JOURNAL OF BACTERIOLOGY, Mar. 1979, p. 1067-1072
0021-9193/79/03-1067/06$02.00/0

Vol. 137, No. 3

Characterization of the Carbohydrate Component of Fraction I in the *Neurospora crassa* Cell Wall

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Received for publication 18 December 1978

The carbohydrate portion of fraction I of the *Neurospora crassa* cell wall has been analyzed for sugar composition by gas-liquid chromatography and colorimetric methods. The analysis was performed comparatively in a wild-type strain (RL 3-8A) and three morphological mutants: *scumbo* (FGSC 49), *peak-2a* (a mutant known to be allelic to *biscuit*), and *ragged* (FGSC 296). Fraction I of all strains studied contains glucose, mannose, and galactose as the main sugars. Uronic acids and amino sugars are also present in small amounts. The glycosidic linkages binding the neutral sugars were analyzed by Lindberg's combined gas chromatography-mass spectrometry techniques for identification of the partially methylated alditol acetate sugar derivatives. The main polymeric portion of fraction I seems to be a linear glucan with the glucose residues linked by 1 → 3 and 1 → 4 bonds. A mannan portion with a branched configuration is also present, with galactose as the sugar residue which serves as branches in the molecule(s). The branched mannan portion appears to increase in amount in correlation with more drastic morphological changes of the mycelia. In this respect, the mutant *ragged* has the lowest mycelial growth rate and the largest amount of mannan. The importance of the polysaccharide structure of fraction I on the colonial morphology of the mycelia is discussed.

In 1961, de Terra and Tatum (3) proposed that the cell wall is an important, perhaps the main, factor in the determination of *Neurospora* morphology. In 1965, Mahadevan and Tatum (10) were able to determine the composition of the *Neurospora* cell wall by removing selectively its different components by chemical or enzymatic degradation. According to these authors, the *Neurospora* cell wall contains four different components, designated as fractions I, II, III, and IV. Fraction I is an alkali-soluble peptide-polysaccharide complex containing glucose, amino acids, and galactosamine. Fraction II is also a glucan with a type of linkage between hexose molecules that is still not determined. Fraction III is a β, 1 → 3 glucan and is easily digested by laminarinase. Fraction IV is composed of chitin. The localization and morphogenetic importance of the different fractions of *Neurospora* cell wall has been explored by Mahadevan and Tatum (11) and Hunsley and Burnett (7). Fractions I and II form an external layer in the cell wall. They usually exhibit quantitative changes in morphological mutants, an observation that indicates their morphogenetic importance (10).

Wrathal and Tatum (16, 17) indicated that the glucan-peptide complex of fraction I has glycosyl-serine and glycosyl-threonine linkages, but the precise linkage of hexose molecules has yet to be determined.

Nevertheless, preliminary results obtained after enzymatic treatment of fraction I indicate the presence of α, 1 → 4 linkages (Pincheira, unpublished data). For additional details on biochemical outputs and morphology in *Neurospora* see the recent review by Mishra (12).

MATERIALS AND METHODS

Wild-type and mutant strains of *Neurospora crassa* were grown for 48 h at 25°C in 1-liter Erlenmeyer flasks containing 400 ml of Vogel minimal medium supplemented with 2% sucrose (15). The culture was constantly shaken on a rotatory agitator at 140 rpm. The mycelia were collected by filtration and lyophilized. Cell walls were obtained by the method of Mahadevan and Tatum (10). The cell walls appeared under microscopic observation to be free of protoplasmatic components. Fraction I was obtained as described by Mahadevan and Tatum (10). The material was lyophilized and kept dried in a vaccum desiccator.

Analytical methods. Fraction I was quantified as the percentage of dry weight of the cell walls.

