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Structure and Physiological Activity of Conidial Polysaccharides of *Mycosphaerella pinodes*. I

MACHIKO MATSUBARA* and HISATORA KURODA

Kobe Women's College of Pharmacy, Motoyama-Kita-Machi, Higashinada-ku, Kobe 658, Japan

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Four polysaccharides were extracted with acetic acid from conidia of a pea brown spot-causing fungus, *Mycosphaerella pinodes*. Fraction II-I showed activity to induce accumulation of pisatin, a phytoalexin. Its molecular weight was about 45000, and it was composed of α-1,2-linked mannose and α-1,4- and α-1,6-linked glucose. The sugar chains were branched with α-1,4,6-linkages and the non reducing terminal was glucose.

Keywords——*Mycosphaerella pinodes*; fungal spore; conidia; polysaccharide; pisatin; phytoalexin; elicitor activity

Vertebrates usually show resistance to pathogens, for example, by an immunoresponse which is highly specific for each pathogen. Plants, on the other hand, sometimes show an immunoresponse-like protective reaction against infecting pathogens by producing plant-specific factors of resistance, although the specificities of the factors for pathogens are not so strict. For example, it has been found in many plants that infection with a pathogen induced the synthesis (*de novo*) of an anti-fungal agent. These protecting agents synthesized by plants are generically named phytoalexins.[1] Production of phytoalexins is induced not only by infection with an intact pathogenic organism, but also by a physical stimulus or contact with a cell-free preparation of the pathogen or with heavy metals. Biological phytoalexin-inducing substances are known as elicitors.[1]

Albersheim *et al.*[2-6] have reported that soybeans start to synthesize glycitol as a phytoalexin when infected with a soybean-specific late blight-causing fungus, *Phytophthora megasperma* var *sojae*, and they showed that the microbial component acting as the elicitor was a β-glucan. Yoshikawa *et al.*[7] have isolated two polysaccharides as elicitors from the fungus of soybean late blight. They were composed of almost equal amounts of mannose and glucose, and their molecular weights were about 40000 and 3000. In addition, Keen and Legrand[8] reported that a glycoprotein isolated from the same fungus showed elicitor activity. Thus, although several different cell components are acting as elicitors, they all appear to be polysaccharide-containing substances. Peas can also resist infection with a pea brown spot-causing fungus, *Mycosphaerella pinodes*, by synthesizing pisatin as a phytoalexin. Oku *et al.*[9] indicated that an elicitor obtained from a culture filtrate of germination culture of *M. pinodes* was a glucan, but its structure was not reported.

The authors have reported on the structure of conidial polysaccharides of a plant-pathogenic fungus, *Cochlibolus miyabeanus*.[10] In the present work, the structures and elicitor activities of conidial polysaccharides of *M. pinodes* were investigated.

Materials and Methods

Microorganism——*Mycosphaerella pinodes* was kindly provided by Prof. Oku of Okayama University. The

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strain was used in this study after subculturing it at 26 °C on a vegetable juice-agar medium which contained 140 ml of DelMonte vegetable juice (a commercial product), 1.4 g of $CaCO_3$, 21 g of agar and 560 ml of distilled water.

Harvesting of Conidia——Conidia of the strain were inoculated on the vegetable juice-agar plate medium and cultured at 26 °C for 6 d. A small amount of cold water was poured onto the fungal growth on the plate medium, and the surface was rubbed gently with a glass rod in order to release the conidia into the cold water. The conidia suspension thus obtained was filtered through eight-ply gauze to remove cell debris. Conidia in the filtrate were harvested by centrifugation at 3000 rpm for 10 min. The conidia were then washed about 4 times with cold water by centrifugation until the washing water showed no sugar reaction (anthrone reaction), and the precipitate of conidia finally obtained was used in this study.

Conidial Polysaccharides——Polysaccharides accumulated in the conidia were extracted and purified through the procedure shown in Chart 1.

