ment of the carbon atom of the resolving agent by a sulfur atom decreased the separation factors of racemic amino acids except for proline derivatives, and the replacement of H atom attached to carbon atom by a OH group increased the separation factors of racemic amino acids. Proline derivative seems to be the best resolving agent because it has a desirable retention times with better separation factors.

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Isolation and Structure of α-1,3-Linked Glucan from the Hyphal Wall of *Phytophthora infestans*[1]

Toshio Miyazaki, Michihisa Yamada, and Takashi Ohno

Tokyo College of Pharmacy[2]

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Phytophthora infestans is a well-known phytopathogenic fungus which belongs to Oomycetes. Chemotaxonomically, it is interesting that the major component of the mycelial cell walls of Oomycetes is not chitin but glucan, and this is quite different from many other filamentous fungi.[3] Glucans from Phytophthora hyphal walls have been examined by a few workers. In *P. cinnamomi*,[4] two different β-glucans comprising nearly 90% of the mycelial wall were isolated and the major component was an extremely water-insoluble and highly branched glucan composed predominantly of β-1,3- and small amount of β-1,4-linkages. The minor component was cellulose. In the case of *P. hevea*[5] presence of similar glucans reported. These results were obtained from enzymic degradation and usual analytical procedures, and suggest that glucans from the hyphal wall of Phytophthora are similar to each other.

Recently, Hodgson, *et al.*[6] reported that water-soluble glucan from *P. infestans* consists of β-1,3-linkage and it completely inhibited local lesion development by potato virus X. However, water-insoluble component of the hyphal wall has not been described. In recent years, histochemical investigation on the hyphal walls of Oomycetes have been made in our laboratory, and an alkali-soluble and water-insoluble glucan was isolated as the major component from the hyphal wall of *P. infestans*. This glucan, contrary to expectation, is highly dextroratatory, $[\alpha]_D^{21}$ +217° (1N NaOH), and showed an absorption band at 844 cm^{-1} for α-glucosidic linkage in its infrared spectrum.

This glucan was methylated by the methods of Hakomori[7] and then of Purdie,[8] and after methanolysis of the methylated glucan was carried out the product was analyzed by gas-liquid chromatography (GLC). As shown in Fig. 1, methyl 2,4,6-tri-O-methyl- and a small amount of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranosides were detected. The

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[1] This constitutes Part XIV of a series entitled "Studies on Fungal Polysaccharides." Part XIII: T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), 22, 1360 (1974). A part of this work was presented at the 92nd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1973.

[2] Location: *20-1, Kitashinjuku, 3-chome, Shinjuku-ku, Tokyo.*

[3] S. Bartnicki-Garcia, *Annu. Rev. Microbiol.*, **22**, 87 (1968).

[4] L.P.T.M. Zevenhuizen and S. Bartnicki-Garcia, *Biochemistry*, **8**, 1496 (1969).

[5] M. Novaes-Ledieu and A. Jimenz-Martin, *J. Gen. Microbiol.*, **54**, 407 (1969).

[6] F.A. Wood, R.P. Singh, and W.A. Hodgson, *Phytopathology*, **61**, 100 (1971).

[7] S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

[8] T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).

methylated D-glucan in $CDCl_3$ provides a proton magnetic resonance (PMR) spectrum (Fig. 2), having a doublet at 5.42 ppm, $J_{1,2}$ 3.0 Hz, for the anomeric protons, H-1, of the α-D-(1—3)-linked[9] residues in the polymer. The anomeric proton signal for linear (1—3) linked glucopyranose residues in the spectrum of permethylated laminarin in $CDCl_3$ has been shown to resonate at 4.7 ppm, $J_{1,2}$ 7.5 Hz (Fig. 3). The glucan was scarcely oxidized by periodate. From these results, it seemed certain that the glucan has a straight chain structure consisting of α-1,3-glucosidic linkage.

