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COMPARATIVE INVESTIGATION OF THE COMPOSITION OF THE PROTEASE INHIBITORS OF WILT-RESISTANT AND WILT-SUSCEPTIBLE VARIETIES OF COTTON

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The amounts and the group, class, and fatty-acid compositions of the feebly- and the strongly-bound lipids of the total protein fraction of cotton seeds that inhibits the growth of the pathogenic fungus Verticillium dahliae *Kleb. have been determined. The main differences of the indices for the seeds of healthy and infected plants of two varieties contrasted by their degrees of susceptibility to verticillium wilt have been established.*

We have previously isolated the total inhibiting fractions (TIFs) from the kernels of seeds of varieties of the cotton plant (*Gossypium hirsutum* L.) resistant and susceptible to verticillium wilt (*Verticillium dahliae* Kleb.) and have determined their partial chemical compositions [1]. The inhibitors suppressed the growth and development of the pathogenic fungus; however, the degree of their activity depended on the variety and was higher for the TIF from the kernels of the resistant variety [2]. According to our results, the total inhibiting fractions of the two varieties consisted of mixtures of not less than three protein complexes [3], two of which might contain lipids.

In the light of the fact that the qualitative and quantitative compositions of the lipids and the set of fatty acids affect the inhibiting properties of some lipoproteins of living tissues [4], we have made a comparative investigation of the lipids of the TIFs of the seed kernels of cotton plants of resistant (175-F) and susceptible (S-4727) varieties. The plants were picked before and after infection by verticillium wilt.

Depending on the structure and nature of the bond, lipids form complexes having different degrees of stability with protein molecules. We therefore first extracted the bound lipids (BLs) from these specimens by the generally adopted Folch method, and for the subsequent isolation of the strongly-bound lipids (SBLs) we used an enzymatic-chemical method permitting their extraction from the seed kernels of oil-bearing and cereal crops with retention of their native nature [5]. We first checked the suitability of enzymolysis by acid and alkaline proteases for the proteins of the TIFs of healthy (sample 1) and infected (sample 2) plants of variety 175-F. The lipids not extracted by proteolysis were isolated after the treatment of the protein residue with a 20% ethanolic solution of KOH and the extraction of the hydrolysis products with diethyl ether (residual lipids (RLs). The results of these experiments are given in Table 1. It can be seen that, as in the case of the protein of the cottonseed kernel investigatd previously [5], a more complete yield of SBLs is achieved on enzymolysis by an alkaline enzyme – protosubtilin G10X.

The yield of RLs increased substantially when enzymolysis was carried out with acid proteases. Analysis of the compositions of the RLs of samples 1 and 2 by TLC in system 1 showed that the largest amount of unidentified substances of nonlipid nature was observed in the case of hydrolysis by pepsin. These substances have not been studied. They did not belong to the known polyphenolic pigments of cotton seeds, since the UV spectrum of the RLs lacked absorption in the 370-380 nm region [6]. A weak maximum was observed in the 270-290 nm interval of the spectrum. It must be mentioned that the TIF was light yellow, the BLs, SBLs and RLs dark brown, and the hydrolyzed protein light gray. The increase in the yield of extractive substances from the products of acid enzymolysis arose through a higher degree of splitting out under these conditions not of lipids but of peptides with aromatic amino acids and/or the aromatic amino acids themselves.

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TABLE 1. Yields of Lipids and Proteins from 100 mg of the Total Inhibiting Fraction of Healthy Seeds of a Cotton Plant of Variety 175-F after Proteolysis by Various Enzymes (mg/g a.d.m)

Enzyme (pH optimum)	Lipids of sample 1			Lipids of sample 2		
	Strongly-bound	Residual	Total	Strongly-bound	Residual	Total
Pepsin (pH 2.0)	66.7	900.0	966.7	66.7	800.0	866.7
Papain (pH 5.0)	133.4	300.0	433.4	200.1	333.0	533.1
Protosubtilin G10X (pH 8.5)	285.7	100.0	385.7	333.3	100.0	433.3

TABLE 2. Compositions of the Total Inhibiting Fractions of the Kernels of Seeds from Healthy and Infected Cotton Plants (mg/g a.d.m.)

