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Note

Structural investigation of a cell-wall galactomannan from *Neurospora crassa* and *N. sitophila*

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Neurospora crassa has been the subject of continuous efforts to ascertain the chemical components of its hyphal walls [1]. Early studies showed that the main components were a β-(1 → 3) branched-chain polymer of glucose, with β-(1 → 6) side branches of some 15 to 30 glucose residues [2,3], a chitin-like substance [4], and a proteinaceous material from which a glycoprotein was isolated [2,5,6]. Later, de Vries [7] described an α-(1 → 3)-glucan and, more recently, Hiura et al. [8] have reported the isolation of a linear β-(1 → 3)-D-glucan, with about 86 residues of glucose, and a smaller β-(1 → 3)-D-glucan, of about 20 residues, with minor branching at positions 6 with two or more (1 → 3)-linked glucose residues. In addition, Nakajima et al. [9,10] have reported the isolation of a proteogalactomannan composed of a skeleton of α-(1 → 6)-D-mannan, highly branched at positions 2 and/or 3, as follows below, with u and v varying between 1 and 4, and n, m, p, q, and the order of substitution being unknown. These chains were in turn bonded to the protein, either through *N*-acetylglucosamine-asparagine and/or serine or threonine residues.

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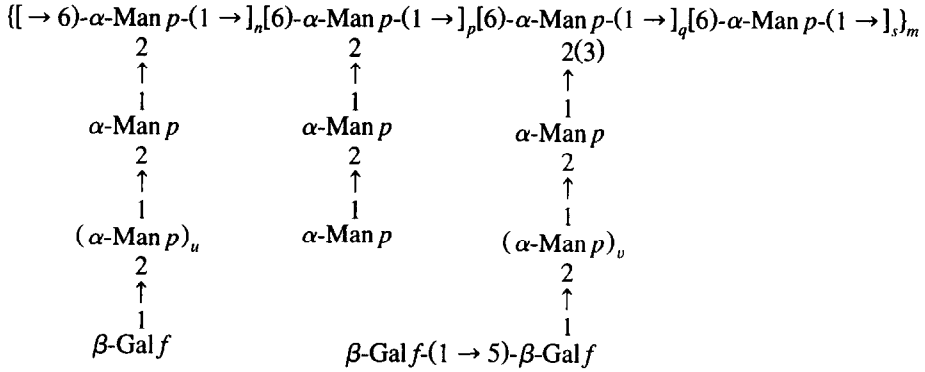


We are engaged in a study of alkali-extractable water-soluble polysaccharides which are minor components (2–8%) of fungal cell walls. On the one hand, it is increasingly clear that, due to their diversity, they could be used as chemotaxonomic markers at the genus or subgenus level [11,12] and, on the other hand, there is growing evidence that they may be involved in cell–cell and/or cell–host recognition mechanisms. Furthermore, many of them (if not all) are antigenically relevant.

As part of these studies we now report on the structure of a galactomannan isolated from the cell walls of *N. crassa* and *N. sitophila*.

1. Experimental

Microorganisms and culture media.—The species of *Neurospora* used were *N. crassa* (CECT 2255) and *N. sitophila* (CECT 2630). The microorganisms were maintained on slants of Bacto potato dextrose agar supplemented with 1 g L^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions have been previously described [13].

Wall material preparation and fractionation.—The preparation of wall material [14] and the fractionation procedure [15] were performed as previously described. The polysaccharide preparation extracted from the dry cell-wall material with 1 M NaOH at 20 °C contained water-soluble polysaccharides (Fraction F1S) and water-insoluble polysaccharides (F1I). Fractions F1S were re-fractionated by treatment with a small portion of water (ca. 50 mg/mL), followed by centrifugation (10,000 *g*, 30 min), giving a solution (F1S-S) and a precipitate (F1S-I).

For preparative chromatography, 100 mg of F1S-S from *N. crassa* were processed according to Leal et al. [16].

Chemical analysis.—Neutral sugars were released by hydrolysis with 2 M H_2SO_4 at 100 °C for 5 h and then converted into their corresponding alditol acetates [17]. Identification and quantification were carried out by gas–liquid chromatography (GLC) using 3% SP-2340 on 100–120 Supelcoport [18]. The absolute configuration of the sugars was determined as described by Gerwig et al. [19].

