*Chemistry of Natural Compounds, Vol. 40, No. 1, 2004*

# ISOLATION AND PROPERTIES OF A BIOCIDAL PEPTIDE FROM *Hibiscus cannabinus* SEEDS

**E. A Pshenichnov, E. M. Sultanova, O. N. Veshkurova, M. V. Ionov, B. A. Salakhutdinov, and Sh. I. Salikhov**

UDC 547.96:577.15

*The fungitoxicity of a biocidal peptide from kenaf* Hibiscus cannabinus *L. seeds was investigated. Its influence on the distribution of calcium ions in mycelial cells of the fungus* Verticilluim dahliae *Kleb. was examined.*

**Key words:** biocidal peptides, defensins, *Hibiscus cannabinus* L., wilt.

Damage to cotton harvests from fungal infections is significant despite improvements in cultivation technology and fungicide application. The development of effective ecologically safe means of protecting plants that are based on phytoimmunity activation is a critical problem.

Therefore, the detection of genes responsible for their biosynthesis with subsequent incorporation into the genome of agricultural plants is especially interesting. This enables the resistance to fungal diseases to be increased.

Data on the fungi- and cytotoxic properties of biocidal peptides isolated from various plants and typical of plant defensins have been reported [1].

Defensins are the principal cysteine-containing peptides and have molecular weights of 5-6 kDa with 43-54 amino acids [2].

Kenaf *Hibiscus cannabinus* L. (Malvaceae) is resistant to fungal pathogens. We isolated a biocidal peptide from its seeds, which was extracted by various methods, and studied its fungicidal activity.

The most effective method was isolation of the peptides by extraction from ground and defatted seeds using $H_2SO_4$ (0.05 N). Separation of the total extract over a Sephadex G-50 column (Fig. 1a) produced three fractions, each of which was investigated for fungicidal activity toward pathogenic strains of *Fusarium oxysporum* Schlecht. and *Verticillium dahliae* Kleb. Turbimetry found that the second fraction was most active. Separation of it over a column of the hydrophobic sorbent butyl-TSK produced four peaks (Fig. 1b). Analysis of the fractions by PAAG-electrophoresis showed the presence of a pure peptide of molecular weight 6 kDa in the fourth fraction (Fig. 2b).

The fungicidal activity of the highly purified peptide at 50 μg/mL was demonstrated by suppression of the growth of *Verticillium dahliae* and *Fusarium oxysporum* by 37 and 64%, respectively.

Data on the influence of plant defensins on the permeability of hyphal cell membranes of *Fusarium oxysporum* and *Verticillium dahliae* for $Ca^{2+}$ ions have been reported [3].

Comparison of the influence of the isolated peptide on the change of membrane-bound $Ca^{2+}$ in mycelial cells of *Verticillium dahliae* has shown that the concentration of membrane-bound $Ca^{2+}$ decreases upon addition of peptide (20 μM) to a suspension of fungal mycelial cells. This is consistent with its possible distribution among intracellular organelles and its migration into the cytosol or out of the cell.

It was also found that the fluorescence intensity of chlorotetracycline (CTC) decreased by 13% upon addition of ethyleneglycol-(bis-aminoethyl ether)-N,N′-tetraacetic acid (EGTA, 2 mM) to a suspension of cells treated with peptide (20 μM).

A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, 700125, Tashkent, fax (99871) 162 70 71, e-mail: ibchem@uzsci.net. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 55-57, January-February, 2004. Original article submitted December 15, 2003.

