

## Characterization of a Virus-Inhibiting Polysaccharide from *Phytophthora infestans*

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### ABSTRACT

A virus inhibitor was isolated from the mycelium of *Phytophthora infestans* and characterized as a water-soluble  $\beta$ -(1  $\rightarrow$  3) linked D-glucan with an average degree of polymerization of 23 glucose units with a single branching point. The yield of polysac-

charide relative to the age of the fungus culture reached a maximum in 7 days. The activity of the inhibitor was not affected by the age of the culture. *Phytopathology* 61:1006-1009.

*Additional key words:* hydrolysis, chromatography, periodate oxidation, methylation.

Although virus inhibitors have been found in a number of species of plants and fungi (5, 8, 16, 21, 23), relatively few have been characterized chemically. Tannins have been identified as the inhibitors in *Pelargonium hortorum* Bailey (7), protein in *Dianthus caryophyllus* L. (18), and polysaccharides in *Trichothecium roseum* Link. (3). An inhibitor of potato virus X (PVX) was demonstrated in extracts of potato leaves infected with *Phytophthora infestans* (Mont.) d By. (12). In a more recent report, a polysaccharide isolated from the mycelium of this fungus (13) was shown to inhibit lesion formation in tobacco by several viruses (22).

The present paper reports on the structure of this polysaccharide, and is part of a continuing study to establish the role of this compound in virus inhibition.

**MATERIALS AND METHODS.—Production and isolation of polysaccharide.**—The culture of the mycelium of *P. infestans* and the isolation of the polysaccharide have been described previously (13). In this study, nucleic acid was precipitated with protamine sulfate (15) instead of  $MnCl_2$ , as  $MnCl_2$  was found to interfere with subsequent periodate oxidation studies.

The relationship between the age of the mycelia and the yield and inhibitory activity of the polysaccharide was determined. Samples of mycelium were removed from the culture at intervals of from 3 to 20 days, the polysaccharide was isolated and weighed, and its inhibitory activity determined as previously described (22).

The yield of polysaccharide expressed as a percentage of the fresh wt of mycelium increased markedly during the first 7 days, resulting in an optimum yield of 4.2%, then declined to 1.2% by the 20th day. However, the inhibitory activity of the polysaccharide was not affected by the age of the culture (Fig. 1).

**Hydrolysis and chromatography.**—Polysaccharide (50 mg) was partially hydrolyzed with 2 ml of 1.0 N  $H_2SO_4$  at 100 C for 30 min, and after neutralization with  $BaCO_3$ , the hydrolysate was concentrated and the products separated by chromatography in *n*-butanol:pyridine:water (6:4:3) for 65 hr on Whatman No. 1 paper. For comparison, a sample of nigeran isolated from *Aspergillus niger* by the procedure outlined by Barker (2) was similarly hydrolyzed, and the products were separated by chromatography. Sugars were detected by spraying the chromatogram with aniline hydrogen phthalate (17).

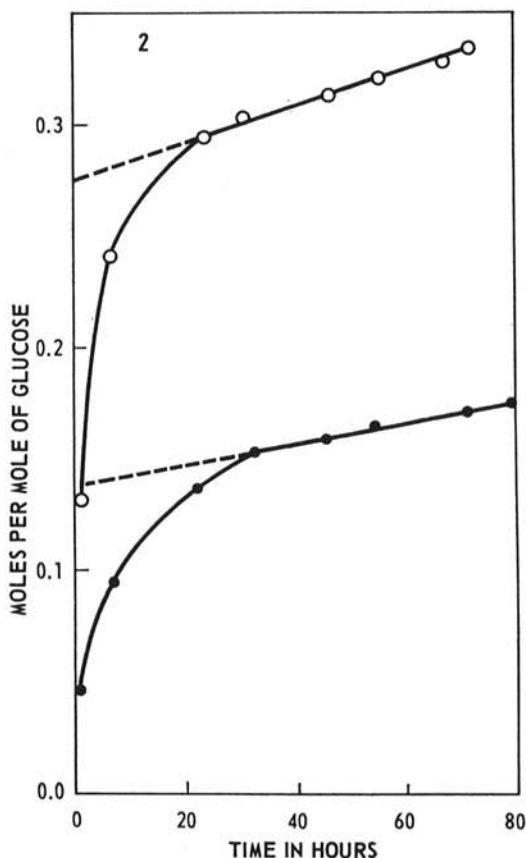
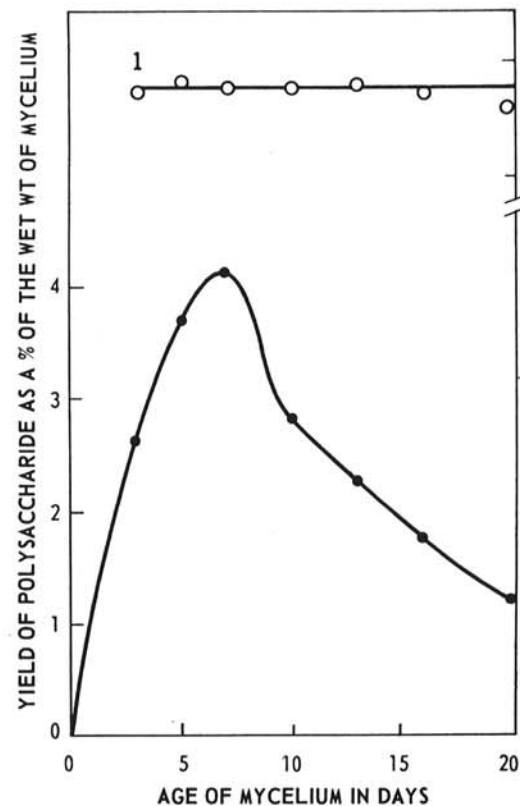
**Periodate oxidation.**—Polysaccharide (800 mg) was dissolved in 500 ml of 0.02 M sodium metaperiodate precooled at 5 C and oxidized at that temperature in the dark. Aliquots were removed at intervals for estimation of periodate consumed and formic acid produced. Unreacted periodate was determined by titration with 0.01 N sodium arsenite (9) and formic acid by titration with 0.01 N NaOH after destruction of excess periodate.