Actually, hyphal wall of *P. infestans* was different from those of other Oomycetes because it was incompletely digested by treatment with snail-gut enzyme.[10]

Occurrence of β-1,3-linked glucans is extensive, particularly in the field of fungi, but the presence of a simple α-1,3-linked glucan is not common, and this type of glucan was isolated only from *Aspergillus niger*[11] (nigerodextran) and *Asp. nidulans.*[12]

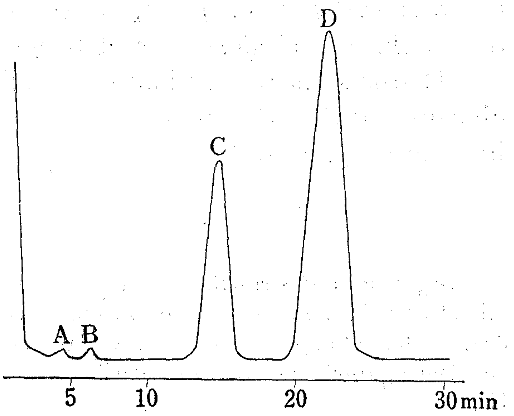

Fig. 1. Gas Chromatogram of Methanolysate of the Methylated PII

A: β-methyl-2,3,4,6-tetra-O-methyl-D-glucoside
B: α-methyl-2,3,4,6-tetra-O-methyl-D-glucoside
C: β-methyl-2,4,6-tri-O-methyl-D-glucoside
D: α-methyl-2,4,6-tri-O-methyl-D-glucoside

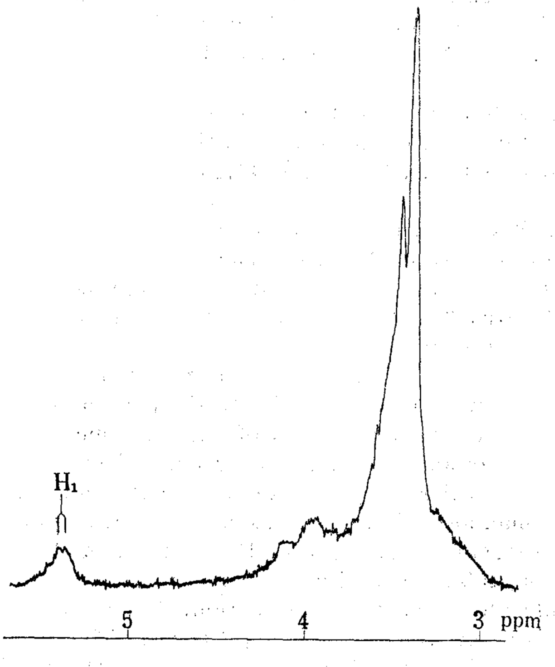

Fig. 2. PMR (60 MHz) in $CDCl_3$ of the Methylated PII

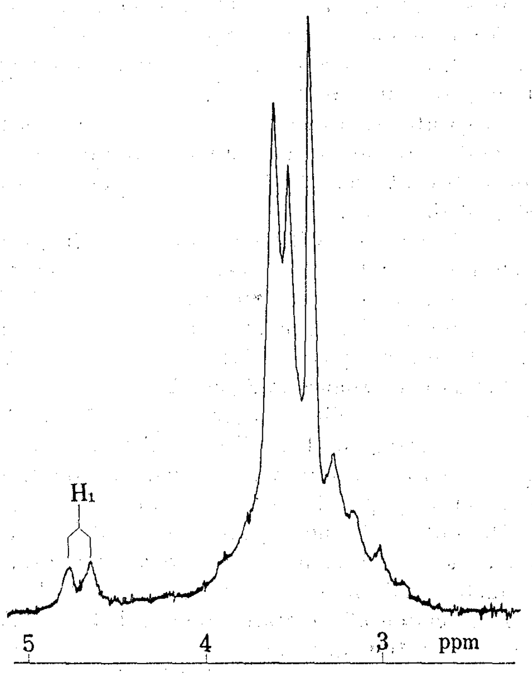

Fig. 3. PMR (60 MHz) in $CDCl_3$ of the Methylated Laminarin

As minor glycans of the hyphal wall, water-soluble glucoserich glycan, which contains β-1,3-linked glucose unit accompanied with galactose and mannose, water- and alkali-insoluble

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[9] B. Casu, M. Reggiani, G.G. Gallo, and A. Vigevani, *Tetrahedron*, **24**, 803 (1968).
[10] T. Ohno, M. Yamada, and T. Miyazaki, unpublished.
[11] I.R. Johnston, *Biochem. J.*, **96**, 659 (1965).
[12] J.M. Zonneveld, *Biochem. Biophys. Acta*, **273**, 174 (1972).

heteroglycan consisting of glucose, mannose, and galactose, and as the third glycan, β-1,4-linked glucan, cellulose, were detected.

