Surface Mannan from *Glomerella cingulata*

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A specific layer at the outermost surface of mycelia of *Glomerella cingulata* G 4-1-4 was observed. The substance isolated from this layer was a polysaccharide ($[\alpha]_D^{20} + 82.6^\circ$) composed of D-mannose (97.6%), protein (2.0%) and a trace amount of phosphate. A high proportion of 3,4-di-O-methylmannose (38 mol%) was found on methylation analysis. Digestion of this mannan with a bacterial α -D-mannosidase released 78% of the D-mannose. In addition, the results of acetolysis, partial acid hydrolysis, and ^1H - and ^{13}C -NMR spectroscopy showed that the *G. cingulata* G 4-1-4 mannan has a backbone joined by α -1,6-linkages, and α -1,2- and 1,3-linked mannose residues are attached to the C-2 position of the main chain segment, giving it a highly branched structure. Although *G. cingulata* G 4-1-4 mannan has the above properties, its basic structure appears to be similar to that of baker's yeast mannan.

We investigated the inhibitory effects on growing mycelia of several phytopathogenic fungi of the concerted actions of lytic enzymes,¹⁾ antibiotics (or agrochemicals) and non-ionic detergents. We found that the mycelia of *Glomerella cingulata* G 4-1-4 have a higher resistance to such concerted actions. Electron microscopic observation showed the presence of a specific layer of about 1 μm in thickness at the outermost surface of the cell wall (Fig. 1). We have found that this layer is composed of a D-mannose homopolymer which has been rarely found in filamentous fungi.²⁾

In this paper, we report the chemical characterization of the *G. cingulata* G 4-1-4 mannan.

MATERIALS AND METHODS

Organisms. *Glomerella cingulata* G 4-1-4 was originally obtained from the National Institute of Agricultural Sciences, Tsukuba, Japan. This phytopathogenic strain was isolated from *Vitis vinifera* (grape). *Serratia marcescens* (IFO 12648) was obtained from the Institute for Fermentation, Osaka, Japan. *Arthrobacter* GJM-1 was supplied by Dr. T. Nakajima of Tohoku University.

Materials. Dextran T-500 and T-110 (Pharmacia Fine

Chemicals, Uppsala, Sweden) and Pullulan PF-30 (Hayashibara Biochemical Laboratory, Okayama, Japan) were kind gifts from Dr. S. Kamimiya of Niigata University. α -D-Mannosidase of *Arthrobacter* GJM-1 was purified by DEAE-cellulose chromatography according to Jones and Ballou.³⁾ The enzyme thus prepared was free from protease and glucosidase activities. The cell wall preparation was obtained as described by Tanaka *et al.*¹⁾ Other materials used in this study were obtained commercially.

Isolation and purification of mannan. A strain of *G. cingulata* G 4-1-4 was grown at 28°C for 48 hr with

FIG. 1. Electron Micrograph of an Ultrathin Section of *G. cingulata* G 4-1-4.

A material of about 1 μm in thickness was observed in the outermost layer of mycelia grown at 28°C for 48 hr.

shaking on Vogel-N sucrose-yeast extract medium⁴⁾ containing peptone (30 g/liter). Mycelia in 5-liter cultures were harvested on a Buchner funnel and washed successively with distilled water. The washed mycelia were extracted with 0.02 M citrate buffer (pH 7.0) by autoclaving at 120°C for 90 min, followed by centrifugation at $10,000 \times g$ for 10 min. The above procedures were repeated for the precipitate obtained. Combined extracts were obtained as described by Raschke and Ballou,⁵⁾ and *G. cingulata* G 4-1-4 mannan was precipitated with Fehling's solution according to Kocourek and Ballou.⁶⁾ The final purification was performed by chromatography on a column (1.6×150 cm) of Sephacryl S-500, and the homogeneity of the mannan was determined by gel filtration and ultracentrifugation.

