POLYSACCHARIDES OF THE PROTEIN–LIPOID–POLYSACCHARIDE COMPLEX OF THE MYCELIUM OF *Verticillium dahliae*.

I. ISOLATION AND FRACTIONATION

T. A. Badalbaeva, N. V. Bykova,
V. K. Lekomtseva, O. S. Otroshchenko,
and A. V. Khotyanovich

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A phytotoxic PLPC has been isolated from the mycelium of the fungus *Verticillium dahliae* Kleb., strain 614. The polysaccharide component has been split out from the PLPC by mild acid hydrolysis. The polysaccharide has been fractionated and the monosaccharide compositions of the fractions have been determined. A phytotoxic polysaccharide homogeneous according to gel chromatography and chromatography on DEAE-cellulose has been isolated which consists of an α-glucan containing a minor amount of D-glucosamine.

Continuing an investigation of the phytotoxic substances of the fungus *Verticillium dahliae* Kleb., we have begun the study of the polysaccharides of the protein–lipoid–polysaccharide complex (PLPC). There is information in the literature on the isolation of a PLPC from the mycelium and the culture liquid of *V. dahliae* and *V. albo-atrum* and the phytotoxic properties of this complex and of its polysaccharide fraction [1-4]. However, the structure of the polysaccharides of the PLPC has scarcely been studied. We give the results of investigations of the isolation, fractionation, and composition of the polysaccharides of the PLPC of *V. dahliae*.

To isolate the PLPC we used Boivin's method [5]. The yield of extract was ∿2% of the weight of the freeze-dried mycelium. The fractional precipitation of the PLPC with a concentration gradient of alcohol yielded three fractions: A, B, and C. The main one quantitatively (yield 88%) was fraction A, and therefore B and C were not studied. Mild hydrolysis with 1% acetic acid of the PLPC (fraction A) gave a polysaccharide component with a yield of ~80%, and this was fractionated by precipitation from aqueous solution with increasing concentrations of alcohol. In this way, fractions I, II, and III were obtained.

The fraction of highest molecular weight, fraction I (yield 88%), which we precipitated at a 40% concentration of alcohol, was found to contain D-glucose and traces of D-glucosamine. A hydrolysate of fraction II (yield 0.4%, precipitation at 52% concentration of alcohol) was found to contain D-glucose, D-galactose, D-mannose, a uronic acid, and traces of L-rhamnose and D-glucosamine. The monosaccharide composition of fraction III was not studied because of its very small amount.

Gel chromatography of the polysaccharide (fraction I) on Sephadexes G-150 and G-200 (Fig. 1a, b) with elution by 1 M NaCl showed that the polysaccharide I issued from the column as one main peak I and several minor peaks. The homogeneity and neutrality of the polysaccharide issuing in peak I was also confirmed by ion-exchange chromatography on DEAE-cellulose in the carbonate form (Fig. 1c). After total acid hydrolysis of the homogeneous polysaccharide (I), D-glucose was identified by PC and TLC in several solvent systems. In addition to this, two ninhydrin-positive compounds were detected in very small amounts, one of which coincided in R_f value with an authentic sample of D-glucosamine while the other was probably its N-acetyl derivative. The high positive specific rotation of the glucan (peak I) before hydrolysis ($[\alpha]_D^{20}$ +251.9° (c 0.43%; water)) and afterwards ($[\alpha]_D^{20}$ +125° (c 0.406%; water)) shows that it has a chain consisting of D-glucose units with the α-configuration of the glucosidic bonds. The glucan is white and readily dissolves in water.

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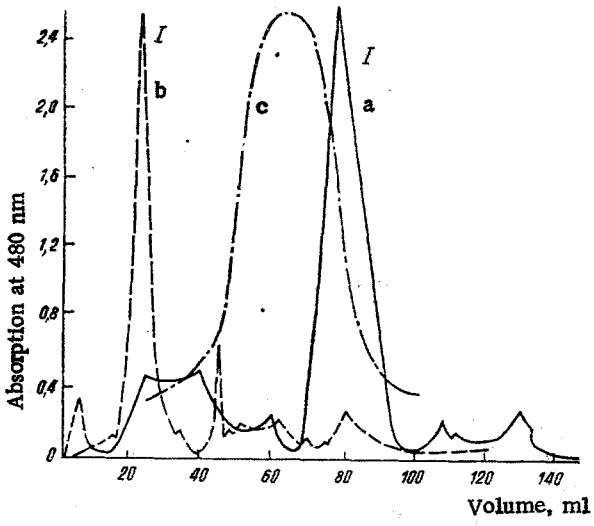

Fig. 1. Elution profiles of the polysaccharide (fraction I) from Sephadex G-150 (a), the polysaccharide (fraction I) from Sephadex G-200 (b), and the glucan from DEAE-cellulose (CO_3^{2-}) (c).

Thus, the polysaccharide component of the PLPC contains a high-molecular-weight α-glucan and a small amount of a heteropolysaccharide. The study of the structure of the α-glucan is continuing.

In biotesting on cotton-plant shoots of varieties 108-F and Tashkent-1 (3-4 true leaves) the PLPC and the α-glucan in concentrations of 1 mg/ml caused the symptoms of wilt.

EXPERIMENTAL

Filtrak FN Nos. 7 and 11 papers, cellulose, and KSK silica gel impregnated with 0.3 M NaH_2PO_4 were used for chromatography.

Solvent systems (by volume): 1) butan-1-ol—pyridine—water (6:4:3); 2) butan-1-ol—acetic acid—water (4:1:5); 3) propan-2-ol—ethyl acetate—water (7.5:13:5); 4a) pyridine—ethyl acetate—water—acetic acid (5:5:3:1); 4b) pyridine—ethyl acetate—water (11:40:6); 5) butan-1-ol—acetone—water (4:5:1); and 6) butan-1-ol—methanol—water (5:3:1).