Histochemical examination, particularly construction and distribution of the component glycans and the other biological behavior of the hyphal wall from *P. infestans* will be discussed in separate papers.

Experimental

Organism and Growth——The organism used in this work, *Phytophthora infestanes* IFO-4872, was supplied by the Institute of Fermentation, Osaka. Incubation was carried out at 25° for 16 days in an Erlenmyer flask of 300 ml capacity containing 100 ml of Sabouraud medium consisting of 2% glucose, 1% polypeptone, and 100 μg/1000 ml of thiamine-HCl. The mycelium was collected by filtration and sufficiently washed with H_2O.

Preparation of Cell Wall Material——The hyphal mycelium was disrupted twice by the French press (200 kg/cm^2), cell wall material was collected by centrifugation, and washed with H_2O several times until freed from cytoplasmic material by repeated differential centrifugation at 2000 rpm. Finally, the isolated wall material was freeze-dried. The cell wall thus obtained was observed not to have any cytoplasmic material by optical and electron microscopy.

Extraction of Polysaccharide from the Cell Wall——1) Extraction with Water at 100°: The cell wall (2 g) was extracted with H_2O for 30 min at 100°. After centrifugation, this procedure was repeated 8 times. The combined supernatant was dialyzed against running water for 2 days, and the internal solution was concentrated *in vacuo*. To the concentrate, 4 volumes of EtOH was added, and the precipitate that appeared was collected by centrifugation, washed with EtOH, acetone, and ether, and dried *in vacuo*. Yield of the water-soluble substance, PI, 120.8 mg (corresponding to 6.03% of the wall).

2) Extraction with 1 N NaOH at Room Temperature: The water-extracted cell wall material (1.7 g) was extracted with 1 N NaOH for 12 hr at room temperature. After centrifugation, this procedure was repeated 7 times. The combined supernatant was dialyzed against running water for 2 days, during which a water-insoluble material appeared in the internal solution. This was collected by centrifugation, washed with H_2O, EtOH, acetone, and EtOH, and dried *in vacuo*. Yield of the material, P II, was 880 mg (corresponding to 43.9% of the wall).

The water-soluble fraction in the tube was concentrated to a small volume *in vacuo*, 4 volumes of EtOH were added to the concentrate, and the precipitate that formed was collected by centrifugation and treated as described above. Yield of the alkali- and water-soluble material, P III, was 41.2 mg (corresponding to 2.06% of the wall).

3) Extraction with 1 N NaOH at 100°: The treated cell wall was further extracted with 1 N NaOH for 30 min at 100°. After centrifugation, this procedure was repeated 7 times. The suoernatant and sediment were treated as described above. Yield of the alkali- and water-soluble material, PIV, was 102.7 mg, yield of the alkali-soluble and water-insoluble material, PV, was 26.5 mg, and that of the sediment was 263.8 mg. These correspond to 5.1%, 1.3%, and 13.2% of the wall, respectively.

Component Sugars of Each Fraction——Each fraction (*ca.* 2 mg) in 2 ml of 2 N H_2SO_4 in a sealed tube was heated in a boiling water bath for 7 hr. After neutralization ($BaCO_3$) and filtration, the hydrolyzate was concentrated and applied to Toyo Roshi No. 50 filter paper for detection of the component sugars. Paper chromatography was carried out by the ascending method using AcOEt–pyridine–H_2O (10: 4: 3). Sugars on the paper chromatogram were detected by spraying solution of $AgNO_3$–NaOH,[13] *p*-anisidine–HCl.[14]

PI contained glucose, galactose, and small amount of mannose; PII, glucose, small amount of galactose and mannose; PIII, glucose, galactose, mannose, PIV, glucose, galactose, mannose; PV, glucose, small amount of galactose, and mannose; and extracted sediment, glucose, galactose, and mannose.