Methylation analyses were performed according to a modification [20] of the

Hakomori method [21]. The permethylated polysaccharides, which showed negligible IR absorption for hydroxyl groups, were hydrolyzed and the resulting monosaccharides converted into partially methylated alditol acetates which were analyzed by GLC–MS [14].

NMR analysis.—Polysaccharides F1S-S (~ 20 mg) were dissolved in D_2O (0.8 mL) followed by centrifugation (10,000 *g*, 20 min). The supernatant solutions (~ 0.7 mL) were used for recording NMR spectra. The sample from *N. crassa* for 2D experiments was lyophilized, redissolved in D_2O (1 mL), and the process repeated twice for further deuterium exchange. The final sample was dissolved in 0.7 mL of D_2O (99.98% D).

^{1}H-NMR spectra for fractions F1S-S were recorded at 40 °C on a Varian XL-300 spectrometer. 1D- and 2D-^{1}H- and ^{13}C-NMR experiments for *N. crassa* (F1S-S) were carried out at 40 °C on a Varian Unity 500 spectrometer. Proton chemical shifts refer to residual HDO at δ 4.61 and carbon chemical shifts to internal acetone at δ 31.07.

The 2D-NMR experiments (DQF-COSY [22], 2D-TOCSY [23], NOESY, and HMQC [24]) were performed as described previously [25].

2. Results and discussion

The proportions of the fractions obtained from the dry cell-wall material of the two species of *Neurospora* were as follows: *N. crassa* fraction F1S (4.7%), fraction F1I (15.7%). Re-fractionation of F1S gave F1S-S (75%) and F1S-I (14%). *N. sitophila* gave F1S (2.7%), F1I (12.8%), F1S-S (85%), and F1S-I (8%). Analyses of fractions F1S-S of *N. crassa* and *N. sitophila* gave galactose (40.8, 39.6), mannose (51.8, 52.3), and glucose (7.4, 8.1%), respectively. All monosaccharides had the D configuration.

Methylation analyses were carried out on the polysaccharide F1S-S from *N. crassa*, giving terminal galactofuranose (42%), 2,6-disubstituted mannopyranose (40%), 6-substituted mannopyranose (7%), terminal glucopyranose (3.8%), and minor amounts of 6-substituted galactofuranose and 2-substituted galactofuranose (ca. 1.5% each). *N. sitophila* gave similar results.

As the ^{1}H-NMR spectra for F1S-S of both species were almost identical, all the NMR experiments were performed on that from *N. crassa*.

The high-resolution ^{1}H-NMR spectrum and the proton-decoupled ^{13}C-NMR spectra of the polysaccharide in D_2O solution showed two major anomeric signals of similar intensity (Fig. 1), indicating that the polymer consists mainly of a disaccharide repeating unit. Four additional minor anomeric signals can also be seen, indicative of contamination or microheterogeneities. The residues have been labelled **A**, **B** (the major ones), and **C**–**E** (the relevant minor ones), according to their anomeric protons, in order of increasing field and importance. Assignment of the signals for the different residues was made by performing DQF-COSY and TOCSY experiments. Coherence transfer to the protons of the monosaccharide residues **A**–**E** was achieved by isotropic mixing, using 2D-TOCSY with a mixing time of 150 ms. The subspectra through the anomeric signals showed clear connectivities to most of the remaining protons (Fig. 2a), in spite of the small values of $J_{1,2}$. Identification of most cross-peaks was also based on the information obtained from the DQF-COSY. The ^{1}H-NMR chemical shifts are listed in Table 1.

