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Physiological and Biochemical Studies on Germinating Fungal Spores. VII.[1] Chemical Composition of Cell Walls in Conidia of *Cochliobolus miyabeanus*

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The chemical structure and composition of conidial cell walls of *Cochliobolus miyabeanus* were studied. Analyses by means of gas liquid chromatography, high performance liquid chromatography, enzymatic digestion and nuclear magnetic resonance and infrared absorption spectroscopy revealed that the acid-soluble neutral polysaccharide was composed of β-1,6- and β-1,3-linked glucosyl residues in the ratio of 2:3, that the branched units connected through C_6 or C_3 of the main-chain glucan, and that nonreducing termini existed in the ratio of one residue to five glycosyl residues. Amino acid analysis indicated the presence of glycine, alanine, aspartic acid, serine, leucine, glutamic acid, proline and threonine in decreasing order of contents. The acid-alkali-insoluble polysaccharide was identified as chitin by enzymatic digestion, X-ray diffraction and infrared spectral analyses.

Keywords——*Cochliobolus miyabeanus*; fungal spore; conidia; cell wall; polysaccharide; chitin; carbohydrate

In the previous study[1] we reported that the polysaccharide fraction extracted with hot 4 N acetic acid from conidia of *Cochliobolus miyabeanus* was consumed as an endogenous substrate for germination and that the structure of the polysaccharide was different from that of mycelial cell walls obtained by similar procedures. Although we clarified in the previous paper[1] some structural properties of the polysaccharide derived from intrasporal storage material, little is known about the polysaccharide of conidial cell walls. In the present study, conidial cell walls were purified and their chemical composition was compared with that of mycelial cell walls. Studies on the chemical structure of mycelial cell walls of fungi such as *Neurospora crassa*[2-4] and *Agaricus bisporus*[5] have shown that the main components of the cell walls generally consist of chitin and β-glucan having proteins and lipids associated with them. We have shown[6] that the main component of mycelial cell walls of *Cochliobolus miyabeanus* consists of a chitin-like substance containing α-*N*-acetylgalactosaminyl residues and β-1,3-glucan with β-glucosyl branched units connected through C_6 and C_1. However, little work has been done[7] on the chemical composition of mycelial cell walls of the fungi and no report has appeared yet on the chemical composition of conidial cell walls of *Cochliobolus miyabeanus*.

Materials and Methods

Organism——The harvesting procedure for conidia of *Cochliobolus miyabeanus* C-37-ATCC 38724 was as described previously.[8]

Preparation of Cell Wall——A suspension of conidia in 1% NaCl was treated four times in a french press at 1000 kg/cm^2 and centrifuged at $1000 \times g$ for 10 min. The precipitate was successively washed with 1% NaCl and distilled water until the supernatant did not show any positive reactions to Lowry's protein–determination method,[9] anthrone reagent and I_2–KI mixture. Complete removal of cell contents from the washed precipitate was verified by

microscopic observation after staining with lactophenol cotton blue reagent. The lyophilized precipitate served as conidial cell wall fraction, amounting to about 38% of the initial dry weight of conidia. The cell wall fraction was further subfractionated according to the procedures shown in Chart 1.

