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CATIONIC PEPTIDES AND PROTEINS FROM SEEDS OF PLANTS OF THE FAMILY MALVACEAE

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Total cationic peptides from seeds of plants of the family Malvaceae were separated by chromatography. The molecular weights of the isolated peptides were determined by MALDI-TOF mass spectroscopy. The antifungal activity of total cationic peptides from seeds of plants of this family against Phytophtora infestans *was demonstrated on potato disks.*

Keywords: antimicrobial peptides, Malvaceae*, Hibiscus cannabinus, Abutilon theophrasti, Malva sylvestris*.

Antimicrobial proteins (AMP) and peptides of multi-cellular organisms are ancient and the most common constituents of protective systems for battling pathogens [1, 2].

Nine classes of protective peptides, the sizes of which vary from 20 to 90 amino acids, have been observed in plants [3]. These include thionins, defensins, hevein- and knottin-like peptides, cyclotides, MBP1, Ib-AMP, so-called lipid-transfer proteins, structurally related 2*S* albumin protein, etc. [4]. They all are positively charged and cysteine-rich peptides with a compact structure and stabilized disulfide bridges, the number of which varies from two to eight [5].

Cationic peptides were isolated by acid extraction from ground defatted seeds of *Hibiscus cannbinus*, *Abutilon theophrasti*, and wild *Malva sylvestris*. The total extract from the seeds was chromatographed using a stepwise gradient of NaCl over a Hi Trap Heparin HP column. The resulting fractions were desalted in a cartridge with reversed-phase Aquapore RP-300 C_8 (4.6 × 220 mm) and lyophilized. The next step in the isolation of the antimicrobial peptides was exclusion chromatography (gel filtration) over a column of Superdex^{TM} Peptide HR. The molecular weights (MWs) in the principal fractions were measured by MALDI TOF mass spectrometry. Fractions containing compounds with MWs in the range characteristic of AMP were separated further into pure components.

Each fraction was separated by HPLC over a Luna C18 Phenomenex column using an CH_3CN gradient.

The MWs of the resulting pure peptides were determined by MALDI TOF mass spectrometry. The peptides were reduced and alkylated in order to determine the number of cysteine residues. The reaction products were separated by reversed-phase chromatography over a Luna C_{18} column and analyzed by mass spectrometry. The numbers of alkyl groups and cysteines were determined from the mass difference of the reduced and alkylated and native pure peptides.

A peptide of MW 9691 Da that contained eight cysteines and was a lipid-transfer protein; peptides presumably belonging to the AMP classes thionins and defensins with MWs 4210, 4297, 4568, 4984, 5116, 5529, and 5856 Da that contained eight cysteines; and peptides with MWs 3645 and 3888 Da containing six cysteines were observed in seeds of *M. sylvestris* according to the mass spectrometric analysis.

A peptide of MW 9425 Da that presumably was a lipid-transfer protein and a peptide of MW 12,694 Da that was a 2*S* albumin protective protein were observed in seeds of *A. theophrasti*. Both peptides contained eight cysteines.

Seeds of *H. cannabinus* yielded two peptides that were AMPs of MW 3134 and 5768 Da and contained six and eight cysteines and two peptides of MW 11,302 and 11,511 Da that were 2*S* albumins.

Extracts of Malvaceae seeds were tested for biological activity by an *in vitro* method using highly aggressive blight *P. infestans* oomycetes. The degree of development and spread of the blight agent on potato disks were determined in the

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concentration range 750–1000 μg/mL. The seed extract of *M. sylvestris* on the sixth day after inoculation showed 20–40% development of blight, assessed as 1 ball on a 4-ball scale of activity. Extracts of *A. theophrasti* and *H. cannabinus* seeds showed 10–20% development of blight (IC_{50}) (2 balls). The ability of the tested varieties of Malvaceae seed extracts to suppress the development and spread of the highly aggressive blight on potato disks at low infection loadings, i.e., the presence of antifungal properties, was established based on the results.

Thus, total cationic peptides from seeds of Malvaceae plants were separated by chromatography. Eight pure peptides from seeds of *M. sylvestris*; two, from seeds of *A. theophrasti*; and four, from seeds of *H. cannabinus* were identified. The MWs of the peptides were determined by MALDI-TOF mass spectroscopy. The isolated peptides were classed as lipid-transfer proteins, defensins, thionins, and 2*S* albumin protective proteins based on the number of cysteine residues. The antifungal activity of the total cationic peptides from seeds of Malvaceae plants against the blight agent *P. infestans* was demonstrated on potato disks.

EXPERIMENTAL

Isolation and Purification of Antimicrobial Peptides. Ground ripe seeds were thoroughly defatted by hexane in a Soxhlet apparatus for 72 h. Defatted seeds (100 g) were extracted by H_2SO_4 (0.05 N) for 3 h with stirring on a magnetic stirrer. The extract was separated by centrifugation for 30 min at 6,000 rpm, neutralized with NaOH (10 N), and held at 8°C for 24 h. The mixture was centrifuged. The precipitate was discarded. The supernatant was lyophilized. The solid was dissolved in TFA (0.1%) and centrifuged to remove insoluble compounds. The dissolved precipitate was desalted by reversed-phase HPLC.