Neutral sugars were analyzed by the method of Albersheim et al. (1) by gas-liquid chromatography of the alditol acetate derivatives as follows. A 2-mg amount of fraction I was hydrolyzed with 1 ml of 2 N trifluoroacetic acid in sealed vials kept at 121°C for 75 min. The sugars were converted to alditol acetates,

and the final solution of acetic anhydride containing the sugar derivatives was directly injected into a column of 0.2% ethyleneglycol succinate–0.2% ethylene glycol adipate–0.4% of silicone (2, 14). Gas chromatography was performed with a Perkin-Elmer model 900 gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) with a temperature program of 3°C increase per min from 160 to 190°C and a nitrogen flow rate of 45 ml/min.

Colorimetric methods. Total sugar determination of fraction I was performed by the anthrone method (4). The quantification of uronic acids was performed by the modified carbazol method of Dische (4), and the amino sugars were determined by the Elson-Morgan test (4, 5).

Methylation analysis for the identification of glycosidic linkages. A 4-mg amount of dried fraction I was methylated by the Hakomori method (6) with the modifications of Sandford and Conrad (13). The methylated products were extracted with a 1:1 mixture of chloroform and methanol. The chloroform–methanol extracts were concentrated to a volume of 2 ml and separated from water-soluble reagents of low molecular weight by dialysis against several changes of distilled water. The methylated products were dried and then hydrolyzed with 1 ml of 2 N trifluoroacetic acid for 75 to 80 minutes at 121°C in sealed tubes. The partially methylated sugars were converted to alditol acetate sugar derivatives (9). For mass spectrometry, the partially methylated sugars were reduced with $NaBD_4$ (Merck Sharp & Dohme Canada, Ltd., Montreal, Canada) instead of $NaBH_4$. The presence of deuterium allows the distinction between C_3- and C_4-linked hexoses, because the presence of deuterium in the anomeric carbon provides a distinguishable difference in the fragmentation patterns derived from these two sugar residues.

Each partially methylated alditol acetate component isolated by gas chromatography was identified according to the following information: (i) the position of the methoxy groups in the methylated alditol acetate as obtained by mass spectrum analysis of fraction I; (ii) the sugar composition of the unmethylated fraction I; (iii) the relative retention times of the components in the ethylene glycol succinate–ethylene glycol adipate–silicone column (9; Cardemil, Ph.D. thesis, Michigan State University, E. Lansing, 1975). Known standards were used for calibration of the column, and all retention times were made relative to terminal glucose. Standards were run at the same temperature program as the sample.

The designation used for each partially methylated alditol acetate indicates the linkages (in addition to C_1) by which the corresponding glycosyl residues of the original sample were connected to other sugars (for instance, 2,6-mannose instead of 1,2,6,5-tetra-*O*-acetyl-3,4-di-*O*-methyl mannitol). The derivatives obtained by sugars that occupy a nonreducing terminal position in the polysaccharide are designated by T, as in T-galactose or T-glucose.

Quantitative analysis was performed by integration of the gas chromatographic peaks with an Autolab Computing Integrator, System IV (Spectra-Physics, Santa Clara, Calif.).

To express the data as moles percent of the recovered neutral sugars of the carbohydrate, each peak area was divided by the molecular weight of the partially methylated alditol acetate derivative, and the sum of these ratios was normalized to 100.

Mass spectrometry was performed with an LKB-9000 mass spectrometer (LKB Instruments Inc., Rockville, Md.). This instrument was interfaced with a PDP 8/1 computer.

A glass tubular column (2-mm ID by 1.22 m long) was used. The stationary phase of the column was Supelcoport (100 to 200 mesh) containing 3% trifluoropropyl silicone SP-2401 (Supelco, Inc., Bellefonte, Pa.). The column was temperature programmed for a 3°C increase per min from 160 to 230°C. The ion source was given at 240°C, and the scan was taken at a voltage of 70 eV. The data were prepared in the form of bar graphs by using a computerized data system. The background was subtracted.

