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CHITIN-BINDING ANTIFUNGAL PROTEIN FROM *Ficus carica* LATEX

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*A low-molecular-weight protein with antifungal activity was isolated from freshly collected latex of the Inzhir tree (*Ficus carica *L.) by successive affinity chromatography over chitin, cation-exchange chromatography over SP-Sephadex C-50, and reversed-phase HPLC. The molecular weight of 6481 and the partial* N*-terminus sequence of the protein were determined (MALDI-TOFMS).*

Key words: chitin-binding protein, antifungal protein.

Antimicrobial peptides and proteins are components of the nonspecific protective system, are responsible for protecting host cells from infection, and provide an effective immune system for higher organisms. They are interesting to medicine, veterinary science, and agricultural biotechnology [1]. Peptides and proteins with antibiotic activity against disease vectors of cultured plants are very important in biotechnology for constructing genetically modified plants with increased resistance to pathogens and pests. Several classes of antibiotic proteins have been reported [1, 2]. Thus, chitin-binding peptides and proteins in addition to animal defensins and plant thionins exhibit antimicrobial activity [3]. The goal of our work was to isolate protein with antifungal activity from latex of the Inzhir tree, which is a mixture of phloem and xylem liquids. The protein content in it varies from 0.1 to 0.5 mg/mL. Collected latex was treated with trichloroacetic acid (TCAA) in order to prevent proteolysis and to precipitate simultaneously protein. Affinity chromatography of latex proteins soluble in acetic acid (50 mM) over a chitin column provided approximately 25-fold purification and a significant increase of biological activity (Table 1). Further fractionation of proteins over a SP-Sephadex column produced a protein fraction with higher antifungal activity. The majority of reported antibiotic proteins is cationic [1].

The cationic protein fraction was separated using reversed-phase HPLC in order to isolate a homogeneous antifungal protein and determine its structure. The chromatography produced three peaks (I, II, and III) exhibiting antifungal activity that had retention times of 12.9, 14.9, and 17.1 min, respectively. The active fractions are strongly hydrophobic proteins according to the elution from the reversed-phase column. The amounts of peaks I and II were insufficient for further chemical characterization.

The molecular weight (7-8 kDa) of the protein with the highest antifungal activity (peak III) was determined by gel-electrophoresis under denaturing conditions (Fig. 1). The exact molecular weight of the protein that was determined using MALDI-TOF mass spectrometry was 6481.3.

The antifungal activity of protein fractions was monitored using growth suppression of two pathogenic cotton-wilt fungi. Of these, the tested strain *F. oxisporum* was highly sensitive. The minimal protein dose (peak III) on the disk that showed reproducible growth suppression on the fungus was 20 μg (Fig. 2).

The *N*-terminus of the antifungal protein was sequenced in order to determine if it was similar to known antibiotic proteins. By analyzing the *N*-terminus of the amino-acid sequence (RPDFFLE) of the protein isolated by us and comparing it with the literature, we confirmed that it was not a plant thionin or defensin. The sequence K(R)XCCK(R) or K(R)XCK(R), where the cysteine units in the 3- and 4-position (thionins) and the 3-position (defensins) are fixed, is characteristic of them [1, 2, 4]. A search for analogs in a database identified hypothetic protein precursors Hhal_2123 from *Halorhodospira halophila* (database No. A1WYX5) (1), HIPL2 from *Arabidopsis thaliana* (database No. Q94F08) (2), and universal stress protein A, USP-A, from *A. thaliana* (database No. 94F08) (3). Figure 3 compares the sequenced portion of the antifungal protein and the aforementioned structures. The correspondence with the domain of USP-A, the structure of which is conserved from bacteria to plants, is especially interesting [5].

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TABLE 1. Protein Yield in Latex Fractions of *Ficus carica*

Fraction	Protein, mg	Antifungal activity*
Latex extract in acetic acid (50 mM)	1000.0	+
Effluent from chitin column by acetic acid (0.5 M)	41.225	++
Cationic proteins eluted from SP-Sephadex	21.450	+++
HPLC peak III (retention time 17.1 min)	1.450	++++

*Growth suppression of *F. oxisporum* at a dose of 20 μm/disk: (+), up to 0.5 cm; (++), up to 1.0 cm; (+++), up to 2.0 cm; and (++++), greater than 2.0 cm around the disk.