**Methylation.**—Freeze-dried polysaccharide (1 g) was further dried for 24 hr in a vacuum desiccator at room temperature, then dissolved in 50 ml of dry dimethyl sulfoxide. Methylsulfinyl-carbanion (10 ml) prepared according to Sandford & Conrad (20) was added, and the reaction mixture incubated with continuous stirring for 6 hr at 50 C in a nitrogen atmosphere. The temperature of the mixture was then lowered to 20 C, and 3 ml of methyl iodide were introduced dropwise over a period of 15 min. After 1 hr, the solution was dialyzed overnight against running tap water, then successively extracted with chloroform.

The methylated polysaccharide was dissolved in ice-cold 72%  $H_2SO_4$  and stirred for 2 hr at room temperature; after dilution to 8% with water it was hydrolyzed in a sealed tube at 100 C for 6 hr. After neutralization with  $BaCO_3$ , the hydrolysate was filtered and evaporated to dryness. The methylated sugars were then redissolved in methanol and separated by chromatography on Whatman No. 3MM paper in *n*-butanol:ethanol:water (4:1:5). The methylated sugars were eluted from the paper with chloroform and methanol, the solvents removed by evaporation, and the methyl sugars determined by the phenol-sulfuric acid procedure (11).

**Oxidation, reduction, and hydrolysis.**—Polysaccharide (400 mg) was dissolved in 100 ml of 0.05 M sodium metaperiodate and oxidized for 72 hr at 5 C in the dark. The periodate and iodate ions were then removed by precipitation with lead acetate, and the filtrate was treated with ion-exchange resin ( $H^+$  form) to remove excess lead ions. The resulting polyaldehyde was precipitated with 2.5 volumes of methanol.

The polyaldehyde was dissolved in 50 ml of water containing 200 mg of sodium borohydride, and reduction allowed to proceed for 48 hr at room temperature. The excess borohydride was destroyed by the addition of ion-exchange resin ( $H^+$  form). The filtered solution



was evaporated to dryness, and boric acid removed by repeated distillations with anhydrous methanol. The polyalcohol produced was hydrolyzed with 1.0 N  $H_2SO_4$  at 100 C for 16 hr; and after neutralization with  $BaCO_3$ , the hydrolysate was filtered, the filtrate concentrated to a small volume, and it was chromatographed in *n*-butanol:ethanol:water (4:1:5) on Whatman No. 1 paper. Glucose, erythritol, and glycerol were detected by spraying the chromatogram with ammoniacal silver nitrate.

*Degree of polymerization.*—Sodium borohydride (50 mg) was added to 50 mg of polysaccharide dissolved in water, and reduction was allowed to proceed for 48 hr at room temperature. The excess borohydride was destroyed by adjusting the pH to 5.5 with acetic acid. The solution was cooled to 5 C, and 5 ml of 0.5 M sodium metaperiodate solution were added, after which the volume was adjusted to 25 ml with  $H_2O$ . The oxidation was allowed to proceed in the dark; and periodically, aliquots were removed and analyzed for formaldehyde by the procedure outlined by Hay et al. (10). Erythritol treated as above was used as a reference compound.