The column was eluted at 1.5 mL/min and 38°C. The required eluent concentration was set using two solutions: TFA (0.1%, solvent A) and TFA (0.1%) containing CH_3CN (80%) (solvent B). Detection was performed at 214 nm. The column was equilibrated with solvent A before placing the sample on it. Salts and other unbound components of the extract were eluted by this same solvent. The protein–peptide fraction was desorbed from the column by 70% solvent B (80% aqueous CH_3CN in 0.1% TFA). The collected fraction was evaporated to dryness in a Speedvac vacuum concentrator (Savant, USA) and redissolved in *Tris*-HCl (10 mM, pH 7.2).

Affinity Chromatography Over a Hi Trap Heparin HP Column (2.5 × 5 cm) (Amersham Biosciences, England) in a Stepwise NaCl Gradient. Desalted extract was separated by affinity chromatography over a column equilibrated with *Tris*-HCl (10 mM, pH 7.2, buffer A). After unbound fractions eluted, proteins and peptides were eluted by a stepwise concentration gradient of NaCl (0.1–0.5–1 M) in *Tris*-HCl (10 mM, pH 7.2) for 2 h at flow rate 1.2 mL/min and room temperature. Proteins and peptides were determined at 214 nm. Collected fractions were evaporated to dryness in a Speedvac vacuum concentrator (Savant, USA) and redissolved in TFA (0.1%).

Gel Filtration over a Superdex™ Peptide HR Column (10 × 300 mm). Desalted fractions were separated by gel filtration over a column equilibrated with CH_3CN (5%) in TFA (0.05%). The flow rate was 15 mL/h. Proteins and peptides were determined at 214 nm.

Reversed-phase HPLC of fractions obtained by exclusion chromatography was carried out over a Luna C18 Phenomenex column (4.6 × 150 mm) using a concentration gradient of CH_3CN (10–50%) over 40 min at flow rate 0.75 mL/min.

MALDI Mass Spectrometry. MWs of isolated compounds were analyzed by MALDI TOF in a Micromass MALDI™ time-of-flight (TOF) mass spectrometer using the MassLynks 4.0 program. The matrix was 2,5-dihydroxybenzoic acid. TOF mass spectra were recorded in direct flight and with a reflector. Mass spectra were processed using the Bruker Data Analysis program. The accuracy of measured masses was 0.015%.

Reduction of Peptides Using Dithioerythritol and Alkylation Using 4-Vinylpyridine. Dried peptide material was dissolved in a solution (35 μL) containing guanidine hydrochloride (6 M) and EDTA (3 mM) in *Tris*-HCl (0.5 M, pH 8.5) and *i*-PrOH (5 μL). The contents were mixed in a vortex stirrer, centrifuged, treated with dithioerythritol (2 μL, 0.7 M) in *i*-PrOH (0.5 mg 1,4-dithioerythritol per 10 μL *i*-PrOH), left in a thermostat at 40°C for 4 h, and treated with vinylpyridine (2 μL, 50%) in *i*-PrOH. The contents were again mixed in a vortex stirrer, centrifuged, left in the dark at room temperature for 20 min, and treated with TFA (30 μL, 0.1%) to stop the reaction. The reaction products were immediately separated by RP-HPLC over a Luna C_{18} column (4.6 × 150 mm).

Determination of Biological Activity by Artificial Inoculation into Plant Tissue. Cultures of phytopathogenic microorganisms of *P. infestans* oomycetes, strain OCB 12, were obtained from Belarus State Agricultural Academy. Strain *P. infestans* (Mont.) de BARY Udacha 2 was isolated from infected leaves of potato variety Udacha by staff members of the Department of Agricultural Phytopathology, RSAU-Timiryazev MAA, in 2006.

The degree of inhibition of the blight agent on tubers was measured using the following method. Two potato tuber disks of about the same thickness (4–5 mm) and area (2,000–2,500 mm^2) were placed into every Petri dish. Tested fractions were mixed in the required concentrations (0.125–2 mg/mL) in a suspension (50 μL) of oomycete zoosporangia in distilled H_2O (inoculum concentration 2×10^4 zoosporangia/mL) in a 1:1 ratio and held at 18–20°C for 2 h. Then, the whole volume (50 μL) was carefully placed on the surface of each potato disk. Dishes with infected potato disks were incubated for 120 h at 16–20°C. Counts were made on the 4^{th}, 5^{th}, and 6^{th} days after inoculation. The number of infected disks relative to their total number was determined for each version (distribution or frequency of infection and degree of development of pathogen spores on the potato disk) (disk area, %). All versions were repeated 10 times. A necessary condition for interpretation of the results was the attainment of a distribution of blight in the control version to less than 60% and more than 40%. Activity was assessed from the degree of blight development on the disk surface calculated on a 4-ball scale as a lack of development (4 balls), <10% development (3 balls), 10–20% development (IC_{50}) (2 balls), and 20–40% development (1 ball).

REFERENCES

1. H. W. Huang, *Biochemistry*, **39**, 8347 (2000).
2. V. Mosolov and T. A. Valueva, *Biochem. Microbiol.*, **41**, 227 (2005).
3. P. Bulet and R. Stocklin, *Protein Pept. Lett.*, **12**, 3 (2005).
4. G.-H. Chen, M.-P. Hsu, C.-H. Tan, H.-Y. Sung, C. G. Kuo, and M.-J. Fan, *J. Agric. Food Chem.*, **53**, 982 (2005).
5. R. E. W. Hancock and R. Lehrer, *Trends Biotech.*, **16**, 82 (1998).