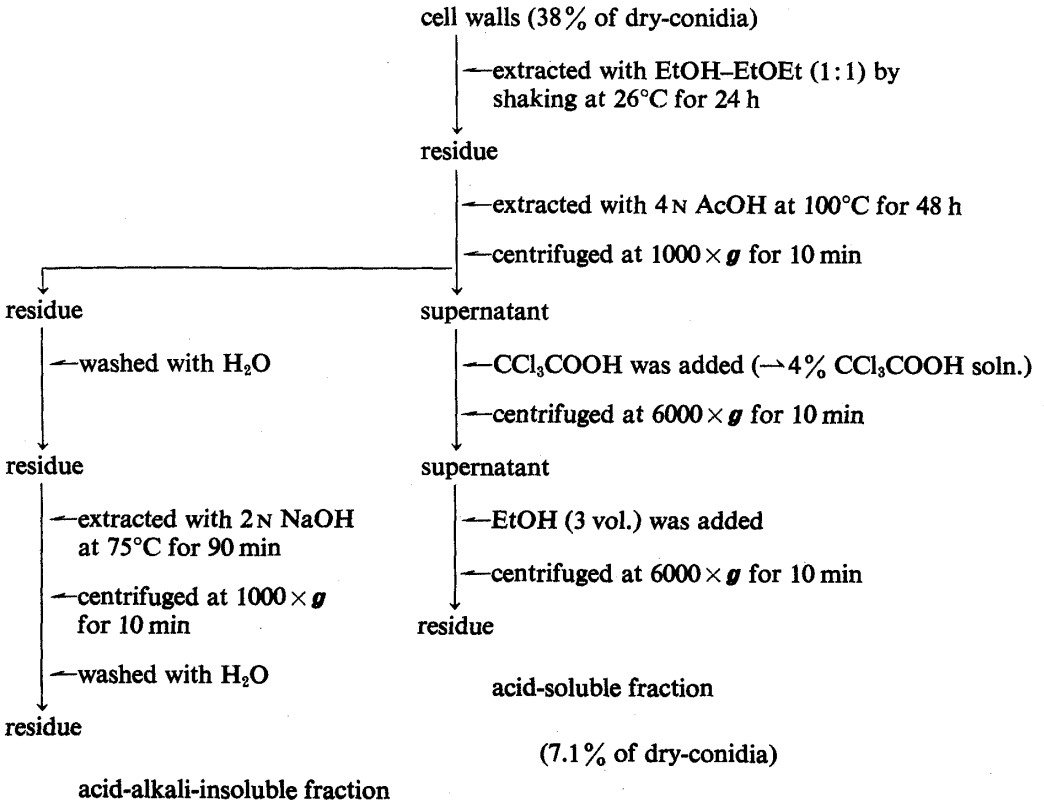

Chart 1. Fractionation of Cell Wall from Conidia of *Cochliobolus miyabeanus*

Fractionation of the Cell Wall Polysaccharide——The polysaccharides were fractionated by means of the procedures shown in Chart 1.

Chromatographic Techniques——i) Column Chromatography: The acid-soluble fraction was applied to a Sephadex G-100 column (2.5 × 25 cm). The column was eluted with distilled water and fractions (4 ml) were collected. Anthrone was used as a color reagent.

ii) High Performance Liquid Chromatography (HPLC): HPLC was performed with a Waters ALC-201 liquid chromatograph system under the following conditions: column, μBondapak CH (0.4 × 30 cm); solvent, CH_3CN–H_2O (85 : 15); flow rate, 2.2 ml/min; detection, RI.

iii) Gas Liquid Chromatography (GLC): GLC was performed under the conditions described in the legends to the figures.

Electrophoresis——For confirmation of the purity of the polysaccharide, paper electrophoresis was carried out on Whatman GF-83 glass fiber paper using the following system: 0.026 M borate buffer (pH 10), 800 V, 4 °C. Aniline hydrogen phthalate was used as a spray reagent.

Enzymatic Digestion——i) Liberation of Glucose: The samples were incubated at 35 °C overnight under the following conditions and the released glucose was determined by the use of Glucostat reagent: a) substrate, 1 mg; β-glucosidase (from almonds, Sigma) 0.3 mg; 0.05 M acetate buffer (pH 5.25), 0.5 ml; b) substrate, 1 mg; α-glucosidase (from yeast, Sigma), 0.3 mg; 1/15 M phosphate buffer (pH 6.80), 0.5 ml.

ii) Liberation of *N*-Acetylglucosamine: The polysaccharide (50 mg) was suspended in 5 ml of 0.01 M phosphate buffer (pH 6.25) and incubated at 37 °C with chitinase (175 mg, Sigma) and toluol (0.03 ml). Aliquots of the reaction mixture were taken at specified times and centrifuged. The supernatant was assayed for *N*-acetylglucosamine according to Reissig's method.[10]

X-Ray Diffraction and Infrared Absorption Analyses——Acid-alkali-insoluble fraction was mixed with 1.5% $KMnO_4$–10% H_2SO_4 (1 : 1). After standing at 4 °C for 48 h, it was decolorized with 10% $H_2C_2O_4$, washed with distilled water and dried. The infrared absorption spectrum of the sample was analyzed in a KBr tablet. X-ray diffraction analysis was carried out in an apparatus (Rigaku Denki, Japan) with Co*K*α radiation filtered through iron

under conditions of 40 kV and 7 mA.

