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Studies of Fungicides. VII.[1] Chemical Composition of Cell Walls of *Cochliobolus miyabeanus*. (1)

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The cell wall fraction was isolated from cytoplasm by sonicating and washing the mycelia of *Cochliobolus miyabeanus*, and its chemical composition was studied. The cell wall was constituted by the polymer of neutral sugars (51.68%), amino sugars (38.03%), peptide (10.75%), lipid (0.57%) and ash (2.33%). A great deal of α- and β-glucose and traces of galactose, mannose, arabinose, rhamnose, fucose and xylose were found in the hydrolysate of poly neutral sugar fraction, and amount of glucosamine and trace of galactosamine in the hydrolysate poly amino sugar fraction by gas liquid chromatography (GLC) and paper parttion chromatography (PPC) analyses. When these two fractions were allowed to react with α- or β-glucosidase and chitinase, glucose was liberated from neutral sugars fraction only when β-glucosidase was used, and acetyl-glucosamine was liberated from amino sugars fraction by chitinase. Sixteen kinds of amino acids were detected from peptide on amino acid analyser. From these results, it is concluded that the cell walls of our fungus are constituted with β-glucan and chitin-like layers associated with a small amount of other sugars and peptides.

Since the fungal hypae contact directly with various exogenous nutrients or drugs through their cell walls, it is necessary to clarify the chemical compositions and their biosynthetic pathways of the cell walls for elucidating the functional mechanisms and developing of new fungicides.

Many reports[3] have been presented on the chemical compositions of cell walls of Ascomycetes and *Phycomycetes* spp., but little is known on their biosynthetic problems. For example, Horikoshi, *et al.*[4] and others[5,6] studied about the chemical compositions of *Aspergillus* spp. (ascomycetes), and Bartnicki-Garcia[7] of *Phytophthora* spp. (phycomycetes).

These authors indicated that cell walls were composed of highly complicated substances, *viz.*, non-cellulosic glucose polymers and protein *etc.*

This paper deals with the chemical compositions of cell walls of the titled fungus, *Cochliobolus miyabeanus* (ascomycetes) which is a typical pathogenic fungus for rice plants.

Material and Method

1) **Preparation of Cell Wall**——The fungal strain was kindly supplied by Dr. Oku, Okayama University. Three-hundred ml of flasks containing 100 ml of 2% popato sucrose liquid medium were inoculated with a suspension of mycelium obtained from a slop culture of the fungus. After shaking at 28° for 72 hr, the colorless mycelium was harvested by filtration through Toyo Roshi No. 2 filter paper and washed thoroughly with distilled water, essentially free from medium components.

Isolation of the cell wall fractions from mycelium was described in Chart 1. The preparations were treated below 4°, and the isolated cell walls were checked for cytoplasmic contamination by I_2-KI reaction, Lowry's method[8] and lactophenol cotton blue staining test.

1) This work was presented in part at The Annual Meeting of The Phytopathological Society of Japan Kochi Branch, 1968.
2) Location: *Motoyamacho, Higashinada-ku, Kobe.*
3) S. Bartnicki-Garcia, *Ann. Rev. Microbiol.*, **22**, 87, (1968).
4) K. Horikoshi and S. Iida, *Biochim. Biophys. Acta*, **83**, 197 (1964).
5) I.R. Johnston, *Biochem. J.*, **96**, 651 (1965).
6) J. Ruiz-Herrera, *Arch. Biochem. Biophys.*, **122**, 118 (1967).
7) S. Bartnicki-Garcia, *J. Gen. Microbiol.*, **42**, 57 (1966).
8) O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **198**, 265 (1951).

mycelium
← blended in homogenizer with 1% NaCl for 20 min
← centrifuged at 3500 rpm for 15 min
residue
← washed several times with 1% NaCl and dist. water
← centrifuged at 3500 rpm for 15 min
residue
← sonicated at 20 kc for 20 min
← centrifuged at 5000 rpm for 15 min
residue
← sonicated at 20 kc for 60 min
← centrifuged at 5000 rpm for 20 min
residue
← lyophilized
cell walls

