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Note

Structure of the β-D-glucan secreted by *Phanerochaete chrysosporium* in continuous culture

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Phanerochaete chrysosporium (Sporotrichum pulverulentum) is a white-rot basidiomycete which is able to degrade all the major structural components of wood material. Recently, this organism has received much attention due to its ability to produce extracellular peroxidases that are involved in the breakdown of lignin[1,2] and different toxic substances[3]. Such degradation properties are a phenomenon of secondary metabolism and can be triggered by nitrogen- or carbon-limited growth conditions. When cells of the fungus are grown in continuous culture, under nitrogen-limited conditions, they secrete a mixture of extracellular polysaccharides[4,5] as a slime-like gel. The characterisation of the major constituent is now reported.

Upon acid hydrolysis, the polysaccharide material isolated from the culture medium by precipitation with 50% ethanol yielded glucose, mannose, galactose, fucose, and xylose in the molar ratios 1.0:0.03:0.01:0.01:0.02. When such material was repeatedly washed with cold water, the insoluble residue was enriched in the glucose-containing polysaccharide. Further purification could be achieved by solubilisation in hot methyl sulphoxide or aqueous 5% KOH, to give fractions yielding >99% glucose on hydrolysis, and hereafter referred to as glucan. No further fractionation was attempted, in order to ensure that the material studied effectively represented that secreted into the culture medium. The glucan contained no uronic acid residues[6], and no protein was detected[7].

When the glucan was incubated with exo-(1→3)-β-D-glucanase from Basidiomycete sp. QM 806, the main products were glucose and gentiobiose in the molar

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ratio ~ 1:1, indicating that the glucan was of the (1→3),(1→6)-β-D type common in fungi[8] and highly branched. This molar ratio remained more or less constant (1:1.18–1:4) during enzymic hydrolysis, indicating that the (1→6) branch-points could be regularly distributed along the backbone. No hydrolysis was detected when the glucan was incubated with the (1→3),(1→4)-β-D-glucanase from *Bacillus subtilis*. The amount of glucose, determined by the D-glucose oxidase method, released from the β-glucan after acid hydrolysis was equivalent to that released after successive treatment with the exo-(1→3)-glucanase and β-D-glucosidase from almond emulsin, thereby showing that all the glucose in the polysaccharide material derived from the putative β-D-glucan.

The glucan was methylated[9], and the product was depolymerised by the formic acid–sulphuric acid method[10]. The resulting methyl sugars were analysed by g.l.c.–m.s. of their acetylated aldononitrile derivatives[11]. Derivatives of 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 2,4-di-*O*-methylglucose were detected in the molar ratios 1:0.89:0.96; unidentified methylated sugar derivatives constituted < 1% of the total ion integral and were probably derived from the traces of contaminant polysaccharides.

When the product obtained on Smith degradation of the β-D-glucan was treated with the exo-(1→3)-β-D-glucanase, only glucose and traces of laminaribiose were obtained, indicating that the side chains were composed of single glucosyl groups. This result was confirmed by methylation analysis of the periodate-resistant polymer which gave derivatives of 2,4,6-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose (trace).

Thus, the β-D-glucan secreted into the culture medium has a structure similar to that reported for many others in the cell walls of fungi[8], *i.e.*, a main chain containing (1→3)-linked β-D-glucopyranosyl residues with single (1→6)-linked β-D-glucopyranosyl groups linked to the backbone. Statistically, every second residue in the main chain is substituted.

Eriksson *et al.*[12] have suggested that the production of glucan by *P. chrysosporium* regulates the concentration of extracellular glucose, since glucose represses glucose-oxidising enzymes. Such enzymes are involved in the production of the H_2O_2 necessary for the degradation of lignin. When the concentration of sugar becomes too low, (1→3)-β-D-glucanases are induced and the β-D-glucan is degraded to glucose. Thus, the d.p. and extent of branching of the β-D-glucan will depend on the state of the culture. The glucan can also function as reserve energy source for the fungus[13].

