

Structural investigations of glucans from cultures of *Glomerella cingulata* Spaulding & von Schrenck

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ABSTRACT

Methylation analysis, enzymic digestion, n.m.r. spectroscopy, and Smith degradation showed that the major extracellular polysaccharide, isolated from cultures of the fungus *Glomerella cingulata*, was a (1→3)- β -D-glucan with side chains of 1–4 (1→3)-linked β -D-glucose residues attached to position 6. A (1→6)- β -D-glucan was produced by the fungus in small proportions. Treatment of the (1→3,1→6)- β -D-glucan (890 315) with >0.05M NaOH at >150°, or Me₂SO–H₂O with a concentration of dimethyl sulfoxide of >80%, irreversibly destroyed the highly ordered structure responsible for the high viscosity of aqueous solutions. The strong shift of the λ_{\max} of aqueous solutions of Congo Red by the degraded glucan, the fact that the mol. wt. of the original glucan was ~4 times higher than that of the degraded polymer, and the suppression of the n.m.r. signals for C-3 indicated that the original glucan had a highly ordered structure, probably built up from single helices.

INTRODUCTION

(1→3,1→6)- β -D-Glucans can form a triple-helical conformation by association of single helices. A complex helical structure was demonstrated for schizophyllan¹ and lentinan², two other fungal (1→3,1→6)- β -D-glucans, by viscosity measurements, n.m.r. and X-ray studies, and the formation of complexes with Congo Red. The triple helix is stabilised by hydrogen bonds, and side chains attached to positions 6 do not prevent its formation. A triple-helical structure is indicated by the finding that the viscosities of solutions of the glucan decrease with increase in alkalinity or concentration of dimethyl sulfoxide and by heat treatment, due to the breakage of hydrogen bonds that stabilise the helix. The glucans shift the λ_{\max} of aqueous solutions of Congo Red to longer wavelength as a result of the formation of a complex with the single-helical structure of the degraded glucan³.

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EXPERIMENTAL

Fermentation. — *Glomerella cingulata*, isolated from a black rot of *Pyrus communis* var. Claps Favorit in Belgium by Hennebert in 1960 and deposited in the Mycothèque Université Catholique de Louvain as MUCL 1232, was taken in culture. The glucan 890 315 was isolated and purified as described⁴.

Methylation. — The glucans were methylated by the method of Harris *et al.*⁵. A completely methylated product was obtained by a second methylation of the polysaccharide isolated and lyophilised after the first methylation. Hydrolysis of the methylated polysaccharide and reduction and acetylation of the products were carried out by the methods described⁵. The partially methylated alditol acetates were analysed by g.l.c.-m.s. on a DB-1701-30W fused-silica column (30 m) with a linear temperature gradient 170° → 210°, a Hewlett-Packard 5890A gas chromatograph, a mass-selective detector 5970B, and an HP work station 300.

Smith degradation. — The Smith degradation was performed according to Blaschek *et al.*⁶.

¹³C-N.m.r. spectroscopy. — The spectra (62.89 MHz) were obtained with a Bruker WM spectrometer for solutions of glucan (15 mg) in 0.5M NaOD (2 mL) at 33° with external Me₄Si and 75,600 scans.

Analyses. — Acetyl groups were determined by the hydroxamate method⁷ and uronic acids by the method of Blumenkrantz *et al.*⁸.

Enzymic digestion. — The glucan 890 315 (50 mg) was digested with cellulase (10 mg; 2312, Merck) in 0.1M acetate buffer (pH 4.8) at 30°.

Physical methods. — Low-angle laser light scattering was performed for solutions (2.14 mg/mL) of the glucan in 0.05M NaCl. Samples (0.2 mL) were injected on to TSK PW 6000 + 5000 + 4000 columns (20°; flow rate, 0.7 mL/min) attached to a Constametric IIIG pump, a KMX 6 scattering detector, and a refracto-monitor (all LDC/Milton Roy). The data were evaluated with a PC/LALLS software package (Milton Roy).

Viscosities were determined with an Ubbelohde viscosimeter at room temperature.

The shift of the λ_{\max} of 0.38 μ M Congo Red (Sigma) was recorded with a Shimadzu double-beam UV 210-A spectrophotometer. NaOH or Me₂SO were removed by dialysis (MWCO 3500) prior to measurement of the λ_{\max} .

RESULTS AND DISCUSSION

Hydrolysis (2M trifluoroacetic acid, 1 h, 121°) of the polysaccharide followed by borohydride reduction of the products and acetylation (acetic acid-acetic anhydride) gave (g.l.c.) only glucitol hexa-acetate. Since no uronic acid was detected by the method of Blumenkrantz *et al.*⁸, the polysaccharide was a glucan.