Revealing substances: 1) aniline phthalate; 2) o-toluidine salicylate; 3) 0.2% solution of ninhydrin in acetone.

The polysaccharides were hydrolyzed with 1 and 4 N HCl for 4 and 6 h at 100°C, respectively. The hydrolysates were neutralized in a vacuum desiccator over NaOH.

The mycelium of the fungus Verticillium dahliae (strain 614, race II, isolated from a cotton plant of the variety Tashkent-1) was grown in a fermenter on a synthetic nutrient medium with Czapek salts and with the addition of a 2% solution of a starch (logarithmic phase, 48 h growth). It was separated from the culture liquid, washed with distilled water, and freeze-dried.

Isolation of the PLPC. The comminuted mycelium (200 g) was treated five times with a 10-fold excess of a 0.25 N solution of trichloroacetic acid by Boivin's method [5]. The extracts were combined and were dialyzed against distilled water. The dialysate was centrifuged and the supernatant was concentrated in vacuum at room temperature in a rotary evaporator and was freeze-dried. Yield ~4g.

Fractional Precipitation of the PLPC with Alcohol. With vigorous stirring until a permanent turbidity appeared, alcohol was added dropwise to a solution of 2 g of PLPC in 100 ml of distilled water up to a concentration of 39%. The precipitate that deposited was separated off by centrifuging and was dissolved in the minimum amount of water and freeze-dried. Yield 1.25 g (fraction A). More ethanol was added to the supernatant liquid until turbidity appeared (49%), and the precipitate was separated off and dried as described above. Yield 0.55 g (fraction B). More ethanol was added to this supernatant (66%), and the resulting precipitate was separated off and freeze-dried. Yield 0.09 g (fraction C).

Isolation of the Polysaccharide Fraction of the PLPC. The PLPC (1.25 g, fraction A) was hydrolyzed with 1% acetic acid (300 ml) at 100°C for 5 h. The precipitate of protein that deposited was separated off by centrifuging, and it was washed and dried in vacuum over P_2O_5. Yield ~0.043 g. The lipids were extracted with chloroform, and the aqueous fraction of the hydrolysate was concentrated in vacuum in a rotary evaporator at 40°C to give a neutral medium, and was then freeze-dried. Yield 1.03 g.

Fractional Precipitation with Ethanol. A 2% solution of 1.03 g of the polysaccharide fraction of the PLPC in distilled water was fractionated with ethanol as described above. This gave three fractions. Fraction I (0.96 g) was precipitated at a 40% concentration of ethanol, fraction II (0.004 g) at 52% of ethanol, and fraction III (0.001 g) at 60% of ethanol. The supernatant liquid was evaporated in vacuum at 40°C to a volume of 5 ml, and the polysaccharides were precipitated with ethanol (20 ml). The precipitate was separated off and dried in the usual way. Yield 0.03 g. D-Glucose and traces of D-glucosamine were detected in a hydrolysate of fraction I (PC in systems 1 and 2, and TLC in systems 3-6). Under similar conditions, D-glucose, D-galactose, D-mannose, a uronic acid, and traces of L-rhamnose and D-glucosamine were identified in fraction II.

The gel chromatography of the polysaccharide (30 mg, fraction I) was performed on a 53 × 2.5 cm column of Sephadex G-150 (fine) with elution by 1 M NaCl. Fractions with a volume of 5 ml were collected, and in 1-ml aliquots of each, the polysaccharides were determined by the phenol–sulfuric acid method [6]. The fractions composing the main peak I, V_e = 80 ml (14-19), were combined, dialyzed against distilled water, concentrated in vacuum, and freeze-dried (Fig. 1a).

When the polysaccharide (8 mg, fraction A) was subjected to gel chromatography in a 45 × 2 cm column containing Sephadex G-200 (fine) under the same conditions, the fractions (7-11) composing the main peak I with V_e = 25 ml were dialyzed against distilled water and freeze-dried (Fig. 1b). In hydrolysates of the main peaks from Sephadexes G-150 and G-200 D-glucose and traces of D-glucosamine were detected (PC in systems 1 and 2 and TLC in systems 3-6) by comparison with markers.

Chromatography on DEAE-Cellulose. DEAE-Cellulose was washed successively with 0.5 N HCl, water to neutrality, 0.5 N NaOH, water, 1 M $(NH_4)_2CO_3$, and water again, after which it was placed in a 33 × 2 cm column (V = 100 ml) and an aqueous solution of 10 mg of the polysaccharide (peak I) was deposited on it and was eluted with water and then with 1 M $(NH_4)_2CO_3$. Fractions with a volume of 3 ml were collected and 2-ml samples were used for the detection of polysaccharides by the phenol–sulfuric acid method (Fig. 1c). The fractions corresponding to the individual peak eluted with water (17-28) were combined, concentrated at 40°C in vacuum, and freeze-dried. In hydrolysates D-glucose and traces of D-glucosamine were detected (PC in systems 1 and 2 and TLC in systems 3-6) in comparison with markers.

SUMMARY

A phytotoxic PLPC has been isolated from the mycelium of the fungus Verticillium dahliae Kleb., strain 614. The polysaccharide component has been isolated from the PLPC by mild acid hydrolysis. The polysaccharide has been fractionated and the monosaccharide compositions of the fractions have been determined. A phytotoxic polysaccharide homogeneous according to the results of gel chromatography and chromatography on DEAE-cellulose which is an α-glucan containing a minor amount of D-glucosamine has been isolated.

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