RESULTS

All of the mutant strains analyzed in this work (Fig. 1) appeared to have a cell wall with a lower content of fraction I as compared with that of the wild-type strain RL-3-8A (Table I). *scumbo* 49 and *ragged* 296 had even less fraction I present in their walls than did *peak-2a* (6.5% for *scumbo* 49 and *ragged* 296 versus 11.2% for *peak-2a*). The total amount of sugars in fraction I, quantified by the anthrone test, did not exhibit significant differences between the wild-type and the mutant strains (Table 2).

The neutral sugar components of fraction I, identified by gas-liquid chromatography of alditol acetate derivatives in all the strains, indicated the presence of glucose, mannose, and galactose (Table 2). However, the amount of glucose decreased from wild type to *ragged* 296 (Table 2), whereas the amount of mannose in-

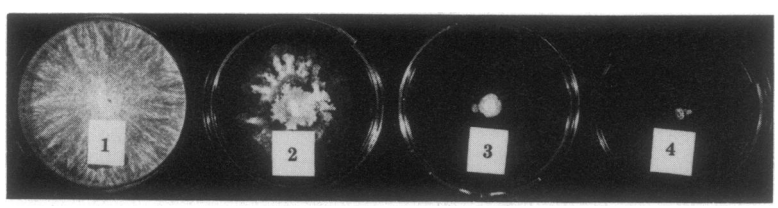

FIG. 1. *Four strains selected for this research. Dish 1 is wild type strain RL 3-8A; dish 2 is scumbo (FGSC 49); dish 3 is peak-2a; and dish 4 is ragged (FGSC 296).*

creased significantly in the mutants compared with the wild type (19.7 ± 0.4, 20.6 ± 0.1, and 37.6 ± 0.1% in the mutants versus 13.2 ± 1.2% in wild type). In *ragged* 296, mannose became the main component, and glucose was only 29.7% of the total weight of the neutral sugars. Galactose was also increased in fraction I of the mutant strains (13.6 ± 0.3, 13.8 ± 0.5, and 26.9 ± 2.3% in the mutant strains versus 4.1 ± 0.4% in wild type). *ragged* 296 was the mutant strain containing the largest amount of galactose (about 6.5 times more than wild type).

The amount of uronic acids decreased gradually in the mutant strains (Table 2). *scumbo* 49 had less than 44.8% of the uronic acids present in wild type. *peak-2a* had 57.8% of *scumbo*, and *ragged* 296 had 55.1% of the uronic acids of fraction I of *peak-2a*.

The amount of hexosamines was substantially reduced in the mutant strains as compared with the wild type (Table 2). However, hexosamines increased from *scumbo* 49 to *ragged* 296.

Methylation of the carbohydrate portion of fraction I gave a soluble product in chloroform-methanol. The recovery of sugar in fraction I after methylation analysis was estimated by the anthrone test and agreed with the total amount of sugars estimated for fraction I before methylation. In most of the experiments, the analysis was performed with a remethylated product, and again the recovery indicated no significant loss of sugars by degradation.

The ethylene glycol succinate–ethylene glycol adipate–silicone column used for gas chromatographic analysis resolved seven peaks of the partially methylated alditol acetate derivatives (Fig. 2). The seven peaks appeared in the same order in the SP-2401 column used for combined gas chromatography-mass spectrometry analysis. The molecular fragments of each of these partially methylated alditol acetate sugars were obtained and were in agreement with those reported by Lindberg (9). In a bar graph of the mass spectrometry results (data not shown) 3-hexose and 4-hexose were easily distinguished because the methyl sugars were reduced by $NaBD_4$; 3-hexose (peak 4) gave fragments of electron mass (*m/e*) 161 and 234, whereas 4-hexose (peak 5) gave fragments of *m/e* 162 and 233.