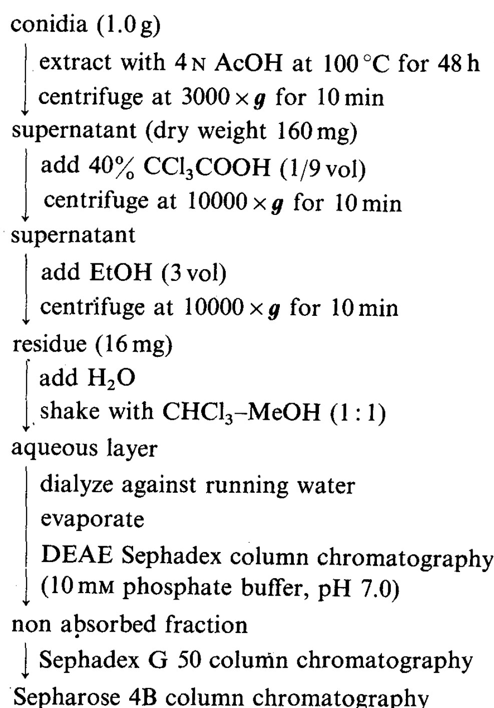

Chart 1. Extraction and Purification of Polysaccharide from Conidia

Enzymatic Digestion——α-Glucosidase (from yeast, Sigma Chemical Co.) and α-mannosidase (from jack bean, Sigma Chemical Co.) were used for polysaccharide digestion. Glucose and monnose liberated were analyzed with the "Glucostat reagent" (Worthington Biochemical Co.) and by gas liquid chromatography (GLC) analysis, respectively. The reaction conditions were as follows: a) substrate, 1.0 mg; α-glucosidase, 1 mg; 1/15 M phosphate buffer (pH 6.8), 0.5 ml; 37 °C for 18 h; b) substrate, 10 mg; α-mannosidase, 1 mg; 0.05 M sodium acetate buffer (pH 5.0), 0.5 ml; 37 °C for 18 h.

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. For detection, 50% H_2SO_4 was sprayed and the plate was heated at 110 °C.

Methylation Analysis——A sample was methylated by the method of Hakomori[11] with sodium methylsulfinyl carbanion and methyl iodide. The fresh carbanion (a 5- to 10-fold excess over free hydroxyl groups of the sample) was prepared according to the method of Corey and Chaykovsky[12] and was added to a solution of the dried sample (5 mg) in dried dimethyl sulfoxide (12.5 ml). The mixture was stirred in a nitrogen stream at 20—25 °C for 5 h. An excess of methyl iodide (6 ml) was dropped into the reaction mixture under stirring, and stirring was continued at 20—25 °C for a further 20—24 h. The reaction mixture was dialyzed against running water, followed by repeated extraction with $CHCl_3$. After being dried with Na_2SO_4, the combined $CHCl_3$ solution was evaporated to dryness *in vacuo*. These procedures were repeated till the infrared (IR) spectrum of the methylated product showed no absorption in the 3200—3700 cm^{-1} region. After checking of the IR spectrum, an aliquot of the permethylated product was subjected to proton magnetic resonance (^1H-NMR) analysis. The rest was methanolyzed with 5% HCl in MeOH in a sealed tube at 100 °C for 6 h, and evaporated rapidly. Toluene–EtOH mixture (1 : 1) was added to the residue, followed by evaporation to dryness *in vacuo*. The residue was dissolved in $CHCl_3$ and analyzed by gas liuid chromatography-mass spectrometry (GLC-MS).

