A Morphological Mutant of *Neurospora crassa* with Defects in the Cell Wall $\beta$-Glucan Structure

Yogo Chiba, Tasuku Nakajima* and Kazuo Matsuda**

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Tsutsumodori-Amamiyamachi, Sendai 980, Japan

Received July 5, 1988

A morphological mutant of *Neurospora crassa*, which showed great changes in cell wall $\beta$-glucan structures, was obtained. The mutant lacked spore-forming ability. Chemical analysis indicated that the mutant cell walls had more carbohydrates and less proteins than the wild type. In the structural polymers of cell walls, heteroglycan and chitin were not apparently changed in their sugar composition and structures. On the other hand, the alkali-soluble $\beta$-glucan of this mutant showed significant changes in the chemical structure, particularly, the number and length of branches. The mutant glucan had about 2.5 times as many branches as that from wild type and the number of 1,3-linked glucose residues was greatly reduced.

Filamentous fungi change their morphology through all their growth stages and these changes may correlate to the chemical changes in cell wall compositions.¹⁻³ In our previous studies, cell wall of *Neurospora crassa* have been shown to be constituted of two types of $\beta$-glucans containing different proportions of $\beta$-1,3-and $\beta$-1,6-linkages,⁴ and the one of the glucans, an alkali-soluble $\beta$-glucan has been shown to change its structure with the growth of the cells.⁵ The *N. crassa* cells produce $\beta$-1,3-glucanase⁶ and $\beta$-1,6-glucanase⁷ which may be involved in structural modification of cell wall $\beta$-glucans.⁵,⁷ Morphological mutants from yeasts, *Saccharomyces cerevisiae* also showed structural alteration in the cell wall $\beta$-glucans.⁸⁻¹⁰

In this study, to elucidate the relation of fungal morphology with the cell wall $\beta$-glucan structures, we isolated a morphological mutant of *Neurospora crassa* and compared the chemical properties of cell walls between the mutant and the wild-type cells.

**MATERIALS AND METHODS**

Materials. Zymolyase 5,000 and 6,000, yeast cell lytic enzymes from *Arthrobacter luteus*,¹¹ was provided by Kirin Brewery Co., Tokyo. Exo-$\beta$-1,3-glucanase from *Basidiomycete* sp. QM-806¹² was donated by Mr. Tabata, Taito Co., Kobe. The series of $\beta$-1,3-, and $\beta$-1,6-glucosyl-oligosaccharides used as markers on chromatographic analysis were obtained from our previous work.¹³ Other chemicals and materials were obtained from commercial sources.

Organism and culture condition. The strain, *Neurospora crassa* IFO-6068 wild type, and its morphological mutants were grown for suitable times at 28°C with shaking in Vogel-N-sucrose medium.¹⁴ Cultures of wild type were maintained on potato agar slants and a morphological mutant, No. 18, was kept in the liquid medium as described above.

Isolation of mutants. Spores (10⁵ ~ 10⁶/ml 0.1 M phosphate buffer, pH 7.0) obtained from the wild-type strain were treated with N-methyl-N′-nitro-nitrosoguanidine (NTG, 800 μM) for 140 min at 25°C. After removal of NTG by washing with phosphate buffer, the washed spores were transferred to Vogel's medium containing 0.1% L-sorbos. The above conditions resulted in 10%
survival. Colonies altered in morphology were selected under microscopic observation.

**Zymolyase treatment.** Mycelia of *N. crassa* wild type and mutant harvested at late logarithmic growth phase were lyophilized and ground. The powdered mycelia (2 mg) were digested with Zymolyase 5,000 (0.15 U) at 37°C for appropriate times.

**Preparation of cell walls.** Mycelia (40 g, wet weight) of the wild type and the mutant No. 18 harvested at late log-phase were suspended in Tris–HCl buffer (0.1 M, pH 8.5, 200 ml) with glass beads (0.2 mm diameter, 150 ml). The mixture was homogenized in a mixer (Hitachi, VA-853) with specifically designed Teflon blades for 10 min below 5°C by addition of powdered dry ice as described previously. The homogenate was centrifuged and the residue was rehomogenized under the same conditions. After confirmation of brakage of the mycelia by phase-contrast microscopic observation (Olympus, BH-2), glass beads were removed by decantation followed by differential centrifugation. After several washings, the cell walls were collected and lyophilized. (Yield: wild type, 1.5 g, mutant No. 18, 0.9 g).