**RESULTS AND DISCUSSION.**—The purified, freeze-dried polysaccharide is a water-soluble powder, reduces Fehling's solution, and has a specific optical rotation

$\left[ \frac{\alpha}{D} \right]_{22} - 7.5$  (4.0 in water). Complete acid hydrolysis followed by paper chromatography revealed glucose as the only monosaccharide, while partial acid hydrolysis revealed a series of oligosaccharides.

The disaccharide of this series was eluted from the paper and rechromatographed along with nigerose (the disaccharide obtained from the hydrolysis of nigeran) and maltose. The disaccharide and nigerose had  $R_{Maltose}$  values of 1.23 and 1.16, respectively, in the *n*-butanol:pyridine:water solvent. Saier & Ballou (19) list only two disaccharides of glucose with  $R_{Maltose}$  values greater than 1.00 in a solvent system based on *n*-butanol:pyridine:water, nigerose  $\alpha$  (1  $\rightarrow$  3), and laminaribiose  $\beta$  (1  $\rightarrow$  3), with laminaribiose having the highest value. Since the disaccharide had an  $R_{Maltose}$  value greater than nigerose in our solvent system, it was tentatively accepted that it is  $\beta$  (1  $\rightarrow$  3) diglucoside.

The graph obtained by plotting the log of  $R_{Maltose}$  against the degree of polymerization showed a linear relationship, indicating the presence of a homologous series of oligosaccharides (14), and consequently one type of glycosidic linkage.

Further evidence of predominately (1  $\rightarrow$  3)-glucosidic linkages in the polysaccharide was found by periodate oxidation. The polysaccharide consumed 0.27 moles of periodate with concomitant liberation of 0.14 moles of formic acid/mole of anhydroglucose (Fig. 2), which is in agreement with the calculated amounts

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**Fig. 1-2.** 1) Yield and inhibitory activity of polysaccharide at various growth periods of the mycelium of *Phytophthora infestans*. 2) Periodate oxidation of the polysaccharide isolated from *Phytophthora infestans*; ○ — ○ = periodate consumption; ● — ● = formic acid released.

(0.26 and 0.13 moles, respectively) for a (1 → 3)-linked glucan. After periodate oxidation, the polysaccharide was reduced with sodium borohydride, hydrolyzed, and chromatographed. The hydrolysate yielded glucose (major spot), glycerol (minor spot), and a trace of erythritol. A (1 → 3)-linked glucan would not be attacked by periodate except at the terminal glucose residues, and on reduction followed by hydrolysis, would yield glucose from the internal and glycerol from the nonreducing terminal glucose residues.

The degree of polymerization (number average) of the polysaccharide was determined from the following evidence. When the reducing terminal of a glucan is converted to an open-chain alcohol by reduction, then followed by periodate oxidation, the primary hydroxyl groups are oxidized and formaldehyde is liberated. If the reducing end is attached at the C<sub>3</sub> position, 2 moles of formaldehyde are liberated for each mole of glucan (10). During the periodate oxidation of the reduced polysaccharide 0.085 moles of formaldehyde were liberated/mole of anhydroglucose, corresponding to a degree of polymerization of 23 glucose units.

The degree of branching was estimated by methylating the polysaccharide, followed by hydrolysis and paper chromatography. The molar ratios of methyl sugars were: tetramethylglucose 2.14; trimethylglucose 22.6; and dimethylglucose 1.00, corresponding to one branching point in the molecule. Infrared analysis of the polysaccharides after two methylations showed no absorption in the hydroxyl region (3,500<sup>cm-1</sup>), indicating that methylation was complete.

The evidence presented characterizes the polysaccharide as a water-soluble glucan having an average degree of polymerization of 23 glucose units, one branching point, and containing β (1 → 3) glycosidic linkages. In the absence of authentic dimethylglucose samples, the linkage of the branching glucose residue was not determined. The specific rotation (-7.5 degrees) approximates that of laminarin (-9 degrees), a β (1 → 3) D-glucan (1). The infrared spectrum of the polysaccharide (KBr pellet) contains a band at 887<sup>cm-1</sup> typical of β-glucans (6) and no band in the 840<sup>cm-1</sup> region, typical of α-glucans (4).

The polysaccharide has been isolated in an essentially pure state, and its structural elucidation will facilitate more effective experimentation on its mode of action as a biologically active compound. Since this work was completed, Zevenhuizen & Bartnicki-Garcia (24) have reported the isolation of a polysaccharide from the mycelium of *Phytophthora cinnamomi* Rands which their results show is an important reserve material of the fungus. Although we have not tested the inhibitory activity of this polysaccharide, it appears to be structurally similar to the one we have isolated, which preliminary results indicate is also an important reserve carbohydrate source.

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