STRUCTURE OF A (1→3)-α-D-GLUCAN (PSEUDONIGERAN) OF *Aspergillus niger* NNRL 326 CELL WALL*

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ABSTRACT

The structure of a fungal (1→3)-α-D-glucan, pseudonigeran (DP 330, $[\alpha]_D^{25}$ +252° in M sodium hydroxide), has been investigated by methylation, periodate oxidation, and partial acid hydrolysis. Pseudonigeran was isolated, by alkali extraction, in 4% yield from the mycellium of *Aspergillus niger* NNRL 326 together with small amounts of nigeran and other polysaccharides. Pseudonigeran was further purified through its acetate. Hydrolysis of the methylated pseudonigeran (1 mole) yielded 2,3,4,6-tetra- (1 mole) and 2,4,6-tri-*O*-methyl-D-glucose (330 moles). Periodate oxidation of reduced pseudonigeran, followed by reduction and partial acid hydrolysis yielded the corresponding degraded pseudonigeran, glycerol, and traces of 2-hydroxyethylidene acetal of glucosylerythritol, indicating the presence of about 1.4% of (1→4) linkages which are either present in pseudonigeran or arise from contaminating material. Partial acid hydrolysis yielded glucose, nigerose, nigerotriose, nigerotetraose, and nigeropentaose. The structure of pseudonigeran is discussed on the basis of these findings.

INTRODUCTION

The presence of glucans containing α-(1→3) linkages has been established in fungal cell walls[1–4] and more recently in *Schizosaccharomyces* and *Cryptococcus* yeast species[5]. Nigeran, a cell-wall polysaccharide of *Aspergillus niger*, has been extensively studied and has been shown to consist of alternating α-D-(1→3)- and α-D-(1→4)-linked glucose residues[6,7].

Johnston[2] reported in 1965 the isolation of a polysaccharide ($[\alpha]_D$ +233° in M sodium hydroxide) from the cell wall of *Aspergillus niger* grown on sucrose. The

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material was shown to contain mannose, galactose, and glucose in the molar ratio 1:4:95 and was characterized by partial acid hydrolysis as a (1→3)-α-D-glucan containing approximately 10% of α-D-(1→4) linkages[2,3].

In our laboratory, a (1→3)-α-D-glucan, pseudonigeran[8], which had properties very similar to that obtained by Johnston, was isolated from the mycelium of the cell walls of *Aspergillus niger* NNRL 326 (grown on D-glucose). The polysaccharide ($[\alpha]_D^{25}$ +252° in M sodium hydroxide) was insoluble in water and contained only D-glucose. The present communication details the purification and the structure of pseudonigeran.

DISCUSSION

Pseudonigeran, a cell wall polysaccharide[2,3,8], was isolated in 4% yield from the mycelium of *Aspergillus niger* NNRL 326. The mycelium was also shown to contain water soluble polysaccharides containing mannose, galactose, and glucose in approximately equal quantities (Table I).

TABLE I

WATER-SOLUBLE POLYSACCHARIDES EXTRACTED FROM *Aspergillus niger* MYCELIUM

Fraction	*Yield (g)*	$[\alpha]_D^{25}$ (M *NaOH*) (*degrees*)
I	0.12	+187
II[a]	5.10	+46.2
III[a]	10.30	−9.0
IIA[a]	1.40	+30.4
IIIA[a]	2.91	−7.1
IV[b]	3.41	+52.3

[a]Contains mannose, glucose, and galactose in approximately equal quantities. [b]Contains in addition a trace of xylose.

Pseudonigeran was purified through its acetate (Table II). After deacetylation, the polysaccharide ($[\alpha]_D$ +252° in M sodium hydroxide) was shown to contain only D-glucose. The structure of pseudonigeran was established by methylation studies.

