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A NEUTRAL MANNAN FROM *CERATOCYSTIS FAGACEARUM* CULTURE FILTRATE

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Abstract—The culture filtrate of *Ceratocystis fagacearum* contains a mannan that produces some symptoms similar to oak wilt in red oak seedlings and cuttings. The mannan has a high molecular weight and a skeleton of α- (1 → 6) linked mannose units with considerable branching. Some similarities to commercial yeast mannan have been observed.

INTRODUCTION

A NEUTRAL polysaccharide composed of mannose has been isolated from the culture filtrate of *Ceratocystis fagacearum* (Bretz) Hunt, the oak wilt pathogen. Chemical characteristics similar to commercial yeast mannan were observed. Leaves wilted and dehydrated when the mannan was introduced into the vascular system of red oak cuttings.

Recognition that cultures of *C. fagacearum* produce phytotoxins responsible for oak wilting has led to chemical investigations. The presence of the oak-wilt-producing properties in the culture filtrate was established by Young.[1] Evidence was presented by White[2] that the culture filtrate contains at least two nonvolatile toxic components, one probably being a polysaccharide.

Polysaccharides having a wilt-inducing effect on tomato plants have been isolated from culture filtrates of *Fusarium solani* f. *eumartii* by Thomas,[3] from *Agrobacterium tumefaciens* and *Xanthomonas phaseoli* by Feder,[4] and from *Pseudomonas solanacearum* by Husain.[5] The wilt-inducing principle was attributed to the high MW polymers by Hodgson.[6]

RESULTS

The neutral polysaccharide, isolated by the procedure described in Experimental represented 25% of the nonvolatile, nondialyzable solutes in the culture filtrate. It had a specific rotation of +78° in water and gave a negative response to Fehling's solution and iodine–potassium iodide in strong calcium chloride. The IR spectrum of the product showed a distinct hydroxyl absorption peak. The aniline-diphenylamine phosphoric acid spray reagent produced a yellow coloration. Procion dyes produced highly colored complexes that were homogeneous on electrophoresis.

[1] R. A. YOUNG, *Phytopathology* **39,** 425 (1949).
[2] I. G. WHITE, *Am. J. Bot.* **42,** 759 (1955).
[3] C. A. THOMAS, *Phytopathology* **39,** 572 (1949).
[4] W. A. FEDER and P. A. ARK, *Phytopathology* **41,** 804 (1951).
[5] A. HUSAIN and A. KELMAN, *Phytopathology* **48,** 155 (1958).
[6] R. HODGSON, W. H. PETERSON and A. J. RIKER, *Phytopathology* **39,** 47 (1949).

PC and GLC revealed that mannose was the sole product resulting from acid hydrolysis of the polysaccharide. Elution data obtained from pore filtration on porous glass columns afforded a MW estimated to be $1{\cdot}07 \times 10^6$ for the material. Periodate oxidation studies showed a consumption of 0·8 mol of sodium metaperiodate with the release of 0·3 mol of formic acid per unit of anhydrohexose. The reduction and hydrolysis of the oxidized polysaccharide produced glycerol. The controlled acetolysis of the neutral polysaccharide produced an acetylated mixture of mannose and oligosaccharides that was chromatographically identical to the components in the acetolyzate of commercial yeast mannan.

Four red oak cuttings, each placed in 0·5% solutions of *C. fagacearum* mannan, took up, on leaf-dry wt basis, less than 16% as much solution as the water-fed control plants. The average dehydration of the cuttings in the mannan solution was 50× greater than the controls after 8 hr, and the leaves were curled and drying after 24 hr. Further studies, with emphasis on the biological significance of this mannan and other substances effective in the oak wilt disease, are being pursued in this laboratory.

DISCUSSION

The purified polysaccharide was homogeneous (immobile on paper chromatograms in systems A, B, and C, and eluted as a single peak on pore filtration) and nonreducing. Complete acid hydrolysis gave only mannose, and methanolysis gave methyl mannoside. The conditions necessary for complete hydrolysis indicate that some units are the more stable mannopyranose.

The mannan showed a positive rotation $[\alpha]_D^{20}$ +78° (0·5 water), which is indicative of a predominance of α-glycosidic linkages.[7] The negative response to iodine-potassium iodide in strong calcium chloride (failure to form a blue coloration or precipitate) indicates the absence of 1 → 4 glycosyl linkages and the presence of considerable branching in the linear chain.[8] When the mannan was spotted on paper, sprayed with aniline–diphenylamine phosphoric acid reagent, then heated, a yellow color was produced. This response has been attributed to 1 → 6 linked glycosidic polysaccharides.[9] Yeast mannan, when similarly tested, gave identical colorations with these reagents.