Purification of the Main Fraction PII——Alkali-soluble and water-insoluble fraction, PII, was extracted with H_2O at 100° and the mixture was centrifuged. This procedure was repeated 3 times. The insoluble material (690 mg) was suspended in H_2O and this was adjusted to pH 7.5 with $NaHCO_3$. To this solution was added pronase (35 mg) to 5% of the content of the crude material, and the mixture was incubated with stirring for 2 days at 35°. The insoluble material was collected by centrifugation, dissolved in 0.5 N NaOH, this alkali solution (150 ml) was shaken vigorously for 30 min with 30 ml of $CHCl_3$–BuOH (4: 1), the mixture was centrifuged, and the aqueous phase was collected. The same procedure was repeated until a gelatinous substances was no longer formed in the mixture. After centrifugation, the supernatant was dialyzed against distilled H_2O in a Visking Cellophane tubing with stirring for 2 days. The resulting precipitate was collected by centrifugation, washed with H_2O, EtOH, acetone, and ether, and dried *in vacuo*. Yield of the material, 331 mg. Paper chromatographic analysis of the acidic hydrolyzate showed the presence of only glucose.

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[13] W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).

[14] L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, **1950**, 1702.

Properties of Purified PII——Purified PII consisted of 98.0% of total hexose by $C_6H_5OH-H_2SO_4$ method, as glucose, and no nitrogen. Iodine reaction, negative. $[\alpha]_D^{21}$ +217° (c=1.02, 1N NaOH). IR ν_{max}^{KBr}: 844 cm^{-1} (α-glucosidic linkage).

Periodate Oxidation of Purified PII——Purified PII (25 mg) was dissolved in 1 ml of 1 N NaOH, the solution was adjusted to pH 5.5 with 0.5 N HCl, and its total volume was made up to 50 ml with 4 ml 0.22 M $NaIO_4$ and H_2O. The mixture was allowed to stand with stirring at room temperature in the dark. A blank solution containing no glucan was processed similarly. An aliquot of 3 ml was taken at different periods for the determination of $NaIO_4$ consumption and formation of HCOOH by the procedures of Maraprade[15] and Whistler.[16] The number of moles of $NaIO_4$ consumed per anhydroglucose unit was <0.1 (72 hr). The value of HCOOH was <0.001 (72 hr).

Methylation of PII——Methylation of the purified PII (50 mg) was carried out by the procedures of Hakomori[7] (twice) and Purdie.[8] The reaction mixture was extracted with $CHCl_3$ and completely methylated PII showed no significant OH bond in the 3500 cm^{-1} region in its IR spectrum.

GLC of the Methanolyzate of Methylated PII and Tri-O-methylsilyl Derivative of the Methanolyzate——Methylated PII was converted into methylglucosides by heating with 1 N MeOH–HCl in a sealed tube for 10 hr in a boiling water bath. MeOH was evaporated and HCl was removed by evaporation in a vacuum desiccator over NaOH. The resulting methylglucosides were dissolved in a minimum amount of MeOH and HCl, the methanolyzate was trimethylsilylated according to the methods of Sweeley, *et al.*[17] and Yamakawa, *et al.*[18] GLC of the methylglucosides and trimethylsilylated derivatives of methylglucosides was effected in a Varian aerograph Model 705, equipped with a flame ionization detector, using a 200×0.95 cm metal column packed with 15% polybutane-1,4-diol succinate on Chromosorb W-80 (100 mesh); column temperature, 165°; N_2 flow rate, 55 ml/min, and column temperature, 140°; N_2 flow rate, 50 ml/min.

PMR Spectrum of Methylated PII——PMR spectra of the methylated PII and methylated laminarin were recorded at 60 MHz with a Varian NV-14 NMR spectrometer at normal operating temperature. Tetramethylsilane was used for the analysis as an internal standard in $CDCl_3$. Sample concentration was about 5%.

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15) L. Maraprade, *Bull. Soc. Chim. France*, **1**, 833 (1934).
16) R.L. Whistler and J.L. Hickson, *J. Am. Chem. Soc.*, 76, 1971 (1954).
17) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
18) T. Yamakawa, N. Ueta, and I. Ishizuka, *Japan J. Exp. Med.*, **34**, 231 (1964).