Peak 1 was identified as a T-hexose. It has the characteristic fragments of the T-hexoses in the pyranose configuration (*m/e*, 45, 117, 161, 205, and 249), and it is T-glucose because it showed chromatographic identity with T-glucose of laminarin. Peak 2 was also a T-hexose because it gave the characteristic fragments of T-hexoses in the pyranose configuration (*m/e*, 45, 117, 161, 205, and 249). It was identified as T-galactose because it has the same relative retention time as T-galactose of arabinogalactan. Peak 3 was a 2-hexose; it gave the characteristic fragments of 2-hexoses (*m/e*, 45, 117, 161, 190, 205, and 234). It had the retention time relative to T-glucose expected for 2-mannose. Peak 4 was identified as a 3-hexose because its fragments were *m/e* 45, 118, 161, 234, and 277, and it was a 3-glucose because it showed chromatographic identity with 3-glucose of laminarin. Peak 5 was a 4-hexose; its fragments were *m/e* 45, 118, 162, 233, and 277, and it showed chromatographic identity with the 4-glucose present in maltose. Peak 6 was a 2,6-mannose. It gave the characteristic fragments *m/e* 45, 162, 189, 206, and 233; it also had the expected retention time relative to T-glucose of 2,6-mannose. Peak 7 was the acetate inositol peak, the internal standard which had a longer retention time relative to T-glucose.

The amount of mannose present in the 2-

TABLE 1. *Weight percentage of fraction I in wild type and mutant strains*

Strain	% of cell wall in fraction I
RL-3-8A (wild type)	13.8 ± 0.4[a]
scumbo 49	6.5 ± 0.6
peak-2a	11.2 ± 1.0
ragged 296	6.5 ± 0.3

[a] Each value is the mean ± standard deviation and was derived by weight determinations of lyophilized material from three different wall preparations.

TABLE 2. *Total amount of sugar and weight composition of the neutral, uronic acid, and amino sugars present in fraction I*[a]

Strain	mg of sugar/mg of fraction I	% of wt in:				
		Glucose	Mannose	Galactose	Uronic acids	Hexosamines
RL-3-8A	0.75 ± 0.01	57.8 ± 5.3	13.2 ± 1.2	4.1 ± 0.4	18.3 ± 2.7	5.9 ± 0.7
scumbo 49	0.73 ± 0.01	58.0 ± 0.5	19.7 ± 0.4	13.6 ± 0.3	8.2 ± 0.2	0.6 ± 0.1
peak-2a	0.78 ± 0.01	59.3 ± 0.5	20.6 ± 0.1	13.8 ± 0.5	4.9 ± 0.2	1.3 ± 0.2
ragged 296	0.72 ± 0.01	29.7 ± 2.5	37.6 ± 0.1	26.9 ± 2.3	2.7 ± 0.1	2.7 ± 0.1

[a] Each value is the mean ± standard deviation and was derived from three different fraction I preparations.

mannose (peak 3) and the 2,6-mannose (peak 6) peaks accounted for the total amount of mannose present in fraction I. T-galactose accounted for the total amount of galactose present in fraction I, and the amount of glucose present in the T-glucose, 3-glucose, and 4-glucose peaks also accounted for the total amount of glucose present in fraction I, as given by sugar composition analysis (Tables 2 and 3). If the data of Table 2 given for neutral sugars are expressed in moles percent (by dividing the peak area of each sugar derivative by its respective molecular weight and normalizing to 100), the data can be easily compared with those of Table 3, and the recovery for each neutral sugar after methylation analysis can be deduced.

DISCUSSION

Fraction I has been described by Mahadevan and Tatum (10) as a complex glycopeptide containing glucose, glucuronic acids, and galactosamine. However, they used analytical methods that have been largely supplanted today. The use of gas-liquid chromatography for neutral sugars permits an easy and efficient identification and quantitation of the sugar components (1, 14). This more efficient technique may explain why mannose and galactose were not detected before in fraction I. These two sugars may not be contaminants in as much as the results are highly reproducible, even when two different methods for purification of fraction I have been