Chart 1. Preparation of Cell Walls from Mycelium of *Cochliobolus miyabeanus*

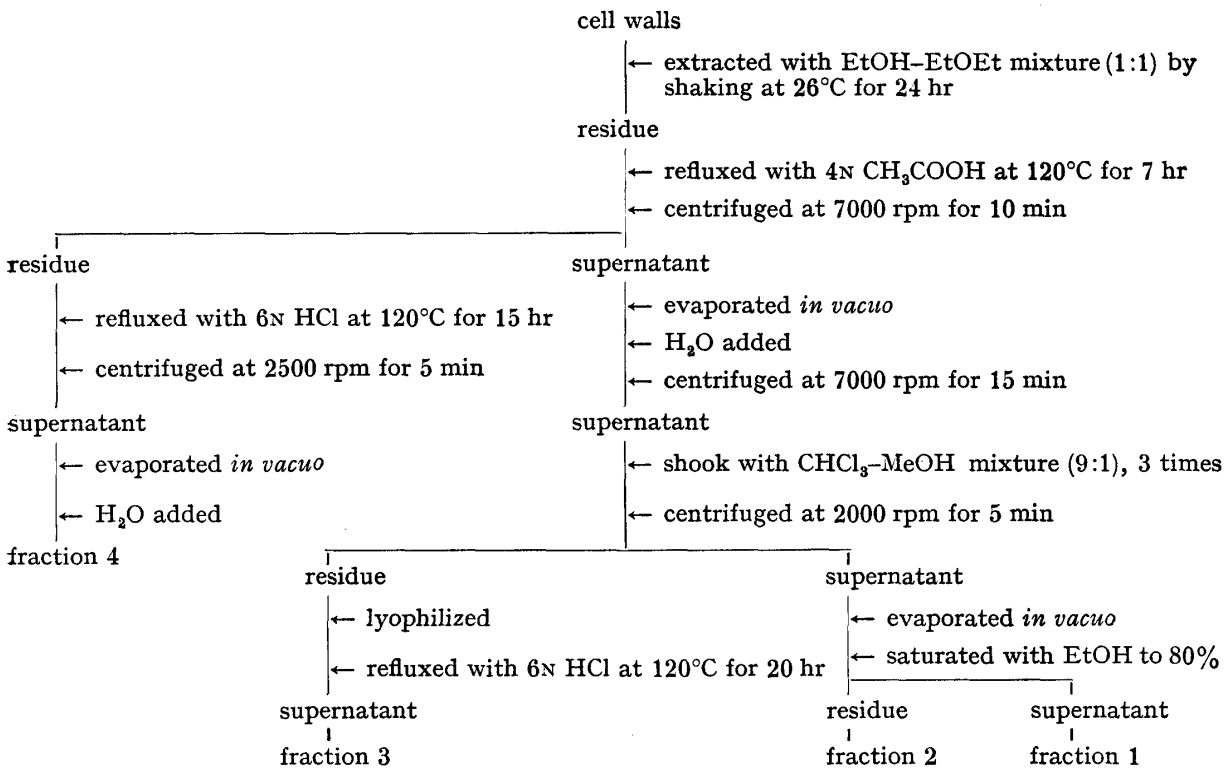

Chart 2. Fractionation of Cell Wall Components

2) **Fractionation of the Cell Wall Components (*cf.* Chart 2)**[9]——The cell walls were defatted by shaking with the ethylalcohol–ether mixture (1:1) at 26° for 24 hr, followed by refluxing with 4N acetic acid at 120° for 7 hr. Peptide was separated from the acid-soluble fraction by treatment with the chloroform–methylalcohol mixture (9:1), and the hydrolysate (fraction 3) was used for amino acid analysis. The residual liquid layer was saturated to 80% with ethylalcohol. After standing for 12 hr at 4°, the pellet was separated (fraction 2) from the supernatant (fraction 1). Both fractions were submitted to sugar analysis.

9) K. Horikoshi and K. Arima, *Nippon Nogeikagaku Kaishi*, 39, 18 (1965).

3) **Analytical Methods**——i) Analysis of Components: Total nitrogen was determined by the micro-Kjeldahl's method, total lipid by Soxhlet's method, and acid and alkali insoluble matter by the Henneberg–Stohmann's method.

ii) Identification of Compounds: Paper Chromatography of Sugars: The aliquot of the fraction 1 was spotted on Toyo Roshi No. 50 paper strips and developed by the following solvent systems. (a) *n*-BuOH: $CH_3COOH:H_2O=4:1:5$ (upper layer), (b) *n*-BuOH:pyridine:$H_2O=6:4:3$.

Sugars were detected by aniline hydrogen phthalate reagent or alkaline silver nitrate reagent.

Gas Chromatography of Sugars: Fraction 1 was evaporated to dryness and treated with the trimethylsilylating agent (TMS-HT Tokyo Kasei Co.).[10,11] The reaction mixture (1—4 μl) was injected on the gas liquid chromatographic column (GLC model SHIMADZU GC-1C) under the condition given in Table I.

