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EFFECT OF COTTON-SEED BIOCIDAL PEPTIDES AND GOSSYPOL ON RESISTANCE TO BIOTIC FACTORS

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Peptide fractions were isolated from seeds of eight cotton varieties differing in resistance to fungal pathogens and cotton bollworm. Their ability to inhibit the growth of V. dahliae *fungal conidiae and α-amylase from various sources was studied. A correlation was found between the fungicidal activity of the peptides and the resistance of the cotton varieties to the pathogens. α-Amylase of cotton bollworm was inhibited less by the isolated peptides than α-amylase of other insects that are not cotton pests. It was shown for the first time that gossypol inhibits α-amylase of insects.*

Key words: α-amylase, enzyme inhibitors, gossypol, cotton bollworm, verticillium wilt, peptides, cotton.

Plant tissues possess various protective mechanisms that are initiated upon attack by pests or contact with microorganisms. For this, the amount of several proteins increases above their baseline level. Such proteins are the inhibitors of amylase, trypsin, hydrolase, and protease and small cysteine-containing antimicrobial peptides.

It has been found that cysteine-containing peptides such as thionins, lectins, knottins, taumatins, etc. often act as inhibitors of insect digestive enzymes, one of the most important of which is α-amylase [1].

Terpenoids, which are always present in plants, can also protect plants from attack by insect pests. For cotton, these are gossypol, hemigossypol, and their derivatives, in particular, gossypol is toxic for cotton aphid, lygaeid bugs (*Lygaeidae*), bollweevil (*Anthonomus grandis*) [2], *Leucania unipuncta*, cotton bollworm (*Heliothis armigera* Hub.), cabbage moth (*Barathra brassicae*), et al. [3]. However, the mechanism of the toxic action of gossypol and compounds related to it has not yet been studied.

Our goal was to isolate total peptides from seeds of eight cotton varieties and lines that differ in degree of resistance to attack by cotton bollworm and fungal pathogens, to determine the degree of growth inhibition of *Verticillium dahliae* Kleb. fungus culture and the degree of inhibition of the digestive enzyme α-amylase of *H. armigera* cotton bollworm larvae by isolated total peptides, to determine the gossypol content in seeds of these same cotton varieties, to study the ability of gossypol to inhibit α-amylase of *H. armigera* larvae, and to find a possible relationship between the gossypol content and the resistance to fungal pathogens.

It can be assumed that the level of biocidal peptides in various cotton varieties will determine their degree of resistance to *V. dahliae* and *H. armigera*, typical cotton pests. We isolated total peptides from seeds of eight cotton varieties and lines with different susceptibility to wilt and cotton bollworm that were developed at the Uzbekistan SRI for Cotton Selection and Seed Breeding: MG-01, MG-02, MG-03, C-2609, C-2610, C-6524, Namangan-77, and the "Gossypol-free" line.

Biocidal peptides were isolated from ground defatted cotton seeds by acid extraction followed by chromatography over Sephadex G-50 and Butyl-TSK-650N sorbent using a stepped gradient of CH_3CN.

The isolated peptides were studied for ability to inhibit α-amylase of *H. armigera* cotton bollworm larvae, walnut sphinx (*Cressonia juglandis*), and for comparison with α-amylase from bovine pancreas (Table 1).

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TABLE 1. Inhibition of *Heliothis armigera* α-Amylase by Cotton-Seed Peptides, Gossypol Content in Seeds, and Resistance of Varieties to Cotton Weevil*

Variety and line	Attack by *H. armigera* larvae**	Yield of total peptides, mass %	Inhibition of α-amylase by cotton-seed peptides, IC_{50}, μg/mL****			Gossypol content, %
			from *Heliothis armigera*	from *Cressonia juglandis*	from bovine pancreas	
Mg-02	Highly resistant***	4.9	53.1	1.8	2.4	2.94
Namangan-77	11	3.5	63.4	2.0	2.5	2.79
Mg-01	15.3	7.5	31.6	2.0	2.5	2.46
C-2609	31	3.8	43.0	2.2	2.6	2.92
C-2610	42	5.9	191.0	2.0	2.5	2.41
C-6524	42	7.3	33.1	2.0	2.6	3.75
Mg-03	45	6.2	146.2	1.4	1.7	1.84
"Gossypol-free "	Poorly resistant***	2.1	26.4	1.7	2.2	1.41

*Varieties are placed in order of decreasing resistance to cotton bollworm.

