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BIOACTIVE PROTEIN COMPONENTS FROM *Hibiscus esculentus* SEEDS

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The fungitoxicity and cancerolytic properties of bioactive proteins from Hibiscus esculentus *seeds were defined and investigated.*

Key words: biocidic proteins, defensins, thionines, *Hibiscus esculentus*, wilt.

Transgenic varieties of agricultural crops with increased resistance to insects and microbial and fungal pathogens are being widely developed and used in practice [1]. Plant genes are intentionally modified by genes from microbes (using bacterial insectotoxin from *Bacillus thuringiensis* as an example) or antibiotic proteins of insect and arthropod poisons.

The long-range consequences of using such genetically modified products (GMP) have not yet been fully determined and cause concern among consumers. The use of biocidal proteins from traditional agricultural plants as gene vectors is proposed for maximal reduction of risk to the environment and human health from use of GMP.

Therefore, cysteine-containing peptide-thionines and plant defensins must be studied to identify genes coding their biosynthesis with subsequent construction of genetically modified cotton varieties with increased fungal resistance. Biocidal proteins are a part of the plant active protective mechanisms. However, pathogens, in turn, developed mechanisms for their detoxification [2]. Therefore, the phytoimmunity of cotton should be activated by introducing genes of biocidal proteins from other *Malva* species in order to increase its resistance.

We isolated and studied the physicochemical properties, fungitoxicity, and cancerolytic properties of bioactive proteins from seeds of *Hibiscus esculentus* L. (okra, Malvaceae), the fruits of which are used as food.

The biocidal peptide from okra seeds was isolated by the literature method [3]. Total proteins isolated by extraction (0.05 N H_2SO_4) from defatted seeds were separated over a column of Sephadex G-50 to produce three fractions I-III (Fig. 1). Each fraction was tested for fungicidal activity toward a virulent strain of the fungus *Verticillium dahliae* Kleb. using a turbimetric method [5] (Table 1).

The investigation showed that fraction II had distinct fungicidal activity. Its subsequent purification over a column of the hydrophobic sorbent butyl-TSK produced three fractions (1-3). Analysis of these by PAAG-electrophoresis detected peptides of molecular weight 6 kDa in two fractions (Fig. 2). Each of these fractions was investigated for fungicidal activity. This showed that the fraction eluted with 60% CH_3CN had the greatest fungicidal activity (Table 1). This fraction was analyzed by HPLC over a Zorbax column. Three peptides were found (He-AFP1, He-AFP2, and He-AFP3) with retention times of 10.5, 14.0, and 16.4 min, respectively.

It was noted previously that the fungicidal activity of thionines and plant defensins is related to their effect on fungal membranes [3]. It can be assumed that compounds with cytotoxic activity will also exhibit cancerolytic properties.

The effect of the prepared fractions and pure peptides on the proliferative activity of murine KML melanoma cells in tissue culture was evaluated in a cytotoxic test for ^{3}H-thymidine incorporation into cellular DNA [4]. The studied proteins suppressed growth of murine melanoma at a concentration of 100 μg/mL in vitro (Table 2).

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TABLE 1. Fungicidal Activity of Isolated Protein Fractions and Pure Peptides of Okra Seeds

Fraction	IC_{50}, μg/mL
I	38.3
II	25.3
III	48.4
1	18.1
2	-
3	50.0
He-AFP1	6.6
He-AFP2	6.8
He-AFP3	7.3

TABLE 2. Growth Suppression of Murine Melanoma Cells by Isolated Protein Fractions and Pure Peptides of Okra Seeds

Fraction	% suppression
I	39.1
II	8.0
III	8.0
He-AFP1	8.8
He-AFP2	8.7
He-AFP3	8.0
GI	0
CII	0
Combined GI and GII	21.0

