

Studies on the Secretion of Maize Root Cap Slime

I. SOME PROPERTIES OF THE SECRETED POLYMER¹

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ABSTRACT

The secreted slime from root cap cells of corn (*Zea mays*, cv. SX-17) was studied. Production of slime by excised root tips is stimulated by the addition of 40 mM sucrose or fucose and half-strength Hoagland's solution to the incubation medium. Secreted slime was recovered from aqueous solution by precipitation with ethanol. The polymer has a molecular weight greater than 2×10^6 daltons and a density of 1.63 g cm^{-3} . Protein is not present in material purified by density gradient centrifugation with cesium chloride. Fucose (39%) and galactose (30%) are the principle neutral sugars found in the purified polymer. Galacturonic and glucuronic acids, arabinose, xylose, mannose, and glucose are also present.

(0.5 mM KH_2PO_4 ; 2.5 mM KNO_3 ; 2.5 mM $\text{Ca}(\text{NO}_3)_2$; 1 mM MgSO_4), 25 μM boric acid, 40 mM sucrose or fucose, 20 $\mu\text{g/ml}$ of chloramphenicol, and 5 $\mu\text{g/ml}$ of streptomycin. In those experiments using entire seedlings rather than excised root tips, the primary root was passed through a hole in a plastic support into the standard incubation medium in a 50-ml beaker. Root tips were excised at the end of the incubation and retained for analysis.

The incubation was terminated by making the medium to 80% (v/v) ethanol by the addition of absolute alcohol and keeping it at 4 C for a minimum of 12 hr. The precipitated material and root tips were collected together on glass fiber discs (Whatman GF/C) previously heated at 500 C for 1 hr to remove any carbohydrate (10). The material was washed five times with 95% (v/v) alcohol to remove soluble sugars. The samples were then dried and transferred to 20- \times 150-mm culture tubes with Teflon-lined screw caps. The internal standard, normally 2 or 4 mg of myoinositol, was added. Then the sample was hydrolyzed and alditol acetates were prepared as described below.

Several investigators have studied the mucilage or slime secreted by corn roots, but there is limited agreement on the chemical composition and properties of this secretion (9, 10, 13, 14). This could result from differences between the varieties of corn studied or from variations in culture methods or purification procedures. Before analyzing the secreted polysaccharide produced by roots of cultivar SX-17 of *Zea mays*, we undertook a detailed study of the culture conditions required for production of the material. We report results of our investigation into the production of the secreted polysaccharide and some of the chemical and physical characteristics of the purified polymer.

MATERIALS AND METHODS

Production and Collection of Mucilage. Seeds of a single crosshybrid maize (*Zea mays*, cv. SX-17), coated with Captan and Malathion, were soaked in aerated tap water for 36 hr at 23 C with three changes of water. Seedlings were then germinated on damp vermiculite or coarse sand covered with 4 layers of damp towelling for 36 hr at 23 C in the dark. Normally, 25 excised root tips were incubated in the dark at 23 C in 10 ml of medium in 50-ml Erlenmeyer flasks shaken at 80 cycles/min in a reciprocating shaker. Except when otherwise specified (Table I), the standard incubation medium consisted of 1 mM sodium acetate, 0.5 Hoagland's solution

The secretory product from root tips of whole seedlings was collected in two ways. The material designated as crude material was collected by wiping the root tip onto a 2.5-cm diameter glass fiber filter disc (Whatman GF/C, heated for 1 hr at 500 C). Discs carrying the adhering material were dried over anhydrous magnesium perchlorate in a vacuum desiccator. Material to be purified was prepared by aspirating the secreted material from the root tip and collecting it in a test tube. There it was diluted with glass-distilled H_2O to reduce its viscosity, and it was centrifuged at 20,000g for 30 min to remove sloughed off cells, cell walls, and other debris. The supernatant was made to 80% (v/v) ethanol and allowed to precipitate overnight at 4 C. The fibrous white precipitate was spooled onto a Pasteur pipette, and transferred to 95% (v/v) ethanol, and washed at least four times with 95% ethanol (8, 13). The precipitated material and the unprecipitated material combined with the washings were retained separately.

Rate of Slime Production. One hundred excised root tips, 1 to 1.5 cm long, were incubated for 20 hr at 23 C in 4 ml of standard incubation medium. At the end of the incubation period, roots were washed four times with H_2O , and the original medium, and washings were pooled and centrifuged at 21,000g for 30 min at 4 C to remove debris. The supernatant was dialyzed at 4 C for 2 days with frequent changes of distilled H_2O . The nondialyzable solution was transferred to a weighed beaker, the dialysis bag was washed three times with H_2O , and the washings were added to the beaker. The material was lyophilized, weighed, and the rate of slime production was calculated.