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Chromatographic Techniques——i) Column Chromatography: Aliquots of a sample or several standard polysaccharides were applied to a Sepharose 4B column (2.5 × 37 cm) or a Sephacryl 200 column (2.5 × 37 cm) and were eluted with distilled water. Fractions (5 ml) were collected. Phenol–H_2SO_4 was used as a color reagent. ii) GLC and GLC-MS: Constituent sugars of polysaccharides were identified by GLC analysis under the following conditions: column length, 3 mm × 1.8 m; packing, 5% SE 30 on Shimalite; temperature, 160 °C; N_2 flow rate, 40 ml/min; detector temp., 250 °C; injection temp., 250 °C; detector, flame ionization detector, H_2 flow rate, 40 ml/min. On GLC analysis methyl 2,3,4,6-tetra-*O*-TMS mannoside (t_R: 12.3, 14.1 min) and methyl 2,3,4,6-tetra-*O*-TMS-glucoside (t_R: 18.1, 20.6 min) were identified. After methanolysis of the polysaccharide with 5% HCl in methanol at 100 °C for 5 h, the methanolyzate was trimethylsilylated and then analyzed. For the identification of methylated sugars, permethylated polysaccharide was methanolyzed with 5% HCl in methanol, dissolved in ether and then subjected to GLC analysis under the following conditions: column length, 3 mm × 1.8 m; packing, 1.5% NGS on Chromosorb W; temperature, 140 °C (10 min) then 2 °C/min to max. 230 °C; N_2 flow rate, 40 ml/min; detector temp., 250 °C; injection temp., 250 °C; H_2 flow rate, 40 ml/min. On GLC analysis, methyl 2,3,4,6-tetra-*O*-methyl glucoside (t_R: 14.2 min), methyl 3,4,6-tri-*O*-methyl mannoside (t_R: 21.2 min), methyl 2,3,4-tri-*O*-methyl glucoside (t_R: 22.5 min), methyl 2,3,6-tri-*O*-methyl glucoside (t_R: 26.1 min) and methyl 2,3-di-*O*-methyl glucoside (t_R: 33.1, 37.2 min) were identified. GLC-MS (JMS-OISG, Nihon Denshi Co.) was performed under the following conditions: column length, 3 mm × 1.0 m; packing, 1.5% NGS on Chromosorb W; temperature, 120 °C (1 min) then 2 °C/min to max. 230 °C; helium flow rate, 30 ml/min.

Elicitor Activity——The activity was measured under the conditions shown in Chart 2.

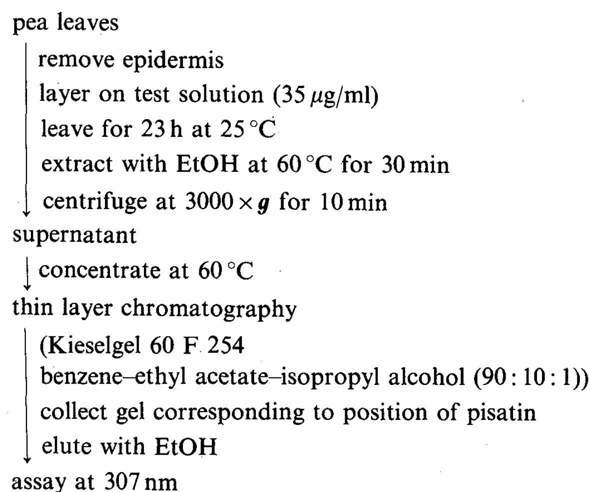

Chart 2. Method of Bioassay for Elicitor Activity

Results and Discussion

Figure 1 shows the result of Sepharose 4B column chromatography of polysaccharide extracted by the procedure indicated in Chart 1. Three peaks (I, II-1 and II-2), as well as a peak (II-3) of low-molecular-weight polysaccharides, were found. These fractions were collected and checked for elicitor activity (tube numbers 11—13 for fraction I, 33—35 for II-1, 39 for II-2 and 42—44 for II-3). The results are shown in Table I. Although fraction I showed the highest activity (and II-3 the lowest), fraction II-1 was selected for further studies on its chemical structure because of the high recovery yield.

As shown in Fig. 2, a single peak was obtained by Sephacryl 200 column chromatography of fraction II-1. No protein was detected in this fraction by Lowry's method. Electrophoresis of the fraction on glass fiber paper revealed a single spot. Fraction II-1 was a water-soluble colorless amorphous powder, and its optical rotation was $[\alpha]_D^{20} + 148°$ ($c = 1$, water). Elicitor activity was also detected in this fraction. As shown in Fig. 3, the molecular weight of the fraction II-1 polysaccharide was estimated to be 4.5×10^4 by a gel filtration method.

