequence of Ar-AMP was determined by Edman degradation in combination with mass spectrometric methods. In addition, the cDNA encoding Ar-AMP was cloned and sequenced. The determined Ar-AMP amino acid sequence was completely confirmed by translation of the cloned Ar-AMP gene. It was shown that Ar-AMP is produced as a precursor with subsequent cleavage of a typical secretion signal peptide and a C-terminal prodomain. Ar-AMP appears to be a potent inhibitor of at least 5 fungi tested. In addition, it shows some inhibitory activity against Gram-positive (*Bacilus subtilis*), but not Gram-negative (*Escherichia coli*) bacteria (Anisimova, Grishin, Egorov, unpublished data). In summary, the antimicrobial peptide Ar-AMP purified from seeds of the weed *A. retroflexus* may be a component of active defense providing a valuable tool for engineering resistance against phytopathogenic fungi in plants.

4. Experimental

4.1. Biological material and chemicals

Seeds of amaranth *A. retroflexus* L. collected in 1967 and barley (*Hordeum vulgare*, cv. Pirkka) were obtained from the All-Russian Institute of Plant Industry, Russian Academy of Agricultural Sciences (St. Petersburg). The following phytopathogenic fungi *A. consortiale* Fr., *B. cinerea* Pers., *H. sativum* Pammel., King et Bakke, *Rhizoctonia solani* Kuhn., *Fusarium culmorum* (Smith) Sacc. All chemicals were of the highest analytical grade commercially available.

4.2. Isolation and purification of Ar-AMP

One hundred milligrams of *A. retroflexus* seeds were ground in a coffee mill, and peptides were extracted with 1 ml of 10% (v/v) acetic acid in the presence of Pepstatin A (1 μg) for 1 h on a magnetic stirrer at room temperature. After centrifugation at 20,817*g* for 5 min and 4 °C proteins and peptides were precipitated overnight at 4 °C with 6 vol of cold acetone. The precipitated fraction was collected by centrifugation at 20,817*g* for 10 min and 4 °C, redissolved in 0.2 ml of 0.1% (v/v) trifluoroacetic acid (TFA) in water (v/v), and injected onto a C18 Vydack RP-300 column (4.6 × 250 mm) after centrifugation. Peptides were eluted with a linear acetonitrile gradient (10–40% of solvent B in solvent A for 60 min) at a flow rate of 1 ml/min and 40 °C. Solvent A: 0.1% TFA in water (v/v), solvent B: 80% acetonitrile in solvent A (v/v). Fractions were tested against *H. sativum* after evaporating to dryness on a SpeedVac concentrator. The total extracts from 1 g seeds were separated on a semi-preparative C18 Nucleosil-100 column (10 × 250 mm) using the same acetonitrile gradient as above.

4.3. Mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) measurements were per-

formed using a Voyager- DE STR BioSpectrometry Workstation mass spectrometer (Perspective Biosystems, Inc., Framingham, MA, USA). One microliter of the peptide solution at a concentration of 1–5 pmol/μl was mixed with 1 μl of a matrix solution consisting of α-cyano-4-hydroxycinnamic acid matrix (10 mg) dissolved in 0.3% TFA in acetonitrile/water (1:1, v/v, 1 ml). From the resulting mixture, 1 μl was applied to the sample plate. Samples were air-dried at 24 °C. Measurements were performed in the reflectron mode at an acceleration voltage of 20 kV, 70% grid voltage and a delay of 150 ns. Each spectrum obtained represented the accumulation of 250 laser shots. For PSD analysis, the reflector voltage was reduced in 25% steps to obtain up to 10 segment spectra, which were pasted to give a single fragment ion spectrum using the Data Explorer software (Applied Biosystems). Mass spectra of the total extract from 1 mg seeds were obtained after desalting of the sample on a C18 ZipTip microcolumn (Millipore, Bradford, USA). Peptides were eluted with the α-cyano-4-hydroxycinnamic acid matrix solution directly onto the target plate, and measurements were performed in a linear mode.

