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Physiological and Biochemical Studies on Germinating Fungal Spores. VI.¹⁾ Functional Polysaccharide as an Endogenous Substrate in Germinating Conidia of *Cochliobolus miyabeanus*

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The chemical structure of the polysaccharide which functions as an endogenous substrate in germinating conidia of *Cochliobolus miyabeanus* was studied. When the germinating conidia were extracted with 1 N NaOH, 30% KOH and 4 N acetic acid successively, the acetic acid-extractable polysaccharide (I) was the only one that decreased after the completion of germ-tube formation. Gas liquid chromatograms of the acid or enzymic hydrolysates of I and the infrared and nuclear magnetic resonance spectra of the methylated I indicated that I is composed of only β -linked glucose (β -glucan). I consists of 129 β -linked glucosyl residues which are composed of β -1,3- and β -1,6-linked glucosyl residues in the ratio of 5:2, and β -glucosyl branched units connected through C6 or C3 of the main chain glucan and non-reducing termini exist in the ratio of one residue to seven glycosyl residues.

Keywords—polysaccharide; *Cochliobolus miyabeanus*; fungal spore; conidia; carbohydrate

Many reports and reviews have been presented on the chemical structures and biosynthetic pathways of mycelial cell wall polysaccharides of various fungi,²⁾ but little is known regarding the polysaccharides stored in fungal conidia.³⁾ The polysaccharides of the mycelial cell wall of the title fungus, *Cochliobolus miyabeanus*, have been clarified already.⁴⁾ However, the properties and structure of the conidial carbohydrates remain to be characterized. In the previous papers,⁵⁾ we reported that the cold acid-soluble sugars stored in conidia (major ingredient: trehalose) were consumed rapidly in the initial stage of germination (incubation time: 0—90 min), while the cold acid-insoluble polysaccharides in conidia began to decrease in the germ-tube elongation stage (incubation time: later than 90 min), and at that time, the content of cold acid-soluble sugars (major ingredient: glucose) increased again. On the other hand, Oku³⁾ proved that the content of 30% KOH-soluble carbohydrate in the same fungus conidia decreased markedly after conidial germination (incubation time: 24 h) and suggested that this carbohydrate was a dextran-like substance. In this paper, we deal with the fine chemical structure of the conidial polysaccharide which functions as an endogenous substrate for germination.

Materials and Methods

Organism and Germinating Conditions—The harvesting procedure for conidia of *Cochliobolus miyabeanus* C-37-ATCC 38724 and the germinating conditions were as described previously.⁵⁾

Reagents and Enzymes—All reagents used were commercial products. α -Glucosidase from yeast and β -glucosidase from almond were purchased from Sigma Chemical Co., and *exo*-type β -1,3-glucanase was obtained from the culture liquid of *Trichoderma* sp. in our laboratory.

Fractionation of the Conidial Polysaccharides—The polysaccharides stored in conidia were fractionated by means of the procedures shown in Chart 1.

Enzyme Digestion—Enzymic digestion of the polysaccharide was done with α -glucosidase, β -glucosidase or β -