Table 1. Procion-dyed polysaccharides

Dye complex	% dye	Anhydromannose units/dye	Electrophoretic mobility (dye = 1)
C. fagacearum mannan-P. red	11·6	29·8	0·50
C. fagacearum mannan-P. blue	14·2	23·2	0·56
Commercial yeast mannan-P. red	18·1	17·9	0·66
Commercial yeast mannan-P. blue	20·2	15·1	0·71

The proportion of primary hydroxyl groups in a polysaccharide has been associated with its capacity to form a complex with Procion dyes.[10] The mannan produced colored complexes which, on purification by chromatography on Sephadex G15, were electrophoretically

[7] R. J. Yu, C. T. Bishop, F. P. Cooper, H. F. Hasenclever and F. Blank, *Can. J. Chem.* **45**, 2205 (1967).
[8] B. D. E. Gaillard, N. S. Thompson and A. J. Morak, *Carbohyd. Res.* **11**, 509 (1969).
[9] S. Schwimmer and H. Bevenue, *Science* **123**, 543 (1956).
[10] W. F. Dudman and C. T. Bishop, *Can. J. Chem.* **46**, 3079 (1968).

homogeneous. The dye content, the number of anhydromannose units to dye, and the electrophoretic mobility are shown in Table 1.

Molecular weight determinations of oligosaccharides and polysaccharides have utilized polyacrylamide gels, dextran gels (Sephadex), and glass of controlled pore size.[11] The neutral polysaccharide, when chromatographed on columns of Bio-Glas-500 and -1000, produced single, sharp elution peaks. Comparison of the elution peak with known dextrans demonstrated that the mannan had a high MW. Applying the generalization (elution volume is inversely proportional to the logarithm of the MW[12,13] to the elution data obtained on Bio-Glas-1000, revealed a MW range of 1·04–1·09 $\times$ 10^6. Yeast mannan similarly applied gave data that revealed a MW range of 1·32–1·35 $\times$ 10^6 (Table 2).

TABLE 2. MOLECULAR WEIGHT DETERMINATION DATA

	Bio-Glas-500 data			Bio-Glas-1000 data		
Polysaccharide	Elution wt	Log. MW	MW from curve ($\times 10^6$)	Elution wt	Log. MW	MW from curve ($\times 10^6$)
Blue dextran 2000	1·80	6·30	—	2·45	6·30	—
Dextran 250	3·30	5·40	—	4·25	5·40	—
Dextran 110	7·00	5·40	—	5·05	5·04	—
Commercial yeast mannan	2·60	5·80–6·10	0·63–0·12	2·80	6·12–6·13	1·32–1·35
C. fagacearum mannan	2·60	5·80–6·10	0·63–0·12	3·0	6·02–6·04	1·04–1·09

Periodate oxidation of the mannan produced a polyaldehyde which, on reduction and acid hydrolysis of the polyalcohol, yielded glycerol. The absence of erythritol in the hydrolyzate confirmed the absence of 1 $\rightarrow$ 4 linked mannose units in the linear portions of the mannan as well as 1 $\rightarrow$ 5 and 1 $\rightarrow$ 6 linked D-mannofuranose units.[7] Mannans of the *Candida* species have been shown to possess mannofuranose units at branch points.

The application of controlled acetolysis of yeast mannan has been utilized to effect cleavage of $\alpha(1 \rightarrow 6)$ linkages in the main chain and to liberate $\alpha(1 \rightarrow 2)$- and $\alpha(1 \rightarrow 3)$-linked side chain oligosaccharide acetates.[14] The procedure applied to the *C. fagacearum* mannan produced a mixture of acetates which, when chromatographed on thin layer silica gel plates, consisted of five components. The acetolysate of yeast mannan, chromatographed similarly, revealed mannose pentaacetate and four acetylated oligosaccharides with corresponding mobility. The deacetylated acetolysate mixtures from both mannans were identical on paper chromatography. On gas chromatography of the trimethylsilylated mixtures from each source, D-mannose and a disaccharide of the same identity, were observed in both mixtures.

