Extracellular Glucan Containing (1→3)- β and (1→6)- β Linkages Isolated from *Monilinia fructigena*

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(Received 17 March 1978)

Monilinia fructigena produced more extracellular polysaccharide when grown in liquid shake culture than in stationary culture, with greater quantities of the polysaccharide being secreted if ascorbic acid was present in the cultures. The polysaccharide was subsequently degraded during autolysis, eventually disappearing completely. After partial purification, the carbohydrate content was 96% and gel chromatography indicated a molecular weight of about 70000. Strong acid hydrolysis of the polysaccharide produced only glucose. Infrared spectroscopy indicated that the anomeric configuration of its D-glucose units was of the β -linkage type only. Periodate oxidation, Smith degradation and enzymic analysis indicated that the polysaccharide was a (1→3)- β -glucan having single (1→6)- β -glucosyl side-chains on every second D-glucose unit.

INTRODUCTION

Several fungi produce extracellular mucopolysaccharides containing (1→3)- β - and (1→6)- β -glucosidic linkages. One of the first polysaccharides of this type, 'sclerotan', was isolated from sclerotia of *Monilinia libertiana* and was shown by Kitahara & Takeuchi (1959) to be a (1→3)- β -linked glucan with single glucose residues attached at intervals by (1→6)- β -linkages. Johnson *et al.* (1963) investigated the structure of a similar polysaccharide produced by *Sclerotium rolfsii*. Analogous polysaccharides have been isolated from *Claviceps purpurea* (Perlin & Taber, 1963), *Pullularia pullulans* (Bouveng *et al.*, 1963), *Claviceps fusiformis* (Buck *et al.*, 1968) and *Schizophyllum commune* (Kikumoto *et al.*, 1970*a, b*).

The accumulation of these polysaccharides in the culture fluid may be due to a structural connection between the walls and the glucan that is produced extracellularly (Buck *et al.*, 1968). The regulation of extracellular macromolecule synthesis is not extraordinary but conforms to that controlling the synthesis of corresponding intracellular polymers (Bull, 1972). Extracellular polysaccharides are important because of their anti-tumour activities (Whistler *et al.*, 1976) and their use as food additives (Gorin & Spencer, 1968).

During the course of our studies on growth and autolysis of *Monilinia fructigena*, we observed the production of a mucilaginous-like substance in the culture filtrate. In this paper we describe this mucilage as a new polysaccharide from *M. fructigena*, containing (1→3)- β - and (1→6)- β -glucosidic linkages.

METHODS

Organism and culture conditions. *Monilinia fructigena* (ATCC 24976) was obtained from Dr R. J. W. Byrde (University of Bristol, Bristol). Conidia were obtained by the method of Byrde & Fielding (1968). To produce the polysaccharide, 60 ml portions of a mineral medium supplemented with Difco yeast extract (1 g l⁻¹) (Reyes & Byrde, 1973) were placed in 300 ml conical flasks, steam-sterilized on three successive days for

30 min each day, and then inoculated with 1 ml of a suspension of 1.6×10^4 conidia ml^{-1} . Cultures were incubated in the dark at 25 °C either unshaken or shaken on an orbital shaker at 80 rev. min^{-1} .

Sampling. The mycelium was separated from the culture medium by filtration. Most of the polysaccharide was found in the culture medium, but a little was retained by the mycelium giving it a gelatinous character. The mycelium was washed with 60 ml distilled water and stirred for 2 h to separate it from the mucilage; it was then dried to constant weight at 70 °C. The polysaccharide was precipitated from the culture filtrate and washed by adding acetone (final concentration 30 %, v/v). The polysaccharide was redissolved in 25 ml distilled water and reprecipitated with acetone three times. It was then dehydrated with acetone and dried in a stream of air.

Physico-chemical properties. Before physico-chemical measurements, polysaccharide solutions were filtered through a no. 2 (Pobel, Madrid, Spain) filter funnel with sintered disc (pore size 40 to 90 μm). The filtrates had a polysaccharide concentration of 0.25 mg ml^{-1} .