**General methods.** Total carbohydrates were estimated by the method of Dubois et al. and reducing sugars by the method of Somogyi. For analysis of component sugars, cell walls were hydrolyzed with 25 N H₂SO₄ for 1 hr at 4°C and then with 8 N H₂SO₄ for 4 hr at 100°C or polysaccharides were hydrolyzed with 2 N H₂SO₄ at 100°C for 1 hr (mannan), and for 8 hr (β-glucan). The acid hydrolyzates were converted to alditol trifluoroacetates and analyzed by gas-liquid chromatography by the method of Imanari et al. using a gas chromatograph (Hitachi, 163) with a glass column containing 1.5% QF-1 on Chromosorb W at 130°C. Protein concentration was measured by the method of Lowry et al. Alkali-soluble β-glucan was methylated by the method of Hakomori four times to obtain a permethylated sample. Hydrolysis of the methylated glucan, and acetylation of the hydrolyzate were done by the procedure of Lindberg. Paper chromatography was done on Toyo No. 50 filter paper by the multiple ascending method with a solvent system of 1-butanol–pyridine–water (6:4:3) at 65°C. Sugars on the chromatogram were detected with alkaline silver nitrate.

**RESULTS**

**Isolation of Neurospora crassa cell wall mutants which defect β-glucan structures**

*Neurospora crassa* IFO-6068, wild type and its mutant cells harvested at stationary phase were treated with Zymolyase, a cell lytic enzyme containing predominantly endo-β-1,3-glucanase. A turbidity assay showed that wild-type cells were more susceptible to the enzyme than mutant cells (Fig. 1). The cells grown on sorbose medium which restricts the growth of hyphae as an inhibitor for cell wall β-glucan synthesis, also showed less susceptibility to Zymolyase (Fig. 1). The Zymolyase digests were separated by gel filtration on a Bio-Gel P-2 column (Fig. 2). General elution patterns obtained from wild-type and mutant cells, showed two clearly separated carbohydrate peaks. On the other hand, only one mutant designated No. 18 strain gave three
peaks with one large and two small saccharides. The difference in the Zymolyase digestion pattern of the mutant No. 18 cells indicates that the cell wall β-glucan might be significantly altered. No 18 was selected for further analysis.

Growth and morphological characteristics of N. crassa mutant, No. 18 cells

The N. crassa mutant, No. 18 showed poorer growth than the wild-type cells. Samples taken in each growth stage were observed under a microscope (Fig. 3). In the wild-type cells, young mycelia taken at early log phase (stage 1), had a shape like strings of beads.
When the cells grew older, the bead-like structure disappeared and the cells became normal mycelial shapes. On the other hand, the mutant cells stopped growing at early log phase and retained the bead-like structures. Moreover, the mutant was defective in spore-formation.

Chemical analysis of the mutant No. 18 cell walls

Table I shows the chemical constituents of cell walls of mutant No. 18 and of wild type. The mutant cell walls had higher carbohydrate and lower protein content than wild-type cells. Chitin content as expressed as hexosamine was not greatly changed. It is notable that the ratio of glucose to mannose was 4 in wild type and 2.5 in the mutant, but the galactose content was not changed between the mutant and the wild type cells.

Cell walls of mutant and wild type were digested with Zymolyase and the digests were fractionated by gel filtration on a Bio-Gel P-2 column (Fig. 4). The polymer fraction on a Sepharose CL-6B column (Fig. 5). Both samples from wild-type and mutant cells eluted at the same position and showed symmetrical peaks. Chemical analysis of the samples showed the same sugar composition with the same ratios indicating the polymer fraction was a heteroglycan whose structure was not changed by the mutation. These results suggest that morphological changes of the mutant cells might be due to structural changes of cell wall β-glucans.