Amino Acid Analysis——The cell wall fraction was suspended in 6 N HCl and hydrolyzed at 105 °C for 17 h in a sealed tube, then analyzed in a Hitachi LB-3B amino acid analyzer.

Methylation Analysis——Methylation analysis was performed as described previously.[1]

Results and Discussion

Chromatography of the acid-soluble fraction on Sephadex G-100 showed that anthrone-positive material was eluted as a single peak (Fig. 1). Fractions 13 to 23 were pooled and concentrated under reduced pressure, then three vol. of ethanol was added. The white amorphous precipitate (compound I) obtained seemed to be homogeneous, since electrophoresis of the precipitate in the presence of boric acid gave a single spot. A solution of the precipitate did not show absorbance at 280 nm and protein was not detected by the method of Lowry.[9]

Compound I was hydrolyzed with 4 N HCl at 100 °C for 3 h and the hydrolysate was analyzed by HPLC (Fig. 2) or GLC after trimethylsilylation (Fig. 3); it contained only glucose. These results indicate that I is a polysaccharide composed of glucose alone. To estimate the molecular weight of I, Sephadex G-200 column chromatography was performed with standard dextrans having molecular weights of 70, 59 and 17.5×10^3. From the results shown in Fig. 4, the molecular weight of I was calculated to be 24×10^3. In order to determine the linkage type of I, the susceptibility of I to α- and β-glucosidase was examined, release of glucose was observed only when I was incubated with β-glucosidase. Figure 5 shows the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of methylated I in $CDCl_3$. On the basis of an anomeric proton signal at δ 4.80 (d, $J=6.0$ Hz) derived from β-linkages, I can be regarded as a β-glucan. Methylation analysis of I was performed in order to determine the linkage sites. I was methylated repeatedly by the method of Hakomori. The progress of the reaction was monitored by checking the infrared (IR) spectra near 3500 cm^{-1} (assigned to free hydroxy groups). Methylated I was subjected to methanolysis at 100 °C for 6 h in 5% HCl in methanol and the methylated derivatives of glucose obtained were identified by gas liquid chromatography-mass spectrometry (GLC-MS) analysis. Eight peaks appeared, as shown in Fig. 6. The methylated derivatives of glucose were identified by comparing the

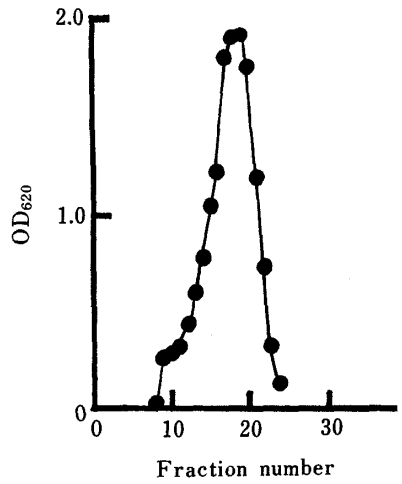

Fig. 1. Sephadex G-100 Column Chromatogram of I

Carbohydrate contents were determined by the anthrone method. Column, 3 × 30 cm.