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Molecular Sieve Chromatography. Chromatography was carried out on a 83.5×2.5 cm column of 100/200 mesh Bio-Gel A-50 and 100/200 mesh Bio-Gel A-150 (Bio-Rad, Laboratories, Richmond, Calif.), with degassed 1 M NaCl as the eluant. The column was packed by gravity and flushed with the equivalent of 2 bed volumes with ascending eluant flow using a 30 cm head. Four milligrams of precipitated material were applied to the column and 3.5-ml fractions were collected and screened by the phenol-sulfuric acid method (7). Void volume was determined using tobacco mosaic virus and total volume was determined with glucose. The column was calibrated with dextran fractions of known mol wt (Sigma Chemicals). Fractions from the columns were pooled, dialyzed against distilled H₂O, and the ratio of methyl hexose to hexose was determined by the cysteine-sulfuric acid method of Dische and Shettles (6).

Equilibrium Density Gradient. Two milligrams of purified material were dissolved in 5 ml of 1.0 M sodium phosphate buffer (pH 7.4) and 5 g of cesium chloride (Metallgesellschaft A.G., West Germany) were added to the buffer. Tubes (12.7×50.8 mm) were filled completely, giving an initial density of 1.55 g cm^{-3} , and the gradient was formed in a Beckman L3-40 centrifuge with a SW50.1 rotor at 40,000 rpm (average 149,000g) for approximately 40 hr.

The concentrations of hexose and methyl-hexose were determined by the cysteine-sulfuric acid method of Dische and Shettles (6), using a 3-min boiling period. Densities were determined by measuring the refractive index with a Zeiss refractometer and applying the equations of Ifft, Voet, and Vinograd (12).

Protease Digestion. Five milligrams of precipitated material dissolved in 4 ml of 3 mM sodium phosphate buffer (pH 7.4) were digested with 10 mg of protease from *Streptomyces griseus* (Sigma Chemicals, repurified type VI) for 24 hr at 34 C (19). The digest was centrifuged at 20,000g for 30 min and the supernatant was dialyzed against distilled H₂O at 4 C for 2 days with frequent changes of H₂O. The nondialyzable material was lyophilized and analyzed in a CsCl gradient.

Viscosity Measurements. Determinations were made in 0.2 M NaCl in an Ostwald (capillary) viscometer at 25 C. Flow time for 0.2 M NaCl was 98.45 sec.

Ultracentrifugal Sedimentation Properties of Polysaccharide. The precipitated material was purified on a CsCl gradient. The fractions having densities of between 1.55 and 1.68 g cm^{-3} were pooled, dialyzed against distilled H₂O, and the nondialyzable material was lyophilized. A solution containing 1 mg/ml of this purified secreted material in 100 mM sodium phosphate buffer (pH 7.2) was centrifuged at 27,690 rpm in a Beckman Model E ultracentrifuge.

Hydrolysis. The materials (crude material, precipitated material, and unprecipitated material) to be analyzed for neutral sugars were placed with 2 mg of myoinositol as an internal standard in 20×150 mm glass culture tubes with Teflon lined screw caps. Preliminary analysis had not detected inositol in corn root cap material. For precipitated material, 2 to 5 mg were dried by lyophilization and then hydrolyzed with 0.1 ml of 72% (v/v) sulfuric acid for 1 hr at 30 C. The sample was shaken to effect solution, made to 3% (v/v) H₂SO₄ by addition of 2.7 ml glass-distilled H₂O, capped tightly, then placed in an autoclave for 1 hr at 15 p.s.i. to hydrolyze the equilibrium reversion products resulting from primary hydrolysis (20).

After cooling, neutralization was achieved by the procedure of Hough *et al.* (11). An equal volume of 10% (v/v) methyl-di-n-octylamine in chloroform was added and shaken vigor-

ously. After separation of the phases, the chloroform layer was removed and discarded. This was repeated three times or until the pH was greater than 6. Excess amine in the water phase was extracted with three changes of an equal volume of chloroform (10).

After neutralization the hydrolysate was filtered by passing through Whatman GF/C fiber filter discs previously heated to 500 C for 1 hr and lyophilized.

Preparation of Alditol Acetates. The lyophilized material was taken up in 1 ml of distilled H₂O containing 10 mg of sodium borohydride. After 1.5 to 2 hr at room temperature, excess borohydride was destroyed by dropwise addition of 1 M acetic acid until effervescence ceased. The contents were dried at 35 C under vacuum and borate ions were removed as trimethylborate by five successive additions and evaporations of methyl alcohol under a stream of air. The samples were dried for 10 to 15 min at 105 C.