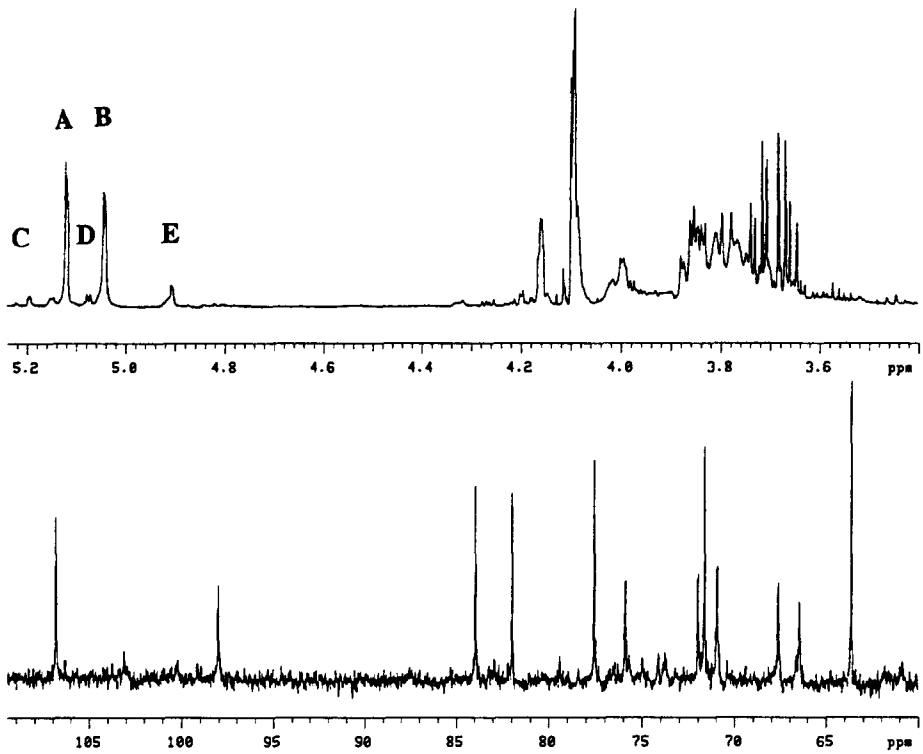


Fig. 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) spectra in D_2O at 40 °C for the cell-wall polysaccharide F1S-S from *N. crassa*. The anomeric protons have been labelled **A**–**E**.

The values of the chemical shifts of H-2, H-3, and H-4 for residue **A** were very similar ($\Delta\delta_{max} < 0.1$), and appeared at relatively low field, $\delta > 4$, indicating the presence of a galactofuranose residue, as also deduced from the methylation analysis. In addition, according to its $J_{1,2}$ value (1.8 Hz), it can be assigned the β configuration [25]. The low chemical shift value of H-2 in residue **B** together with the small value of its coupling constant ($J_{1,2} < 1.5$ Hz) suggest that **B** is a mannopyranose.

In order to confirm the substitution of the residues, we recorded an HMQC experiment (Fig. 2b), which maps the connectivities between carbon atoms and their directly bonded protons. The assignment of most of the signals in the ^{13}C-NMR spectrum was straightforward, since almost all the ^{1}H-NMR chemical shifts were already known. Carbon chemical shifts are also listed in Table 1. The values for C-2 and C-6 of monosaccharide units **B** and **E**, compared with those of the corresponding methyl glycosides [26], allow us to conclude that **B** is a 2,6-disubstituted mannopyranose and **E** a 6-substituted mannopyranose. The chemical shifts of the anomeric carbons indicate unequivocally [26,30] that monosaccharide unit **A** is a β-Gal*f* residue (compare, for instance, with the observed 100–104 ppm for α-Gal*f* units in similar structures [30]). Furthermore, the $^{1}J_{C\text{-}1,H\text{-}1}$ values, obtained from a coupled HMQC experiment, gave **A**,