TABLE II

FRACTIONATION OF PSEUDONIGERAN ACETATE

Fraction	*Petroleum ether (%)*	*Yield (g)*	$[\alpha]_D^{25}$ ($CHCl_3$) (*degrees*)
A	48	trace	
B	50	1.45	+162
C	52	2.33	+151
D	55	8.18	+153
E	60	1.84	+153

On hydrolysis, the fully methylated pseudonigeran (1 mole) yielded only 2,4,6-tri-*O*-methyl-D-glucose (330 moles) and 2,3,4,6-tetra-*O*-methyl-D-glucose (1 mole). These results indicate that pseudonigeran is a linear (1→3)-linked D-glucan (DP 330).

Periodate oxidation studies also indicated that reduced pseudonigeran was (1→3)-linked. However, the periodate consumption and the formic acid and formaldehyde production were higher than expected for a linear (1→3)-D-glucan having DP 330. In order to obtain further information on the fine structure, the polymer was subjected to successive Smith degradations (Table III). The presence of a few (1→4)-linked D-glucose residues (1.4–1.6%) in pseudonigeran or in a contaminating polysaccharide was demonstrated by the liberation of the 2-hydroxyethylidene acetal of glucosylerythritol (14–15.5 mmoles per glucose residue) during the first Smith degradation. No glucosylerythitol was found as such and this fact remains to be explained.

TABLE III

SMITH DEGRADATIONS OF PSEUDONIGERAN

Degradation	*Periodate reduced*		*Formaldehyde produced* (*mmoles per* D-*glucose residue*)		*Formic acid produced*		*Glycerol produced*	
	Obs.	*Calc.*[a]	*Obs.*	*Calc.*[a]	*Obs.*	*Calc.*[a]	*Obs.*	*Calc.*[a]
I	56	15.1	11	6.1	14	6.1	3.5	3.0
II	26	6.1	8.9	0	6.4	6.1	4.0	3.0
III	16	6.1	3.2	0		6.1	3.0	3.0
IV	10	6.1	2.8	0		6.1	2.8	3.0
V	10	6.1	2.8	0		6.1		3.0

[a]Calc. for a linear reduced (1→3)-D-glucan (DP 330).

Dialdehyde polymers derived from some (1→4)-linked polysaccharides are known to give 2-hydroxyethylidene acetals by partial, acid hydrolysis. However, the parent glycoside is normally the major component[9]. The origin and significance of small amounts of formaldehyde or "formaldehyde-like material" generated by the third, fourth, and fifth Smith degradations is not known. It seems likely, however, that some type of overoxidation occurred, as the periodate uptake for the successive Smith degradations was higher than would be expected for a (1→3)-linked glucan having DP 330 (Table III).

The presence of α-D-(1→3)-linked glucose residues in pseudonigeran was confirmed by the isolation of oligomers of nigerose on partial acid hydrolysis of pseudonigeran. No trace of maltose or other oligosaccharides could be detected by chromatography.

In conclusion, methylation, partial acid hydrolysis, and periodate oxidation studies indicated pseudonigeran to be a linear glucan containing at least 98% α-D-(1→3)-linked glucose residues. It is impossible to decide conclusively if pseudonigeran was contaminated by a small amount of other polysaccharides or contained a few (1→4)-linked D-glucose residues.

EXPERIMENTAL

General methods. — Evaporations were performed under reduced pressure at 30°. Paper and thin-layer chromatography was performed using the following solvent systems: (A) 2:5:7 pyridine–ethyl acetate–water (upper phase)[10], (B) 65:10:25 propyl alcohol–ethyl acetate–water[11], (C) 3:2:1 butyl alcohol–ethanol–water[12], (D) 9:6:3:1 butyl alcohol–acetic acid–ethyl ether–water [13], (E) 2-butanone–water azeotrope[14], and (F) 200:47:15:1 benzene–ethanol–water–acetic acid (upper phase)[15]. Silica gel G was used for t.l.c.; R_{Glc} and R_{TMG} refer to the mobility relative to that of D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively. Ammoniacal silver nitrate[16] (reagent A) was used to detect reducing sugars and some methylated sugars. Reagent B was *p*-anisidine[17] containing phosphoric acid (one drop per ml). Silica gel chromatograms were sprayed with M sulfuric acid and heated at 100–150° for 5–10 min. Sugars were quantitatively determined by the phenol–sulfuric acid method[18] and glycerol and erythritol by the chromotropic acid method[19]. Melting points were determined on a Fisher–Johns block and are uncorrected. Optical rotations were measured with a Bendix ETL-NPL automatic polarimeter, type 143 A.