Methylation analysis (Table I) showed that, on average, every third glucose residue of the main chain of the glucan was branched at position 6. The partial loss of

TABLE I

Results of the methylation analysis

| Alditol acetate ^a | Linkage | Mol % | Retention time | Main fragments (m/z) |
|------------------------------|-----------|-------|----------------|----------------------|
| 2,3,4,6-Me ₄ | Terminal | 23 | 0.73 | 205, 129, 101 |
| 2,4,6-Me ₃ | (1→3) | 43 | 1 | 233, 129, 117 |
| 2,3,4-Me ₃ | (1→6) | 8 | 1.09 | 233, 189, 101 |
| 2,4-Me ₂ | (1→3,1→6) | 26 | 1.45 | 233, 189, 117 |

^a 2,3,4,6-Me₄ means 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc.

terminal glucose may have been due to volatility during the methylation procedure⁵.

Four consecutive Smith degradations were necessary for complete degradation of the side chains of the glucan. A non-dialysable (MWCO 3500) and water-insoluble product was obtained after each Smith degradation. Thus, the main chain of the glucan consisted exclusively of (1→3)-linked residues, and the side chains had various lengths with ~50% as single glucose residues and the remainder as (1→3)-linked di-, tri-, and tetra-saccharides (Table II). On average, every 12th glucose residue carried an acetyl group (ν_{\max} 1740 cm⁻¹) as determined by the hydroxamate test⁷. After saponification, the solubility of the polysaccharide decreased.

For ¹³C-n.m.r. spectroscopy, solutions in 0.5M NaOD were used because of the better solubility of the glucan and the absence of highly ordered structures which result in broad signals of low intensity⁹. The β configuration of the D-glucose residues was indicated¹⁰ by the C-1 resonance at 103.3 p.p.m., and branchings at C-6 by signals for C-6 at 68.8 p.p.m. and for unsubstituted C-6 at 61.4 p.p.m. The multiplicity of the signals and the broad C-3 signal at 86.6 p.p.m. could be ascribed to the presence of (1→3)- and (1→3,1→6)-linked residues, and terminal residues (Table III). The weak C-3 resonance at 86.6 p.p.m. could be due to the immobilisation caused by the participation of C-3 in the glycosidic bond, the position of C-3 in the pyran ring, and the ordered structure of the glucan^{2,11,12} (see below).

The glucan contained ~8% of (1→6)-linked residues that were removed by the Smith degradations, which left a water-insoluble product. Therefore, the (1→6)-linked portion was not part of the (1→3)-linked main chain. In order to confirm this conclusion, the glucan was digested with a cellulase that could hydrolyse (1→3) and (1→6) linkages, and which was tested on laminarin and pustulan. A (1→3)- β -D-glucanase (laminarinase) did not hydrolyse the glucan due to the ramified structure. The ratio of (1→3)- to (1→6)-linked glucose during the enzymic degradation, determined by methylation analysis at intervals of 24 h, decreased with increasing time of digestion (Fig. 1). Therefore, the cellulase cleaved (1→3) linkages more rapidly than (1→6) linkages. After 7 days, no (1→3) linkages were detectable and only (1→6) linkages were present. The final product had a mol. wt. of ~3000 as determined by gel-permeation chromatography. Since the enzyme also cleaved (1→6) linkages, the original mol. wt. of the

TABLE II

Smith degradations^a of the glucan

| Linkage type | Original | 1st | 2nd | 3rd | 4th |
|----------------------------|----------|------|-----|-----|-----|
| Terminal | 23 | 5 | — | — | — |
| (1→3) | 43 | 83 | 95 | 98 | 100 |
| (1→6) | 8 | — | — | — | — |
| (1→3,1→6) | 26 | 12 | 5 | 2 | — |
| Branching (%) ^b | 37.6 | 12.6 | 5 | 2 | — |

^a Values represent mol%. ^b Given by [mol% (1→3,1→6)/mol% (1→3,1→6) + (1→3)] x 100.

TABLE III

¹³C-N.m.r. data for the glucan

| Glep | Chemical shifts (p.p.m.) | | | | | |
|------------|--------------------------|------|-------------------|------|------|-------------------|
| | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| Terminal | 103.5 | 75.1 | 76.8 | 70.5 | 75.9 | 61.4 |
| 3,6-Linked | 103.3 | 73.7 | 86.6 ^a | 68.8 | 76.6 | 68.8 ^a |
| 3-Linked | 103.3 | 73.3 | 86.6 ^a | 68.8 | 76.6 | 61.0 |

^a Carbon atoms involved in glycosidic linkages.