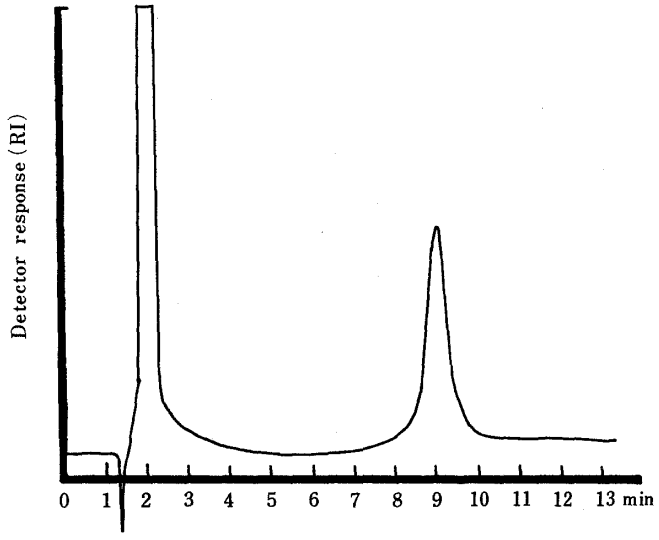

Fig. 2. High Performance Liquid Column Chromatogram of Constituent Sugar of I

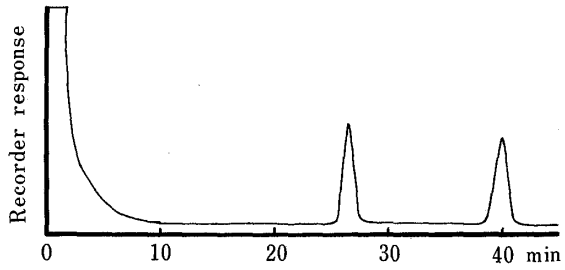

Fig. 3. Gas Chromatogram of Constituent Sugar of I

Conditions: column size, 3 mm × 1.8 m; column temp., 170 °C; packing, 5% SE 30 on Shimalite; N_2 flow rate, 40 ml/min; detector, FID; detector temp., 250 °C; injection temp., 250 °C; H_2 flow rate, 40 ml/min.

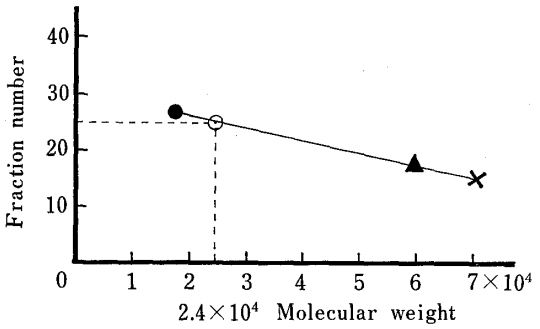

Fig. 4. Estimation of the Molecular Weight of I by Column Chromatography

I (⊙) and dextran [(×, mol. wt. 7×10^4), (▲, mol. wt. 5.9×10^4), (●, mol. wt. 1.75×10^4)] were applied to a Sephacryl 200 column (2.5 × 37 cm). Eluted compounds were detected by the anthrone method.