Structural analysis of cell wall β-glucans

Polymers in the cell walls were extracted successively with hot water and alkali (Fig. 6). Finally, four fractions, namely, a hot water extractable fraction (F-I), a 1N alkali extractable and water soluble fraction (F-II), a 1N alkali extractable and water insoluble fraction (F-III), and a 1N alkali unextractable fraction (F-IV) were obtained. The monosaccharide composition of each fraction from mutant and wild-type cell walls (Table II) showed that F-III and F-IV had predominantly glucose indicating β-glucan fractions. F-III is soluble in

**Table I. Analysis of Cell Walls of Neurospora crassa IFO-6068 Wild Type and Mutant No. 18**

<table>
<thead>
<tr>
<th>Components</th>
<th>Wild type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(weight %)</td>
<td>(mol %)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>54.5</td>
<td>100</td>
</tr>
<tr>
<td>Glucose</td>
<td>61.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Mannose</td>
<td>15.2</td>
<td>21.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>21.4</td>
<td>22.1</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>8.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Protein</td>
<td>14.1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

a Cell walls were hydrolyzed with 6N HCl at 100°C for 4 hr. Hexosamine content was assayed by the method of Johnson.28

b Cell walls were hydrolyzed with 6N HCl for 24 hr at 100°C. Protein content was measured by the ninhydrin method.29
Fig. 5. Gel Filtration of the Polymer Fractions from Zymolyase Digests.
Polymer fractions eluted in void volume on Bio-Gel P2 column (Fig. 5) were further purified by gel filtration on Sepharose CL-6B column (1.4 x 70 cm).

Table II. Yields and Monosaccharide Compositions of the Fractions Obtained from Cell Walls

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Strain</th>
<th>Yield (wt%)</th>
<th>Sugar composition (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fuc  Ara  Man  Glc  Gal</td>
</tr>
<tr>
<td>I</td>
<td>Wild type</td>
<td>9.3</td>
<td>7.2  41.1  23.5  28.1</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>8.1</td>
<td>—    2.5  37.2  31.8  25.6</td>
</tr>
<tr>
<td>II</td>
<td>Wild type</td>
<td>4.9</td>
<td>0.8  2.2  37.3  22.4  37.3</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>8.5</td>
<td>1.1  1.6  38.5  18.4  40.4</td>
</tr>
<tr>
<td>III</td>
<td>Wild type</td>
<td>8.7</td>
<td>—    —    0.9  91.7  7.3</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>9.0</td>
<td>—    —    1.7  85.1  13.2</td>
</tr>
<tr>
<td>IV</td>
<td>Wild type</td>
<td>7.35</td>
<td>—    9.1  73.3  17.7</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>78.0</td>
<td>—    18.4  65.7  16.0</td>
</tr>
</tbody>
</table>

* Trace.

Fig. 6. Scheme for Sequential Extraction of Polymers from Neurospora crassa Cell Walls.

alkali, on the other hand F-IV is not soluble in alkali and other solvents. Although F-IV could not be purified further, F-III was further fractionated by gel filtration on Sepharose CL-6B column (Fig. 7). Both fractions obtained from wild-type and mutant cell walls showed similar elution patterns which were heterogeneous in molecular size, so F-III was fractionated into four subfractions, namely, F-IIIa, F-IIIb, F-IIIc, and F-IIIId (Fig. 7). Among these subfractions, F-IIIb and F-IIIc was further analyzed, since F-IIIa and F-IIIId were still heterogeneous in size.

Methylation analysis of F-IIIb and F-IIIc clearly showed differences in the ratio of branching (2,3,4,6-tetramethyl Glc/2,3,4-+ 2,4,6-trimethyl Glc) and also in the ratio...
Table III. Methylation Analysis of β-Glucan Fractions

<table>
<thead>
<tr>
<th>Alditol acetate</th>
<th>Linkage</th>
<th>Fraction IIIb</th>
<th>Fraction IIIc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild</td>
<td>Mutant</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-Me-Glc*</td>
<td>Glc (1-)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2,4,6-Tri-O-Me-Glc</td>
<td>-3)Glc (1-)</td>
<td>44.0</td>
<td>14.2</td>
</tr>
<tr>
<td>2,3,4-Tri-O-Me-Glc</td>
<td>-6)Glc (1-)</td>
<td>6.3</td>
<td>3.6</td>
</tr>
<tr>
<td>2,4-Di-O-Me-Glc</td>
<td>-3)Glc (1-)</td>
<td>1.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* 3,5-Di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, etc.