Analysis of Amino Acids: Analysis of amino acids contained in the fraction 3 was performed on the Hitachi automatic recording amino acid analyzer, model KLA-3B.

Table I. Condition of Gas Liquid Chromatography

Column L. 3 mm$\phi \times$ 1.8 m	detector FID
Temp. 150°C temp. program 4°C	detector temp. 250°C
Packing 3% OV-17 on Shimalite W	injection temp. 250°C
N_2 flow rate 46 ml/min	H_2 flow rate 40 ml/min

Result

As shown in Table II, carbohydrates were the main components of the cell walls and were studied forward.

Table II. Composition of Cell Walls

Component	Percent of dry weight	Component	Percent of dry weight
Total-N	1.72	Soluble matter (neutral sugar)	51.68
Peptide (N × 6.25)	10.75	Total lipid	0.57
Insoluble matter (amino sugar)	38.03	Ash	2.33

Sugar Components in Fraction 1

Typical paper chromatograms of fraction 1 were given in Table III. In addition to the identified spots of glucose, mannose, galactose, arabinose, xylose and rhamnose, three unidentified spots were detected. The results of GLC analysis clarified the presence of glucose, galactose, laminaribiose and traces of mannose, arabinose, fucose and xylose, as shown in Fig. 1. The mole ratio of sugars was given in Table IV.

Table III. Paper Chromatograms of Sugars in Fraction 1

Compound	*Rg*		
	Solvent a	Solvent b	Authentic compound
Fraction 1	4.20		
	1.10	1.17	mannose
	1.00	1.00	glucose
	0.89	0.91	galactose
	0.80	1.15	arabinose
	0.63	1.35	xylose
	0.47	1.58	rhamnose
	0.33	0.41	
	0.10	0.15	

10) C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
11) C.C. Sweeley and B. Walker, *Anal. Chem.*, **36**, 1461 (1964).

Table IV. Mole Ratio of Sugars in Fraction 1

Sugar	Mole ratio	Sugar	Mole ratio
α-Glucose	29.2	Rhamnose	0.83
β-Glucose	19.8	Fucose	0.23
Galactose	11.3	Xylose	0.44
Mannose	1.0	Laminaribiose	4.26
Arabinose	0.30		

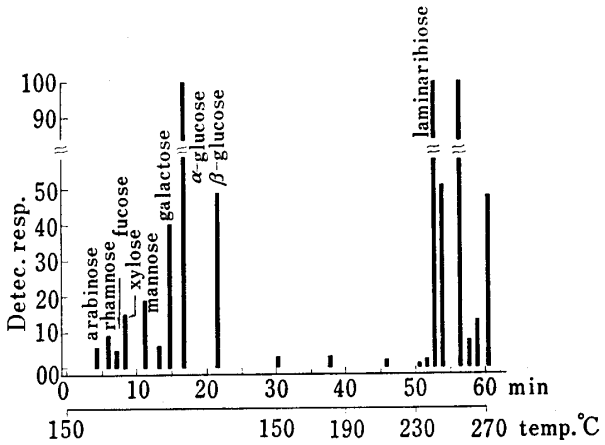

Fig. 1. Gas Liquid Chromatograms of Sugars in Fraction 1

Sugars were treated with trimethylsilylating agent as described in the text.

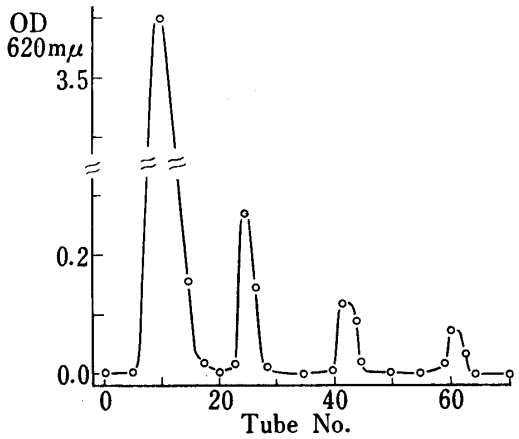

Fig. 2. Fractionation of Oligosaccharides by Sephadex G-10 Column Chromatography: Sephadex G-10 (25×790 mm), detected by Anthrone Method

Chemical Composition of the Oligosaccharides

In the PPC and GLC analysis, some spots or peaks showing low *Rg* values (0.10—0.41) or long retention time (53—58 min) were observed. Since these results suggested that the fraction 1 contained some oligosaccharides, the separation of oligosaccharides by Sephadex column and hydrolysis (1N HCl, 110°, 10 hr or enzyme treatment) of the fraction 1 were undertaken.

