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Note

Structure of the extracellular β-D-glucan from *Botrytis cinerea*

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The fungus *Botrytis cinerea*, a ubiquitous plant-pathogen, produces an extracellular (1→3,1→6)-β-D-glucan responsible for clarification difficulties in wine technology[1–3]. This was confirmed by Montant and Thomas[4,5], who isolated a similar D-glucan from liquid cultures, and Leal *et al.*[6] investigated the effect of carbon and nitrogen sources on the production of this polysaccharide. We now report on the structure of *Botrytis cinerea* β-D-glucan.

Gel filtration of the β-D-glucan on Sepharose 6B indicated a range of molecular weights from 10^5 up to $>10^6$, with a preponderance of material of high molecular weight. Ultracentrifugation analysis indicated the average molecular weight to be 912,000.

Six products were identified after methanolysis of the methylated glucan (Table I), with retention times corresponding to methyl 2,3,4,6-tetra-, 2,4,6-tri-, and

TABLE I

G.L.C. ANALYSIS OF METHYL ETHERS OBTAINED FROM METHYLATED GLUCAN OF *Botrytis cinerea*

Methyl D-*glucoside*	O-*Methyl derivatives*			O-*Acetyl*-O-*methyl derivatives*		
	T *(min)*	*Area ratio*	*Molar ratio*	T *(min)*	*Area ratio*	*Molar ratio*
2,3,4,6-Tetra-*O*-methyl-α-	8.2	31	1	8.4	(18)	
-β-	6.0			6.1		
2,4,6-Tri-*O*-methyl-α-	18.2	47	1.51	22.8	48	1.45
-β-	14.8			18.1		
2,4-Di-*O*-methyl-α-	28.8	(22)		33.4	33	1
-β-	24.8			28.6		

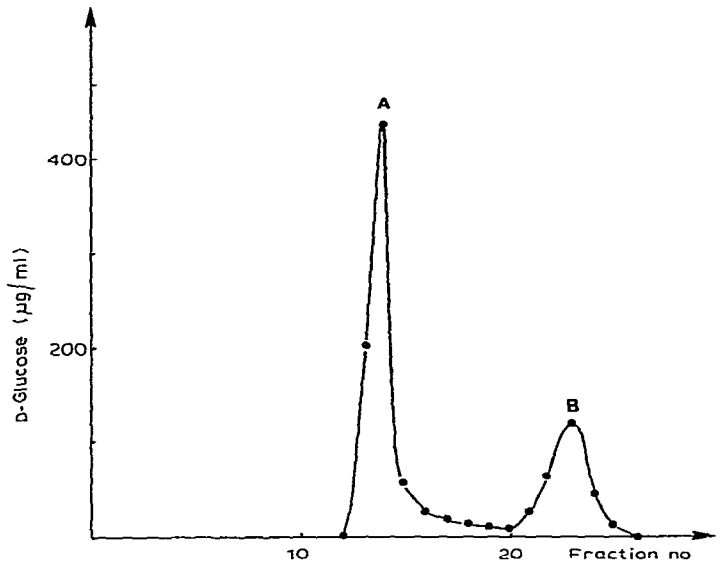


Fig. 1. Separation of the acetolysis products of the β-D-glucan of *Botrytis cinerea* on a column (1.6 × 64 cm) of Sephadex G-15.

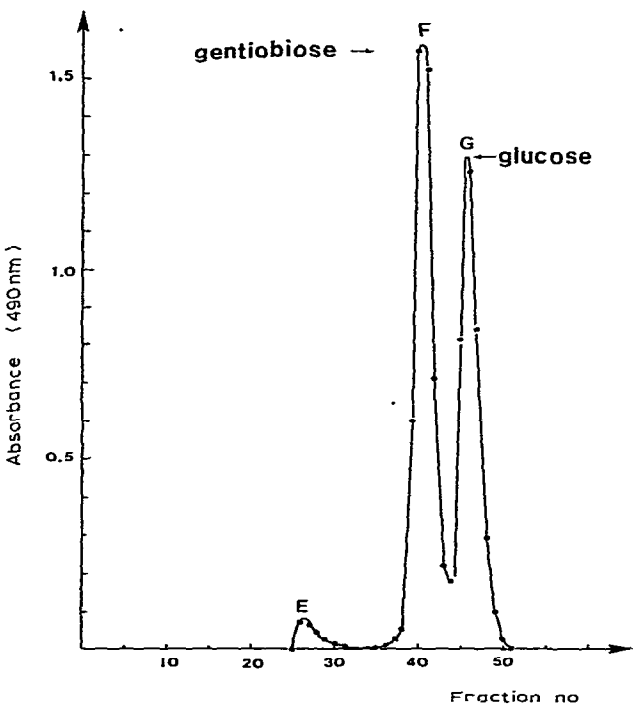


Fig. 2. Separation on a column (1.6 × 120 cm) of Sephadex G-15 of the products obtained by treatment of the glucan of *Botrytis cinerea* with exo-(1→3)-β-D-glucanase.