**Data from field tests at the Uzbek SRI for Plant Protection.

***Data for percent susceptibility to cotton bollworm are missing. This quantity can be estimated only qualitatively.

****IC_{50}: concentration of a compound at which enzyme activity is suppressed by 50%.

TABLE 2. Growth Inhibition of *Verticillium dahliae* Fungus by Peptide Extracts from Seeds of Various Cotton Varieties

Peptide source	Attack by *V. dahliae*, %**	Yield of total peptides, mass %	IC_{50}, μg/mL***
Mg-01	3	7.5	5.8
Mg-02	5	4.9	6.1
Mg-03	6	6.2	5.6
C-2609	7	3.8	5.7
C-2610	12	5.9	6.4
"Gossypol-free"	12	2.1	18.1
Namangan-77	18	3.5	8.3
C-6524	25	7.3	Not inibit.

*Studied cotton varieties are placed in order of decreasing resistance to fungal pathogens.

**Data from field tests at the Uzbekistan SRI for Cotton Selection and Seed Breeding

***IC_{50}: concentration of a compound at which enzyme activity is suppressed by 50%.

We found during the study of the *in vitro* activity of gossypol on α-amylase that gossypol inhibited the activity of α-amylase isolated from *H. armigera* larvae (IC_{50} 27.7), *Taxoptera graminum* aphids (IC_{50} 7.2), and *Culex pipiens* common mosquito nymphs (IC_{50} 70.4). α-Amylase from aphids was the most sensitive to gossypol.

We determined the gossypol content and the ratio of its enantiomers in seeds of eight cotton varieties and lines in order to find a correlation between the gossypol content in cotton and its resistance to *H. armigera* and *V. dahliae* (Table 1). Previous *in vivo* experiments on feeding of *H. armigera* cotton bollworm larvae from the third stage to the imago stage with a diet containing (+)- and (-)-gossypol found that larvae whose food contained (+)-gossypol developed more slowly and developed less to the imago stage [4]. However, the mechanism of action of gossypol on the insect organism is poorly studied.

A comparison of IC_{50} data for α-amylase from various sources showed that α-amylase from *H. armigera* larvae was weakly inhibited by the biocidal peptides. Substantially higher peptide concentrations were needed to suppress its activity than to inhibit α-amylase of walnut sphinx and mammals. Obviously cotton bollworm, the main food of which is cotton leaves, developed during evolution resistance to biocidal cotton peptides.

The results showed that the gossypol-free line, which had the lowest gossypol content, was most susceptible to cotton bollworm. However, a clear correlation between the gossypol level and the resistance to cotton bollworm was not observed.

Table 2 gives results for the ability of the isolated peptides to suppress growth of *V. dahliae* fungus.

The results suggest that the resistance of a variety was correlated with the fungicidal activity of the peptides isolated from its seeds. On the other hand, the gossypol content did not affect the resistance of the plants to this fungus species.

The gossypol level probably did not determine the insect- and fungicidal resistance of the cotton. This agrees with previous results that other terpenoid aldehydes related to gossypol, namely heliocides H_1, H_2, H_3, and H_4, were the most toxic to insects [5] whereas hemigossypol and hemigossypolone protected against fungal pathogens [6].

EXPERIMENTAL

Cotton seeds of the studied varieties were supplied by Candidate of Agricultural Sciences Sh. E. Namazov of the Uzbekistan SRI for Cotton Selection and Seed Breeding.

HPLC of Peptides was performed on an Agilent 1100 series chromatograph (Agilent Inc., USA) using a gradient of CH_3CN/TFA (1%) 0–40% and a Zorbax SB-C18 (4.6 × 250 mm) column at flow rate 1 mL/min with UV detection at 226 nm.

Electrophoresis of proteins and peptides was carried out using the Laemmli method [7].

Protein concentration was determined by the Bradford method [8] using trypsin and ovalbumin as standards.

Isolation and Purification of Biocidal Peptides. Ground seeds were defatted by hexane in a Soxhlet apparatus for 48 h and extracted with H_2SO_4 solution (0.05 N) on a magnetic stirrer for 3 h. The extract was separated by centrifugation for 30 min at 6,000 rpm and neutralized with NaOH solution (10 N). The resulting precipitate was separated by centrifugation. The supernatant was lyophilized, placed on a column with Sephadex G-50 (1.5 × 85 cm) in NH_4HCO_3 (0.01 M), and eluted with the same buffer. The isolated fractions were analyzed by electrophoresis in a thin layer of PAAG (15%).

Isolation of α-Amylase. Enzymes were isolated from medium- and old-aged larvae grown under laboratory conditions. Larvae were frozen and ground in buffer (20 mM CH_3CO_2H/NaOH, pH 5.4, 5 mM $CaCl_2$). The homogenate was centrifuged. Total proteins were precipitated from the supernatant by $(NH_4)_2SO_4$, from 20 to 60% saturation). The resulting precipitate was dialyzed against buffer and centrifuged. The supernatant was placed on an anion-exchange column with DEAE-cellulose and eluted by a linear gradient of NaCl (0-500 mM). Fractions were combined and concentrated. Proteins were precipitated by $(NH_4)_2SO_4$ (up to 100% saturation). The resulting suspension was stored at 0-4°C. The enzyme was purified 25 times compared with the initial extract. The activity yield was 15%.