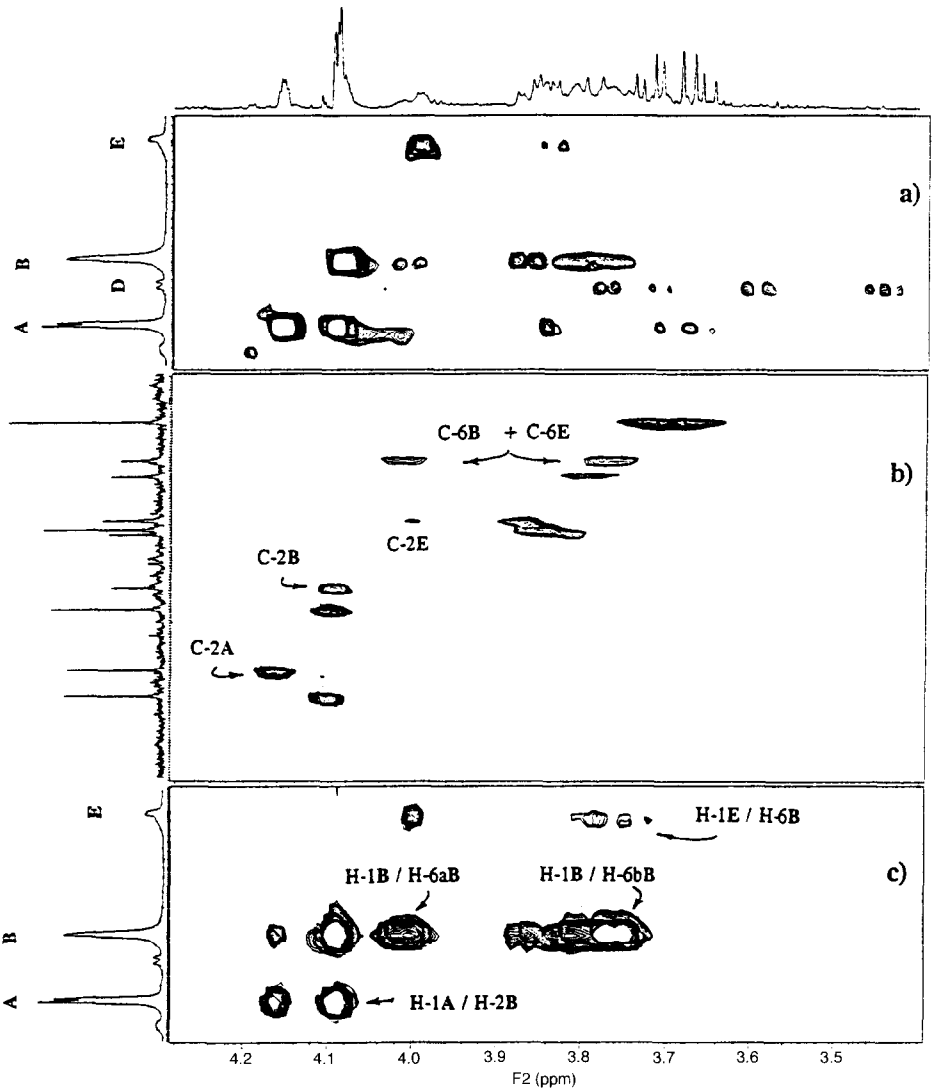


Fig. 2. Selected regions of the 2D-TOCSY (a), HMQC (b), and 2D-NOESY (c) spectra for fraction F1S-S of *N. crassa*, showing the connectivities with the rest of the protons. For the sake of clarity, the spectra (b) and (c) have been registered showing only cross-peaks corresponding to residues **A** and **B**. Relevant cross-peaks have been labelled.

174.2; **B**, 176.2; **E**, 176.0 Hz; supporting a β configuration for **A** (found for α configuration, 182.0 Hz [30]), and α configurations for **B** and **E** [27].

With this substitution of the residues **A** and **B**, two possible structures are consistent with the results thus far discussed: a chain of Man*p*-(1 → 6)-Man*p* with galactofuranosyl groups at position 2 of each unit, or a chain of Man*p*-(1 → 2)-Man*p* with the galactofuranosyl groups attached to position 6 of each mannose.

Table 1
^{1}H- and ^{13}C-NMR chemical shifts (δ) and coupling constants (J, Hz, in brackets) for the cell-wall polysaccharide F1S-S from *N. crassa*

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
F1S-S							
A	5.12 (1.8)	4.16	4.10	4.10	3.85	3.73	3.87
B	5.05 (< 1.5)	4.09	3.87	3.80	3.81	4.01	3.76
C	5.20 (< 2)	4.20	n.d. [a]	n.d.	n.d.	n.d.	n.d.
D	5.08 (3.4)	3.60	3.72	3.45	3.78	n.d	n.d.
E	4.91 (1.5)	4.00	3.84	3.75	n.d.	n.d.	n.d.

