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IDENTIFICATION OF β-GALACTOFURANOSYL RESIDUES AND THEIR RAPID INTERNAL MOTION IN THE PENICILLIUM OCHRO-CHLORON CELL WALL PROBED BY ^{13}C NMR

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SUMMARY

Polysaccharides, which come into resonances in the ^{13}C NMR spectrum of Penicillium ochro-chloron intact mycelium and give anomeric carbon signals at 107.5 and 108.3 ppm, are associated with the cell wall. By ^{13}C NMR and gas liquid chromatography analysis, it is shown that the polysaccharides are two types of β-galactofuranosyl residues, one of which has (1→2)-β-galactofuranosyl linkages. Both β-galactofuranosyl residues, which are minor cell wall components, experience rapid internal motion in the cell wall.

INTRODUCTION

The fungal cell wall is a complex structure composed mainly of polysaccharides. The crystalline polysaccharides, such as chitin, cellulose and glucan, constitute the inner skeletal portion of the cell wall, while the amorphous homo- and heteropolysaccharides are components of the outer wall matrix (1). However, little is known about minor components and dynamic property of the cell wall polysaccharides.

Nuclear magnetic resonance (NMR) spectroscopy provides information on the molecular motion of the compounds as well as the chemical structure and has been applied to the studies of intact cells to analyze intracellular molecular components in vivo (2). The ability to exhibit narrow resonances depends strongly on the motional flexibility of the molecules.

Previously we have reported that well resolved, natural abundance ^{13}C NMR spectra of mycelia of a heavy metal tolerant fungus, Penicillium ochro-chloron, were successfully obtained and that the resonances of mannitol, triacyl glycerols and polysaccharides were observed (3).

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In the present communication, we describe the results of studies on the location in the cell and on the structure of the polysaccharides, designated PS B, which give anomeric carbon resonances at 107.5 and 108.3 ppm in the ^{13}C NMR spectrum of P. ochro-chloron cells. The polysaccharides PS B proves to be located in the cell wall and identified as β-galactofuranosyl residues. The dynamic property of the β-galactofuranosyl residues in the cell wall is also discussed.

MATERIALS AND METHODS

Culture condition: Penicillium ochro-chloron, strain ATCC 36741, was grown at 30°C with aeration to the early stationary phase on the chemically defined medium (4) which contained 4% glucose as a sole carbon source. The mycelium was harvested on a suction filter and washed three times with deionized water.

Preparation of the cell walls: The mycelium was suspended in deionized water and disintegrated three times with a MANTON GAULIN homogenizer at 4°C. The mixture was then centrifuged and the residue was lyophilized and extracted with chloroform:methanol (2:1, v/v) to remove contaminating lipid. The air dried preparation was washed by centrifugation first with 5% sodium dodecyl sulfate (SDS) six times and then with deionized water till the lather of SDS disappeared. The washed cell walls were lyophilized and the yield was about 40% of the dry weight of the mycelium used. Each step of the cell wall preparation was monitored using a phase contrast microscope and ^{13}C NMR.

Isolation of the cell wall polysaccharides PS B: Two g of the cell walls was treated with 50 ml of deionized water for 20 min at 100°C and then extracted with 50 ml of 0.1 N H_2SO_4 for 20 min at 100°C. The H_2SO_4 extract was neutralized with 0.2 N $Ba(OH)_2$, and $BaSO_4$ precipitate was removed by centrifugation. The supernatant was lyophilized and dissolved in 5 ml of deionized water. Nine volumes of ethanol were added to the solution to afford a precipitate which was collected by centrifugation and lyophilized. These crude polysaccharides were dissolved in deionized water and fractionated by fractional precipitation with ethanol. The polysaccharide PS B1 was found in 80-90% ethanol fraction.

NMR measurement: ^{13}C NMR spectra were obtained at 25.1 MHz on a JEOL FX-100 spectrometer operating in the Fourier transform mode. The deuterium resonance of external D_2O was used as a frequency lock. Chemical shifts are given relative to external tetramethylsilane at 0 ppm.

Sugar analysis: The sugars hydrolyzed by treatment of 1 mg of a sample with 1 N H_2SO_4 for 2 hr at 100°C were trimethylsilylated (5) and separated by gas liquid chromatography on a Shimadzu GC-5A equipped with a hydrogen flame ionization detector. A glass column coated with OV-101 was used at 175°C.