The reduced material was acetylated by the addition of 1 ml of acetic anhydride and 0.1 ml of 98% (w/w) sulfuric acid as a drying agent. The reaction was allowed to proceed for 1 hr at 50 to 60 C. The reaction mixture was cooled and poured into an ice/H₂O mixture of at least 15 ml in a 125-ml separatory funnel. The alditol acetates were precipitated and extracted by shaking three times with 10-ml volumes of dichloromethane (1, 4), which was then evaporated under a stream of air at 40 C. Two milliliters of water were added and similarly evaporated.

Gas-Liquid Chromatography. Alditol acetates were separated on a 183×0.318 cm stainless steel column packed with 3% ECNSS-M on (100/200 mesh) Gas Chrom Q, or a 122×0.318 cm stainless steel column packed with 3% OV-225 on (100/120 mesh) Gas Chrom Q. Columns were prepared by Applied Sciences Laboratories (State College, Pa.). A dual column Varian Aerograph Model 204B gas-liquid chromatograph was used with N₂ as the carrier gas at 30 ml/min. Hydrogen flame ionization detectors were kept at 245 C with a H₂ flow rate of 27.5 ml/min. Injector temperature was 275 C and the column operated isothermally at temperatures between 180 C and 205 C.

Peaks on the chromatograms were identified by comparison of retention times with known standards under similar conditions. The areas under the peaks were determined by measuring height and width at half the height (21).

Table I. Effect of Salts on Production of Fucose-containing Polysaccharide by 1-cm Long Root-tip Sections

Medium ¹	Fucose-containing Polysaccharide
	moles fucose $\times 10^{-6}$
25 μ M Boric acid	0.433
2.5 mM Calcium nitrate	0.647
0.5 Hoagland's microsals	0.674
Distilled H ₂ O	0.814
2.55 M Calcium nitrate + 40 mM sucrose	1.939
2.5 mM Calcium nitrate + 40 mM sucrose + 25 μ M boric acid	2.275
0.5 Hoagland's + 40 mM sucrose	3.027
0.5 Hoagland's + 40 mM sucrose + microsals	3.313

¹ All incubation media contained 1 mM sodium acetate, 20 μ g ml⁻¹ of chloramphenicol, and 7 μ g ml⁻¹ of streptomycin. The 0.5 Hoagland's solution was: 0.5 mM KH₂PO₄, 2.5 mM KNO₃, 2.5 mM Ca(NO₃)₂, and 1 mM MgSO₄. The microsals were: 5 μ M MgCl₂, 2 μ M ZnSO₄, 0.5 μ M CaSO₄, 0.5 μ M NaMoO₄, 25 μ M H₃BO₃.

The amount of neutral sugar was calculated as follows:

% neutral sugar in polysaccharide

$$= \frac{\text{component area}}{\text{myoinositol area}} \times \frac{\text{weight of inositol (mg)}}{\text{oven dry weight of sample (mg)}} \\ \times \frac{0.88 \text{ pentoses or } 0.9 \text{ hexoses}}{\text{hydrolysis survival}} \times \frac{1}{\text{molar adjustment factor}}$$

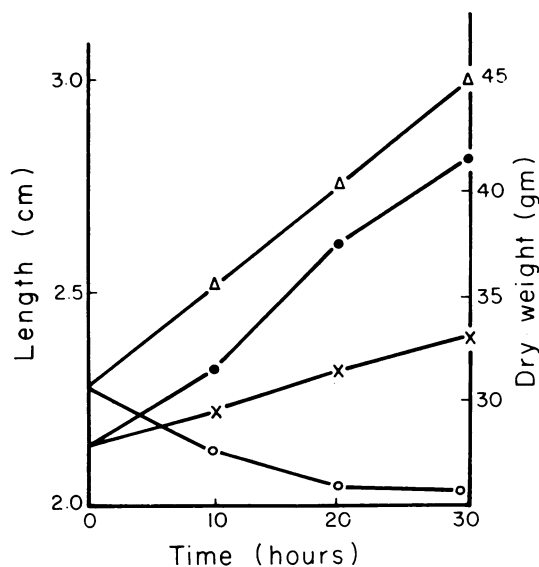


FIG. 1. Changes in length and dry weight of maize root tips incubated for 30 hr in 0.5 Hoagland's solution with and without 40 mM sucrose. Length, sucrose (●); length, without sucrose (×); dry weight, sucrose (Δ); dry weight, without sucrose (○).