Viscosity was measured using a Cannon-Fenske type capillary viscometer at 37 °C. Results are shown as intrinsic viscosity, and relative to the viscosity of water (η_{rel}).

Specific optical rotation was measured using a Dr Steeg & Reuter model SR-6 polarimeter.

Density was calculated using standard pycnometric procedures (James, 1967).

Infrared spectra of samples dispersed in KBr discs were obtained using a Perkin Elmer 457 spectrophotometer by Dr J. Bellanato (Instituto de Optica, C.S.I.C., Madrid, Spain).

Elementary analyses were performed in the Departamento de Análisis y Técnicas Instrumentales (Instituto de Química Orgánica General, C.S.I.C., Madrid, Spain).

Gel filtration and chemical analyses. Samples (1 mg ml^{-1}) were applied to columns (Pharmacia K 26/40 and K 26/100) of Sephadex G-100 or G-75 previously equilibrated with 0.05 M-borate/citrate/phosphate buffer, pH 5.5, or distilled water. The elution rate (descending flow) was 14 ml h^{-1} and fractions of 3.5 ml were collected. Columns were calibrated with Dextran Blue 2000000 (Serva) and dextrans T-10, T-40 and T-70 (Pharmacia). The carbohydrate content of each fraction was determined by the anthrone method of Trevelyan & Harrison (1952) using glucose as standard. In some experiments the polysaccharide was subjected to ultrasonic treatment for 8 min in an ultrasonic disintegrator (MSE model MK2, 150 W).

Protein content was measured using bovine serum albumin as standard (Lowry *et al.*, 1951).

Total reducing substances were determined by the Somogyi method (1945) in conjunction with that of Nelson (1944) using glucose as standard.

Free glucose in the enzymic and chemical hydrolysates was estimated with glucose oxidase (De la Fuente & Sols, personal communication). The glucose oxidase reagent was a solution of 50 mM-phosphate buffer, pH 6.5, with 0.4 mM- MgCl_2 containing (per ml) 0.4 μg Triton X-100, 100 μg *o*-dianisidine, 5.4 units glucose oxidase and 3 units peroxidase (both enzymes from Boehringer, grade I). Sample, in a volume of 0.2 ml, was incubated with 1 ml glucose oxidase reagent for 1 h at 37 °C before measuring the absorption at 425 nm.

Free glycerol in chemical hydrolysates was estimated with glycerokinase using the method of Wieland (1963) as adapted by Dr Lagunas (personal communication). The reaction was carried out using a sample volume of 1 ml containing 0.125 M-Tris, 0.2 M-hydrazine, 0.2 M- MgCl_2 , 1 mM- NAD^+ and 1.2 mM-ATP plus 0.5 units glycerol-3-phosphate dehydrogenase (final pH 9.0). Absorbance was measured at 340 nm; then 0.5 units glycerokinase were added and the change in absorbance was determined after 1 h.

Sugar composition. Polysaccharide samples were hydrolysed in 0.5 M- H_2SO_4 at 100 °C in sealed tubes for between 0.5 and 7 h. Prior to chromatography, hydrolysates were neutralized with $\text{Ba}(\text{OH})_2$ and filtered through a Millipore filter (GS 0.22 μm pore size).

Exploratory thin-layer chromatography of sugars was done on cellulose plates (Merck, TLC Al sheets of cellulose, without fluorescent indicator, layer thickness 0.10 mm). Descending paper chromatography was done on Whatman no. 1 paper using the solvent system butan-1-ol/acetic acid/water (40:10:22, by vol.) (Partridge, 1948). Reducing compounds were detected with silver nitrate in acetone, followed by ethanolic sodium hydroxide (Trevelyan *et al.*, 1950).