Component	Variety 175-F		Variety S-4727	
	1	2	1	2
Lipids				
bound	100.0	107.1	94.3	431.4
strongly-bound	285.7	200.1	33.3	20.0
residual	100.0	100.0	625.0	250.0
total	485.7	407.2	752.6	701.4
Protein				
hydrolyzed	71.4	100.0	166.7	164.7
unhydrolyzed	257.1	300.0	20.0	13.2
total	328.5	400.0	186.7	177.9
$\frac{\Sigma \text{ lipids}}{\Sigma \text{ proteins}}$	1.48	1.02	4.03	3.84

In the following experiments, we used subtilin 10GX for the isolation of the SBLs. The amounts of the individual groups of lipids and of protein in the TIF samples of the two varieties of cotton plant are given in Table 2.

The TIFs 1 and 2 of the resistant variety contained 1.5-1.7 times less total lipids than the TIFs of the susceptible variety. In the TIF 1 of variety 175-F the SBLs predominated, the ratio of the the three groups of lipids (BLs:SBLs:RLs) being 1:1.8:1, and it changed little as the result of infection (1:2:1 in the TIF 2). In the TIF 1 from variety S-4727 the RLs predominated, this ratio being 2.8:1:18.8, while after infection the proportion of BLs had risen substantially and and the level of RLs had fallen (21.6:1:12.5 in the TIF 2). Common for both varieties was a fall in the total lipids in the TIFs under the influence of wilt infection.

The same marked differences were also shown in the ratio of the protein and its hydrolyzable and nonhydrolyzable fractions (Table 2). The TIF 1 from the resistant variety contained 1.7 times as much total protein as the TIF from the susceptible variety, and its level rose somewhat after infection. In the case of the sensitive variety, infection led to a fall in this index. Variety-dependent differences were observed in the degree of hydrolyzability of the protein of the TIFs by an enzyme: in the TIFs of variety 175-F the protein was more hydrolyzable than in those of variety S-4727.

The compositions of the three groups of lipids of the TIFs were determined with the aid of TLC in systems 1, 2, and 3. The sets of components of the BLs, SBLs and OLs of the inhibitors in the two varieties were similar, and this both in the healthy and the infected plants. However, the quantitative ratios of the individual classes within the neutral lipids and the phospho- and glycolipids depended on the degree of resistance of the variety to wilt infection.

In the BLs, among the neutral lipids we reliably identified hydrocarbons, triacylglycerols, free fatty acids, and hydroxy acids, and, not completely reliably, hydroxyacylmonoacylglycerols, di- and monoacylglycerols, and fatty alcohols; among the glycolipids, esters of steryl glycosides; among the phospholipids, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol, and also two unidentified weakly polar phospholipids (R_f 0.70, 0.82, system 3). Judging from the ratio of the areas of the spots, triacylglycerols, sterols, and phosphatidylcholine predominated in the TIFs of the resistant variety, and free fatty acids, hydroxy acids, and phosphatidylcholine with phosphatidylethanolamine in the TIFs of the susceptible variety.

TABLE 3. Fatty Acid Composition of the Lipids of the Total Inhibiting Fractions from Kernels of the Seeds of a Healthy and an Infected Cotton Plant of Variety 175-F, % GLC

Acid	Bound lipids		Strongly bound lipids						Residual lipids					
			pepsin		papain		protosubtilin		pepsin		papain		protosubtilin	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
10:0	—	—	—	—	—	—	—	—	0.8	1.8	Tr.	1.2	1.1	—
12:0	—	—	Tr.	0.6	0.8	0.5	0.5	Tr.	1.0	1.7	—	0.9	0.9	Tr.
13:0	—	—	—	Tr.	1.7	2.4	2.7	1.4	1.2	1.5	—	0.9	2.5	—
14:0*	0.5	0.4	12.9	12.8	18.3	17.3	23.9	18.1	3.6	3.4	3.2	2.9	7.3	5.1
15:0	0.7	0.6	Tr.	0.7	0.9	Tr.	1.4	2.1	4.3	1.9	0.8	1.9	2.3	Tr.
16:0	17.7	17.4	20.5	27.2	25.7	27.3	26.8	24.9	27.7	35.9	39.3	34.5	32.9	29.8
16:1	0.9	1.3	0.9	1.3	2.3	3.4	3.7	3.2	3.8	3.5	2.3	2.4	7.1	2.5
17:0	Tr.	Tr.	Tr.	Tr.	1.2	0.8	0.5	0.9	2.4	1.9	Tr.	1.4	Tr.	Tr.
18:0	1.2	1.8	5.9	2.7	8.4	10.8	12.3	13.1	11.8	8.0	10.4	11.5	6.3	13.9
18:1	27.2	27.9	24.9	22.0	20.5	21.8	17.4	21.7	20.9	16.9	19.3	18.9	16.7	17.6
18:2	51.8	50.6	34.9	32.7	20.1	15.7	10.8	14.6	22.5	23.5	24.7	23.6	22.9	31.1
20:0	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	—	—	Tr.	Tr.
20:1	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	—	—	—	—
Σ_{sat}	20.1	20.2	39.3	44.0	57.1	59.1	68.1	60.5	52.8	56.1	53.7	55.1	53.3	48.8
Σ_{unsat}	79.9	79.8	60.7	56.0	42.9	40.9	31.9	39.5	47.2	43.9	46.3	44.9	46.7	51.2