(1→6)-linked moiety must have been >3000. Since there were no long sequences of (1→6) linkages in the main chain of the (1→3,1→6)- β -D-glucan and long (1→6)-linked side chains were unlikely, it was concluded that a small proportion of a (1→6)- β -D-glucan was present in addition to the (1→3,1→6)- β -D-glucan.

Application of size-exclusion chromatography and low-angle laser light scattering indicated the original glucan to be a mixture with mol. wts. in the range 70,000–1,800,000 with a distribution maximum at 681,000. Heating an aqueous solution of the glucan (30 min, 150°) decreased the mol. wt. by a factor 4.7 to 145,000. (1→3,1→6)- β -D-Glucans, *e.g.*, schizophyllan¹, in aqueous solution form a triple-helical structure, disruption of which by cleavage of the stabilising hydrogen bonds decreases the mol. wt. to one-third¹³. The triple helix is stabilised by an interstrand O-2···O-2' hydrogen-bond network, as in (1→3)- β -D-xylan and schizophyllan. In (1 \rightarrow 3 \rightarrow 3 \rightarrow 3)-linked polysaccharides, in contrast to the linear structure of such (1 \rightarrow 3 \rightarrow 4 \rightarrow 4)-linked polysaccharides as cellulose, two intramolecular hydrogen bonds are possible^{14,15}, namely, O-2···O-2' and/or O-5···O-4'. Thus, a single-helical structure for (1→3,1→6)- β -D-glucans can be explained on the basis of hydrogen bonding. The loss of hydrogen bonds can be achieved by heating^{16,17} (150°), a dimethyl sulfoxide concentration of >80% in Me₂SO-H₂O mixtures^{1,13}, or treatment¹⁸ with alkali (>0.05M NaOH).

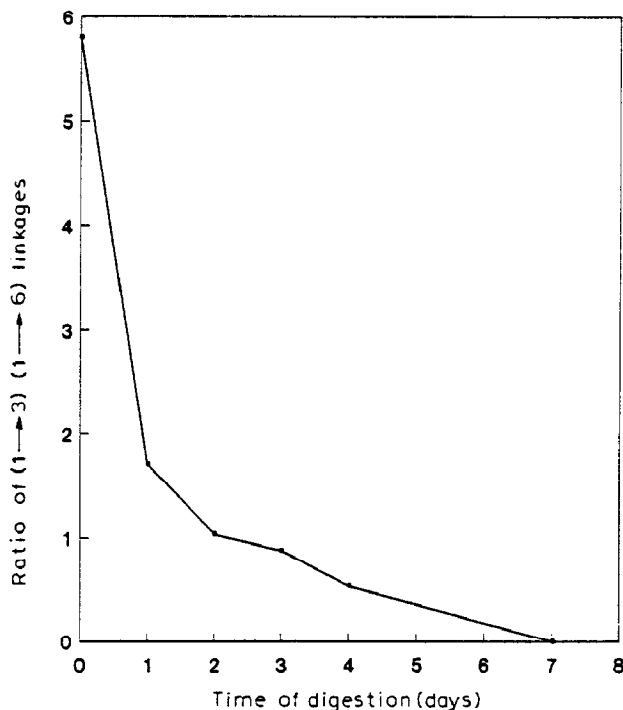


Fig. 1. Dependence of the ratio of (1→3)- to (1→6)-linked glucose residues on the extent of digestion of glucan with a cellulase.

The mol. wt. of the degraded glucan was 4.7 times smaller than that of the parent glucan. Theoretically¹³, the factor after melting of the triple helix to single helices should be 3, but at 150° some covalent bonds can be cleaved¹⁹.

In order to investigate the more highly ordered structure, the effects of NaOH, Me₂SO, and heating on the glucan were examined. Ordered structures of glucans increase the viscosity of aqueous solutions and, thus, their breakdown will reduce the viscosity^{18,20}. Congo Red forms complexes with single-helical structures of glucans³ in aqueous solutions and results in a large shift of the λ_{max} from 497 nm to longer wavelength.

Increasing concentrations of NaOH caused a marked decrease of the viscosity from 7.93 cSt in water to 1.32 cSt in 0.5M NaOH (Fig. 2). In the range 0–0.2M NaOH, the viscosity of solutions of the glucan decreased until a constant value was reached at 0.2M NaOH.

Heating of aqueous solutions of glucan (1 mg/mL) from 20° to 160° reduced the viscosity continuously but, on cooling to room temperature, there was no increase in viscosity; hence, the destruction of the ordered structure was irreversible (Fig. 3).