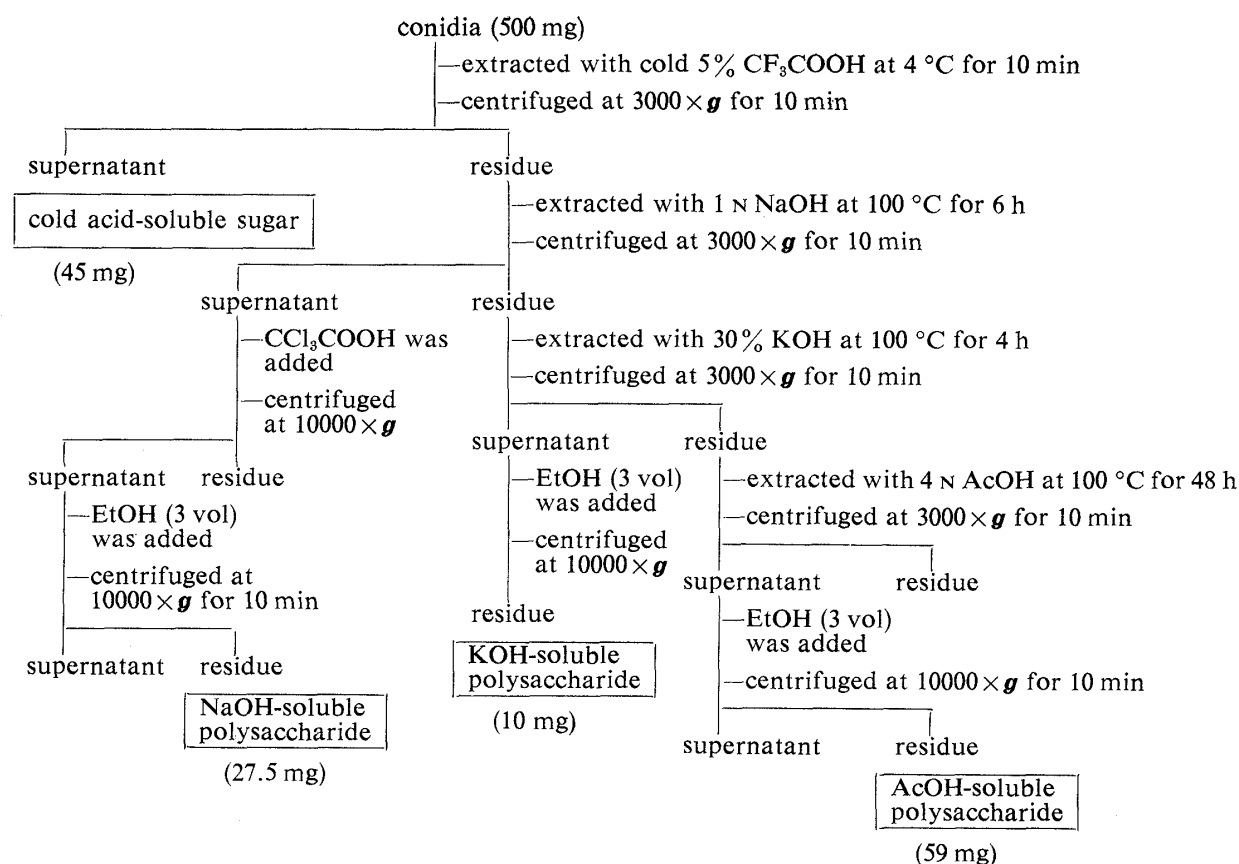


Chart 1. Extraction and Fractionation of Polysaccharides of *C. miyabeanus* Conidia

1,3-glucanase. The polysaccharide was incubated with the enzyme under the following conditions and the quantity of released glucose was determined by use of the Glucostat reagent (Worthington Biochemical Co.). a) Substrate, 2 mg; α -glucosidase, 80 μ g; 1/15 M phosphate buffer (pH 6.8), 1 ml; at 35 °C for 18 h. b) Substrate, 2 mg; β -glucosidase, 80 μ g; 1/20 M acetate buffer (pH 5.25), 1 ml; at 35 °C for 18 h. c) Substrate, 2 mg; β -1,3-glucanase, 50 μ g; 1/20 M acetate buffer (pH 5.0), 1 ml; at 50 °C for 18 h.

Partial Hydrolysis—After refluxing of the polysaccharide (fract. A, 2 mg) with 0.03 N H_2SO_4 (2 ml) at 100 °C for 20 min, the solution was poured into ice water, then neutralized with 1 N NaOH solution and evaporated to dryness *in vacuo*. The residue was extracted with pyridine and the extracted carbohydrates were subjected to further analyses after removal of pyridine by evaporation.

Acetolysis—Acetolysis of the polysaccharide was performed according to the method described previously⁴⁾ except that the sample was dissolved in acetic acid–acetic anhydride–conc. H_2SO_4 mixture (10:10:1.5) at 30 °C for 4 d, and the products were identified by paper partition chromatography (PPC) or gas–liquid chromatography (GLC).

Methylation Analysis—A sample was methylated by the method of Hakomori⁶⁾ with sodium methylsulfinyl methide 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 Chaykovsky⁷⁾ and was added to the dried sample (6 mg) in dried dimethyl sulfoxide (12.5 ml). The mixture was stirred in a nitrogen stream at 20–25 °C for 4.5 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 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 (OH groups of carbohydrates). After checking of the IR spectrum, an aliquot of the permethylated product was subjected to proton magnetic resonance (^1H -NMR) analysis. The rest was methanolized 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 liquid chromatography–mass spectrometry (GLC-MS).

Chromatographic Techniques—i) PPC: PPC was performed with Toyo Roshi No. 50 paper and the following solvent systems. Solvent A, 1-BuOH–pyridine–water (6:4:3); solvent B, water-saturated collidine. Sugars were

TABLE I. Conditions of Gas Chromatography of Sugar Derivatives

	a. For monosaccharides	b. For oligosaccharides	c. For methylated glucoses
Column length	3 mm × 1.8 m	3 mm × 35 cm	3 mm × 1.8 m
Temperature	160 °C	100 °C (3 min) temp. rate 10 °C/min max. 300 °C	140 °C (12 min) temp. rate 8 °C/min max. 180 °C
Packing	5% SE 30 on Shimalite	1% OV-1 on Celite 545	1.5% NGS on Chromosorb W
N ₂ flow rate	40 ml/min	40 ml/min	40 ml/min
Detector	FID	FID	FID
Detector temp.	250 °C	300 °C	270 °C
Injection temp.	250 °C	270 °C	270 °C
H ₂ flow rate	40 ml/min	40 ml/min	40 ml/min

detected on paper with aniline hydrogen phthalate spray reagent.