General procedures. The carbohydrate content of the mannan was estimated by the phenol sulfuric acid method of Dubois *et al.*,⁷⁾ and reducing sugar was assayed by the method of Nelson-Somogyi.⁸⁾ Protein was determined by Lowry's method,⁹⁾ and phosphorus by the modified Allen method reported by Nakamura.¹⁰⁾ Hexosamine was determined by the Elson-Morgan method as modified by Arima *et al.*¹¹⁾ Optical rotation was measured with a UNION-PM 101 digital polarimeter. Thin-layer chromatography (TLC) was carried out on Avicel SF plates with the following solvent systems (v/v): a) 1-butanol-pyridine-water (6:4:3), and b) 1-butanol-ethanol-water (4:1:1.5). Sugars on the chromatograms were detected with alkaline silver nitrate and/or aniline-HCl reagents.

Methylation analysis. The mannan (10 mg) was methylated three times, repeatedly, by the method of Hakomori¹²⁾ as described by Lindberg.¹³⁾ The methylated product was hydrolyzed with 0.5 N H_2SO_4 at 100°C for 15 hr. The solution was neutralized with barium carbonate and then the precipitate was filtered and washed successively with distilled water. The filtrate was deionized with Dowex 50 (H^+) and then concentrated to dryness. The hydrolyzate was converted to alditol acetate derivatives which were analyzed by gas chromatography (GLC). The acetolysis fragments were also subjected to methylation analysis in a similar manner. In this case, the removal of dimethyl sulfoxide from the reaction mixture was performed by the method of Lindberg.¹³⁾

Acetolysis. Acetolysis was performed according to Kocourek and Ballou.⁶⁾ The mannan (55 mg) was acetylated in a mixture (10 ml) of dry pyridine and acetic anhydride (1:1) at 100°C for 12 hr. After acetylation, the sample was acetolyzed in 10 ml of acetolysis reagent (acetic acid, acetic anhydride and sulfuric acid, 10:10:1) at 40°C for 15 hr. The acetolysis products were deacetylated with about 0.5 N sodium methoxide and then neutralized with Dowex 50 (H^+). The deacetylated products were separated on a Bio-Gel P-2 column (1.5×170 cm).

Hydrolysis of the mannan and the cell wall. Complete acid hydrolysis of the mannan (5 mg) was performed with 1.0 ml of 1 N H_2SO_4 at 100°C for 12 hr. After neutralization with barium carbonate, the hydrolyzate was filtered and deionized with Dowex 50 (H^+). Partial acid hydrolysis was performed with 0.1 or 1 N HCl at 100°C for 0.5, 1, 2 and 3 hr. The hydrolysis products, after the removal of HCl, were examined by TLC and GLC. Enzymatic hydrolysis with *Arthrobacter* α -D-mannosidase was performed as follows; a reaction mixture containing the mannan (50 mg), $CaCl_2$ (2.5 μ mol) and the enzyme (160 units) in 25 ml of 0.05 M potassium phosphate buffer (pH 6.8) was incubated at 37°C. At 1 hr intervals, an aliquot of the reaction mixture was taken and assayed for released reducing sugar. The amount of reducing sugar liberated was estimated as mannose. Hydrolysis for liberation of component sugars of the cell wall was performed by the method reported by Nakajima *et al.*¹⁴⁾ Hydrolysis for determination of hexosamine of the cell wall was carried out by the method of Sakurai *et al.*¹⁵⁾

Gas chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS). GLC of the alditol acetate derivatives of sugars was carried out with a GC-4APF or GC-4CPF (Shimadzu, Kyoto) chromatograph equipped with a flame ionization detector. The samples were injected into a 3 mm $\times$ 2 m glass column packed with 3% Silicone OV-225 on Supelcoport (80~100 mesh) or Chromosorb W (60~80 mesh). The operating temperature of the column was 200°C. GLC with a capillary column was performed as follows; a glass column (0.28 mm $\times$ 20 m) of G-SCOT Silicone OV-225 was used at 165°C. A GC-MS combination system (M-60, Hitachi, Tokyo) was used. The mass spectra were obtained at an ionization energy of 30 eV, an ion source temperature of 190°C and an accelerating voltage of 3.2 kV. A computer equipped with a data management system (002B 8DK) was used to analyze the mass spectral data and to prepare mass chromatograms.