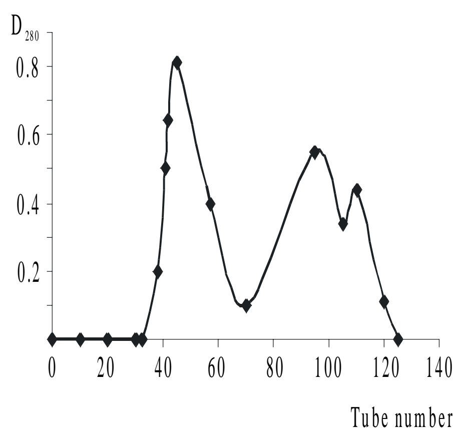

Fig. 1

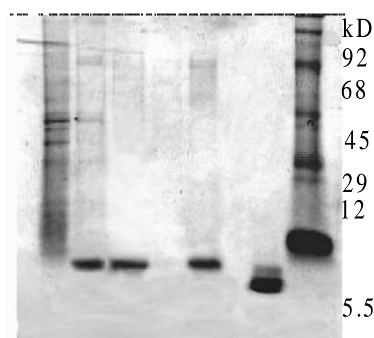

Fig. 2

Fig. 1. Gel filtration of total protein extract of okra seeds over a column (1.5 × 85 cm) of Sephadex G-50 in NH_4HCO_3 buffer (0.01 M) at pH 8.2. Flow rate 44 mL/h.
Fig. 2. Electrophoretic analysis of isolated fractions: I and II, respectively, after separation over Sephadex G-50; eluted from a butyl-TSK column by 60, 80, and 100%, respectively, CH_3CN (1-3); marker proteins (4, 5).

It can be seen that fraction I has the highest cancerolytic properties. According to PAAG-electrophoresis, this fraction contains proteins with molecular weights in the range 12-45 kDa. These are assumed to be glycoproteins because they react on the electrophoregram with phenol—sulfuric reagent to give brown bands.

Bioactive proteins were isolated preparatively by extraction with Tris-HCl buffer (25 mM) at pH 7.5 containing protease phenylmethylsulfonylfluoride (PMSF) as an inhibitor. The fraction of high-molecular-weight proteins was precipitated with trichloroacetic acid (TCA, 20%). The total glycoproteins were precipitated by adding three volumes of cold acetone and separating over a column of Sephadex G-50 into two fractions designated GI and GII. The fractions produced this way were studied for cancerolytic activity (Table 2).

These fractions did not have cancerolytic activity. However, combining them gave a synergetic effect and suppressed ^{3}H-thymidine incorporation by 21%.

EXPERIMENTAL

Isolation and purification of proteins from seeds was carried out as described previously [3].

Isolation of Total Glycoproteins. Seeds were ground with polyvinylpyrrolidone (1:10) and defatted with hexane in a Soxhlet apparatus for 72 h. Defatted seeds were extracted with Tris-HCl buffer (25 mM) at pH 7.5 containing sodium azide (5 mM) and PMSF (2 mM) with vigorous stirring on a mechanical stirrer for 3 h. The total extract was treated with TCA (20%). The precipitate was separated by centriguation at 6000 rpm for 30 min. The supernatant was treated with five volumes of cold acetone. The precipitate was collected by centrifugation and separated into two fractions by gel-filtration over a column of Sephadex G-50 (85×1.5 cm) in Tris-HCl buffer (25 mM) at pH 7.5.

HPLC was performed in an Agilent-1100 instrument over a Zorbax Eclipse XDB C_8 column (4.6×150 mm, 5 μm) using an CH_3CN gradient and 215 nm wavelength.

Cytotoxic Activity. KML cell culture [6] was obtained from a murine melanoma strain. Cells were placed into vials (4×10^4 cells/mL) containing RPMi-1640 nutrient medium (3 mL) with fetal calf serum (10%), glutamine (200 mM), and antibiotics and cultivated in a thermostat at 37°C. The studied proteins were added after 24 h at a concentration of 100 μg/mL. The exposure time was 24 h. Then ^{3}H-thymidine (10 μCi/vial) was added for 1 h. The label was removed from the medium. Versene solution (1 mL, 0.2%) as added for 3-5 min to remove cells from the glass. The cell suspension was transferred onto GFC filters, stabilized, washed with TCA (5%, 3×10 min) and distilled water (3×10 min), and dried.

The cytotoxic activity of the studied proteins was evaluated using the amount of ^{3}H-thymidine incorporation into cellular DNA according to Beta-1 and ZhS-106 liquid scintillation counters. The results were expressed in percent of a control for suppressing label incorporation. The experiments were repeated three times. The control was cells without the studied protein.

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