Determination of α-amylase activity and α-amylase inhibition (adapted amyloclastic photometric method of Smith and Roe [9]).

Solution P_{SEI}. A solution of soluble starch (0.6%, Lintner) in buffer (20 mM CH_3CO_2H/CH_3CO_2Na, 6 mM $CaCl_2$, pH 5,5, 100 μL) was treated with inhibitor solution (20 μL) in a suitable solvent [H_2O, $CHCl_3$, CH_3CN, $(CH_3)_2SO$, $(CH_3)_2CO$], thermostatted at 37°C with stirring for 10 min, treated with α-amylase solution (80 μL) in buffer, thoroughly mixed, and held for exactly 30 min at 37°C.

Solutions P_{SI}, P_{SE}, P_S, and P_0 were prepared analogously.

	P_{SEI}	P_{SI}	P_{SE}	P_S	P_0
Starch solution in buffer (100 μL)	+	+	+	+	
Inhibitor solution in solvent (20 μL)	+	+			
α-Amylase solution in buffer (80 μL)	+		+		
Buffer (100 μL)					+
Solvent for inhibitor (20 μL)		+	+	+	+
Buffer (80 μL)		+		+	+

The reaction was stopped by adding CH_3CO_2H (100 μL, 1 N), cooling, adding distilled water (up to 10 mL), adding I_2 in 50% aqueous ethanol (100 μL, 0.3%), stirring, and holding the temperature constant (10 min). Optical density of the resulting solution vs. P_0 was determined at 570 nm (or 540 nm) (1 cm path length).

The inhibitory activity was calculated using the formula

$$I = [A(P_{SI}) - A(P_{SEI})] \times 100/[A(P_S) - A(P_{SE})],$$

where I is the suppression of α-amylase activity (%); $A(P_{SEI})$, $A(P_{SE})$, $A(P_{SI})$, and $A(P_S)$, the optical density of the corresponding solutions measured vs. P_0.

Determination of Gossypol Content [10]. Purified seeds were ground in liquid nitrogen. A portion (20 mg) was treated with oxalic-acid solution (2 mL, 0.1 M) in 70% $(CH_3)_2CO$, stored with a loose cover for 6 h at 55°C, cooled, treated with $BaCl_2$ solution (100 μL, 0.5%), treated with acetone (70%, up to 2 mL), and centrifuged after 10 min. An aliquot (0.5 mL) was taken and treated with $(CH_3)_2CO$ (70%, up to 5 mL). Optical density was measured at 380 nm.

Solutions were prepared for constructing a calibration curve [20, 40, 60, 80, and 100 μL; 0.01% gossypol in $(CH_3)_2CO$ (70%, up to 5 mL)].

Fungicidal activity toward *V. dahliae* was studied by a turbidimetric method by measuring after incubation (24 h) the optical density decrease of suspensions of fungus conidial spores at 540 nm in the presence of the studied peptide compared with a control. Peptide concentration in the culture liquid was 6 μg/mL.

REFERENCES

1. O. L. Franko, D. J. Rigden, F. R. Melo, and M. F. Grossi-de-Sa, *Eur. J. Biochem.*, **269**, 397 (2002).
2. G. T. Bottger, E. T. Sheehan, and M. J. Lukefahr, *J. Econ. Entomol.*, **57**, 283 (1964).
3. G. T. Bottger and R. Patana, *J. Econ. Entomol.*, **59**, 1166 (1966).
4. W.-H. Yang, L.-N. Ma, H.-Q. Zhu, and S.-K. Xiang, *Acta Gossypii Sinica*, **11**, 31 (1999).
5. R. D. Stipanovic, D. W. Altman, D. L. Begin, G. A. Greenblatt, and J. H. Benedict, *J. Agric. Food Chem.*, **36**, 509 (1988).
6. L. S. Puckhaber, M. K. Dowd, R. D. Stipanovic, and C. R. Howell, *J. Agric. Food Chem.*, **50**, 7017 (2002).
7. U. R. Laemmli, *Nature (London)*, **227**, 681 (1970).
8. M. M. Bradford, *Anal. Biochem.*, **72**, 341 (1976).
9. R. W. Smith and J. H. Roe, *J. Biol. Chem.*, **179**, 53 (1949).
10. R. W. Storherz and R. T. Holley, *J. Agric. Food Chem.*, **2**, 745 (1954).