Electron microscopy. For ultrathin sectioning, mycelia of *G. cingulata* G 4-1-4 were washed three times with distilled water, and then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 50 ppm of $CaCl_2$ at 0°C for 2 hr followed by post-fixation with 1.3% osmium tetroxide in the buffer at 0°C for 2 hr. The fixed materials were dehydrated with a graded ethanol series (50 to 100%) and then embedded in Epon 812. Serial sections were picked up on formbar coated grids. Ultrathin sections were stained for 20 to 30 min in a saturated aqueous solution of uranyl acetate followed by staining for 15 min with a lead staining solution according to Sato.¹⁶⁾ Observation was carried out with a transmission electron microscope (JEM 100-B, JEOL Ltd., Tokyo) operated at 80 kV.

Nuclear magnetic resonance spectroscopy (NMR). 1H -

(PMR) and ^{13}C -nuclear magnetic resonance (^{13}C -NMR) spectra of the mannan were measured at 70°C in D_2O according to the method described by Gorin and Spencer.¹⁷⁾ Chemical shifts are given relative to the external tetramethylsilane. The PMR and ^{13}C -NMR spectra were obtained with standard JEOL FX 200 and FX 90Q apparatus (Tokyo), respectively.

RESULTS

Isolation and purification of mannan

Crude mannan (4.3 g) was isolated as a white powder from wet mycelia (270 g) of *G. cingulata* G 4-1-4 with Fehling's reagent. GLC analysis indicated that the crude mannan obtained was composed of 99% D-mannose and a trace amount of D-glucose. This crude mannan was dissolved in distilled water and then chromatographed on a Sephacryl S-500 column. It was separated into three fractions (F-I ~ III) (Fig. 2). The eluate corresponding to the major peak (F-I) was collected and lyophilized. The yield of the purified mannan was 3.8 g.

Table I shows some physical and chemical properties of this mannan. This sample was found to be homogeneous on analytical ultracentrifugation. The sedimentation coefficient was calculated to be 2.7S at 20°C . This sample

was composed of carbohydrate (97.6%), protein (2.0%) and a trace amount of phosphate. No sugar other than D-mannose was detected on GLC. The molecular weight was estimated to be about 8×10^6 from the elution profile on Sephacryl S-500 (Fig. 2). The optical rotation was $[\alpha]_{\text{D}}^{20} + 82.6^\circ$ (c, 1.00 in water).

Methylation analysis

The mannan was methylated and hydrolyzed. The hydrolyzate was converted to alditol acetates, and then these derivatives were identified and quantitated by GLC and GC-MS. A capillary GLC chromatogram is shown in Fig. 3, and the results of GC-MS analysis are summarized in Table II. Peaks I, II, III, IV and V corresponded to 2,3,4,6-tetra-O-methyl-, 3,4,6-tri-O-methyl-, 2,4,6-tri-O-

TABLE I. PROPERTIES OF THE PURIFIED MANNAN FROM *G. cingulata* G 4-1-4

Carbohydrate content	97.6%
(D-Mannose)	>99%
Protein content	2.0%
Phosphate content	0.2%
Molecular weight	8×10^6 ^a
$S_{20,w}$	2.7
Optical rotation	+82.6 ^{cb}
Extent of digestion with α -D-mannosidase	78%

^a Determined by Sephacryl S-500 gel filtration.

^b $[\alpha]_{\text{D}}^{20}$ (c, 1.00 in H_2O).

FIG. 2. Elution Profile on Sephacryl S-500 Column Chromatography of *G. cingulata* G 4-1-4 Mannan Isolated by Fehling's Method.

The column ($1.6 \times 165\text{cm}$) was loaded with the crude mannan and eluted with distilled water at a flow rate of 18 ml/hr. Cells of *Serratia marcescens* (Vo), Dextran T-500 (MW 5.0×10^5), Pullulan PF-30 (MW 2.8×10^5) and Dextran T-110 (MW 1.1×10^5) were used as references.

FIG. 3. Gas-liquid Capillary Column Chromatogram of the Partially Methylated Alditol Acetates from the Purified Mannan.

