[Chem. Pharm. Bull. 19(3) 448—452 (1971)]

UDC 547.458.02.05 : 581.192 : 582.282

Studies on Fungicides. VIII.[1] Chemical Structure of Polysaccharides of Cell Walls from *Cochliobolus miyabeanus*. (2)

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(Received April 15, 1970)

The chemical structure of the polysaccharide, a main component of cell walls of *Cochliobolus miyabeanus*, was studied. The polysaccharide, isolated from the fungal cell walls, was methylated by Hakomori's method and, after hydrolysis, the products were determined by paper chromatography, thin-layer chromatography, gas-liquid chromatography and paper electrophoretic chromatography. The molar ratio of the determined methyl sugars in the hydrolysate was as follows: 2,3,4,6-tetramethyl-: 2,4,6-trimethyl-: dimethyl- (probably 2,4-dimethyl-) glucose=3:10:2.

These results demonstrated that the polysaccharide in this fungal cell wall is composed of the β-1,3 linked glucan having branched units connected through C-6 and C-1.

Introduction.

The main components of the cell walls of *Ascomycetes* spp. (*Neurospora crassa, Aspergillus oryzae*) were recently characterised as glucan,[3-5] while many other fungal cell walls were composed of α- or β-glucan. In the previous paper,[1] the authors reported the presence of a β-glucosyl-linked polysaccharide in the cell walls of *Cochliobolus miyabeanus*. This paper deals with the fine chemical structure of this polysaccharide.

Material and Method

1) **Isolation of the Polysaccharide**——The cell wall fraction free from cytoplasmic contamination was prepared from colorless mycelia of *Cochliobolus miyabeanus* as described in the previous paper.[1]

EtOH was added to the acid-soluble fraction to give 80% alcohol solution and the sediment produced was used as the polysaccharide fraction.

2) **Acetolysis of the Polysaccharide**——Acetolysis of the polysaccharide was performed according to the method of Matsuda, *et al.*[6,7] The polysaccharide (500 mg) was dissolved in the AcOH–Ac_2O–H_2SO_4 mixture (3.2:4.8:0.6, 4 ml). This mixture was kept at 25° for 72 hr and at 80° for 0.5 hr, then poured into ice water (100 ml), and neutralized with $NaHCO_3$ to pH 6.5. The oily product was extracted several times with $CHCl_3$ and the $CHCl_3$ layer was washed, dried over Na_2SO_4 at 4° for 12 hr, and evaporated *in vacuo*. The residue was dissolved in 0.05 N MeONa in MeOH (20 ml) and kept at 26° for 1 hr. Neutralisation of the solution with Amberlite IR-120, followed by evaporation *in vacuo* gave the deacetylated mono and oligo-saccharides. These saccharides were identified by paper partition chromatography (PPC).

3) **Methylation of the Polysaccharide** ——Methylation of the polysaccharide was performed according to the method of Hakomori.[8] The polysaccharide (50 mg) was dissolved in Me_2SO (5 ml) by agitating with a magnetic stirrer at 20—25° for 5 hr. On the other side, NaH (1.5g) washed with petr. ether was suspended in Me_2SO (16ml) stirred in nitrogen stream at 20—25° for 0.5 hr and then at 50—60° for 1.5 hr. Two solutions described above were mixed and incubated under continuous stirring at 20—25° for 4 hr. An excess of MeI (8 ml) was added and stirring was continued at 20—25° for further 20—24 hr. After the reaction mixture was washed, followed by repeated extraction of the methylated products with $CHCl_3$, the solution was evaporated *in vacuo*. The residue was dropped into petroleum ether and the pellet pro-

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2) Location: *Motoyama-cho, Higashinada-ku, Kobe.*
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8) S. Hakomori, *J. Biochem.*, (Tokyo), **55**, 205 (1964).

duced from the mixture was harvested by centrifugation. The infrared spectrum showed no absorption at 3200—3700 cm^{-1} region responsible for OH group in carbohydrates (Fig. 1).

4) **Methanolysis and Acid Hydrolysis of the Methylated Polysaccharide** ——The methylated compound, dissolved in 5% MeOH–HCl, was heated at 120° for 4 hr in a sealed tube. After evaporating the solution *in vacuo*, aliquot of methanolyzed compound was submitted to gas-liquid chromatographic analysis.

The rest was dissolved in 72% H_2SO_4 at 0—4° for 1 hr, then diluted to 8% with distilled water, and heated at 100° for 4 hr. After the reactant was neutralized with $Ba(OH)_2$, followed by centrifugation at 5000 rpm, the supernatant was evaporated and the residue was submitted to paper, thin-layer, and electrophoretic chromatographic analyses.