RESULTS AND DISCUSSION

Fig. 1 shows the natural abundance ^{13}C NMR spectra of P. ochro-chloron intact mycelium and isolated cell walls at stationary phase. While, in the ^{13}C NMR spectrum of intact cells, the resonances of mannitol, triacyl glycerols and

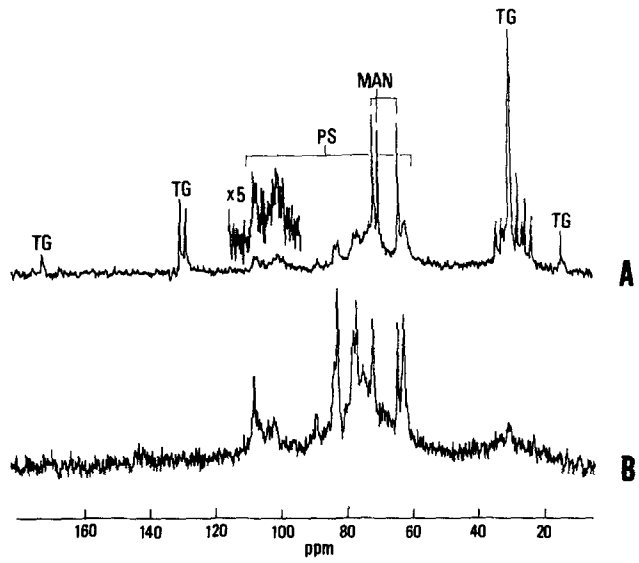

Figure 1. ^{13}C NMR spectra of P. ochro-chloron: (A) intact cells, (B) cell walls. Intact cells and cell walls suspended in deionized water were packed into 10 mm diameter NMR tubes. Spectrometer conditions were as follows: spectral width 6 kHz, 8 k data points, 13 μsec (90°) pulse width, 0.8 sec repetition time, 4,500 scans, 27°C. The abbreviations: MAN, mannitol; TG, triacyl glycerols; PS, polysaccharides.

polysaccharides were observed, the ^{13}C spectrum of cell walls exhibited mainly the resonances of polysaccharides, designated PS B, whose anomeric carbons resonated at 107.5 and 108.3 ppm. This observation indicates that the polysaccharides PS B is a cell wall constituent and that mannitol, triacyl glycerols and the polysaccharide having an anomeric carbon at 101.0 ppm (PS A) are probably located in cytoplasma. The polysaccharide PS A was assigned to (1→4)-α-glucan, because only the ^{13}C NMR signals of PS A were disappeared when the lyophilized cells were treated with amylase and because the chemical shifts of PS A corresponded to the reported value of (1→4)-α-glucan (6).

Isolation of the polysaccharides PS B was carried out to identify the structure of the polysaccharides. Treatment of the cell walls with 0.1 N H_2SO_4 at 100°C for 20 min released a sugar fraction which was composed of 82.0% galactose, 17.5% glucose and trace amount of mannose. The yield of this extract was 6.2 ± 0.3% (n= 4) of the total cell walls. The residue of H_2SO_4 extraction gave no resonances of the polysaccharides PS B. As shown in Fig. 2(A), the ^{13}C NMR spec-

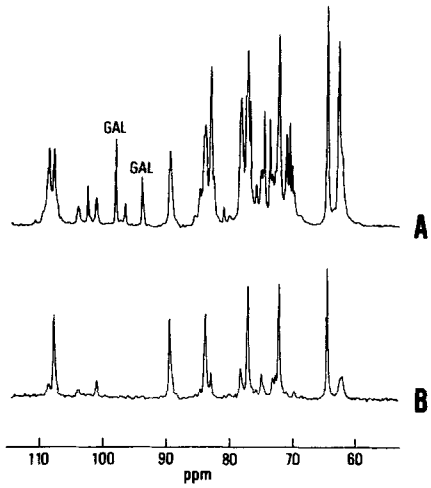

Figure 2. ^{13}C NMR spectra taken during the purification of polysaccharides PS B from P. ochro-chloron cell walls: (A) H_2SO_4 extract, (B) 80-90% ethanol precipitate. Experimental conditions: spectral width 6 kHz, 8 k data points, 7 μsec (45°) pulse width, 0.8 sec repetition time, accumulation (A) 47,517, (B) 68,605, 27°C, concentration (A) 126 mg/ ml of H_2O, (B) 16 mg/ ml of H_2O. The abbreviation: GAL, galactose.

trum of H_2SO_4 extract exhibited two anomeric carbon signals arised from the polysaccharides PS B at 107.5 and 108.3 ppm in the anomeric region, together with the resonances of galactose and some accompanying polysaccharides. The H_2SO_4 extract was further purified by fractional precipitation with ethanol. Fig. 2(B) shows the ^{13}C NMR spectrum of 80-90% ethanol fraction, which is composed of 77.3% galactose and 22.7% glucose. Six strong peaks including an anomeric carbon signal at 107.5 ppm were observed, showing a homopolysaccharide having a single type of glycosidic linkage was isolated. These results indicate that the polysaccharides PS B consists of two types of polysaccharides, namely PS B1 and PS B2, whose anomeric carbons resonate at 107.5 and 108.3 ppm, respectively, and that PS B1 and PS B2 are galactan. The purification of the polysaccharide PS B2 is now in progress.