GLC analysis was performed after methanolysis of the sample. Only mannose and

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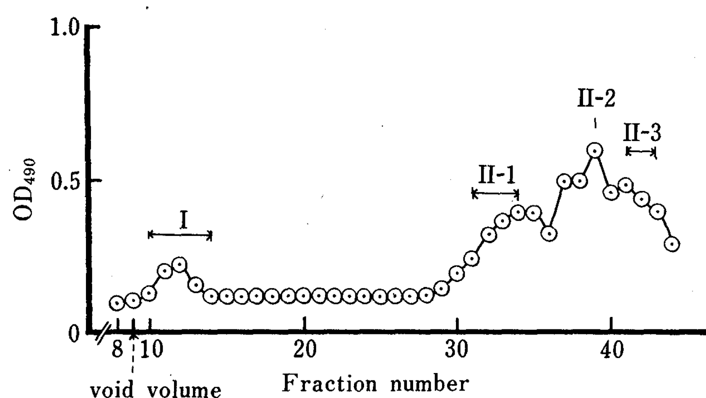

Fig. 1. Sepharose 4B Column Chromatogram

Table I. Elicitor Activity of Fractions

Fraction	Accumulated pisatin (μg/g of fresh leaves)
H_2O (control)	9.5
I	22.5
II-1	19.9
II-2	20.1
II-3	10.6

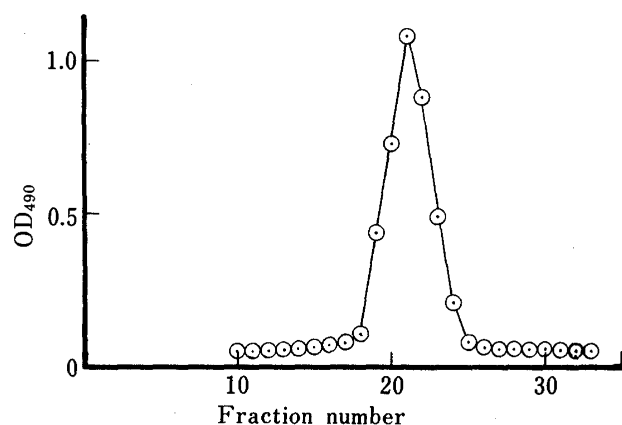

Fig. 2. Sephacryl 200 Column Chromatogram of Fraction II-1

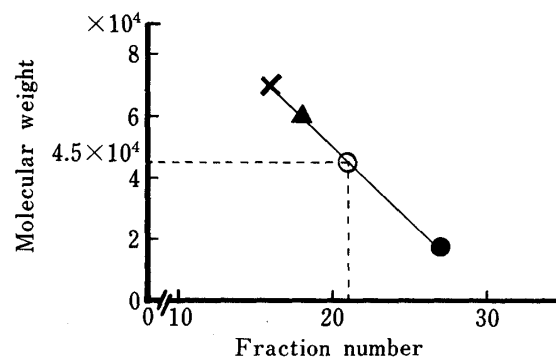

Fig. 3. Estimation of the Molecular Weight of Fraction II-1 by Column Chromatography

Fraction II-1 (○) and dextran [(●, M_r 1.75×10^4), (▲, M_r 5.9×10^4), (×, M_r 7×10^4)] were applied to a Sephacryl 200 column (2.5 × 37 cm).

glucose were detected as sugar constituents of the polysaccharide. In order to identify the binding positions of the sugars, fraction II-1 was methylated according to Hakomori's method, methanolyzed and then subjected to GLC analysis. The GLC-MS of the peaks obtained are shown in Table II. The binding points were estimated by comparing the results shown in Table II with some reported data.[13] The molar ratios of the glucosides and mannosides (summarized in Table III) were calculated from the peak areas. It could not be decided whether the peak (t_R: 14.2 min) in Table II, was methyl 2,3,4,6-tetra-*O*-methyl glucoside or methyl 2,3,4,6-tetra-*O*-methyl mannoside.

In order to identify the linkages of the sugars, a KBr tablet of fraction II-1 was subjected to IR spectroscopic analysis. The absorption peak of α-D-glucopyranose was found at

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TABLE II. Relative Intensity of Main Fragment Ions in GLC-MS[a)]