The peaks identified by mass spectrometry are (I) 2,3,4,5-tetra-O-methylmannitol, (II) 3,4,6-tri-O-methylmannitol, (III) 2,4,6-tri-O-methylmannitol, (IV) 2,3,4-tri-O-methylmannitol and (V) 3,4-di-O-methylmannitol.

TABLE II. METHYLATION ANALYSIS OF THE PURIFIED MANNAN

Peak	Molar ratio ^a	Major mass fragments (<i>m/z</i>)	<i>O</i> -Acetyl- <i>O</i> -methylalditol
I	6.0	43 45 71 87 88 101 102 118 129 145 161 162 205	2,3,4,6-Tetra- <i>O</i> -methylmannitol
II	6.1	43 45 87 88 100 101 129 130 161 190 205 234	3,4,6-Tri- <i>O</i> -methylmannitol
III	7.3	43 45 101 118 129 161 202 203 234 277	2,4,6-Tri- <i>O</i> -methylmannitol
IV	1.0	43 87 99 102 113 118 129 162 189 233	2,3,4-Tri- <i>O</i> -methylmannitol
V	12.4	43 87 88 99 100 129 130 189 190 233 234	3,4-Di- <i>O</i> -methylmannitol

^a Calculated from the peak areas.

FIG. 4. Gel Filtration on Bio-Gel P-2 of the Acetolysis Products Obtained from the Purified Mannan.

Acetolysis products from 55 mg of the purified mannan were applied to a column (1.5 × 170 cm) and eluted with distilled water at a flow rate of 11.8 ml/hr. The elution positions of the references (I to V; mannose to mannopentaose) are shown in the figure.

methyl-, 2,3,4-tri-*O*-methyl- and 3,4-di-*O*-methylmannitol acetates, respectively. The molar ratio was 6:6:7:1:12. This ratio is markedly different from that found on methylation analysis of baker's yeast mannan.

Acetolysis of the mannan and methylation analysis of the acetolysis fragments

The mannan was acetolyzed at 40°C for 15 hr, under conditions with which 1,6-mannosidic linkages are cleaved preferentially. Resolution of the acetolysis products on a column of Bio-Gel P-2 (Fig. 4) revealed

TABLE III. MOLAR RATIO OF ACETOLYSIS PRODUCTS

	Fraction	Molar ratio ^a
I	Mannose	0.78
II	Mannobiose	1.00
III	Mannotriose	0.46
IV	Mannotetraose	0.57
V	Mannopentaose	0.13

^a Relative to mannobiose.

mono-, di-, tri-, tetra- and pentasaccharides. The main fraction as to weight is mannopentaose. The five peaks were pooled separately and lyophilized. Table III shows the molar ratios of the acetolysis products. The presence of mannopentaose suggests that this mannan has side chains of the mannopentaose unit.

The linkages in these four oligosaccharides were investigated by methylation according to Hakomori.¹²⁾ GLC analysis of the acid hydrolyzates of all the methylated oligomannosides revealed that they consist of 1,2- and 1,3-linked mannose residues. Table IV shows that the ratio of 1,3-linkages to the total linkages gradually increases from the mannobiose to the mannopentaose fragment.

Partial acid hydrolysis

The purified mannan was treated with 0.1 or 1 N HCl at 100°C for various times. Each hydrolysis product was analyzed by TLC on Avicel SF plates. The conditions under which only mannose was released from the mannan

TABLE IV. METHYLATION ANALYSIS OF ACETOLYSIS FRAGMENTS

Partially methylated alditol acetate	Molar ratio			
	Mannobiose	Mannotriose	Mannotetraose	Mannopentaose
2,3,4,6-Tetra- <i>O</i> -methylmannitol	1.00	1.00	1.00	1.00
3,4,6-Tri- <i>O</i> -methylmannitol	0.88	1.38	1.66	1.73
2,4,6-Tri- <i>O</i> -methylmannitol	0.12	0.59	1.27	2.24

FIG. 5. Gel Filtration on Bio-Gel P-2 of the Mild Acid Hydrolysis Products from the Purified Mannan.