Fraction 1 was fractionated by Sephadex G–10 column (Fig. 2) and was assayed by the anthrone method. The fractions from tube No. 9 to 16 were collected and concentrated *in vacuo*. After the absence of monosaccharide was confined by GLC, PPC and glucostat reagent (Worthington Biochemical Co. New Jersey U.S.A.) analysis, the concentrated fractions were refluxed with 3N HCl at 110° for 10 hr. As shown in Table V, the hydrolyzate gave the sole compound glucose which was characterized by PPC or GLC analyses. Consequently, these oligosaccharides were regarded as "glucan" which were consisted only of glucose molecule.

Table V. Paper and Gas Liquid Chromatograms of Acid Treated Oligosaccharides

Compound	*Rg* (PPC)	*Rt* (GLC)
Glucose	1.00	23
Acid-treated Oligosaccharide	1.00	23

The separated oligosaccharide fraction on Sephadex G-10 column from fraction 2 was hydrolyzed with 3N HCl at 110° for 10 hr, and monosaccharide in the hydrolysate was analyzed as described in Material and Methods.

In order to determine whether the linkage type of this glucan was α or β, the glucan was dissolved in 0.01 M McIlvaine buffer, pH 6.15 and α- or β-glucosidase was added at a concentration of 83.5 μg/ml. After incubated at 30° for 24 hr, the liberated glucose was detected by PPC, GLC and glucostat reagent (Table VI). As shown in Table VI, β-glucosidase separated some glucose from glucan, while α-glucosidase did not. This result suggested that this glucan would contain β-glucosidic linkages.

Table VI. Detection of Glucose from α- or β-Glucosidase Treated Glucan by PPC, GLC and Glucostat Analyses

Material	*Rg*	*Rt*	Glucostat
α-Glucosidase	—	—	—
β-Glucosidase	—	—	—
Glucose	1.00	17.5	+
Glucan[a)]	—	—	—
Glucan[a)] + α-glucosidase	—	—	—
Glucan[a)] + β-glucosidase	1.00	17.5	+

a) glucose free

Table VII. Paper and Gas Liquid Chromatograms of Amino Sugars in Fraction 4

Compound	*Rg*	*Rt*
Glucosamine	0.73	16.0
Galactosamine	(0.69)	(14.0)
Fraction 4	0.73	16.0
	(0.69)	(14.0)

The condition of analyses were described in Material and Method.

Hydrolysate of Acetic Acid–Insoluble Matter (Fraction 4) (*cf.* Chart 2)

Aliquot of the fraction 4 was chromatographed on Sephadex G-25 column and the eluates (tube No. 19 to 25), positive to Elson–Morgan's reagent[12)] were collected, evaporated *in vacuo* and analyzed by PPC and GLC separation. As shown in Table VII, glucosamine and small amount of galactosamine were identified. The result of PPC the Ehrlich reagent as a detector demonstrated the presence of these two hexosamines together with several kinds of amino acids. On the other side, the insoluble matter from the cell wallls was incubated with chitinase at 38—40° for 72 hr under the following condition: 25 mg substrate, 1000 μg chitinase (Biochemical Co.) in 3.5 ml of 0.01 M McIlvaine buffer (pH 6.55) and toluene. After incubation, the

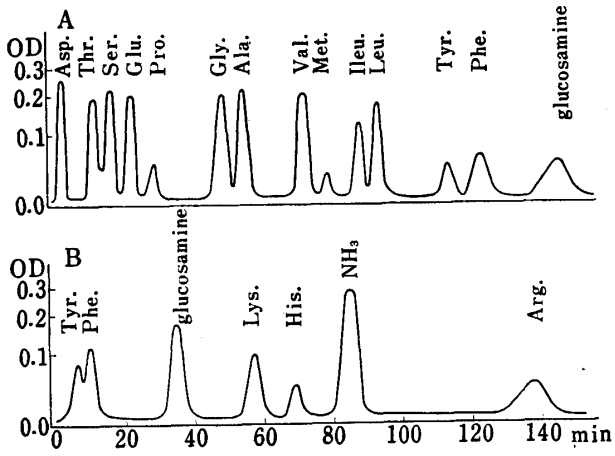

Fig. 3. Comparison of Amino Acid Composition of the Cell Wall

A: neutral and acidic amino acids
B: basic amino acids

12) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).

liberated N-acetylglucosamine was detected in the supernatant fraction by GLC and PPC analyses. These results suggested that a chitin–like substance layer would exist in the fungal walls.