Solutions of the glucan in Me₂SO–H₂O mixtures with >80% of Me₂SO had dramatically reduced viscosities due to breakdown of the network of hydrogen bonds. Schizophyllan¹ showed similar behaviour at >85% of Me₂SO. The reason for the

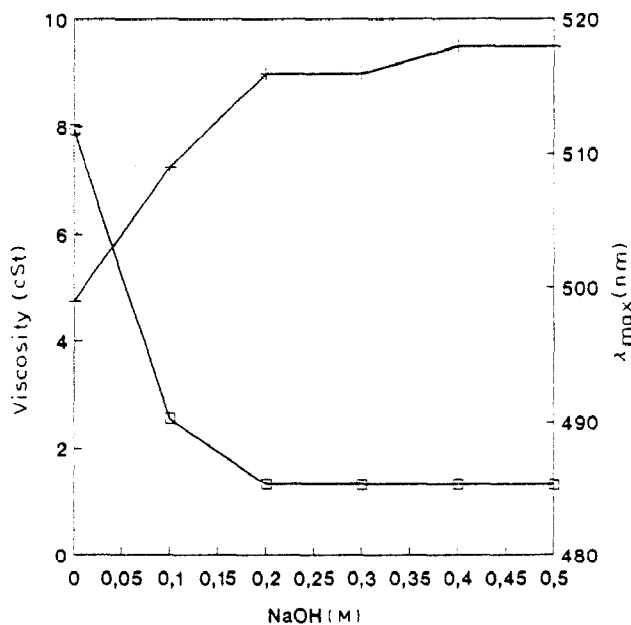


Fig. 2. Dependence on the concentration of NaOH of the viscosity (\square) of, and the λ_{\max} of Congo Red (+) in, solutions (1 mg/mL) of glucan.

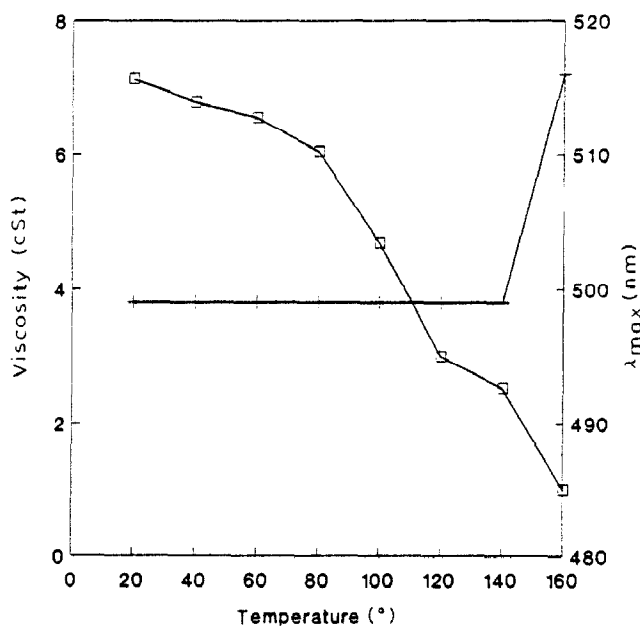


Fig. 3. Effect of heating on the viscosity (\square) of, and the λ_{\max} of Congo Red (+) in, solutions (1 mg/mL) of glucan.

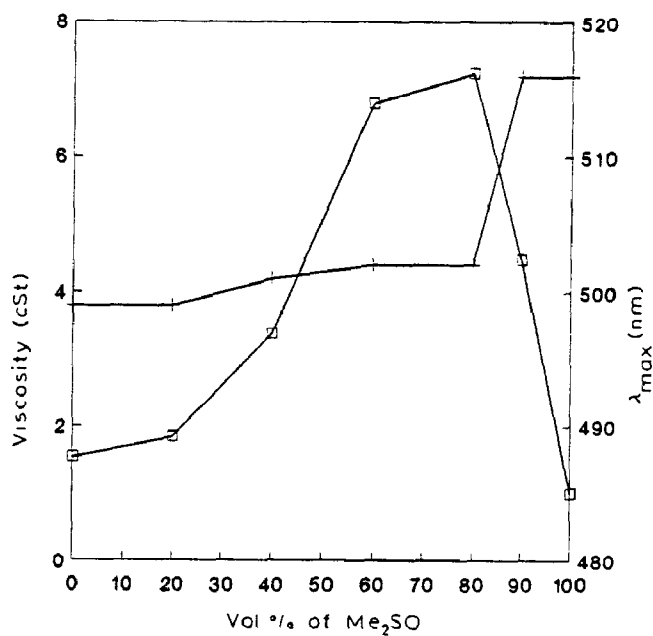


Fig. 4. Dependence on the concentration of Me_2SO (\square) of, and the λ_{max} of Congo Red (+) in. solutions (1 mg/3 mL) of glucan.