5) **Identification of the Methylated Sugars**——a) Gas-Liquid Chromatography(GLC): Methylated polysaccharide was subjected to methanolysis and analyzed by GLC (Shimadzu model GC-1C) equipped with a flame ionization detector. The conditions of GLC analysis were as follows: a steel column (3×1800 mm) packed with 60—80 mesh 1.5% neopentyl glycol succinate on Chromosorb, 140° column temperature, and N_2 flow rate 40 ml/min.

b) Paper Chromatography (PC): PC analysis on Toyo Roshi No. 50 paper was carried out on mono- and oligo-saccharides obtained in (2) using BuOH–AcOH–H_2O (4:1:5) and AcOEt–pyridine–H_2O (12:6:5) systems, and on methylated sugars obtained in (4) using BuOH–EtOH–H_2O (5:1:4).

c) Thin-Layer Chromatography (TLC): The methylated sugars were developed on a Kieselgel G plate (0.25 mm thick) using benzen–EtOH–H_2O–NH_4OH (200:47:15:1) as a solvent.

d) Paper Electrophoretic Chromatography (PEC): PEC was performed according to the method of Foster.[9,10] The methylated sugar solution was spotted on Toyo Roshi No. 50 paper (250×20 mm), equilibrated with 0.01M NaB_4O_7 solution (pH 9.0). Developing was carried out at a constant potential gradient of 300 V and current of 20—30 mA at 4° for 3 hr. Before the reagent was applied to the paper, the strip was washed with acetic acid and dried.

e) Determination of Separated Sugars: The chromatograms obtained by PPC, TLC, and PEC were stained with aniline hydrogen phthalate reagent or alkaline silver nitrate reagent.

6) **Quantative Determination of the Methylated Glucoses**——The methylated compounds which were hydrolyzed with after methanolysis were spotted on a Kieselgel G plate (0.25 mm thick) and developed repeatedly 5 times. The separated methyl glucose was extracted and determined by the anthrone method. [11,12]

Result and Discussion

In the proceding paper, the authors reported that β-glucan and chitin layer were the main components of the cell walls of *Cochliobolus miyabeanus*. Therefore, the fine chemical structure of the β-glucan was studied in detail.

TABLE I. Paper Chromatograms of Deacetylated Products from the Acetolysate of Polysaccharide in Cell Wall

Substance	*Rg*	
	Solvent a	Solvent b
	1.00	1.00
Laminarin in *Eisenia bicyclis*	0.10	0.75
	0.00	0.20
Polysaccharide in cell wall	1.00	1.00
	0.10	0.75
Authentic glucose	1.00	1.00
Authentic laminaribiose	0.10	0.75

solvent a: BuOH:AcOH:H_2O=4:1:5
solvent b: AcOEt:pyridine:H_2O=12:6:5
detector: aniline hydrogen phthalate

9) A. B. Foster, *J. Chem. Soc.*, **1953**, 982.
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12) R. Dreywood, *Ind. Eng. Chem. Annl. Ed.*, **18**, 499 (1946).

Since the PPC analysis indicated that one of the oligosaccharides in the hydrolysate corresponded to authentic laminaribiose, acetolysis of the polysaccharide was carried out and the liberated glucose and laminaribiose were detected by PPC analysis (Table I).

Peat,[13] Anderson[14] and their co-workers indicated that laminarin is a polysaccharide consisting of β-1,3 and β-1,6 glucosyl linkages. Since the polysaccharide in the present study corresponded closely to laminarin (Table I), it was suggested that the main structure of the fungal polysaccharide was composed of β-1,3 glucosyl linkage.

In order to confirm the existence of β-1,3 glucosyl linkage, the polysaccharide from the cell wall was methylated. After repeated methylation by checking with infrared spectrum, the completly methylated compound was submitted to methanolysis in a sealed tube with 5% methanolic HCl at 120° for 4 hr. The aliquot of the product was used for GLC analysis and the rest was submitted to PPC, TLC, and PEC analyses after demethylation by acid hydrolysis.

The following methylated sugars were prepared by the same methylation procedure as authentic samples: 2,3,4,6-tetramethylglucose from D-glucose, 2,4,6-trimethylglucose from laminarin (β-1,3 bonds), 2,3,4-trimethylglucose from dextran (α-1,6 bonds), 2,3,6-trimethylglucose and 2,3-dimethylglucose from glycogen (α-1,4 and α-1,4,6 bonds).