It is possible to determine chemical structures of polysaccharides on the basis of the shift value (7). The chemical shift data for the galactan PS B1 and methyl galactosides are listed in Table 1. The resonances of PS B1 at 107.5 and 64.2 ppm were readily assigned to C-1 and C-6, respectively, by comparison

Table 1

Assignments of signals in the ^{13}C NMR spectrum of a polysaccharide PS B1. Chemical shifts are expressed in ppm from tetramethylsilane.

compound	C-1	C-2	C-3	C-4	C-5	C-6	OCH_3-1
PS B1	107.5	89.2	76.8	83.5	71.8	64.2	
Methyl α-D-galacto-pyranoside[a]	100.5	69.4	70.6	70.4	71.8	62.3	56.3
Methyl β-D-galacto-pyranoside[a]	104.9	71.8	73.9	69.8	76.2	62.1	58.3
Methyl α-D-galacto-furanoside[a]	103.1	77.4	75.5	82.3	73.7	63.4	56.1
Methyl β-D-galacto-furanoside[a]	109.2	81.9	77.8	84.0	72.0	63.9	56.1

[a] Taken from ref. 8.

with methyl galactosides. Galactan are thought to have four types of anomeric configuration: α- and β-pyranosyl, and α- and β-furanosyl linkages. By comparing the C-1 chemical shift of PS B1 with those of methyl galactosides, the galactan PS B1 was shown to have β-galactofuranosyl linkages. The occurrence of furanose ring form was also supported by the chemical shift of C-6. When the chemical shifts of PS B1 were compared with those of methyl β-D-galactofuranoside, the resonances of PS B1 at 71.8, 76.8 and 83.5 ppm corresponded well to the C-5, C-3 and C-4 resonances of methyl β-D-galactofuranoside, respectively, and the resonance at 89.2 ppm was shifted downfield by 7.3 ppm relative to the C-2 resonance of methyl β-D-galactofuranoside. It is well known that on formation of a glycosidic linkage the resonance of the linked carbon is deshielded by approximately 6-9 ppm relative to its position in a monomer and the resonances of the neighboring carbons are not significantly affected (6). Accordingly, the resonances at 71.8, 76.8, 83.5 and 89.2 ppm were respectively assigned to C-5, C-3, C-4 and C-2, demonstrating C-2 is involved in the glycosidic bond, and it is, therefore, concluded that the structure of PS B1 is (1→2)-β linked galactofuranosyl residues. The galactan PS B2 which afforded the C-1 resonance at 108.3 ppm was also identi-

fied as β-galactofuranosyl residues by comparison of the chemical shift of the anomeric carbon with the data for methyl galactosides given in Table 1.

Azuma et al. showed that a glycopeptide (galactomannan-peptide) was obtained from Aspergillus fumigatus cells and the galactomannan had D-galactofuranosyl side-chains, which were joined to the main chain of (1→2)-α linked D-mannopyranosyl residues (9). Gander et al. reported a polymer composed primarily of galactofuranosyl and glucosyl residues from Penicillium charlesii cell walls (10). ^{13}C NMR spectroscopy demonstrated clearly the occurrence of galactofuranosyl residues in the cell wall of P. ochro-chloron and their β anomeric configuration. It is possible that the β-galactofuranosyl residues exist in the cell wall as a branch of a polymer like the macromolecules described above.

Ascomycetes, the class to which Penicillium, Aspergillus and Neurospora species belong, contains chitin-glucan as the major cell wall polysaccharide (11). In the ^{13}C NMR spectrum of P. ochro-chloron cell walls, however, only the β-galactofuranosyl residues which were no more than 6% of the cell wall gave resonances and the resonances of chitin and glucan were not observed. Considering that a molecule which has a high degree of mobility yields NMR resonances, this result indicates that the β-galactofuranosyl residues experience fast internal motion, while the skeletal crystalline polysaccharides, chitin and glucan, which constitute inner part of the cell wall, have rigid structures. The galactofuranosy residues are known to contribute to the antigenic specificity of Penicillium species (12). The rapid internal motion of the β-galactofuranosyl residues in the cell wall may be related to their immunological activity.

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