Compound	t_R (min)	45	71	73	74	75	87	88	89	101	102
						Relative intensity					
Me 2,3,4,6-tetra-*O*-Me glucoside	14.2	27	14	14	3	45	1	100	9	45	5
Me 3,4,6-tri-*O*-Me mannoside	21.2	61	94	17	62	94	25	100	22	43	15
Me 2,3,4-tri-*O*-Me glucoside	22.5	36	9	21	7	38	5	100	9	44	3
Me 2,3,6-tri-*O*-Me glucoside	26.1	42	20	24	16	59	16	100	10	23	2
Me 2,3-di-*O*-Me glucoside	33.1	52	34	39	26	72	30	100	11	29	4
	37.2	49	36	35	28	59	26	100	12	31	4
2,3-Di-*O*-methyl-glucose[13)]		39	—	39	21	72	36	100	13	32	—

(m/e values in column headers)

a) The following sugars were methylated by the same procedure to obtain authentic compounds: glucose, mannose, gentiobiose, kojibiose, cellobiose, laminarin, glycogen, mannan from *S. cerevisiae*.

TABLE III. Molar Ratio of Methylated Sugars

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

840 cm^{-1} (characteristic C_1-anomer region). The absorption due to α-D-mannopyranose was also detected around 810 cm^{-1}. These results indicated that the polysaccharide is α-linked. The ^{1}H-NMR spectrum of fraction II-I in D_2O also showed signals due to α-linkages at 5.0, 5.14 (d, J=3 Hz) and 5.22 ppm (d, J=1.5 Hz). Since Miyazaki *et al.*[14)] reported that a signal at 5.28 ppm (d, J=3 Hz) is due to α-(1→2)-linked mannose residues, other α-linkages are also presumed to be present in the fraction II-1 polysaccharide, in addition to α-(1→2)-linked mannose residues.

Finally, enzymatic digestion of the polysaccharide was performed in order to identify the non-reducing terminal sugar. Glucose was released by an exo-type α-glucosidase, and no mannose was detected after treatment with an exo-type α-mannosidase. These results clearly indicate that the non-reducing terminal sugar is glucose. In addition, these results enabled us to identify the peak (t_R: 14.2 min) in Table II as that of methyl 2,3,4,6-tetra-*O*-methyl glucoside.

From all the results described above, the structural characteristics of the elicitor-active polysaccharide of fraction II-1 extracted from conidia of *M. pinodes* may be summarized as follows.

1) It is an α-glycan composed of mannosyl and glucosyl residues in a molar ratio of 1:4, and its molecular weight is about 45000.

2) It has a sugar residue ratio of 1:6:3:3:1 of α-glucosyl (as the non reducing terminal sugar), 1,4-disubstituted α-glucosyl, 1,6-disubstituted α-glucosyl, 1,2-disubstituted α-mannosyl and 1,4,6-trisubstituted α-glucosyl residues. Based on the assumption that this polysaccharide contains orderly repeated structures of subunits, it may be composed of α-1,4-linked glucose, α-1,6-linked glucose and α-1,2-linked mannose in a ratio of 6:3:3. The molecule may consist of about 20 repeating units, with branching points at the 6th or 4th position of α-1,4- or α-1,6-linked glucose in the main sugar chain and having one side chain whose

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terminal is α-glucose.

Oku *et al.*[9] suggested that an elicitor found in culture filtrate of germinating conidia of *M. pinodes* was a polysaccharide composed of only glucose. We considered that intraconidial and/or conidial wall polysaccharides might be partially hydrolyzed into small molecules and secreted outside the conidia during the germination stage. Therefore, as an initial step, polysaccharides in intact conidial cells were evaluated. Of four polysaccharide fractions obtained from the cell extract, fraction II-1 was studied structurally because it showed elicitor activity with a high recovery yield. Unlike the polysaccharide of Oku *et al.*, it was a heteroglycan composed of mannose and glucose and having side chains. Fractions I and II-2 were also checked, but only for sugar composition. They were, like fraction II-1, heteroglycans which contained mannose and glucose in a molar ratio of 1:4 (data not shown).

As described above, all the elicitor-active polysaccharides extracted directly from ungerminating conidia were heteroglycans containing mannose and glucose, and were different from the glucan found by Oku *et al.* Since these intraconidial heteroglycans may be hydrolyzed during the germination stage and the resulting glucans, having elicitor activities, may be released outside the cells, we intend to investigate polysaccharides which are produced in the culture filtrate of germinating conidia. Determination of the sequence of sugar residues of the fraction II-1 polysaccharide is also in progress.

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