Periodate oxidation and Smith degradation. The procedure was carried out using methods described by Sietsma & Wessels (1977). Polysaccharide solutions in water (25 ml, 1 mg ml^{-1}) were treated with 0.03 M-sodium periodate (25 ml) at room temperature in the dark for 96 h. The periodate consumption was followed by measuring the decrease in absorbance of IO_4^- at 223 nm with a correction for absorbance of IO_3^- . Controls with glucose and blanks were carried out. The production of formic acid was determined by titration with 0.001 M-NaOH (standardized against potassium hydrogen phthalate to a methyl red endpoint). A calculated amount of ethylene glycol was added to destroy the excess periodate and the suspension was dialysed against distilled water for 24 h to remove inorganic salts. NaBH_4 (174 mg) was added to the dialysed solution, containing the oxidized polysaccharide, and reduction was allowed to proceed at room temperature for 20 h. A previously determined amount of acetic acid was added to neutralize the solution which was then dialysed against distilled water for 24 h. Samples were taken for hydrolysis after periodate oxidation and Smith degradation.

Table 1. *Production and degradation of polysaccharide by Monilinia fructigena*

Monilinia fructigena was grown in a mineral medium supplemented with Difco yeast extract having ammonium tartrate as nitrogen source and glucose as carbon source, in the presence of ascorbic acid. Results are the mean of four replicates in stationary cultures and two replicates in shaken cultures.

Stationary cultures				Shaken cultures			
Incubation time (d)	pH	Poly-saccharide (mg dry wt per flask)	Mycelium (mg dry wt per flask)	Incubation time (d)	pH	Poly-saccharide (mg dry wt per flask)	Mycelium (mg dry wt per flask)
4	4.1	2.7	42.9	3	3.6	trace	62.5
5	3.7	4.5	108.9	5	3.9	10.1	113.7
8	3.3	10.2	114.9	7	4.1	21.5	131.0
10	3.2	10.5	115.2	8	4.1	14.0	135.7
13	3.3	10.2	240.6	10	4.1	14.5	110.0
15	3.3	9.0	232.5	11	4.2	12.5	106.8
18	4.1	7.2	291.0	15	4.3	9.1	110.0
32	5.3	7.2	186.3	23	4.9	7.9	110.0
41	5.4	2.6	91.0	36	5.6	trace	119.6
56	5.8	0	78.5	44	5.7	0	121.9
82	5.9	0	80.1				

Table 2. *Effect of ascorbic acid concentration in the culture medium on the formation of polysaccharide in stationary cultures (7 d) of Monilinia fructigena*

Ascorbic acid (mg per flask)	Polysaccharide (mg per flask)
1	2.5
2	2.5
3	2.5
4	6.9
5	8.2
8	10.5
12	8.0

Enzymic hydrolysis. A lytic enzyme preparation, containing (1→3)- β -glucanase was obtained from autolysed cultures of *Monilinia fructigena* (Reyes & Lahoz, 1977). The (1→3)- β -glucanase was partially purified by gel filtration on Sephadex G-75 or G-100 equilibrated with borate/citrate/phosphate buffer as described above. Fractions containing the required enzyme activity were used without further purification. This enzyme preparation showed exo-(1→3)- β -glucanase activity with laminarin (Koch-Light) as substrate, but showed no activity on carboxymethylcellulose (Na salt, Serva), amylose (Merck) or (1→6)- β -glucan (pustulan, prepared by us as described by Reese *et al.*, 1962). A little gentiobiase (6 % of the initial activity) remained in the fractions of highest (1→3)- β -glucanase activity, but was absent from the subsequent fractions.

Polysaccharide solutions (1 ml, containing 1 mg ml⁻¹) were incubated with 1 ml of the enzyme preparation at 37 °C for between 30 min and 5 h. For paper and thin-layer chromatography, the resulting solution was dried at 60 °C, extracted with pyridine at 30 °C for 15 min and filtered.

RESULTS

The fungus grew as a mat in stationary cultures and in shaken cultures as a homogeneous biomass formed by filamentous aggregates.