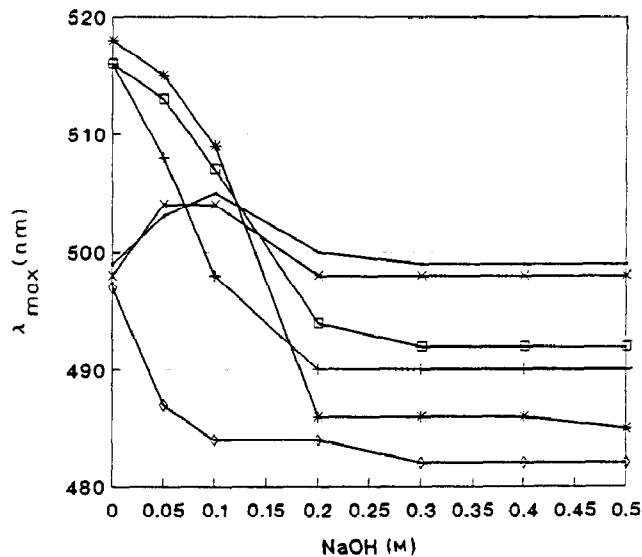


Fig. 5. Dependence on the concentration of NaOH of the λ_{max} of Congo Red in solutions of the original (\bullet), NaOH- ($*$), heat- (150°C \square , 20°C-140°C \times), or Me_2SO -degraded (+) glucan; (\diamond) blank.

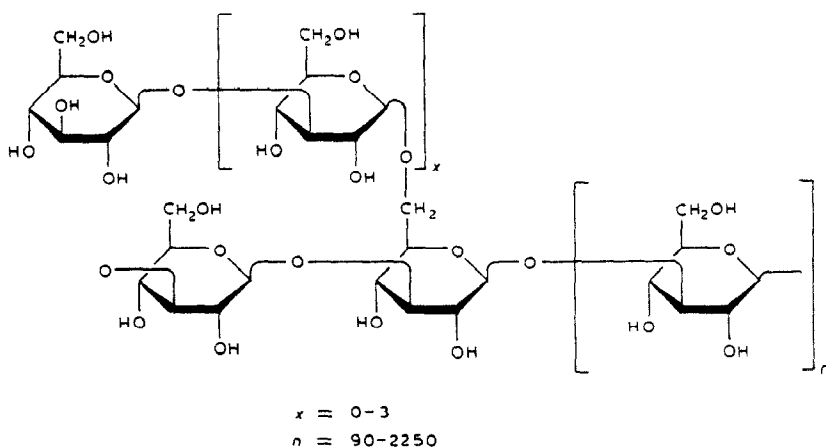


fig. 6. Proposed structure of the glucan.

increase in viscosity in the range 0–80% of Me_2SO for the glucan is unclear (Fig. 4).

The λ_{max} of Congo Red ($0.38\mu\text{M}$) in aqueous solutions that contained the original glucan was 498 nm, and 516–518 nm for the degraded glucan. Stepwise addition of NaOH decreased the λ_{max} until blank values were obtained (Fig. 5). The degraded glucan had a single-helical structure, the complexes of which with Congo Red were destroyed by the addition of NaOH, due to the breakdown of stabilising hydrogen bonds.

Heating from 20° to 140° reduced the viscosity, but did not change the λ_{max} . Therefore, the ordered structures responsible for the high viscosity were destroyed, but no single-helical structures were generated. Thus, only heating to $>140^\circ$ completely converted the ordered structure into single helices, detected by the shift of λ_{max} from 498 to 516 nm.

The same phenomenon was observed by Me_2SO -mediated disaggregation of the glucan, where only $>80\%$ Me_2SO caused a shift of λ_{max} to 516 nm. Likewise, the λ_{max} of Congo Red was shifted to 518 nm for samples of the glucan treated with $>0.2\text{M}$ NaOH. Stepwise addition of NaOH to the original glucan caused a shift of λ_{max} only to 504 nm in the range 0.05–0.2M. At higher concentrations, λ_{max} decreased to 499 nm. Therefore, the conformation of the glucan–Congo Red complex is changed in the presence of NaOH.

Thus, cultures of *Glomerella cingulata* produce an extracellular (1 \rightarrow 3,1 \rightarrow 6)- β -D-glucan with an average mol. wt. of 681,000, which, in aqueous solution, has a highly ordered structure that consists of triple helices, and the structure in Fig. 6 is proposed.

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