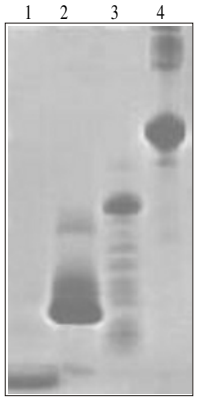

Fig. 1

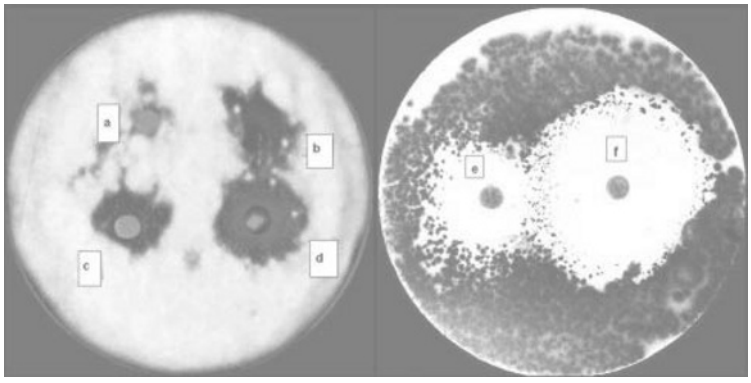

Fig. 2

Fig. 1. Electrophoresis of antifungal protein in polyacrylamide gel (12%): fraction corresponding to peak III (1), cytochrome C (12,400) (2), DNA-ase I (31,000) (3), and human albumin (68,000) (4).

Fig. 2. Growth suppression of pathogenic cotton-wilt fungi by the protein fraction from peak III [left: *V. dahliae*, 5 μg (a), 10 (b), 20 (c), 50 (d); right: *F. oxisporum*, 20 μg (e) and 50 (f)].

The antifungal activity of the isolated protein can be explained by its chitin-binding ability. According to accepted thinking [1], protein binding to the chitin component of the cell wall suppresses fungal hyphae growth. However, the structure of the sequenced portion was different from those of known chitin-binding proteins, for example, hevein. Because the partial amino-acid sequence was different from those of classical chitin-binding domains, the affinity for chitin and the distinct antifungal activity can be ascribed to a second protein subtype PR-4, which lacks the characteristic chitin-binding domain but exhibits antifungal activity [7].

Short polypeptides with distinct antimicrobial activity have been reported [2, 3]. For example, the sequenced portion of the antifungal protein and the structure of the antimicrobial protein misgurin from the fish *Misgurnus anguillicaudatus* (database registration No. NCBI-P81474, 21 units RQRVEELSKFSKKGAAARRRK) were similar in the presence of basic and polar amino acids at the amine terminus, which neighbors the hydrophobic domain.

The isolated homogeneous antifungal protein was not acutely toxic to mice upon i.v. administration at a dose of 50 mg/kg and i.p. administration up to 100 mg/kg. These doses are 5-10 times greater than the concentration at which antifungal activity appears.

Thus, a protein of molecular weight 6481 kDa was isolated and purified from latex of *F. carica*. It exhibited antifungal activity against *Verticillium* and *Fusarium* fungi. The ability of the protein to bind chitin and the analysis of its partial amino-acid sequence classified it as a PR-4/type II protein [6].

FLAFP	1	**RPDFFLE**	7
A1WYX5	40	**RPDFFLE**GFTMVDFDEQGQRR	60
Q8LGG8	124	**RPDF**LVVGSRGLGRFFQKVFVG	144
94FO8	467	**RPD**Y**FL**CADVGKDTYEEVII	487

Fig. 3. Comparison of the partial amino-acid sequence of antifungal protein (FLAFP) and the structure of precursor of proposed protein HALHL from *Halorhodospira halophila* (A1WYX5) and two protein precursors from *Arabidopsis thaliana*, HIPL2 (Q94F08) and universal stress protein (94F08). Number to the left and right of the sequences are the number of units of the amine and carboxylic terminuses, respectively, of the compared portions. Identical amino-acid units are bracketed and written in bold.

EXPERIMENTAL

Latex was collected in early morning at the beginning of fruiting. Young branches of a local variety of *F. carica* were cut with a scalpel. The resulting latex drops were collected in a flask containing TCAA solution (20%). When a volume of latex equal to the starting volume of TCAA solution was collected, the mixture was centrifuged for 10 min at 6,000 rpm. Then the precipitate was washed twice with cold acetone (3 mL for every mL of precipitate, -20°C). The precipitate was dried in a vacuum desiccator and stored at -70°C until the next protein-isolation step.