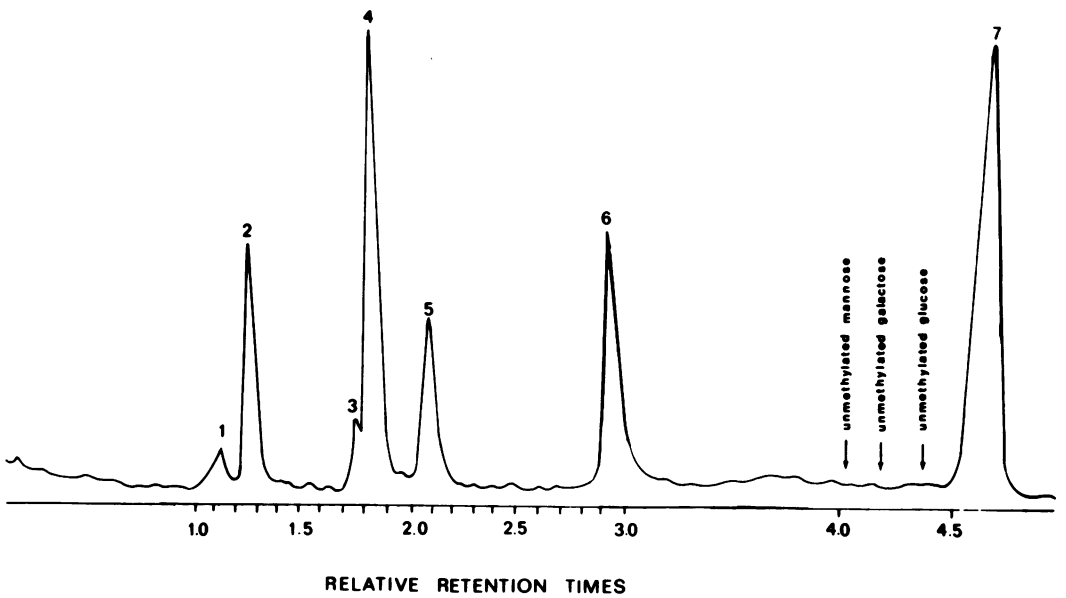

FIG. 2. *Gas chromatogram of the partially methylated alditol acetate derivatives obtained from the carbohydrate portion of fraction I of the wall of the colonial mutant ragged 296. The gas chromatogram of the mutant was chosen for illustration in this figure because it is the one that shows the best T-galactose and 2,3-mannose peaks. Peak 7 is acetate inositol, used as internal standard. The time scale is expressed as relative retention time relative to T-glucose. The arrows show the relative retention times for unmethylated mannose, galactose, and glucose (alditol acetate derivatives). The chromatogram was obtained from the ethylene glycol succinate–ethylene glycol adipate–silicone column. The program used for the column and the identification of each peak of the chromatogram are described in the text.*

TABLE 3. *Moles percent composition of the partially methylated alditol acetate components of fraction I*

Strain	Mol% of the following partially methylated alditol acetate components:						
	T-glucose	T-galactose	2-Mannose	3-Galactose	4-Glucose	2,6-Mannose	Total
RL-3-8A	3.3 ± 0.2[a]	5.1 ± 0.2	18.2 ± 0.2	58.7 ± 0.8	9.3 ± 0.2	5.3 ± 0.3	99.9
scumbo 49	5.4 ± 0.8	15.0 ± 2.8	6.9 ± 2.5	45.4 ± 3.9	10.6 ± 0.4	16.4 ± 5.3	99.7
peak-2a	2.7 ± 1.1	18.5 ± 3.2	6.7 ± 0.4	43.5 ± 4.4	11.0 ± 0.1	17.3 ± 1.4	99.7
ragged 296	3.5 ± 0.1	28.7 ± 1.5	8.5 ± 3.0	19.1 ± 0.2	10.1 ± 1.0	30.0 ± 1.2	99.9

[a] Each value is the mean ± standard deviation of three independent fraction I preparations.

used (10).

Fraction I is present in a smaller amount in the mutants here analyzed than in the wild-type strain analyzed (Table 1), but the amount of the glycopeptide in the walls seems not to be correlated with the colonial morphology. When similar studies have been performed before (10, 11) in other colonial strains, greater amounts of fraction I have been found in the wild type.