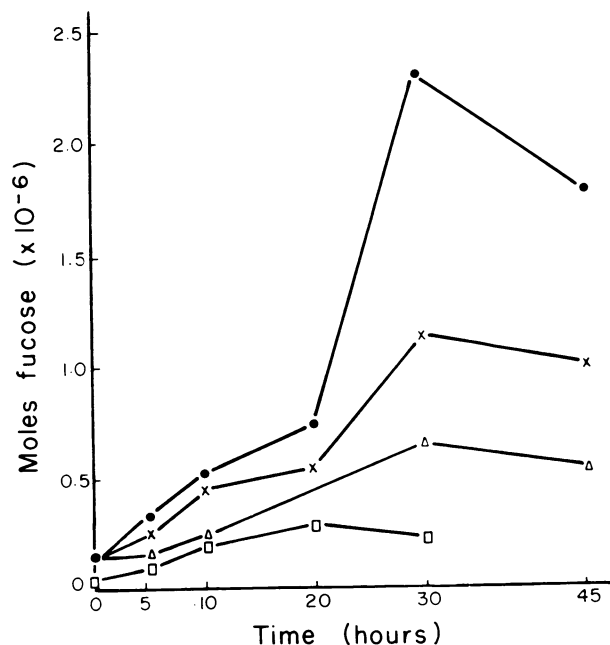


FIG. 2. The production of fucose-containing polysaccharides, expressed as moles of fucose, by maize root tips under various conditions. Intact seedlings incubated with 40 mM sucrose (●); 1.5-cm root tips with 40 mM sucrose, (×); 1.5-cm root tips with 20 mM sucrose (Δ); 0.5-cm root tips with 40 mM sucrose (□). All incubation media contained 0.5 Hoagland's solution.

Fucose has a molar adjustment factor of 0.882 with a hydrolysis survival of 91% under the conditions employed. The 0.88 pentoses or 0.9 hexoses adjust for mol wt change upon hydrolysis.

Qualitative Uronic Acid Determination. The procedure used was that of Harris and Northcote (10), except that Whatman No. 3MM paper strips, 55 × 3.8 cm, were used. The positions of the sugars and uronic acids were determined with freshly prepared aniline hydrogen phthalate (22).

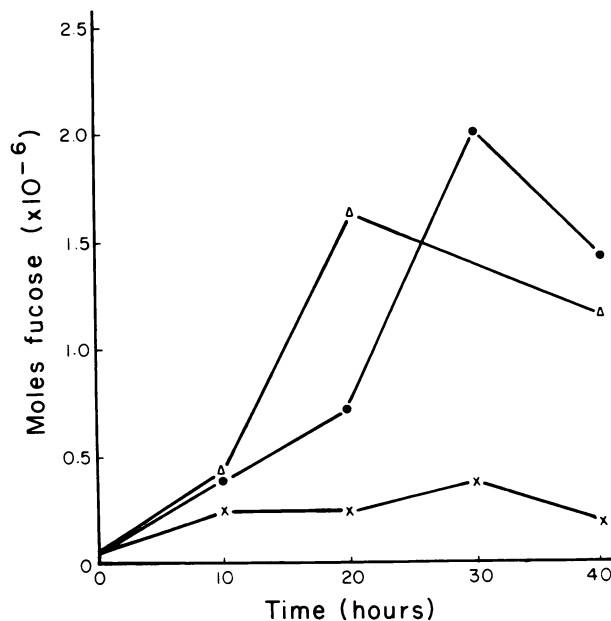


FIG. 3. The production of fucose-containing polysaccharide, expressed as moles of fucose, by 1.5-cm long maize root tips in the presence of either 40 mM sucrose (Δ) or 40 mM fucose (●). The control (×) was incubated in 0.5 Hoagland's solution.

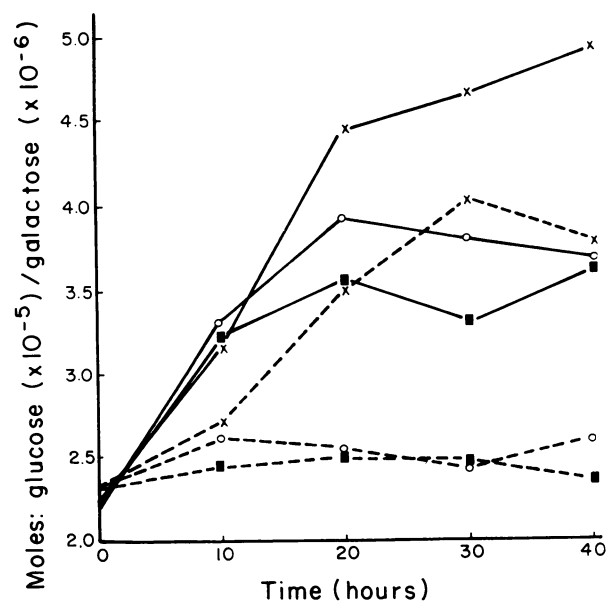


FIG. 4. The production of glucose- and galactose-containing polysaccharides by maize root tips (1.5 cm) with either 40 mM sucrose (×) or 40 mM fucose (○) in the incubation medium. The control incubation medium (■) contained only 0.5 Hoagland's solution. Galactan (—); glucan (---).