![Fig. 8. Gel Filtration on Bio-Gel P2 of the Products Obtained on Exo-β,1,3-glucanase Digestion of Fractions IIIb and IIIc.](image)

Fig. 8. Gel Filtration on Bio-Gel P2 of the Products Obtained on Exo-β,1,3-glucanase Digestion of Fractions IIIb and IIIc. Samples (500 µg) were hydrolyzed with Basidiomycete QM-806 exo-β,1,3-glucanase (0.2 U) at 30°C for 12 hr. The solubilized products were fractionated on the Bio-Gel P2 column (1.5 x 100 cm). The elution position of glucose-oligosaccharides are shown in the figure.

of 1,3-linked glucose (2,4,6-trimethyl Glc) to 1,6-linked glucose (2,3,4-trimethyl Glc) between the wild type and the mutant (Table III). The alkali-soluble β-glucan from the mutant No. 18 cell walls has about 2.5 times as many branches as that from wild-type cell walls and the number of 1,3-linked glucose residues is greatly reduced. Gel filtration of exo-β,1,3-glucanase digests from F-IIIb and F-IIIc also showed different elution profiles between them (Fig. 8). The mutant showed three peaks corresponding to monosaccharide, disaccharide, and trisaccharide positions and two of them (di- and trisaccharides) were not seen in the wild type. The oligosaccharides were glucose, gentiobiose, and gentiotriose, as checked by paper chromatographic analysis (data not shown).

These results indicate that the alkali-soluble β-glucan from N. crassa mutant No. 18 has significant modifications in its structure, especially the number and length of branches.

**DISCUSSION**

It has not been well established what determines the shape of fungal cells. Inhibition of chitin synthesis by Polyoxin D causes significant morphological changes in a filamentous fungus, *Mucor rouxi*,24,25 and the antibiotics also affect the cell wall β-glucan to inhibit the formation of hyphae.25) 2-Deoxyglucose, a inhibitor of β-glucan synthesis, also leads to morphological changes in the yeast *Saccharomyces cerevisiae*.26) This suggests that chitin and β-glucan which are the main structural components in fungal cell walls, may be important in fungal morphogenesis. Chemical analysis of the cell walls from colonial mutants of *Neurospora crassa*1) showed appreciable changes of the amount of chitin, β-glucan, and peptide-polysaccharide, and the latter two
polysaccharides have been shown to be important to morphology. An *N. crassa* morphological mutant obtained in this study showed an alkali-soluble β-glucan being altered with no appreciable changes in chitin and cell surface proteo-heteroglycan. The results suggest that reduction of the amount of β-1,3-linked glucose residues with a concomitant increase of β-1,6-linked glucose residues may inhibit the hyphal wall extention. It is of interest that the *N. crassa* β-glucan mutant in this study had higher resistance to Zymolyase than wild-type cells, on the other hand, β-glucan mutants from yeasts obtained in our previous study showed great sensitivity to Zymolyase. This difference may be based on the differences of cell wall composition between filamentous fungi and yeasts. The hyphal walls are composed usually of two main skeletal polysaccharides, chitin and β-glucan, but yeast cell walls contain less chitin which localizes only in bud scars, and more β-glucans which are cementing materials as well as skeletal polysaccharides. *N. crassa* wild type grown on sorbose showed some restriction of the hyphal growth and Zymolyase resistance, but no prominent change in the Zymolyase digestion pattern. This indicates that sorbose actually affects the growth of hyphae, probably on the cell wall β-glucan synthesis, but the change of the β-glucan structure caused by sorbose may be small.

Finally, it is not clear whether the morphological changes of the mutant No. 18 reflect a single genetic event or not; genetic analysis is needed.

**Acknowledgments.** We wish to thank Dr. K. Morimoto, Kirin Brewery Co., for providing Zymolyase and Mr. K. Tabata, Taito Co., for the *Basidiomycetes* sp. QM-806 exo-β-1,3-glucanase.

**REFERENCES**