Polysaccharide production

More polysaccharide was produced in shaken cultures than in stationary cultures (Table 1). Degradation of polysaccharide in both cultures always started before maximum growth was attained (taken as the starting point of autolysis). During autolysis, degradation of the

Fig. 1. Gel chromatography of *Monilinia fructigena* polysaccharide on Sephadex G-100 (Pharmacia 26/100 column). Arrows indicate (from left to right): V_0 of the column; V_e of dextrans having average molecular weights of 70000, 40000 and 10000 respectively.

polysaccharide and mycelium took place simultaneously in stationary cultures, while in shaken cultures there was a further small increase in the dry weight of the mycelium. When the pH of the medium reached values of about 5.5 the polysaccharide disappeared from the culture medium.

Experiments carried out in the presence of ascorbic acid indicate that the formation of polysaccharide was always favoured by the presence of this substance (Table 2).

For analytical purposes, the polysaccharide was harvested from shaken cultures after incubation for 7 d.

Physico-chemical properties

The specific optical rotation, $[\alpha]_D^{20}$, was zero. The intrinsic viscosity was 48.9 dl g^{-1} and η_{rel} was 2.23 at 37°C . The density was 0.997 g ml^{-1} at 23°C .

The infrared spectrum resembled those of laminarin and paramylon (Michell & Scurfield, 1970) with bands at 890 , 1200 , 1248 , 1370 and 2920 cm^{-1} , but with an additional band at 1730 cm^{-1} . This band was probably caused by some impurity because it disappeared when the polysaccharide was subjected to further purification (dissolution and reprecipitation a further five times). The spectrum did not show significant absorption in the region of 1550 to 1535 cm^{-1} which might result from protein contamination.

Gel filtration

During gel filtration, polysaccharide that had not been treated ultrasonically was partially retained by the column and was always eluted unpredictably in volumes of six or seven times the elution volume of the column. The polysaccharide which had been subjected to ultrasonic treatment was eluted from a Sephadex G-100 column in borate/citrate/phosphate buffer at a volume corresponding to an average molecular weight of about 70000 (Fig. 1).

Chemical analysis

Chemical analysis revealed 96% carbohydrate and a protein concentration of less than 1.5%. Free glucose was not detected in the polysaccharide solution. Elementary analysis gave (%): C, 38.67; H, 5.89; N, 0.06; O (by difference), 55.30; P, 0.00; S, 0.00. The proportions of C, H and O are close to the theoretical values for a glucan of 96% purity.

Table 3. *Sugars and oligosaccharides detected by paper chromatography in the hydrolysate of a polysaccharide from Monilinia fructigena and from laminarin*

Results show the mobilities (R_G) of spots in the chromatograms relative to that of glucose (R_G 1.00), and their relative intensities (+ to + + +). The spot at R_G 0.55 is gentiobiose.

Polysaccharide	Laminarin
—	0.14 +
0.27 +	0.26 +
0.34 +	0.33 +
0.40 +	0.41 +
—	0.46 +
0.55 + +	0.55 + +
0.78 +	0.76 + +
1.00 + + + +	1.00 + + + +
1.11 +	1.10 +

Sugar composition

Paper and thin-layer chromatography of the acid hydrolysates of the polysaccharide showed that glucose alone was obtained after hydrolysis for 7 h or more. After hydrolysis for 2 to 3 h, glucose and gentiobiose were obtained. No significant quantity of polysaccharide was hydrolysed in less than 1 h. Hydrolysis for between 1 and 1.5 h yielded a variety of substances; their relative mobilities are shown in Table 3, together with those for substances obtained by gentle hydrolysis of laminarin (0.5 h, 0.25 M-H₂SO₄). In both hydrolysates there were traces of a substance with an R_G of 1.10 or 1.11, possibly mannose. The substances with R_G values less than that of gentiobiose (0.55) are probably oligosaccharides of laminarin (laminaribiose, triose, tetrose, etc.) which disappear with longer hydrolysis.

Enzymic hydrolysis with the purified (1 → 3)- β -glucanase from *M. fructigena* yielded only glucose and gentiobiose in a molar ratio of 1:1.