Table II. Gas Chromatograms of Methylated Sugars derived from the Polysaccharide in Fungal Cell Wall

Compound	*Rt* (min)	
2,3,4,6-Tetramethylglucose (from D-glucose)	3.4	
2,3,4-Trimethylglucose (α-1,6 bond from dextran)	7.5	10.5
2,3,6-Trimethylglucose (α-1,4 bond from glycogen)	10.0	13.5
2,4,6-Trimethylglucose (β-1,3 bond from laminarin)	9.5	14.5
Cell wall (polysaccharide)	3.4	8.0
	9.5	14.0
	14.5	

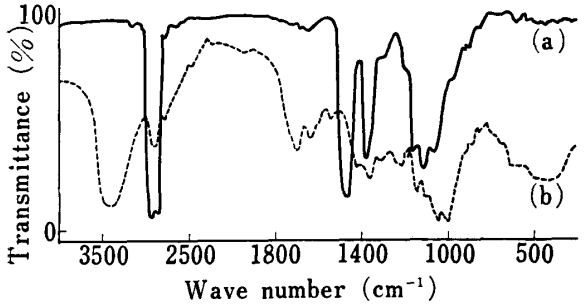

Fig. 1. Infrared Spectra of Methylated Polysaccharide

(a) methylated polysaccharide
(b) non-methylated polysaccharide

Laminarin was extracted from *Eisenia bicyclis* by the method of Handa, *et al.*,[15] and the commercial products (Seikagaku Kogyo Co., Tokyo) were used as the carbohydrate samples.

On GLC analysis (Table II), several peaks appeared and some of them corresponded to those of 2,3,4,6-tetramethyl (*Rt* 3.4) and 2,4,6-Trimethyl (*Rt* 9.5, 14.5) glucoses. Since two peaks (*Rt* 8.0, 14.0) remained unclarified, PPC and TLC analyses were carried out.

As shown in Table III and IV, PPC and TLC analyses revealed three spots. The *Rg* values of the two spots corresponded to authentic 2,3,4,6-tetramethyl and 2,4,6-trimethylglucose, while the third spot agreed closely with authentic 2,3-dimethylglucose.

Hay, *et al.*[16] reported that the *Rg* values of 2,4-dimethyl- and 2,3-dimethylglucoses were very similar (0.04—0.03), and Aspinal[17] indicated that both these dimethyl derivatives

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15) N. Handa and K. Nisizawa, *Nature*, **192**, 1078 (1961).
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Table III. Paper Chromatograms of Methylated Sugars derived from the Polysaccharide in Fungal Cell Wall

Compound	*Rg*				
2,3,4,6-Tetramethylglucose(from D-glucose)	1.00				
2,3,4-Trimethylglucose (α-1,6 bond from dextran)		0.85			
2,3,6-Trimethylglucose (α-1,4 bond from glycogen)			0.83		
2,4,6-Trimethylglucose (β-1,3 bond from laminarin)				0.80	
2,3-Dimethylglucose (1,4,6 bond from glycogen)					0.56
Cell wall (polysaccharide)	1.00		0.80		0.56

filter paper: Toyo Roshi No. 50
solvent: BuOH:EtOH:H_2O=50:10:40
detector: aniline hydrogen phthalate

Table IV. Thin-Layer Chromatograms of Methylated Sugars derived from the Polysaccharide in Fungal Cell Wall

Compound	*Rf*				
2,3,4,6-Tetramethylglucose (from D-glucose)	0.50				
2,3,4-Trimethylglucose (α-1,6 bond from dextran)		0.22			
2,3,6-Trimethylglucose (α-1,4 bond from glycogen)			0.20		
2,4,6-Trimethylglucose (β-1,3 bond from laminarin)				0.14	
2,3-Dimethylglucose (1,4,6 bond from glycogen)					0.03
2,4-Dimethylglucose[16]					0.04
Cell wall (polysaccharide)	0.50			0.14	0.02

plate: Kieselgel G (0.25 mm thick)
solvent: benzene: EtOH:H_2O:NH_4OH=47:15:1
detector: aniline hydrogen phthalate

appeared to have nearly the same retention time) 2.30—3.22 and 2.46—3.22). Although no direct comparison could be made between the methylated sugars obtained from the cell wall and authentic 2,4-dimethylglucose could be performed, it was likely in all respects that the unidentified methylated sugar would be 2,3-dimethyl- or 2,4-dimethylglucose.

The behavior of methylated sugars on the paper electrophoresis was examined by Foster[9,10] and 2,3-dimethylglucose (*Mg* 0.15) was found to move much faster than 2,4-dimethylglucose (*Mg* 0.04).

From the results in Table V, which was obtained under the same experimental condition as Foster's reference, our unidentified methylated sugar corresponded to 2,4-dimethylglucose and not to 2,3-dimethylglucose.