The column (1.5 × 160 cm) was loaded with 20 mg of the hydrolysis products. The hydrolyzate was eluted with distilled water at a flow rate of 10.5 ml/hr.

were 0.1 N HCl at 100°C for 30 min. The product obtained under these hydrolysis conditions was subjected to the following treatments successively. The hydrolyzate was neutralized with 0.1 N NaOH and then chromatographed on a column of Bio-Gel P-2 (Fig. 5). The eluate corresponding to the degraded mannan was collected and lyophilized. The acid-degraded mannan was methylated and then hydrolyzed. This hydrolyzate was analyzed by GLC as alditol acetates. Only the proportion of 2,4,6-tri-*O*-methyl-mannose in the methylated sugars from the mannan treated as above was considerably decreased as compared with that in the original mannan. This indicates that 1,3-linkages are located at the sites of the non-reducing ends of the side chains.

Enzymatic hydrolysis

The mannan was treated with *Arthrobacter* α-D-mannosidase by the method described

FIG. 6. PMR Spectra (199.5 MHz) of *G. cingulata* G 4-1-4 Mannan and Baker's Yeast Mannan.

Each spectrum was obtained at 70°C for 10% mannan dissolved in a D₂O solution.

A, *G. cingulata* G 4-1-4 mannan; B, baker's yeast mannan.

above. On reaction for 2 hr, the mannan was easily digested to the extent of 78% (on the basis of reducing sugar). Both longer incubation and further addition of enzyme (80 units) did not increase the extent of hydrolysis. After 6 hr, the reaction was stopped by boiling the mixture and then the solution was concentrated with a rotary evaporator. After that, the sample was applied to a 1.5 × 170 cm column of Bio-Gel P-2 and eluted with distilled water. The fragment which was enzyme-resistant was methylated and hydrolyzed. As a result, the fragment was found to be a linear mannan consisting of 1,6-linkages. This confirmed that the backbone chain of *G. cingulata* G 4-1-4 mannan consists of 1,6-linkages, similar to that of baker's yeast mannan.

PMR and ¹³C-NMR spectra of the mannan

The PMR spectrum (Fig. 6) of the mannan

FIG. 7. ^{13}C -NMR Spectrum (22.49 MHz) of *G. cingulata* G 4-1-4 Mannan.

The spectrum was obtained at 70°C for 1 ml of a D_2O solution containing 130 mg of the purified mannan in a 10 mm tube.

showed a distinctive fingerprint in the δ 5.0~6.0 ppm region assignable to H-1 signals. The signals at δ 5.83, 5.71, 5.63 and 5.38 ppm probably arise from the H-1s of 2-*O*-, 3-*O*-, 2,6-di-*O*- and 6-*O*-substituted α -D-mannopyranosyl units, respectively.^{17~19)} The signal at δ 5.71 ppm corresponding to the 3-*O*-substituted α -D-mannopyranosyl unit was intenser than that in baker's yeast mannan.

The ^{13}C -NMR spectrum of this mannan showed signals at δ 103.7, 102.2, 101.5 and 100.1 ppm, which are attributable to C-1s of non-reducing α -D-mannopyranosyl end units or to 3-*O*-substituted mannose units, and 2-*O*-, 6-*O*- and 2,6-di-*O*-substituted α -D-mannopyranosyl units, respectively (Fig. 7).^{18~22)} Under the same experimental conditions, the chemical shifts of the C-1 region in this spectrum were similar to in the case of baker's yeast mannan, except for the signal at δ 101.5 ppm. The pattern of the C-2 to 6 regions of this spectrum was almost the same as that of baker's yeast mannan.²¹⁾

Component sugars of the cell wall

The cell walls of fungi consist mainly of polysaccharides. The composition of the neu-

tral carbohydrate from *G. cingulata* G 4-1-4 was 92% D-glucose, 4.5% D-galactose and 3.6% D-mannose. No other sugars were detected on GLC analysis. This sugar composition is very similar to that of the cell wall polysaccharide of *Pyricularia oryzae* P₂.¹⁴⁾ The content of hexosamine was 4.9%. This value is lower than those for several other phytopathogenic fungi (ranging from 11.3 to 37.4%).²³⁾