0009-3130/04/4001-0063 

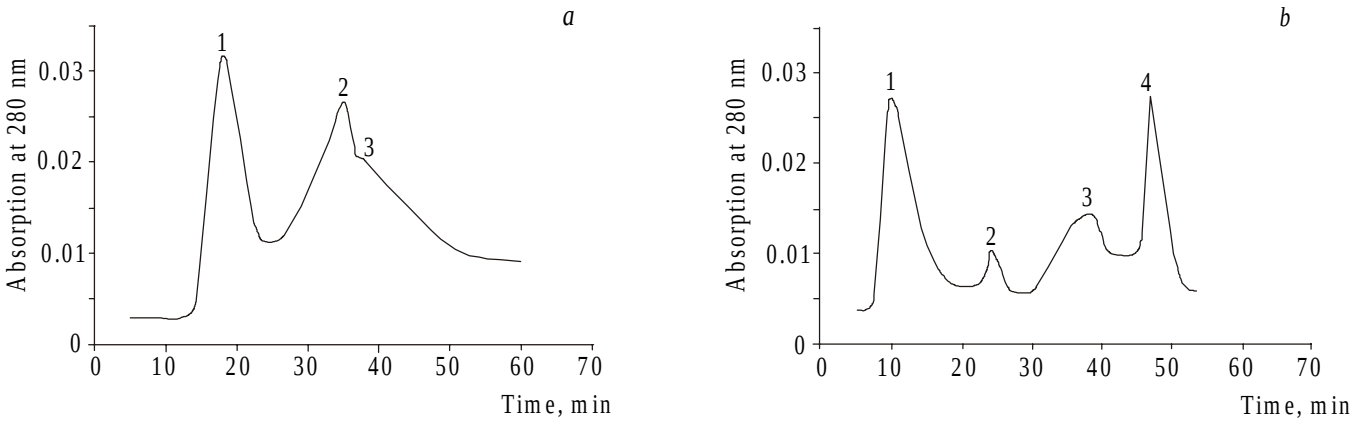


Fig. 1. Elution of total extract from a Sephadex G-50 column by $NH_4HCO_3$ (0.01 M) (a) and of the fraction containing the biocidal peptide from a butyl-TSK column by a stepped gradient of $CH_3CN$ (b).

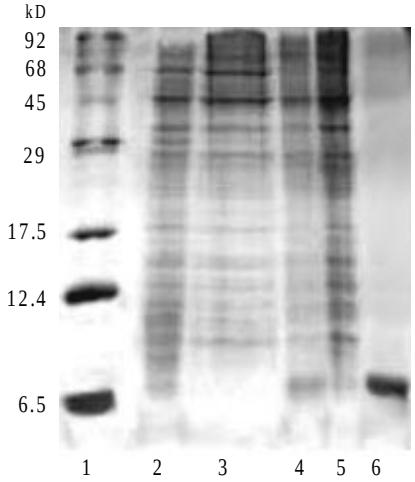


Fig. 2

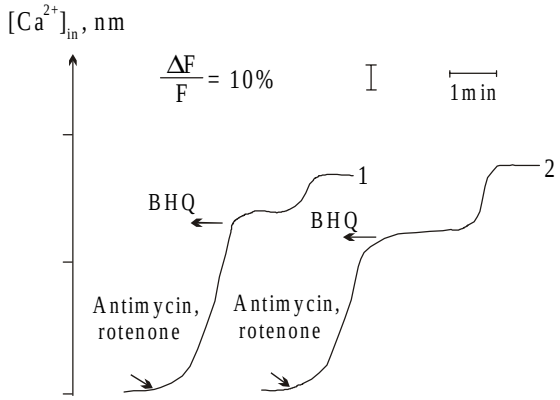


Fig. 3

Fig. 2. Electrophoretic analysis of isolated fractions: protein markers (1), total extract (2), after separation over Sephadex G-50 (3, 4), after separation over butyl-TSK (5, 6).

Fig. 3. Influence of biocidal peptide from kenaf on fluorescence intensity of CTC upon addition of antimycin A + rotenone and BHQ: control (1) and with investigated peptide (20 μM).

Addition of EGTA, which does not reach the cytosol, decreases CTC fluorescence through binding of plasmalemmal $Ca^{2+}$. Therefore, it can be concluded that the investigated compound decreases just this pool of $Ca^{2+}$.