Anderson and his co-workers[14] reported the presence of dimethyl compounds in the methylated sugars from laminarin and they ascribed this fact to a demethylating reaction which might occur during the hydrolysis procedure. However, the authors did not obtain any dimethyl sugar after hydrolysis of the methylated laminarin under the same or even more drastic condition.

Table V. Paper Electrochromatogram of Methylated Sugars derived from the Polysaccharide in Fungal Cell Wall

Compound	*Mg*		
2,3,4,6-Tetramethylglucose(from D-glucose)			0.00
2,3,4-Trimethylglucose (α-1,6 bond from dextran)			0.00
2,3,6-Trimethylglucose (α-1,4 bond from glycogen)			0.00
2,4,6-Trimethylglucose (β-1,3 bond from laminarin)			0.00
2,3-Dimethylglucose (1,4,6 bond from glycogen)	0.15		
2,4-Dimethylglucose[9,10]			0.04
Cell wall (polysaccharide)		0.04	0.00

running condition: 0.01M $Na_2B_4O_7 \cdot 10H_2O$ (pH 9.2) Toyo Roshi No. 50, 3 hr, 4° 300 V, 20—30 mA
detector: aniline hydrogen phthalate

It is established that the three kinds of methylated sugars, *i.e.*, 2,3,4,6-tetramethyl, 2,4,6-trimethyl, and probably 2,4-dimethylglucose, are derived from the polysaccharides in the cell walls of *Cochliobolus miyabeanus* by methylation experiments.

The molar ratio of each of the methylated sugars was determined by the anthrone method and is given in Table VI.

Table VI. Molar Ratio of Methylated Glucoses derived from the Polysaccharide in Fungal Cell Wall

Compound	Molar ratio[a)]
2,3,4,6-Tetramethylglucose	3
2,4,6-Trimethylglucose	10
2,4-Dimethylglucose	2

a) determined by the anthrone method

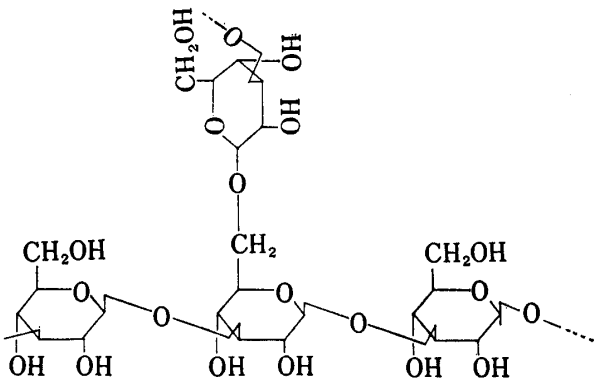

Fig. 2. Chemical Structure of Glucan in the Cell Wall of *Cochliobolus miyabeanus*

The presence of 2,4,6-trimethyl- and 2,4-dimethylglucose in the methylated product indicates the presence of 1,3 disubstituted glucopyranose and 1,3,6 trisubstituted glucopyranose in the polysaccharide, and 2,3,4,6-tetramethylglucose is derived from non-reducing terminal glucopyranose.

All experimental results demonstrate that the polysaccharides in the present fungal cell walls are composed of β-1,3 linked glucan having branched units connected through C-6 and C-1, as shown in Fig. 2.

As shown in Table VII,[3,17–21)] several papers reported the presence of α- or β-1,3 and β-1,6 glucosyl linkages in the polysaccharides of some kinds of fungal cell walls by applying the enzymic digestion method. The polysaccharides of these fungal cell walls were to be composed of only straight-chained glucans, while the present fungus was characterized by the presence of branched glucans. Recently, Zevenhuizen[22)] reported that the insoluble hypal wall glucan of *Phytophthora* spp. was a branched type andconnected through C-6 and C-1. Though *Cochliobolus miyabeanus* is not a sole fungus which has branched in the cell walls, this is the first case in *Ascomycetes* spp. Further studies on periodate oxidation of polysa ccharides are in progress.

TABLE VII. Chemical Structures of Polysaccharides in the Fungal Cell Wall

Fungus	Polysaccharide	Reference
Asp. niger	α-1,3 linked glucan (nigeran type)	18
Asp. oryzae	β-1,3 linked glucan (laminarin type)	5
Neurospora	β-1,3 and β-1,6 linked glucan	19
Phytophthora	β-1,3 and β-1,6 linked glucan	20
Pythium	β-1,3 and β-1,6 linked glucan	21
C. miyabeanus	β-1,3 linked glucan with branched units through 1–6 linkage	present paper

Acknowlegement The authors are greatful to Prof. Dr. K. Tsukida for reading of this manuscript.

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