Periodate oxidation and Smith degradation

The results obtained by periodate oxidation were calculated with reference to the molar concentration of glucose in the polysaccharide: 0.67 μ mol periodate were reduced per μ mol anhydro-glucose unit and 0.33 μ mol formic acid were produced per μ mol anhydro-glucose unit, indicating that the percentages of (1 → 3), (1 → 6) and (1 → 4) linkages, respectively, were 66, 33 and 1 (Jeanes, 1965).

After degradation, the remaining polysaccharide was subjected to hydrolysis. The unhydrolysed polysaccharide was recovered, dried and weighed; the degree of hydrolysis was 39% after 7 h. By paper chromatography, only glucose and glycerol were detected. These products were confirmed enzymically. The molar ratio of glucose to glycerol after 7 h hydrolysis was 1:2.08. Linkages of the (1 → 6) type were not detected.

DISCUSSION

The production of extracellular glucans by filamentous fungi may be related to the existing glucans which form part of the wall, as in *Schizophyllum commune* (Sietsma & Wessels, 1977) and *Claviceps* species (Buck *et al.*, 1968). Therefore, it is not surprising that the same fungus can produce structurally different types of extracellular exopolysaccharides under different conditions, which also lead to structurally different glucans in the wall. This could explain the difference between the exopolysaccharide previously obtained from *Monilinia fructigena* (Archer *et al.*, 1977) and that described here. However, comparisons of results are difficult since the culture media employed were different and ascorbic acid may also increase the production of the polysaccharide. In view of the multinucleate nature of *M. fructigena*

hyphae and conidia, culture on a rather unusual medium may lead to preferential selection of those nuclei associated with specific areas of metabolism (R. J. W. Byrde, personal communication). Furthermore, ascorbic acid may have some lytic effect on fungal wall polymers. The data of Table 1 indicate that the polysaccharide is degraded during autolysis and may be re-metabolized as indicated by the later slight increase in dry weight in shaken cultures.

The infrared spectrum with absorption at 890 cm^{-1} and the lack of absorption at 840 cm^{-1} indicated that the anomeric configuration of the D-glucose units is of the β -type.

The viscosity of a solution of a macromolecule is a simple measure of its asymmetry. The intrinsic viscosities for extracellular fungal polysaccharides are high (Wallen *et al.*, 1965; Archer *et al.*, 1977). Similarly this polysaccharide has a high viscosity indicating a certain asymmetry or a high degree of hydration, or both.

Periodate oxidation and enzymic analysis indicated the presence of 66% (1 $\rightarrow$ 3)- β linkages and 33% (1 $\rightarrow$ 6)- β linkages in the polysaccharide. The presence of only glycerol and glucose after Smith degradation confirmed the existence of these linkages. Thus the polysaccharide can be considered as a (1 $\rightarrow$ 3)- β -D-glucan having single (1 $\rightarrow$ 6)- β -D-glucosyl side-chains on every second D-glucose unit (Marshall, 1974; Gorin & Spencer, 1968).

This polysaccharide resembles that described by Feather & Malek (1972) from *Monilinia fructicola* in having 66% (1 $\rightarrow$ 3) linkages, but differs considerably in the content of (1 $\rightarrow$ 4) linkages; the polysaccharide from *M. fructicola* has a relatively high content (33%) of (1 $\rightarrow$ 4) linkages but this type of link is absent in the glucan secreted by *M. fructigena*. Therefore, the polysaccharide described here has the most common linkage type for branched fungal glucans, that is, (1 $\rightarrow$ 3) linkages with branching via (1 $\rightarrow$ 6) linkages (Archer *et al.*, 1977). It differs from the glucan isolated from *M. fructigena* by Archer *et al.* (1977) in which the main linkage was of the (1 $\rightarrow$ 4) type, with a low proportion of (1 $\rightarrow$ 2) linkages.

We thank Dr S. A. Archer for helpful advice and for critical reading of the manuscript. F.S. is greatly indebted to the Departamento de Microbiología de la Facultad de Ciencias de la Universidad Autónoma de Madrid for allowing him to do this work. We acknowledge with thanks the technical assistance of Miss Teresa Raposo.

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