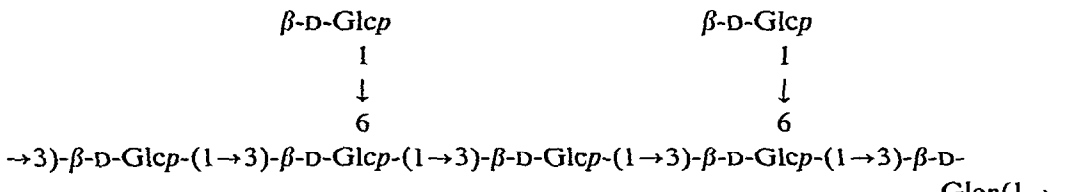
2,4-di-*O*-methyl-α,β-D-glucopyranoside, indicating a (1→3)-linked backbone with branches attached by (1→6) linkages. The molar ratios between tetra-, tri-, and di-*O*-methyl derivatives were 1 : 1.5 : 1 (Table I).

(1→6) Linkages in oligo- and poly-saccharides are preferentially cleaved during acetolysis[7], and the relative rate for (1→3)-β and (1→6)-β linkages in disaccharides[8] is 1 : 29. The glucan rapidly dissolved in the acetolysis mixture. After reaction for 18, 24, or 36 h, gel filtration gave only two products (A and B, Fig. 1). G.l.c. of the trimethylsilyl derivative of B revealed only glucose, which is compatible with single-unit, side chains in the glucan. Peak A corresponded to the void volume of the column, and methylation followed by methanolysis gave methyl 2,3,4,6-tetra- and 2,4,6-tri-*O*-methyl-α,β-D-glucopyranoside in the molar ratio 1 : 6. Thus, the glucan was completely debranched by acetolysis, but some cleavage of (1→3) linkages also occurred.

The insoluble glucan obtained by Smith degradation of the natural glucan was hydrolysed by the exo-(1→3)-β-D-glucanase of Basidiomycete QM 806. The elution profile of the digest on Sephadex G-15 showed mainly glucose and a small proportion of intact glucan. Thus, the side chains of the glucan are single, (1→6)-linked D-glucosyl groups.

Complete degradation of the glucan with a purified exo-(1→3)-β-D-glucanase gave mainly gentiobiose and D-glucose in the molar ratio 3 : 2 (Fig. 2).

The foregoing data indicate the β-D-glucan to have the structure:



Several fungi produce extracellular, or reserve, (1→3,1→6)-β-D-glucans, which may be classified according to the frequency of the side chains. Thus, the less-branched (1→3,1→6)-β-D-glucans are found in mycelium and zoospores of *Phytophtora palmivora* and *P. cinnamoni*[7]; they possess a relatively low d.p. (~30) and have only 1 or 2 side chains per molecule.

The extracellular β-D-glucan of *Botrytis cinerea* is structuraly related to another group of more highly branched (1→3,1→6)-β-D-glucans, such as sclerotan isolated from the sclerotia of *Sclerotinia libertiana*[8] and from the culture medium of *Sclerotium rolfsii*[9], which have single, (1→6)-linked β-D-glucosyl groups attached on average to every third unit of the main chain. *Claviceps purpurea*[10] and *Pullularia pullulans*[11] produce β-D-glucans of the same general type, but the branches occur every fourth and second unit, respectively. *Claviceps fusiformis*[12] and *Schyzophyllium commune*[13] form similar glucans, and the relationship between the molecular structure of these polysaccharides and antitumor activity against transplanted tumors has recently been investigated[14].

EXPERIMENTAL

Microbiological techniques. — The wild strain (C_{77}-4) of *Botrytis cinerea*, isolated from grape, was grown on a Czapek Dox liquid-medium containing 5% of D-glucose. A fermentor vat (8 L) was inoculated with a spore suspension from a 10-day culture grown on a Czapek Dox agar-medium. The vat was incubated at 25°, mechanically stirred (200 r.p.m.), and aerated (2 L/min). After 20 days, the mycelium was removed by centrifugation and the supernatant solution was filtered through glass wool.

β-D-Glucan. — (*a*) *Isolation.* The glucan was obtained from the clear, culture filtrate (1 L) by precipitation with ethanol (0.5 L). The filamentous mass was collected by centrifugation, washed several times with 50% aqueous ethanol, dispersed in water, and freeze-dried, to give fibrous material (270 mg).