Isolation of pseudonigeran. — *Aspergillus niger* NNRL 326 mycelium (received from the Clinton Processing Company, Clinton, Iowa, U. S. A., suspended in ethanol; dry weight 260 g) was dispersed in 0.1M sodium borohydride and left for several h at room temperature with occasional shaking. The mycelium was washed and extracted with hot water. Crude nigeran precipitated from the cooled water extract and was reprecipitated from a M sodium hydroxide solution with methanol (Fraction I, 0.12 g). Fractions II and III were obtained from the supernatant of the extract by precipitation with 1 and 2 vol. of acetone. The hot-water extraction of the mycelium was repeated under the same conditions yielding Fractions IIA and IIIA, but no precipitate of nigeran (Table I).

The residual mycelium was treated twice with M sodium hydroxide (2 l, 0.5 h, 90°), and crude pseudonigeran was precipitated from the combined extracts by addition of 3 vol. of methanol. The precipitate was dissolved in hot M sodium hydroxide (2 l) and was reprecipitated by neutralisation with dilute acetic acid. From the supernatant, Fraction IV was obtained by precipitation with methanol (3 vol.).

After two further hot-alkali purifications, pseudonigeran was treated twice with hot water, and centrifuged while still warm. The polysaccharide was then dissolved in M sodium hydroxide (2 l) containing sodium borohydride (3 g). After being stirred for 1 h at room temperature, the solution was neutralized with dilute acetic acid and centrifuged, and the precipitate washed with water, methanol, ethanol, and petroleum ether. Pseudonigeran was dried *in vacuo* (yield; 14.0 g), $[\alpha]_D^{25}$ +248.5° (*c* 0.73, M sodium hydroxide).

Purification of pseudonigeran. — Pseudonigeran (13.5 g) was acetylated twice in formamide (200 ml) and pyridine with acetic anhydride by the method of Carson[20]. The acetate was heated at reflux in acetone (1 l) for 4 h. A precipitate (1.9 g) was obtained from the supernatant by addition of petroleum ether. The acetone extrac-

tion was repeated and afforded 0.3 g of material soluble in hot acetone. The acetone-insoluble fraction was dried *in vacuo* to give pseudonigeran acetate (17.8 g) which was fractionated from a chloroform solution (1.5 l) with petroleum ether (Table II). Fraction D was deacetylated in chloroform with methanolic potassium hydroxide to give a pseudonigeran of $[\alpha]_D^{25}$ +252° (*c* 0.5, M sodium hydroxide). Pseudonigeran was hydrolyzed in M sulfuric acid for 24 h at 100°. Examination of the hydrolyzate by paper chromatography (solvent A, B, and C) revealed only glucose which was identified as D-glucose by conversion to methyl α-D-glucopyranoside. The glucose content was determined after hydrolysis of pseudonigeran (50 mg) under the same conditions. Found: 55 mg as D-glucose.

Methylation of pseudonigeran. — Pseudonigeran (Fraction D, 100 mg) was methylated four times by the liquid ammonia procedure[21] to give a fully methylated product (81 mg), $[\alpha]_D^{27}$ +206° (*c* 0.34, chloroform). Found: OCH_3, 45.5.