Amino Acid Composition of the Fraction 3 (*cf.* Chart 2)

The amino acid composition of the fraction 3 was determined by Hitachi amino acid analyser, model KLA-3B. The neutral, acidic and basic amino acids composition of the fraction 3 was illustrated in Fig. 3.

Discussion

It was clarified from the results given in Table II that the chemical compositions of *Cochliobolus miyabeanus* cell walls were 50% of glucan, 40% of chitin–like substance, 10% of peptide and 2.5% of mineral. These values did not correspond closely with those of fungal cell walls of different species as shown in Table VIII. It is likely that such deviations are caused by differences of fungal species of cultural ages.

TABLE VIII. The Chemical Compositions in Different Species of Fungal Cell Walls

Compound	Fungi			
	Asp. niger	*Asp. spp.*	*Phytophthora spp.*	*Pythium spp.*
Protein	0.5— 2.5	8.3	3.6 — 5.6	1.24— 2.02
Neutral sugar	73 —83	45.5—51.3	86 —90	81 —82.4
Hexosamine	9 —13	14.7—18.2	0.3 — 2.3	0.5 — 1.3
Lipid	2 — 7	7.4	0.2 — 2.5	2.6
Ash	0.1	4.0	0.26— 0.4	0.44— 2.4
Reference	5	6	7, 18	19, 20

As shown in Table III and IV, glucose, galactose, mannose, arabinose, rhamnose, fucose and xylose were detected in the case of *Cochliobolus miyabeanus*, while some different compositions were characterised in other fungi belonging to the same class "ascomycetes." No xylose and fucose were found in *Aspergillus niger*, *Botrytis cinerea* and *Penicillum notatum*,[13] while xylose was detected in *Penicillum chrysogenum*,[14] *Aspergillus oryzae*[15] and fucose in *Sporobolomyces*, *Rhodotorula*[13] and *actinomyces*.[16] Except for *actinomyces*, these organisms belong to the same class "ascomycetes." Since even though some species of fungi belonged to the same genus, *Aspergillus niger* and *Aspergillus oryzae* have different sugar compositions, it was not surprising that the neutral sugars described above appeared in the cell walls of *C. miyabeanus*.

As shown in Tables V and VI, the existence of β-glucosidic linkage in our fungal cell walls is evident. This is not peculiar, because β-glucan compounds have been reported to exist in the cell walls of several fungi belonging to ascomycetes or phycomycetes, *e.g.*, *Neurospora crassa*,[17,18] *Aspergillus oryzae*,[19] *Pythium deberyanum*[7] and *Phytophthora spp.*[3] *etc.*

13) E.M. Crook and I.R. Johnston, *Biochem. J.*, **83**, 325 (1962).
14) P.B. Hamilton and S.G. Knight, *Arch. Biochem. Biophys.*, **99**, 282 (1962).
15) K. Horikoshi, "I.A.M. Symposia on Microbiol," No. 3, ed. by The Institute of Applied Microbiol. Univ. Tokyo, p. 124
16) L. Pine and C.J. Boone, *J. Bacteriol.*, **94**, 875 (1967).
17) M.S. Manocha and J.R. Colvin, *J. Bacteriol.*, **94**, 202 (1967).
18) M.J. Potgieter and M. Alexander, *Can. J. Microbiol.*, **11**, 122 (1965).
19) B.A. Cooper and J.M. Aronson, *Mycologia*, **59**, 658 (1967).

Chitin (a polymer of N-acetylglucosamine) has been found in the cell walls *Aspergillus*, *Neurospora*, *Penicillum* and many other kinds of fungi.[3] As was shown in Table VII, the fraction 4 contained glucosamine as a major and galactosamine as a minor, and it is certain that our fungal cell walls have a chitin-like layer.

Sixteen kinds of amino acids were detected (Fig. 3), and some differences were recognized compared with those of other fungal cell walls. Although E.M. Crook[13] reported the absence of methionine in ascomycetes, this amino acid was detected in our fungus (classified in ascomycetes). While E.M. Crook[13] and Bartnicki-Garcia[7] reported the presence of hydroxy proline in phycomycetes and γ-amino butyric acid in yeast, no such amino acids were found in our fungus.

From these results, it is concluded that the cell walls of *Cochliobolus miyabeanus* are constituted of β-glucan and a chitin-like layer associated with a small amount of galactose, mannose, other sugars and peptides.

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20) M. Novaes-Ledieu, A. Jimenez-Martinez and J.R. Villanueva, *J. Gen. Microbiol.*, **47**, 237 (1967).