Isolation of Antifungal Protein. Dry latex precipitate (10 g) was suspended in acetic acid (100 mL, 50 mM). The suspension was filtered through filter paper. The filtrate was centrifuged for 3 min at 10,000 rpm. The clarified latex solution at room temperature was placed on a column of ground chitin (1.2 × 10 cm) that was equilibrated beforehand with 10 column volumes of acetic acid (50 mM). The chitin for the affinity column was prepared from fish scales as before [3]. The column was eluted with acetic acid (5 column volumes, 50 mM) to remove nonbonded proteins. Chitin-binding proteins were eluted by acetic acid (10 mL, 0.5 M). Total proteins in the effluent were precipitated by adding acetone (40 mL) containing sodium acetate (0.1 M). The precipitated proteins were stored for 1 h at -20°C, collected by centrifugation at 6,000 rpm for 10 min, washed twice with cold acetone, and dried in a vacuum desiccator.

The chitin-binding protein fraction was dissolved in sodium-acetate buffer (10 mL, 50 mM, pH 4.5) and placed on a column (1.2 × 5 cm) of SP-Sephadex C-50 equilibrated with the same buffer. The column was eluted by buffer (10 mL) to fix the sample. Cationic proteins were eluted by sodium acetate (5 mL, 0.2 M, pH 8.0). Then proteins were precipitated by adding acetone (20 mL) as described above.

The protein fraction eluted from SP-Sephadex C-50 was dissolved in TFAA (0.1%) to give a concentration of 10 mg/mL and was separated by reversed-phase HPLC over a column (1.0 × 25 cm) containing C_{18}-silica gel (Vydac, USA). HPLC was performed using a Beckman (USA) chromatograph at 45°C. The mobile phase consisted of TFAA (0.1%, A) and CH_3CN (80%, B). The composition of the mobile phase was monitored using a programmed pumping regime of 10% B (initial), at the 2-min mark a linear gradient of B from 10 to 60% in 1 min; 9 min holding the content of B at 60%; at the 12-minute mark a linear gradient of B from 60 to 75% in 2 min. The chromatography time was 20 min; flow rate, 1.5 mL/min. The chromatograms were detected at 230 nm. Separate fractions were collected according to the chromatogram peaks and dried using a rotary vacuum evaporator (SpeedVac, USA) at 30°C.

Electrophoresis of antifungal protein was performed under denaturing conditions in polyacrylamide gel (12%) [7]. Protein was determined by a modified Bradford method [8].

Antifungal activity of fractions was estimated using pathogenic cotton-wilt fungi by growth suppression of the fungal mycelium. Phytopathogenic strains *V. dahliae* and *F. oxisporum* were obtained from the microorganism collection of the Institute of Plant Genetics and Experimental Biology of the Academy of Sciences of the Republic of Uzbekistan and were placed

onto Chapek agar medium at a dose of 10,000 spores/mL. The protein fraction was inoculated as a sterile disk onto the medium surface after 2 h. Fungi were germinated at 28°C until a continuous pad appeared in control dishes (1-2 d).

***N*-Terminus amino-acid sequence** of chromatographically homogeneous antifungal protein was determined by automated degradation in a 470 A sequencer (Applied Biosystems, USA).

MALDI-TOF mass spectrum of protein was obtained in a VISION 2000 instrument (Termo Bioanalysis, USA) using 3,5-dimethoxy-4-hydroxycinnamic (sinapic) acid as the matrix.

A search for analogs of the sequenced antifungal protein was conducted by comparing it with protein database servers at http://www.ncbi.nlm.nih.gov and http://www.expasy.org using the BLAST X algorithm.

The partial amino-acid sequence of the antifungal protein was compared with the structures of identified analogs from the database using the Clustal X program for collocating numerous sequences [9].

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REFERENCES

1. W. F. Broekaert, B. P. Cammue, M. F. De Bolle, K. Thevissen, G. W. de Samblanx, and R. W. Osborn, *Crit. Rev. Plant Sci.*, **16**, 297 (1997).
2. F. Garcia-Olmedo, A. Molina, J. M. Alamillo, and P. Rodrigues-Palenzuela, *Biopolymers*, 479 (1998).
3. H. K. No, P. Meyers, and K. S. Lee, *J. Agric. Food Chem.*, **37**, 575 (1989).
4. T. Iwai, H. Kaku, R. Honkura, S. Nakamura, H. Ochiai, T. Sasaki, and Y. Ohashi, *Mol. Plant-Microbe Interact.*, **15**, 6, 515 (2002).
5. D. Kerk, J. Bulgrein, D. W. Smith, and M. Gribskov, *Plant Physiol.*, **131**, 1209 (2003).
6. C. P. Selitrennikoff, *Appl. Environ. Microbiol.*, **67**, 2883 (2001).
7. R. J. Simpson, *Protein and Proteomic*, CSHL Press, (2003), p. 83.
8. F. Gonzalez, G. Cardenas, and N. Marmolejo, *Anal. Biochem.*, **197**, No. 1, 276 (1991).
9. J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, *Nucleic Acid Res.*, **25**, 4876 (1997).