Uronic acids decrease as changes in colonial morphology are more evident (from wild type to *ragged* 296) (Table 2 and Fig. 1). The quantitative correlation between uronic acids and colonial morphology suggests that uronic acids may play a role in regulating linear growth of the hyphae of the mycelia. Uronic acids containing polysaccharides are molecules that generally hold a greater amount of water. The retention of water by fraction I might give more plasticity to the cell wall and therefore allow a larger extention growth rate of the hyphae; this would explain why growth is mainly longitudinal without branching.

The hexosamines are diminished in the mutants as compared with the wild-type strain (Table 2). They increase from *scumbo* 49 to *ragged* 296. They double from one mutant to the next. The hexosamines are generally components that link the sugars of the carbohydrate portion to the amino acids of the protein in a glycoprotein molecule. They act as bridge components in the glycoprotein. Our results suggest that in the *ragged* 296, these bridge sugars are more numerous than in the other strains analyzed.

From the methylation analysis of neutral sugar components of fraction I, we reached the following conclusions. (i) In the wild-type strain, the main portion seems to be a linear glucan, where glucose is linked by $1 \rightarrow 3$ bonds (Fig. 2). (ii) 4-Glucose is present in only about 10% of the total sugar component derivatives found. This amount does not change from wild-type to mutant strains (Table 3). (iii) All of the galactose present is terminal. There is also a small amount of T-glucose that remains constant in wild type and mutants (Table 3). (iv) Mannose is an internal sugar. There is some linear mannose linked through C_2. The 2-mannose decreases in amount in the mutants (18.2% in wild type versus 6.95, 6.68, and 8.50% in the mutants). The branched 2,6-mannose increases considerably from wild type to *ragged* 296 (5.30 versus 32.17%; Table 3). The larger amount of branched mannose in the colonial mutants is not an artifact of undermethylation, as its amount is correlated with the amount of T-galactose and there are no peaks of unmethylated alditol acetate sugars (Fig. 2, arrows). Furthermore, the results of methylation analysis are highly reproducible. In Table 3 each value is a mean value of three different experiments. (v) The increase of T-galactose, along with the rise of branched 2,6-mannose, suggests that galactose is the branch sugar that 2,6-mannose is holding (Table 3). The presence of linear 2-mannose leads to the possibility that T-galactose might be linked to C_6 of the branched mannose.

The analyses we have performed cannot tell us whether there is only one polysaccharide present or more than one, a glucan and a mannan. Nor can we identify the glycosidic linkages of the hexosamines and uronic acids or where they are located in the one or more polymers, since uronic acids and hexosamines are not detected by methylation analysis.

Whether the branching mannan component is causally related to morphology is unknown. This is strongly suggested by our results, but it remains to be proved. It may be envisaged that a branching polysaccharide is adding complexity to the cell wall structure. This complexity might make the wall more rigid, providing more bonding interactions between the polymers that constitute fraction I. Branching mannans in other *Ascomycetes*, such as yeast, become the main polysaccharide component of their walls (8). We are in the process now of analyzing fraction I carbohydrate in a double mutant that we have obtained by crossing *peak-2a* with *ragged* 296. The results of this analysis may answer whether the colonial phenotype is related to a partial replacement of a linear glucan component by a branching mannan component in fraction I. It might well be that the dichotomous branching in the mutants is a consequence of a preferential deposition of branching mannan in a specific area of the wall which could be surrounded by areas where the uronic acid components of fraction I are present in larger amounts.

ACKNOWLEDGMENTS

We thank C. C. Sweeley, Department of Biochemistry, Michigan State University, for the use of the mass spectrometer and Adrian Srb, Cornell University, and Deborath Delmer, Michigan State University, for their critical comments on the manuscript.

This research was supported by grant 1543 from the Servicio de Desarrollo Científico, Artístico y de Cooperación Internacional, Universidad de Chile.

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