RESULTS AND DISCUSSION

Effects of Salts and Sugars on Polysaccharide Production. Twenty-five root tips (2 cm) were incubated for 35 hr in a medium with various inorganic salts as indicated in Table I, and the level of 80% (v/v) alcohol-insoluble fucose was determined. Root tips incubated in 0.5 Hoagland's solution with microsalts and 40 mM sucrose produced the greatest amount of polysaccharide (Table I) and increased linearly in

length and dry weight during 30 hr of incubation (Fig. 1). Root tips in salts but no sucrose produced less polysaccharide (Table I), increased only slightly in length, and decreased in dry weight (Fig. 1). Fucose-containing polysaccharide production was considerably higher in intact seedlings than in excised 0.5- and 2-cm root tips (Fig. 2). In excised tips, production increased with time up to 30 hr, then declined or remained constant. Both 40 mM fucose and sucrose stimulated fucose polysaccharide production above minus-sugar controls in 1.5-

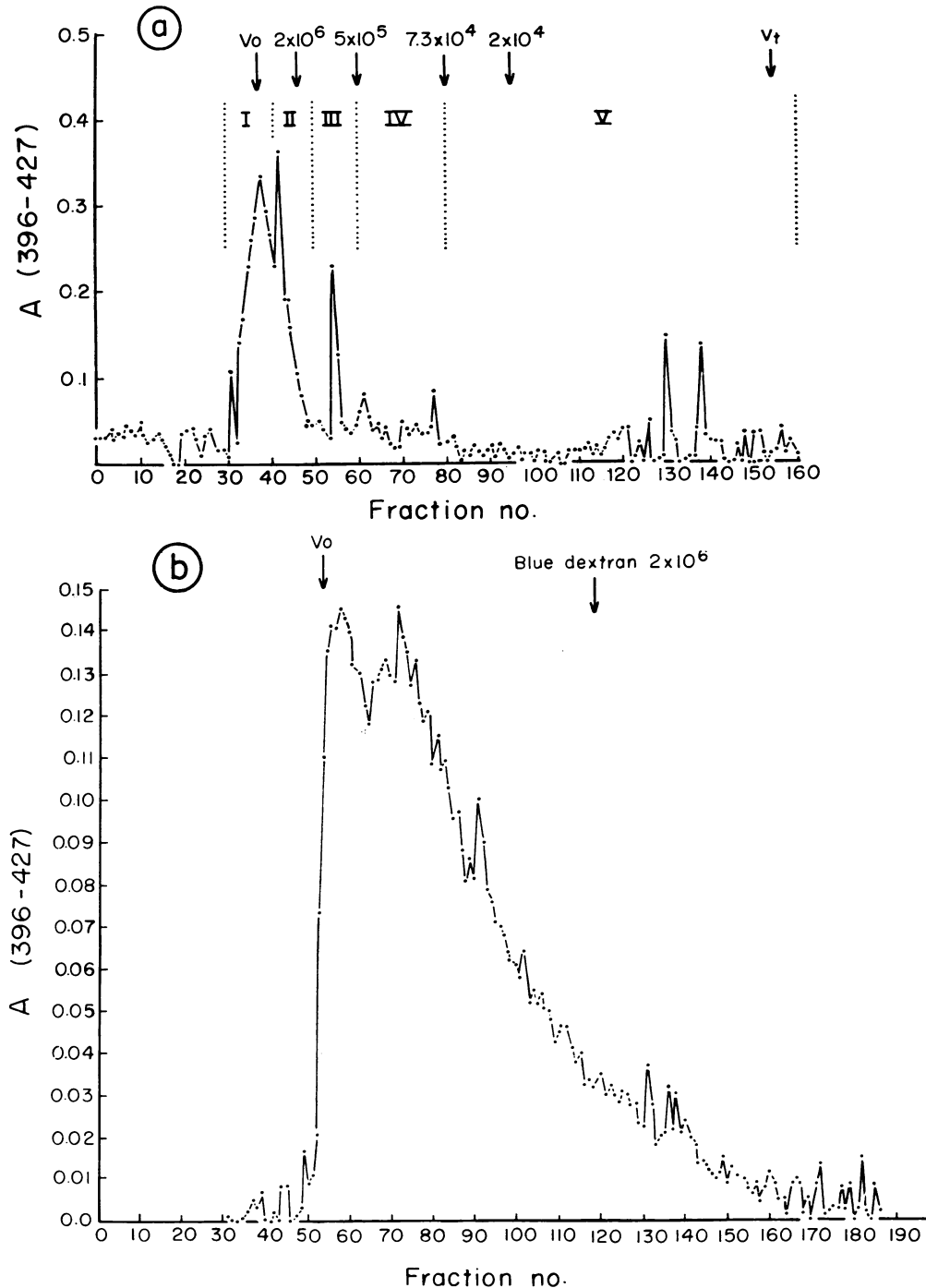


FIG. 5. The elution pattern of carbohydrates from Bio-Gel A-50 (a) and Bio-Gel A-150 (b) columns (83.5×2.5 cm). The crude mucilage was applied to the bottom of the column and eluted upwards with 1 M sodium chloride. The fractions were assayed by the procedure of Dubois *et al.* (7). The positions of dextran standards and void (V_0) and total volumes (V_t) are indicated by arrows. Roman numerals refer to fractions pooled and assayed for methyl hexose and hexose (Table II).

Table II. Ratio of Methyl Hexose to Hexose in Pooled Fractions from A-50 Column as Shown in Figure 5

Pooled Fraction	Methyl Hexose/Hexose
I	2.23
II	1.89
III	1.33
IV	0.867
V	0.67
Fucose Standard (20 μg) ¹	11.42

¹ The fucose standard gives the maximum ratio using the procedure of Dische and Shettles (6).

cm excised root tips. Whereas fucose stimulated production of a fucose containing polysaccharide (Fig. 3), it did not stimulate glucan synthesis and only slightly stimulated galactan synthesis (Fig. 4). Sucrose increases the production of all three polymers. As a result of our investigations of culture conditions, our standard incubation medium for the production of polysaccharide slime from excised root tips contained 40 mM sucrose, 0.5 Hoagland's solution plus boric acid. Root tips incubated for 20 hr in this medium produced the polysaccharide slime at a rate of 0.79 $\mu\text{g hr}^{-1}$ root tip⁻¹.