EXPERIMENTAL

General Methods. The aqueous *C. fagacearum* culture filtrates were dialyzed. The nondialysable portion containing 1·55 g of solute per gallon was precipitated from concentrated solution by addition of EtOH. The precipitate was fractionated on a column of microcrystalline cellulose by washing with 90% EtOH

[11] W. HALLER, *Nature, Lond.* **206,** 693 (1965).
[12] H. DETERMANN and W. MICHEL, *J. Chromatog.* **25,** 303 (1966).
[13] M. JOHN, G. TRÉNEL and H. DELLWEG, *J. Chromatog.* **42,** 476 (1969).
[14] T. S. STEWART, P. B. MENDERSHAUSEN and C. E. BALLOU, *Biochem.* **7,** 1843 (1968).

followed by elution with water. The copper complex was formed by adding the crude polysaccharide in 1 N NaOH solution to Fehling's solution, and the mannan was regenerated by stirring the precipitate in a suspension of a strong cation exchange resin, Dowex ($50H^+$). Complete acid hydrolysis was performed by heating samples 2 hr with 1 N HCl in sealed tubes or by refluxing samples 24 hr in methanolic hydrogen chloride followed by refluxing 16 hr in 2 N HCl.

PC of the neutralized hydrolysates was performed on Whatman No. 1 using: (A) EtOAc–pyridine–H_2O (10:4:3, v/v); (B) *n*-BuOH–pyridine–H_2O (9:5:7, v/v); and (C) *n*-BuOH–EtOH–water (40:11:19, v/v) with detection by aniline diphenylamine phosphate, sodium metaperiodate-ammoniacal $AgNO_3$, or *p*-anisidine. HCl in *n*-BuOH. Acetolysates were chromatographed on silica gel plates with benzene–EtOAc (2:1, v/v) and detected by naphthoresorcinol–H_2SO_4–EtOH or ammonium bisulfate. Oligosaccharides were chromatographed on paper using EtOAc–HOAc–H_2O (2:1:1, v/v/v) with *p*-anisidine detection. GLC of TMS derivatives was made isothermally using a 5-ft column of 20% SE 30 on 60–80 mesh Chromosorb W with a thermal conductivity detector.

MW Determination. Equal amounts (1·6 mg in 0·5 ml H_2O) of dextrans of known MWs, yeast mannan, and *C. fagacearum* mannan were chromatographed on silanized Bio-Glas-500 and -1000 columns. Fractions, 0·13–0·14 g, collected using a drop-counter, were diluted to 1 ml and treated with 2 ml of 0·2% anthrone in 95% H_2SO_4 (w/v). This mixture was heated for 10 min in boiling water. The polysaccharide content was measured by reading the absorbance at 620 nm. The MW range was determined from elution curves (absorbance vs. elution wt) and a plot of elution wt against the logarithm of the MW.[12,13]

Procion dye complex.[10] The dye complex was prepared by treating 50 mg of the mannan in 5 ml H_2O with 50 mg of Procion dye in 5 ml H_2O, shaking the mixture 5 min, adding 0·2 g of NaCl and, after 30 min shaking, introducing 0·01 g of Na_2CO_3. After standing overnight, the solution was applied to a column of Sephadex G15, and the dyed polysaccharide was collected in the void volume.

Periodate oxidation studies. Samples of polysaccharide were oxidized with sodium metaperiodate and the consumption was followed spectrophotometrically.[15] The uptake was constant after 28 hr, and formic acid released during the oxidation was constant after 48 hr as found by titration.[16] Oxidized polysaccharide was dialyzed, reduced with $NaBH_4$ and the resulting neutral polyalcohol hydrolyzed. The hydrolysate was freed from acid and examined by paper and gas chromatography. Glycerol was the only identifiable component; no trace of erythritol or mannose was discernible.

Acetolysis. Acetolysis reactions were carried out by occasionally shaking a mixture of 136 mg of polysaccharide in 8 ml of a mixture of Ac_2O–HOAc–H_2SO_4 (10:10:1, v/v) at room temp. for 5 days, then warming at 60° to insure complete solution and quenching the reactions by adding 2 vol. of pyridine. The solution was evaporated to a small volume, and $CHCl_3$ was added. The $CHCl_3$ extract was water-washed, dried, and used for silica gel chromatography. Deacetylation with methanolic NaOMe provided the free oligosaccharides.

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[16] J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.* **78**, 5907, 5910 (1956).