TABLE 4. Fatty Acid Composition of the Lipids of the Total Inhibiting Fractions from Kernels of the Seeds of a Healthy and Infected Cotton Plant of Variety S-4727, % GLC

Acid	Bound lipids		Strongly bound lipids		Residual lipids	
	1	2	1	2	1	2
12:0	2.0	1.9	0.4	0.5	0.5	0.8
13:0	3.0	2.0	2.5	4.6	5.8	7.4
14:0	Tr.	Tr.	3.2	3.9	1.4	2.5
15:0	—	—	2.4	1.5	2.4	2.9
15:1	—	—	Tr.	0.8	1.7	1.5
16:0	35.6	34.3	35.0	30.0	27.9	34.2
16:1	2.4	2.5	4.8	6.2	2.5	3.6
17:0	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.
18:0	3.0	1.8	13.1	12.1	10.4	12.3
18:1	35.3	35.7	24.9	26.7	32.1	23.2
18:2	18.7	21.8	13.7	13.7	15.3	11:6
18:3	Tr.	Tr.	—	—	—	—
20:0	—	—	Tr.	Tr.	Tr.	Tr.
20:1	—	—	Tr.	Tr.	Tr.	Tr.
Σ_{sat}	43.6	40.1	56.6	52.6	48.4	60.1
Σ_{unsat}	56.4	60.0	43.4	47.4	51.6	39.9

*In admixture with an unidentified component.

The qualitative compositions of the SBLs and RLs were identical and included free fatty acids, sterols, and, possibly, fatty alcohols and traces of triacylglycerols, and also a number of unidentified components not revealed by iodine vapor or by heating with H_2SO_4. In the TIFs of the resistant variety the SBLs were enriched with sterols and in those of the susceptible variety with free fatty acids.

All the groups of lipids isolated had a brown coloration, and, on chromatography by TLC, the pigments possessed no chromatographic mobility in the system for neutral lipids, while in the system for glycolipids they were separated as a diffuse spot at the front of the plate, which made it difficult to identify the components. No appreciable differences were detected in the compositions of the TIFs of the infected plants of the two varieties.

The compositions of the fatty acids of the lipids of the samples under investigation were then determiend by GLC and mass spectrometry. Table 3 gives the fatty acid compositions of the lipids of TIFs 1 and 2 from variety 175-F, including the SBLs and the RLs isolated after acid enzymolysis, while Table 4 gives the analogous information for variety S-4727.

The basic composition of the acids was determined by GLC, while mass-spectrometric analysis revealed additionally, as minor components, the 17:0, 18:3, 20:0, and 20:1 acids. Likewise, in addition to the acids listed in Table 4, in the mixture of acids of TIFs 1 and 2 from the nonresistant variety we identified a hydroxylinoleic (OH-18:2) acid with the aid of its mass spectrum. Peaks of ions with m/z 310 (M^+), 292 $(M - 18)^+$, 279 $(M - 31)^+$, and 278 $(M - 32)^+$ were ascribed to the breakdown of its methyl ester, and fragments with m/z 123, 153, 155, and 157 showed the possible structure of the acid as 9-OH-18:2(10,12) [7]. This acid was present in trace amounts in the acids of the TIFs from variety 175-F.

It can be seen from Table 3 that the conditions of enzymolysis substantially affected the fatty acid compositions of the lipids isolated. Hydrolysis by acid proteases liberated more highly unsaturated SBLs 1 than hydrolysuis by an alkaline enzyme; in the case of the acid proteolysis of an infected sample, a fall in the total degree of unsaturation of the acids was observed, while in an experiment with an alkaline protease the total unsaturation of the SBLs 2 was higher than that of the SBLs 1. Apparently, the cleavage of protein takes place under acid conditions more specifically than under alkaline conditions.