4.4. Alkylation and N-terminal sequencing of Ar-AMP

The N-terminal amino acid sequence was determined on a model 492 Procise sequencer (Applied Biosystems). The peptide was preliminarily reduced and alkylated as described by Thomsen and Bayne (1988) with some modifications. Approximately 100 pmol of peptide were dissolved in 40 μl of the alkylation buffer (6 M guanidine hydrochloride, 3 mM EDTA in 0.5 M Tris buffer, pH 8.5) and reduced with 2 μl of freshly prepared solution of DTT in 50% 2-propanol, containing 1.4 μmol of DTT, for 4 h at 40 °C under nitrogen. Alkylation was performed by adding 2 μl of 4-vinylpyridine diluted in 2-propanol (1:1 v/v). After incubation under nitrogen at room temperature for 20 min, the reaction mixture was diluted twofold with 0.1% TFA and immediately injected onto the reversed-phase column (3.2 × 150 mm). Peptides were eluted with 50% aq. acetonitrile in 0.1% aq TFA (v/v) after washing out salts and by-products with 0.1% aq TFA (v/v). The number of cysteine residues and disulphide bridges in Ar-AMP were determined by mass measurements of the intact, alkylated intact and reduced and alkylated peptide.

4.5. Isolation and sequencing of cDNA

One gram seeds of *A. retroflexus* were homogenized in liquid nitrogen. Total RNA was isolated as described (Feramisco et al., 1982). Five micrograms of total RNA were reverse transcribed in a total volume of 20 μl for 60 min at 42 °C (Sambrook et al., 1989) using the RLdT primer (5′-GAGAATTCGGATCCCTGCAGAAGC-$(T)_{19}$V-3′). The 3′-end of Ar-AMP cDNA was specifically amplified by PCR in a total volume of 25 μl, containing: 1 × reaction buffer (10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton X-100, and 2 mM $MgCl_2$), 0.05 μg first strand cDNA, 0.2 mM of each dNTP, 1.5 U of Taq DNA polymerase, 5 pmol of the RL oligonucleotide primer (5′-GAGAATTCGGATCCCTGCAGAAGCTT-3′), and 100 pmol of the specific degenerate oligonucleotide primer AMP-3′ RACE (5′-GCTGGNGARTGTGTNCARGGNMG-3′). A strategy for selecting the degenerate primers and cloning procedures were described in Lipkin et al. (2002) and Kozlov et al. (2000). Amplification of the 5′-end of Ar-AMP1 cDNA was performed by PCR using specific oligonucleotide primers AMP-5′RACE1 (5′-AAGATACTAGTATTTATACAGGAGC-3′) and AMP-5′RACE2 (5′-CTAAGAAAACAGTACTAAAGCAAAGC-3′) as described (Matz et al., 1999).

4.6. Biological assays

The inhibitory activity of peptides on the growth of phytopathogenic fungi was assayed by a modification of a radial diffusion method. After cultivation of a fungus on 1.5% (w/v) agar medium containing the Nestle 5 cereal (20 g/l) in a centre of Petri dish for two days at 26 °C, 50 μl of the peptide solution to be tested were applied to the wells formed in agar at a distance of 3 cm from the centre. Following two days of incubation at 26 °C, the inhibition of fungal growth and morphological changes in the fungus induced by the sample tested were assayed using an arbitrary rating system (see notes to Table 1).

The protective effect of Ar-AMP against common root rot of barley seedlings. Seeds of the barley variety Pirkka highly sensitive to root rot were surface sterilized for 1 min in 70% (v/v) ethanol, 5 min in 0.5% (w/v) solution of $KMnO_4$, and washed several times with sterile water. Seeds were germinated on a Nestle medium for 24 h at 24 °C. Immediately after the appearance of seedlings, germinated seeds were placed in Petri dishes filled with 2% (w/v) agar close to membrane filter discs preliminarily soaked in the *H. sativum* conidium suspension (10^6 spores/ml) and Ar-AMP solution. The Petri dishes were incubated in a horizontal position in a thermostat for 24 h, then in the vertical position for another 24 h in and finally at day light in a vertical position at room temperature (22 °C) for three days. After that, root length and the height and mass of seedlings were measured, and the infection of roots with *H. sativum* was scored.

Acknowledgements

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