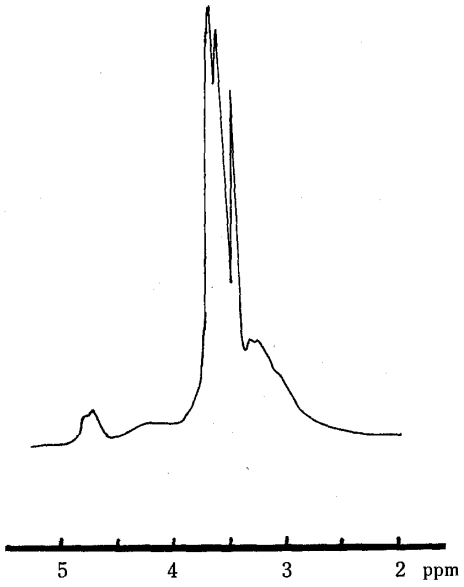

Fig. 5. NMR Spectrum of Permethylated I

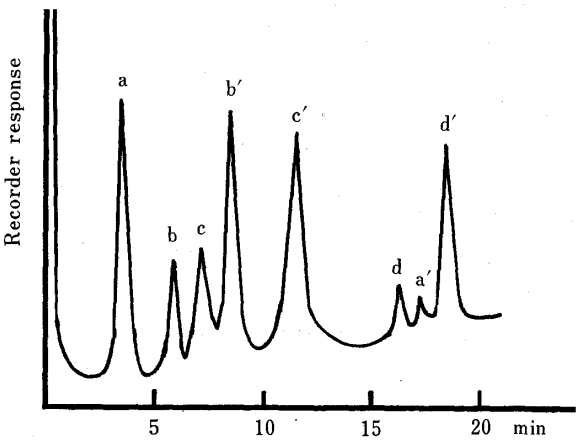

Fig. 6. Gas Chromatogram of Permethylated I

Conditions: column size, 3 mm × 1.0 m; column temp., 140 °C (12 min) then increasing at 8 °C/min to 180 °C; packing, 1.5% NGS on Chromosorb W; N_2 flow rate, 40 ml/min; detector, FID; detector temp., 270 °C; injection temp., 270 °C; H_2 flow rate, 40 ml/min. Peaks: a, a′, methyl 2,3,4,6-tetra-*O*-methyl glucoside; b, b′, methyl 2,3,4-tri-*O*-methyl glucoside; c, c′, methyl 2,4,6-tri-*O*-methyl glucoside; d, d′, methyl 2,4-di-*O*-methyl glucoside.

TABLE I. Molar Ratio of Methylated Sugar

Methylated sugar	Molar ratio
Methyl 2,3,4,6-tetra-*O*-methyl glucoside	1
Methyl 2,3,4-tri-*O*-methyl glucoside	2
Methyl 2,4,6-tri-*O*-methyl glucoside	3
Methyl 2,4-di-*O*-methyl glucoside	1

retention times and relative intensities of the main fragments in the mass spectra with those of authentic compounds as described previously[1] and the molar ratio of each methyl ether of glucose was calculated from the peak area. The results of the analysis are listed in Table I; methylglucosides of 2,3,4,6-tetra-*O*-methyl, 2,3,4-tri-*O*-methyl, 2,4,6-tri-*O*-methyl and 2,4-di-*O*-methyl were obtained from the completely methylated I in the ratio of 1:2:3:1. The presence of such methylated derivatives indicates that (I) consists of 1,6-disubstituted, 1,3-disubstituted and 1,3,6-trisubstituted and non-reducing terminal glucose residues.

Since the molecular weight of I is assumed to be about 24×10^3 (Fig. 4), one molecule of I would contain about 148 glucosyl residues, consisting of 21 non-reducing terminal glucose, 42 1,6-disubstituted glucose, 63 1,3-disubstituted glucose and 21 1,3,6-trisubstituted glucose (branching points). Therefore, it is concluded that I consists of a main-chain glucan with β-1,3- and β-1,6-linked glucosyl residues and with branching units connected through C_6 or C_3 in the ratio of 6 glucosyl residues to one branched unit. The structure of the polysaccharide obtained from conidial cell walls in the present study was very similar to that obtained from conidial cell content[1] with respect to sugar composition, molecular weight and branching frequency. However, the ratio of β-1,3-linked glucosyl residue to β-1,6-linked glucosyl residue differed considerably between the cell wall polysaccharide (3:2) obtained in the present study and the cell content polysaccharide (5:2) described previously.[1] The β-glucan in the mycelial cell wall of the present fungus[6] differed greatly from the polysaccharide in conidial cell walls in that the former has no β-1,6-linkage and exhibits a higher branching frequency in the ratio of 13 glucosyl residues to 3 branched units. Trocha[7] reported that the chemical compositions of uredo spore walls and germ tube walls in *Uromyces phaseoli* var. *typica* were different. The present study revealed that in *Cochliobolus miyabeanus* the chemical composition and structure of glucans are different among conidial and mycelial cell walls and intrasporal storage material.

The amino acid composition of the cell wall protein is shown in Table II. In the case of conidial cytoplasmic proteins, the glutamic acid content was highest, followed by aspartic acid, glycine, alanine and leucine in decreasing order.[8] The serine content in the cell wall was considerably higher than that in the cytoplasm. The amino acid composition shown in Table II is similar to that of the protein of mycelial cell walls of the present fungus except for the higher content of proline in the present results.