For each variety, the fatty acids of the three groups of lipids of TIFs 1 and 2 had differences in their qualitative and quantitative compositions. The simplest with respect to the set of components were the acids of the BLs of the resistant variety (Table 3); at the same time, it contained the largest amount of the 18:2 acid. In this variety, with an increase in the strength of binding of the lipids, their fatty acid composition became more complicated through the appearance of the 10:0-13:0 acids with medium molecular masses; the level of total 10:0-15:0 acids in the SBLs 1 exceeded 28%, while among the unsaturated components in them the 18:1 acid predominated in them, and in the RLs 1 the 16:0 acid acid predominated. Infection caused the most considerable changes in the levels of the 14:0, 18:1, and 18:2 acids in SBLs and the 18:0 and 18:2 acids in the RLs.

In the lipids of the TIF 1 from variety S-4727 (Table 4) the total unsaturation of the fatty acids of the BLs was lower, while in the SBLs and RLs it was higher, than for variety 175-F; the proportion of fatty acids having medium molecular masses in the SBLs and RLs was smaller; the main unsaturated acid in all the lipids was the 18:1 species, and infection changed mainly the levels of the 16:0 and 18:1 acids in the RLs.

Thus, the TIFs of the seed kernels of cotton plants of varieties resistant and susceptible to verticillium wilt differ sharply in their contents of lipids strongly bound with protein and lipids isolatable after its far-reaching denaturation, in their fatty acid compositions, and in the predominance of individual classes of substances in the lipids.

The consequences of the infection of a cotton plant by wilt were shown more acutely in the composition of the fatty acids of the TIFs bound to protein by stronger bonds for the resistant variety, and in the amounts in the TIFs of individual groups of lipids – particulary lipids weakly bound with protein – for the nonresistant variety.

EXPERIMENTAL

The conditions for taking UV and mass spectra and performing GLC have been described in [6]. TLC was conducted on silica gel L 5/40 (Czechoslovakia) with the addition of 10% of $CaSO_4$, and on Silufol (Czechoslovakia) in the systems: 1) hexane–diethyl ether–acetic acid (70:30:1) for the NLs; 2) acetone–benzene–water (91:30:8) for the GLs; and 3) chloroform–methanol–20% ammonia (65:25:4) for the PLs and Gls. The chromatograms were revealed as described in [8]. The lipids were identified by comparison with standard compounds isolated previously from plant materials [9] and with literature information [8].

Enzymatic hydrolysis and the isolation of the lipids were carried out in accordance with [5], with the exception of the fact that the reaction mixture was not separated into liquid and solid phases but was subjected to freeze-drying, and the hydrolysis products were extracted from the dry residue with diethyl ether three times.

For the identification of the FAMEs we used literature information on C_{sp} [10], model FAMEs and natural mixtures of them [4], and the values of M^+ and of the ions $[M - 29]^+$, $[M - 31]^+$, and $[M - 43]^+$ in the mass spectra of the FAMEs [11].

REFERENCES

1. L. G. Mezhlum'yan, É. F. Redina, and P. Kh. Yuldashev, Khim. Prir. Soedin., 444 (1993).
2. L. G. Mezhlum'yan, É. F. Redina, and P. Kh. Yuldashev, Khim. Prir. Soedin., 274 (1994).
3. T. D. Kasymova, L. G. Mezhlum'yan, and P. Kh. Yuldashev, in: Abstracts of the 5th International Symposium on Verticillium [in Russian], Leningrad (1990), p. 54.
4. M. I. Gurr, Progr. Lipid Res., **22,** No. 4, 257 (1983).
5. S. D. Gusakova, I. P. Nazarova, A. I. Glushenkova, D. A. Khashimov, Kh. G. Alimov, and P. Kh. Yuldashev, Khim. Prir. Soedin., 621 (1991); 713 (1991).
6. S. D. Gusakova, S. G. Yunusova, T. V. Chernenko, I. P. Nazarova, and A. I. Glushenkova, Khim. Prir. Soedin., 677 (1986).
7. N. Natale, Lipids, **12,** No. 10, 847 (1971).
8. M. Kates, Techniques of Lipidology, American Elsevier (1972).
9. S. G. Yunusova, S. D. Gusakova, Kh. G. Mirzaazimova, A. I. Glushenkova, S. A. Usmanov, and Yu. Ikramov, Khim. Prir. Soedin., 285 (1989).
10. R. Ackman, J. Am. Oil Chem. Soc., **40,** No. 10, 558 (1963).
11. H. Budzikiewicz, C. Djerassi, and D. Williams, Interpretation of Mass Spectra of Organic Compounds, Holden-Day, San Francisco (1964).