Molecular Sieve Chromatography. The elution pattern of the 80% (v/v) ethanol-precipitated material from a Bio-Gel A-50 column shows a single major peak of carbohydrate at or near the void volume and a smaller peak at the total column volume, with a number of very minor peaks between these two extremes (Fig. 5). A fucose-rich polymer elutes near the void volume as shown by the ratio of methyl hexose to hexose for the pooled fractions (Fig. 5; Table II). Thus, molecular sieve chromatography on Bio-Gel A-50 indicates a mol wt of 2×10^6 or greater. Chromatography of the precipitated material on Bio-Gel A-150 under similar conditions also shows elution of methyl hexose near the void volume (Fig. 5). Because carbohydrate standards of high mol wt are not available, estimates of mol wt of the sample from Bio-Gel A-150 cannot be determined.

Ultracentrifugal Analysis. In the analytical ultracentrifuge, the polysaccharide sediments as a large peak with a calculated sedimentation coefficient of 19.48S indicating a mol wt greater than 2×10^6 . There is a very small heavier component just behind this main peak.

Viscosity. A 0.1% (w/v) solution of ethanol-precipitable material has a relative viscosity of 7.1 and an intrinsic viscosity of 4.25. In a sequence of concentrations down to 0.003% (w/v), the intrinsic viscosity extrapolates to 2.4 at zero concentration. This intrinsic viscosity measurement suggests a mol wt higher than that indicated by exclusion chromatography. Using a light scattering technique, Floyd and Ohlrogge (8) reported a mol wt of 9×10^7 for nodal root slime of corn. The higher values obtained by viscometric and light scattering techniques probably reflect a high degree of intra- and intermolecular interactions rather than a high mol wt.

Density Gradient Equilibrium. The localization of methyl hexoses (fucose) and hexoses on a CsCl gradient is presented in Figure 6a. There is one major fucose-containing peak at a density of 1.63 g cm⁻³. Hexoses similarly occur at the same density but a smaller peak of hexose-containing material occurs at 1.39 g cm⁻³. Since protein bands at a buoyant density of 1.3 g cm⁻³ (5) and carbohydrates band at 1.6 g cm⁻³, sedimentation of the fucose-containing material at 1.6 g cm⁻³, suggests that it is mainly carbohydrate in composition, a protein component, if present, being small (3). Figure 6b compares the

equilibrium density of secreted mucilage with similar material after protease digestion. Protease digestion of the secreted mucilage does not cause the peak densities to shift, although it may cause a slight broadening of the peak.

Protein Composition. The ethanol-precipitable material has 8.3% to 10.8% (mean 9.2%) protein as determined by the method of Lowry *et al.* (16). Following purification of the secreted material on a cesium chloride gradient, the protein component of the 1.6 g cm⁻³ band was reduced to below the level detectable by this assay indicating, as did the banding of the material at density 1.6 g cm⁻³ in a CsCl gradient, a material principally carbohydrate in nature with little or no protein (Fig. 6).