(*b*) *Molecular weight.* Gel filtration of the glucan was performed on a column (2.6 × 80 cm) of Sepharose 6B calibrated with Dextrans T-500, T-70, T-40, and T-10 (Pharmacia). The average molecular weight was also determined by using a Beckman Model E analytical ultracentrifuge equipped with a Schlieren system and a Rayleigh interferential-optical system.

(*c*) *Methylation.* The glucan (2 mg) was methylated by the Hakomori method[15,16], and the product was purified by elution from a column of Sephadex LH-20 with ethanol–chloroform (1 : 1). The dry product was treated with 0.5M methanolic hydrogen chloride at 80° for 24 h. The products of methanolysis were separated by g.l.c. before and after acetylation (pyridine–acetic anhydride, 1 : 1; 100°, 30 min), using a capillary glass column (60 m × 0.35 mm) wall-coated with Carbowax 20-M, a nitrogen pressure of 0.4 bar, and a temperature programme from 140→225° at 2°/min. Two reference mixtures[17–19] containing methyl ethers of methyl α- and β-D-glucopyranoside were used. The identities of products were confirmed by g.l.c.–m.s. (methylated glucitol acetates). G.l.c. of the methylated derivatives of D-glucose enables estimation of the molar ratio of tetra-*O*- and tri-*O*-methylglucosides, but not quantification of di-*O*-methylglucosides, which have a lower mass-response. The molar ratio of tri-*O*- and di-*O*-methyl derivatives was obtained by g.l.c. of the acetylated, methylated glucosides, but most of the tetra-*O*-methyl-D-glucoside was lost by this method.

(*d*) *Enzymic hydrolysis.* Exo-(1→3)-β-D-glucanase from[20] Basidiomycete QM 806 was prepared according to the procedure of Peterson and Kirkwood[21]. Absence of (1→6)-β-D-glucanase activity in the preparation was ascertained by using a pustulan solution (0.2%) and the usual technique[9]. (1→3)-β-D-Glucanase activity was determined by the same method, but with laminaran as the substrate. A suspension of the glucan (10 mg) in a solution (10 mL) containing 5 units of (1→3)-β-D-glucanase[21] in 50mM sodium acetate buffer (pH 4.8) was stored at 40° for 24 h. After heat inactivation (100°, 5 min), centrifugation, and concentration of the supernatant solution, the digest (3 mL) was applied to a column (1.6 × 180 cm)

of Sephadex G-15 and eluted with water. Fractions (4 mL) were collected, and the sugar content was determined by the phenol–sulfuric acid method[22].

(*e*) *Acetolysis.* Suspensions of D-glucan (10 mg) in 10:10:1 acetic anhydride–acetic acid–sulfuric acid (10 mL) were stored in sealed tubes at 20° for 18, 24, or 36 h. Each mixture was poured on to ice (5 mL) and neutralised to pH 4–6 with sodium hydrogencarbonate. Acetylated sugars were extracted with chloroform (5 × 4 mL), and the combined extracts were washed with conc. aqueous sodium hydrogencarbonate, dried ($CaCl_2$), and concentrated. Each residue was dissolved in acetone (4 mL), and 0.2M sodium hydroxide (4 mL) was added. After 30 min at 4°, the reaction was stopped by adding Dowex 50-X8 (H^+) resin to pH 5. The resin was removed, and the filtrate was concentrated at 40° under diminished pressure. The carbohydrates present in the residue were eluted from a column (64 × 1.6 cm) of Sephadex G-15 with water. Appropriate fractions were combined and freeze-dried. Polysaccharides excluded from the gel were methylated, and mono- or oligo-saccharides were characterised by g.l.c. of the *O*-trimethylsilyl derivatives.

(*f*) *Smith degradation.* Periodate oxidation, reduction, and mild hydrolysis with acid were performed by the procedure of Johnson *et al.*[23]. To a solution of the glucan (50 mg) in water (20 mL) was added 50mM sodium periodate (40 mL), and the mixture was stored for 8 days at 4° in the absence of light. Excess of ethane-1,2-diol (240 mg) was added and, after 30 min, the mixture was dialysed against water for 48 h. A gelatinous mass formed which dissolved during reduction with sodium borohydride (500 mg, 24 h, 20°). The solution was dialysed and then treated with 50mM sulfuric acid at 20° for 24 h. The precipitate was washed several times with water and collected by centrifugation. The precipitate was digested by QM 806 (1→3)-β-D-glucanase, as described above.

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