The infrared absorption spectrum of the acid-alkali-insoluble fraction (Chart 1, compound II) was taken in a KBr tablet to check for the presence of chitin-like substances. Figure 7 shows that II exhibited a spectrum similar to that of authentic chitin from crab shell. X-ray diffraction analysis of II and authentic chitin showed (Fig. 8) similar patterns with a high peak at 19° and a low peak at 23°. Figure 9 shows the effect of chitinase on the release of *N*-acetylglucosamine from II. The release of *N*-acetylglucosamine was almost linear up to 30 h

Table II. Amino Acid Composition of Cell Wall

Amino acid	Molar ratio	Amino acid	Molar ratio
Asp	33.5	Met	1.0
Thr	18.5	Isoleu	6.5
Ser	25.5	Leu	23.0
Glu	19.5	Tyr	5.0
Pro	19.0	Phe	6.5
Gly	42.5	NH_3	20.0
Ala	37.5	Rys	17.5
Cis	—	His	1.5
Val	9.5	Arg	2.5

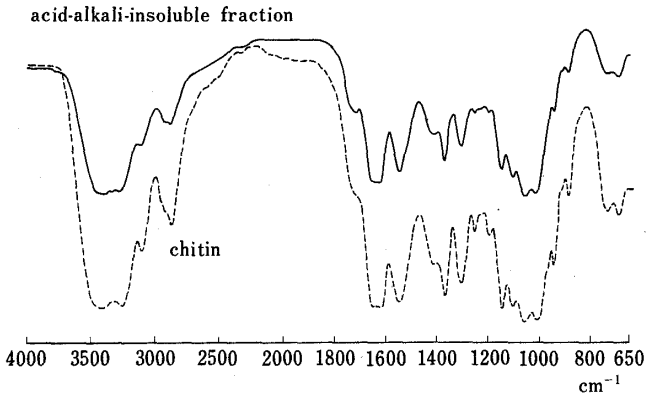

Fig. 7. Infrared Spectra of II and Authentic Chitin

——, II; – – – –, authentic chitin.

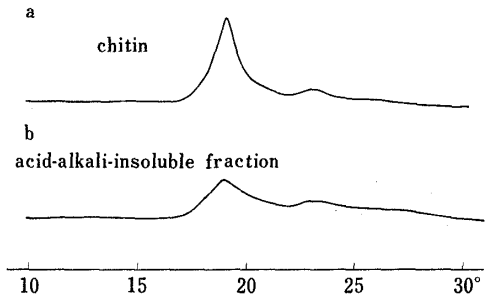

Fig. 8. X-Ray Diffractometry of II and Authentic Chitin

a, authentic chitin; b, II.

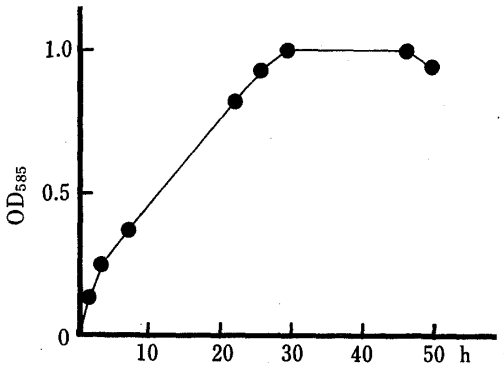

Fig. 9. Release of *N*-Acetylglucosamine from II by Chitinase Digestion

of incubation. These results (Figs. 7—9) clearly indicate that the major component of II consists of chitin. We also prepared from mycelial cell walls a chitin-like substance composed of branches of α-*N*-acetylgalactosamine residues associated with a main-chain of the chitin,[6] though we could not detect any galactosamine derivatives in the aicd-alkali-insoluble fraction of conidial cell walls in the present study.

From the present results, we conclude that although the mycelial and conidial cell walls both consist mainly of β-glucan and chitin-like substance, the fine chemical structures of the two cell walls are evidently different.

References and Notes

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