Neutral Sugar Composition. A typical GLC chromatogram of neutral sugars from an hydrolysate of the secreted ethanol-precipitable material shows that fucose, galactose, and arabinose are the predominant sugars with smaller proportions of xylose, glucose, and mannose (Fig. 7). Calculation of percentage of neutral sugar composition from the peak areas of chromatograms confirms that fucose and galactose are the major sugars, comprising up to 69% of the total neutral sugars

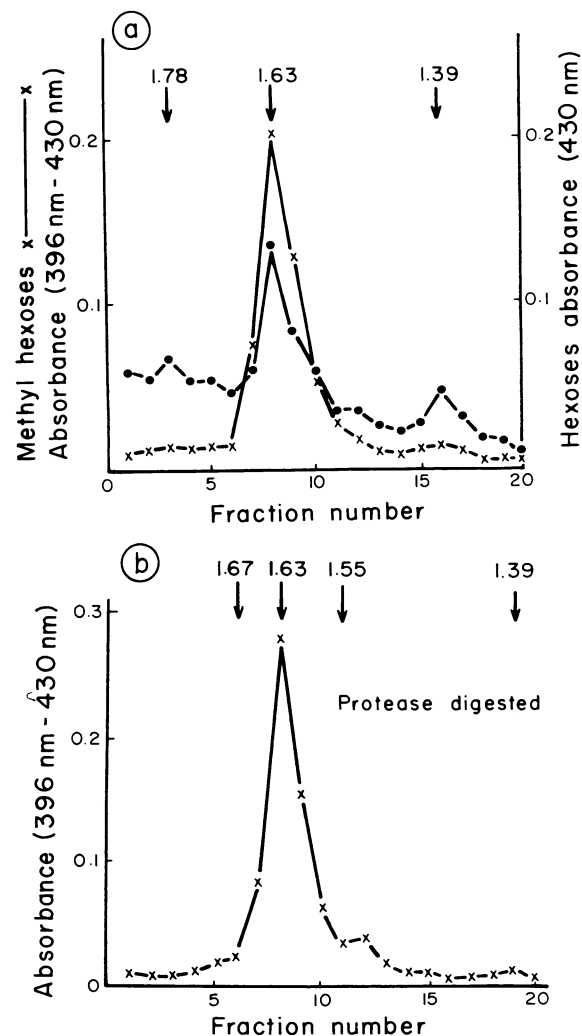


Fig. 6. a: Centrifugation of 80% (v/v) ethanol-precipitable material from maize root tips on a cesium chloride gradient. b: Centrifugation of ethanol-precipitable material on cesium chloride gradient after digestion with protease. Densities (g cm⁻³) indicated by arrows.