DISCUSSION

A mannan was isolated from mycelia of *G. cingulata* G 4-1-4, the pathogenic fungus in grape blast disease. As shown in Fig. 1, this mannan was located in the outermost layer of the fungal cell wall, irrespective of the age of the fungal mycelium. The purified mannan consists of carbohydrate (97.6%) and protein (2.0%) with a trace amount of phosphate. The only component sugar is D-mannose. On the other hand, the cell wall of *G. cingulata* G 4-1-4 is composed of D-glucose (92.0%), D-galactose (4.5%) and D-mannose (3.6%). These results strongly support that the mannan extracted with 0.02 M citrate buffer (pH 7.0) is derived from the surface polysaccharide of *G. cingulata* G 4-1-4.

The high proportion of 3,4-di-*O*-methylmannose (38 mol%) found on methylation analysis indicates that this mannan has a highly branched structure. When this mannan was subjected to Smith degradation,²⁴⁾ glycerol (75 mol%) and mannose (25 mol%) were produced (unpublished results). This result is consistent with that of methylation analysis. Digestion of this mannan with *Arthrobacter* α -D-mannosidase released 78% of the D-mannose. This enzyme cleaves most of the α -1,2- and 1,3-mannosidic linkages, producing free mannose and leaving a resistant polymer containing mainly α -1,6-linkages.²⁵⁾ Methylation analysis of the enzyme-resistant fragment showed it consisted mainly of α -1,6-linkages. Partial acid hydrolysis showed that the proportion of α -1,3-linked mannose residues in the acid degraded mannan was lower than that in the original mannan. Analysis of

the acetolysis fragments showed that the mannan has side chains consisting of α -1,2- and 1,3-linkages, and that the ratio of α -1,3-linkages to the total linkages increases with the length of side chains.

Galactomannan has often been found in the polysaccharides obtained from filamentous fungi. Extracellular galactomannans have been obtained from *Penicillium charlesii*²⁶⁾ and *Neurospora crassa*.²⁷⁾ On the other hand, as to fungal cell wall polysaccharides, galactomannan in *Aspergillus niger*,²⁸⁾ and glucogalactomannans in both *Pyricularia oryzae*²⁹⁾ and *Dactyrium dendrois*¹⁹⁾ have been reported. Accordingly, most polysaccharides from filamentous fungi are heteropolymers. However, only one report of fungal mannose homopolymers has been published.¹⁸⁾ Such mannans have been found in *Ceratocystis paradoxa* and *Ceratocystis fimbriata*, and are similar to those synthesized by *Saccharomyces cerevisiae* with respect to the nature of the side chains. Many common yeasts have a D-mannan similar to baker's yeast mannan, consisting of a linear backbone chain of α -1,6-linked D-mannose residues to which short side chains of α -1,2- and 1,3-linked residues are attached.³⁰⁾

The PMR and ¹³C-NMR spectra of *G. cingulata* G 4-1-4 mannan were compared with those of baker's yeast mannan. The C-1 signal at δ 101.5 ppm in the ¹³C-NMR spectrum, which was found only for the *G. cingulata* G 4-1-4 mannan, possibly arises from 6-O-substituted mannopyranosyl units. Comparison of both PMR spectra showed that the amount of α -1,3-linkages in *G. cingulata* G 4-1-4 mannan is greater than that in baker's yeast mannan. These data are in agreement with the results of methylation analysis.

The molecular weight of *G. cingulata* G 4-1-4 mannan was estimated to be 8×10^6 by gel filtration. This weight was significantly greater than that of common yeast mannans, but its sedimentation coefficient is small (2.7S). This discrepancy is probably due to the molecular conformation in the aqueous solution.

All the facts described above led us to the following conclusion; *G. cingulata* G 4-1-4

mannan has a backbone joined by α -1,6-linkages, and α -1,2- and 1,3-linked mannose residues are attached to the C-2 position of each main chain segment, giving it a highly branched structure. The fundamental structure appears to be similar to that of common yeast mannans. Both the relationship between the surface mannan and the cell wall polysaccharide and that between the pathogenicity and the fungal surface layer containing this mannan will be investigated.

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