Hydrolysis of the methylated pseudonigeran and identification of the cleavage products. — Methylated pseudonigeran (50 mg) was hydrolyzed by the 72%–8% sulfuric acid method[22]. Examination of the hydrolyzate by paper chromatography (solvent E) revealed, in addition to a trace of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, which was recrystallized from acetone, m.p. 123°, $[\alpha]_D^{25}$ 102→74° (*c* 1.0, methanol); lit.[23]: m.p. 126°, $[\alpha]_D$ +111→70° (methanol). 2,4,6-Tri-*O*-methyl-D-glucose was further identified, as the only tri-*O*-methylglucose present, by paper electrophoresis in 0.1M borate buffer at 600 V (reagent B).

A portion of the hydrolyzate (corresponding to 16.9 mg of methylated pseudonigeran) was separated by t.l.c. (solvent F). The band corresponding to 2,3,4,6-tetra-*O*-methyl-D-glucose was eluted with ethanol and the sugar content was determined. Found: 54.9 μg as 2,3,4,6-tetra-*O*-methyl-D-glucose (DP 330). A duplicate experiment indicated DP 325. Penta-*O*-methylglucitol (R_{TMG} 1.05) was tentatively identified when the tetra-*O*-methyl-D-glucose solution was further examined by t.l.c. (solvent F).

Periodate degradation of pseudonigeran. — Pseudonigeran (0.744 g) was oxidized with 0.1M sodium periodate (100 ml) at 5° in the dark. At suitable intervals, aliquots were taken and the periodate consumption was determined by the arsenite method[24]; formic acid was determined by potentiometric titration with sodium hydroxide and formaldehyde by the chromotropic acid reagent[19] after dialysis of the suspension. The oxidation was complete after 10 days (Table III), and the polysaccharide was then degraded by the Smith procedure[19] (Table III).

The supernatant of the Smith degraded pseudonigeran was examined by paper chromatography (solvent A, reagent A). Only glycerol (R_{Glc} 2.8, blue-gray) and the 2-hydroxyethylidene acetal of glucosylerythritol[24] (R_{Glc} 1.9, brown) were revealed. No erythritol (R_{Glc} 2.0) and no glucose were detected. Glycerol was determined in the supernatant by the chromotropic acid method[19]; found: 3.53 mmoles of glycerol per D-glucose residue. A portion of the supernatant was also hydrolyzed in 0.5M sulfuric acid for 4 h at 100°. The solution was deionized with Duolite A-4 and evaporated to a small volume. On paper chromatography (solvent A), three spots corresponding to

glycerol, erythritol, and glucose were separated and quantitatively determined; found: 3.60, 15.5, and 14.0 mmoles per D-glucose residue, respectively.

The degraded pseudonigeran was further submitted to four Smith degradations. Glycerol was the only product detected in the mild-acid hydrolyzates of degraded pseudonigeran (Table III).

Partial acid hydrolysis of pseudonigeran. — Pseudonigeran (500 mg) was stirred in 72% sulfuric acid for 10 min at room temperature. The polysaccharide dissolved completely. Water (400 ml) was added, and the solution was deionized with Duolite A-4 and Amberlite IR 120 (H^+), and evaporated *in vacuo* (yield, 295 mg). This low yield is explained by precipitation of material in the Duolite A-4 column. A portion of the sirup was chromatographed on paper (solvents A and B) revealing the presence of glucose, nigerose, nigerotriose, nigerotetraose and nigeropentaose having R_{Glc} 1.0, 0.76, 0.54, 0.36, and 0.23 (solvent B), respectively. No trace of other oligosaccharides could be detected. The different components were separated by paper chromatography (solvent B) and were shown to be homogeneous by paper chromatography (solvent B) and by paper electrophoresis in 0.1M borate buffer (600 V, 3 h) having M_{Glc} 1.0, 0.57, 0.53, 0.42, and 0.37, respectively.

A solution of each oligosaccharide in water containing Amberlite IR 120 (H^+) was heated at 75–80°. Aliquots were removed every h and chromatographed on paper (solvent B). All possible oligomers were shown to occur after a 3-h hydrolysis. No trace of other di-, tri- or tetra-saccharides could be detected.

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