The change of the membrane-bound $Ca^{2+}$ concentration was studied as a function of the investigated compound in the presence of the respiration inhibitors antimycin A (1 μM) and rotenone (10 μM). Judging from the smaller CTC-fluorescence increase compared with the control, the biocidal peptide of kenaf *H. cannabinus* enhances the release of $Ca^{2+}$ from mitochondria (Fig. 3). Thus, the mitochondrial pool reacts to antimycin and rotenone 7% more than the control under the influence of the peptide (20 μM).

Release of $Ca^{2+}$ from mitochondria under the influence of the biocidal peptide may have led to its redistribution into the endoplasmatic reticulum (ER) because the CTC-fluorescence increases relative to the control upon addition to the incubation medium of the ER $Ca^{2+}$-ATPase inhibitor 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ, 10 μM) (Fig. 3).

Thus, a biocidal peptide with fungicidal activity was isolated for the first time from kenaf seeds. It has been found that it can change the distribution of membrane-bound $Ca^{2+}$ in cells of a *V. dahliae* culture.

## EXPERIMENTAL

**Isolation and Purification of Peptides.** Ground kenaf seeds were defatted with hexane in a Soxhlet apparatus for 48 h and then extracted with $H_2SO_4$ (0.05 N) on a magnetic stirrer for 3 h. The extract was separated by centrifugation and neutralized with NaOH (10 N). The resulting precipitate was separated. The supernatant was lyophilized, placed on a Sephadex G-50 column (1.5 × 85 cm) in $NH_4HCO_3$ solution (0.01 M), and eluted with this same buffer. The fraction exhibiting fungitoxic activity was purified over a butyl-TSK column (1 × 5 cm) using a stepped gradient of $CH_3CN$. The isolated fractions were studied by electrophoresis in a thin layer of PAAG (15%) by the literature method [4].

**Fungicidal activity** toward strains of the fungi *F. oxysporum* and *V. dahliae* was studied by placing disks on fungal plates and by turbimetry [5] by measuring the decrease of optical density of conidial-spore suspensions at 540 nm in the presence of the biocidal peptide and comparing with a control.

**Influence of Peptide on $Ca^{2+}$ Distribution.** The amount of membrane-bound $Ca^{2+}$ was measured by adding to a suspension of cells in the isolation medium containing $NaNO_3$ (2 mM), $KH_2PO_4$ (7.3 mM), KCl (6.7 mM), saccharose (58 mM), $FeSO_4$ (trace), and HEPES (10 mM) at pH 7.2 the fluorescence probe CTC (100 μM) and incubating for 1 h at 23°C until the interaction of CTC with membrane-bound $Ca^{2+}$ in plasmatic and intracellular membranes reached a maximum.

The excitation wavelength was 405 nm; detection, 530 nm. Measurements were made by known methods [6, 7] on a TURNER-430 spectrofluorimeter. The final cell concentration in the fluorimeter cell was 100,000 cells per mL.

## REFERENCES


1. F. R. G. Terras, K. Eggermont, V. Kovaleva, N. V. Raikhel, R. W. Osborn, A. Kester, S. B. Rees, S. Torrekens, F. van Leuven, J. Vanderleyden, B. P. A. Cammue, and W. F. Broekaert, *Plant Cell*, **7**, 573 (1995).
2. W. F. Broekaert, F. Terras, B. P. A. Cammue, and R. W. Obsorn, *Plant Physiol.*, **108**, 1353 (1995).
3. K. Thevissen, A. Ghazi, G. W. De Samblanx, C. Brownlee, R. W. Osborn, and W. F. Broekaert, *J. Biol. Chem.*, **271**, 15018 (1996).
4. U. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
5. M. S. Almeida, K. S. Cabral, R. B. Zingali, and E. Kurtenbach, *Arch. Biochem. Biophys.*, **378**, 278 (2000).
6. V. F. Peresypkin, *Agricultural Phytopathology* [in Russian], Agropromizdat, Moscow (1989), pp. 183-189.
7. Yu. A. Dobretsov and G. E. Vladimirov, *Fluorescent Probes in Biological Membrane Research* [in Russian], Nauka, Moscow (1980), pp. 53-57.