Unit	C-1	C-2	C-3	C-4	C-5	C-6
F1S-S						
A	106.8	82.0	77.5	84.0	71.6	63.6
B	98.0	75.9	70.9	67.6	72.0	66.5
C	106.5	80.2	n.d.	n.d.	n.d.	n.d.
D	97.8	71.6	73.6	70.3	73.2	n.d.
E	100.0	70.7	71.4	67.4	ca. 71	n.d.

[a] n.d., Not determined.

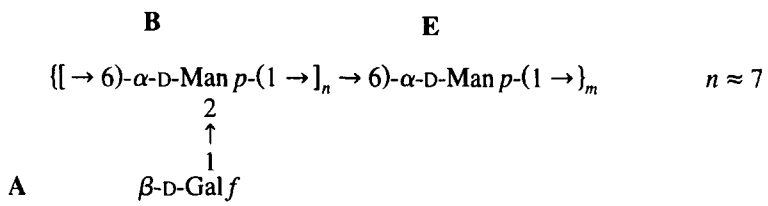
Information concerning the connection **A**–**B** was obtained from a 2D-NOESY experiment (mixing time 300 ms). The existence of cross-peaks between signals of different residues indicates their proximity in space and, probably, their connection. Thus, cross peaks between the anomeric proton of unit **B** and the H-6a and H-6b of a second residue **B** could be observed, along with others connecting H-1 of residue **A** with H-2 of unit **B** (Fig. 2c). Although NOE cross peaks are dependent on the conformation around the glycosidic bonds, and therefore the existence of these cross peaks does not necessarily indicate the exact position of the linkage, in this case it seemed obvious that Gal*f* **A** was (1 → 2)-linked to Man*p* **B** which was attached (1 → 6) to a second unit of Man*p* **B**.

Conclusive additional evidence was obtained by using an HMBC experiment which shows long-range connectivities between carbon atoms and their coupled protons through two or three bonds. Using this technique, coupling across the glycosidic linkages was detected unequivocally. Thus, cross peaks H-1**B**/C-6**B** and H-1**A**/C-2**B** could also be seen.

Concerning the identity and linkages corresponding to the minor signals, a detailed study of the 2D spectra mentioned above led to the identification of most of the cross peaks of units **D** and **E** (see Table 1 and Fig. 2). On the basis of their proton and carbon chemical shifts, the values of their anomeric coupling constants (**D**: $J_{1,2}$ 3.4 Hz; **E**: $J_{C\text{-}1,H\text{-}1}$ 176.0 Hz), comparison with model compounds [28–30], and also methylation analysis, it was concluded that **D** was terminal α-glucopyranose, and confirmed that **E**

was 6-substituted mannopyranose. A cross peak H-1**E**/C-6**B** in the HMBC spectrum demonstrated that **E** was part of the main chain, which means that there is no galactofuranosyl substituent on about one out of every eight mannose residues. On the other hand, although the chemical shifts of the anomeric proton and the corresponding carbon seem to indicate that **C** is a β-galactofuranose residue, the very limited information obtained for **C** does not allow the assignment, by NMR, of the position of substitution by the terminal α-glucopyranosyl group **D**. **C** and **D** together amount to about 6% of the residues. However, taking into account the results of the methylation analyses, the terminal α-glucopyranosyl groups seem to be randomly attached to one out of every 14 galactofuranose residues, either at position 2 or at position 6.

The above connectivities and substitutions are in agreement with the analytical results, and allow us to propose an idealized basic structure (> 95%) of the *N. crassa* polysaccharide as being composed of the repeating unit:



We have found a similar structure in the cell wall of *T. flavus*, but the glycosidic linkage of the galactofuranose moiety was α instead of β [30].

Since this polysaccharide differs from those isolated by a similar procedure from other genera [12,14–16,30,31], it could be used as a chemotaxonomic marker for the genus *Neurospora*.

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