EXPERIMENTAL

Culture conditions. — The white-rot fungus *Phanerochaete chrysosporium* (ATCC 24725) was cultured as described previously[5], but with agitation and aeration rates which varied between 300–600 r.p.m. and 0.05–0.4 v/v/min, respectively. The nitrogen source was diammonium tartrate at 0.44 g/L. With a dilution rate of 0.02

$\pm$ 0.002 h^{-1}, the biomass concentration (1.34 g/L) and the amount of polysaccharide secreted[5] (140 mg/L) were independent of the agitation and aeration rates.

Isolation of the extracellular polysaccharide. — The fungal mycelia were collected and the filtrate was either concentrated five-fold by ultrafiltration on an Amicon PM-10 membrane or the polysaccharide was precipitated with ethanol (1 vol.), washed with aqueous 50% ethanol, suspended in water, and then freeze-dried. The freeze-dried residue was extracted with water (100 mL/g) in an autoclave (1 h, 121°), to yield material (3%) which was not studied further. The insoluble residue was extracted further with methyl sulphoxide (100 mL/g) in an autoclave, as above, to yield, after dialysis and freeze-drying, the β-D-glucan (60% of the freeze-dried material) and a residue which was finally extracted with aqueous 5% KOH under N_2 to give another fraction (13% after neutralisation, dialysis, and freeze-drying).

Sugar analyses. — Polysaccharides were hydrolysed according to the procedure of Saeman *et al.*[14], and the free sugars were determined by g.l.c. of the acetylated aldononitrile derivatives[15] on columns (15 m × 0.53 mm) of DB-225 (J. & W. Scientific), using a Hewlett-Packard 5890A chromatograph equipped with a flame-ionisation detector. Enzymic hydrolysates were analysed by g.l.c. of the trimethylsilylated oxime derivatives[16] by chromatography on columns (15 m × 0.53 mm) of DB-1 (J. & W. Scientific). Hydrolysates were routinely examined by p.c. on Schleicher & Schüll No. 2043b paper, using ethyl acetate–pyridine–water (8:2:1) or 1-propanol–ethyl acetate–water (7:1:2) and detection with alkaline silver nitrate[17].

Methylation analysis. — Samples (10 mg) of the glucan and the Smith-degradation product were methylated as described by Harris *et al.*[9], using lithium methylsulphinylmethanide[18], and each product was depolymerised using formic acid/sulphuric acid[10]. The methyl sugars were converted into their acetylated aldononitrile derivatives[11] and subjected to g.l.c.–m.s., using a Hewlett-Packard 5890A gas chromatograph equipped with a column (25 m × 0.33 mm) of DB-225 coupled to a 5970B mass spectrometer. Derivatives were identified by comparison of their spectra with reported data[11] and with the spectra of the appropriate standard compounds. The peak areas obtained from the total ion chromatogram were corrected by using effective carbon response factors to give the molar ratios[19].

Smith degradation. — A suspension of the glucan (50 mg) in 25mM $NaIO_4$ (25 mL) was stirred for 2 days at room temperature. An excess of ethylene glycol was then added and the polyaldehyde was reduced with $NaBH_4$. The insoluble product was washed free of inorganic material with water and treated with 0.5M sulphuric acid for 6 h at room temperature. The product was washed with water and an aqueous suspension was freeze-dried (yield 20 mg). Samples of the product (~ 5 mg) were taken for methylation analysis and hydrolysis studies.

Enzymic hydrolysis. — Samples (5 mg) of the glucan were suspended in 50mM acetate buffer (pH 5.5, 2 mL), and purified exo-(1→3)-β-D-glucanase (EC 3.2.1.58) from the Basidiomycete sp. QM806 (1 mg) was added. Incubation was carried out at 37° for 1–6 h. Hydrolysates were examined by p.c. and g.l.c.

The *B. subtilis* glucanase was obtained by partial purification of the bacterial

amylase BAN 1000S supplied by Novo Industri (Copenhagen, Denmark), and the β-D-glucosidase from almond emulsin was obtained from Fluka (Buchs, Switzerland).

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