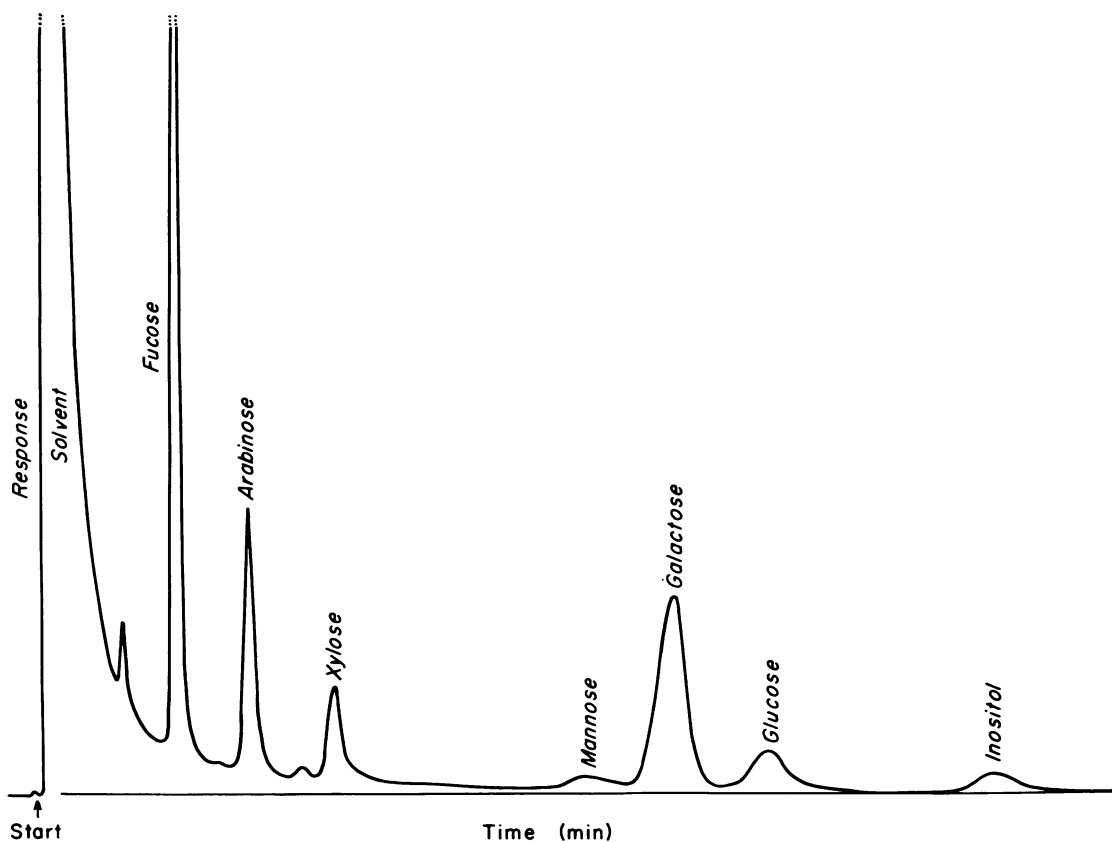


FIG. 7. GLC elution pattern of alditol acetates prepared from hydrolyzed, 80% (v/v) ethanol-precipitated slime from maize root tips

(Table III). Arabinose is present to 14.5% and other sugars are present at less than 7.5%. The small amounts of other sugars in this polysaccharide suggest a very definite structure based on two major neutral sugars and possibly galacturonic acid. Analysis of crude, nonpurified secreted material collected on glass fiber discs agrees closely with the results of Harris and Northcote (10) and with the analysis of the water-soluble, ethanol-insoluble fraction, but shows a high glucose percentage (Table III). Glucose represents 91% of the neutral sugars found in the ethanol-soluble fraction, after alcohol precipitation of secreted material, which would indicate that the glucose is a contaminant of the crude-secreted material (Table III). At no time was ribose detected in the water-soluble, ethanol-insoluble secreted fraction, though it was present to 1% in the ethanol-soluble washings.

The detection of fucose (6 deoxy-L-galactose) is significant because it provides a useful marker for the secreted material. Fucose is a widespread constituent of plant polysaccharides but is rarely found in high concentrations (17). Fucose is not a major component of root cap slime from wheat or other species analyzed (18), but occurs in all maize cultivars analyzed to date. The exudate on nodal roots of maize shows a similar neutral sugar composition with 23% galactose, 26% arabinose, 16% xylose, and 35% fucose (8). Although arabinose and xylose are higher in this analysis, fucose is still a major component. Jones and Morr  (14) reported only 8% fucose in secreted material from maize root tips. This result may indicate a varietal difference in the fucose percentage or a difference in incubation and purification techniques.

Uronic Acids. Both galacturonic and glucuronic acids were present in the water-soluble, ethanol-insoluble fraction. More intense stain at the position of galacturonic acid may suggest

Table III. Neutral Sugar Composition of the Hydrolysate of Maize Root Tip Slime and Washings

Results are adjusted for hydrolysis losses and the molar adjustment factor.

Sugars	Crude Mucilage	Water-soluble, Ethanol-insoluble Fraction	Ethanol-soluble Fraction
		%	
Fuc	24.07	38.58	0.32
Ara	9.36	14.51	1.17
Xyl	10.29	7.45	0.217
Man	1.8	3.07	5.02
Gal	35.63	30.41	1.23
Glc	18.85	5.68	90.91
Rib	0.0	0.0	1.09

it is present in larger quantities than glucuronic acid. Uronic acid has been detected by a number of workers in secreted root cap slime (8, 10, 14, 15), and Bowles and Northcote (2) showed galacturonic acid to be the major sugar acid, based upon studies with incorporation of glucose [¹⁴C].

CONCLUSIONS

This study has confirmed and extended the information on the production and characterization of maize root cap slime. Incubation of excised root tips in a medium containing sucrose stimulates the synthesis of glucan- and galactan-rich polymers whereas incubation in fucose results in the preferential production of a fucan-rich polymer. Molecular sieve chromatography and analytical ultracentrifugation of the slime indicate a mol

wt greater than 2×10^6 daltons, and viscometric determinations suggest a high degree of inter- and intramolecular interactions. The equilibrium density of slime purified by ethanol precipitation is 1.63 g cm^{-3} , a value which is unaffected by digestion with protease before centrifugation. Slime purified by ethanol precipitation is 9.2% protein. However, after centrifugation to equilibrium in CsCl, no protein can be detected in the polymer. Both buoyant density value and protein analysis suggest that secreted slime is composed primarily of carbohydrate. Fucose and galactose make up 69% of the neutral sugars of this polymer and arabinose and xylose comprise 22%.

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