ii) Column Chromatography: Aliquots of a sample or several standard polysaccharides were applied to a Sephacryl 200 column (2.5 × 37 cm) or a Sephadex G 100 column (3 × 30 cm) and were eluted and fractionated with distilled water. Anthrone was used as a color reagent.

iii) GLC or GLC-MS: To identify monosaccharides, the dried sugars were treated with trimethylsilylating reagent (TMS-HT, Tokyo-Kasei Co.) at 25 °C for 10 min under shaking, and an aliquot of the reaction mixture was tested under the conditions given in Table Ia (Shimadzu GC-1C GLC machine). To identify oligosaccharides obtained by partial hydrolysis or enzyme digestion, the sugars were dissolved in pyridine (0.5 ml), GLC-S1 (hexamethyldisilazane, HMDS in pyridine, Nakarai Chemicals Ltd., 0.1 ml) and GLC-S2 (trimethylchlorosilane, TMCS, in pyridine, Nakarai Chemicals Ltd., 0.1 ml) mixture and kept at 50 °C for 30 min. The reaction mixture was shaken vigorously with CHCl₃ (3 ml) and water (2 ml), and the aqueous layer was removed. These procedures were repeated three times, then the CHCl₃ layer was evaporated to dryness. The residue was treated again with pyridine-GLC-S1-GLC-S2 mixture (1 : 1.3 : 0.8) at 50 °C for 30 min, then centrifuged, and an aliquot of the supernatant was subjected to GLC under the conditions given in Table Ib. To identify methylated glucose, samples were dissolved in CHCl₃ and subjected to GLC under the conditions given in Table Ic. GLC-MS (JMS-OISG, Nihon Denshi Co.) was carried out under the same conditions but using helium as a carrier gas.

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, 2 h, 4 °C. Aniline hydrogen phthalate was used as the spray reagent.

Results and Discussion

When the ungerminated conidia were extracted with 20 ml each/100 mg of conidia of 1 N NaOH (under reflux), 30% KOH (under reflux) and 4 N acetic acid (in a sealed tube at 100 °C) successively, the amounts of extractable polysaccharides in each step leveled off within 6, 4 and 45 h extraction times, respectively, and further extraction in the same way in each step gave no material which reacted positively against anthrone reagent. From these results, the conditions shown in Chart 1 were chosen for the fractionation of polysaccharides from the germinating conidia. In order to investigate the role of carbohydrate as an endogenous substrate, the changes in content of each fraction were measured during the course of germination within 24 h (Table II). Table II indicates that the content of cold acid-soluble sugar increased after 24 h of incubation, as reported in the previous paper,⁵⁾ almost no difference was detected in alkali-soluble carbohydrate content, but the acetic acid-soluble polysaccharide content decreased markedly during germination. In apparent contradiction to the present results, Oku³⁾ demonstrated a marked decrease of alkali-soluble carbohydrate during conidial germination. Although the reason for this difference is unclear, the results in Table II suggest that what plays an important role as an endogenous substrate in the germ-tube elongation stage is not the alkali soluble polysaccharide but the acetic acid-soluble polysaccharide. Therefore, characterization of the acetic acid-soluble polysaccharide was performed. Fig. 1 shows the elution profile of the acetic acid-soluble polysaccharide from a Sephadex G 100 column. The polysaccharide was eluted as a single peak with a small

TABLE II. Carbohydrate Content of Each Fraction in Conidia before or after Germination

Fraction	Cold acid-soluble sugar (%)	NaOH-soluble polysaccharide (%)	KOH-soluble polysaccharide (%)	AcOH-soluble polysaccharide (%)
Ungerminated conidia	9.0	5.5	2.0	11.8
Germinated conidia (incubated for 24 h)	12.0	5.25	2.2	5.5

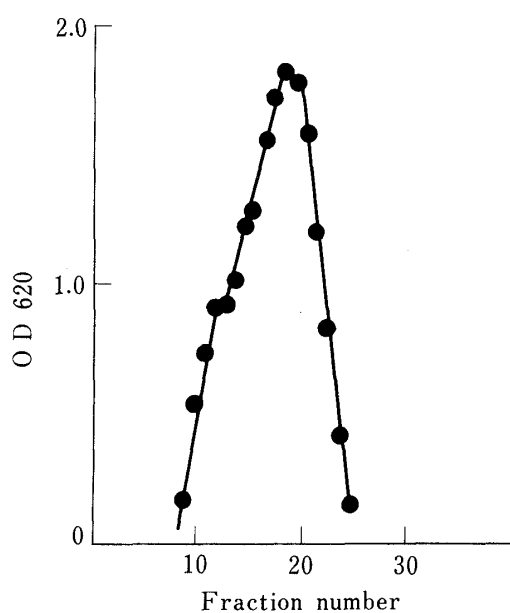


Fig. 1. Sephadex G-100 Column Chromatogram of Fraction A

Carbohydrate contents were determined by the anthrone method.
Column, 3 × 30 cm.

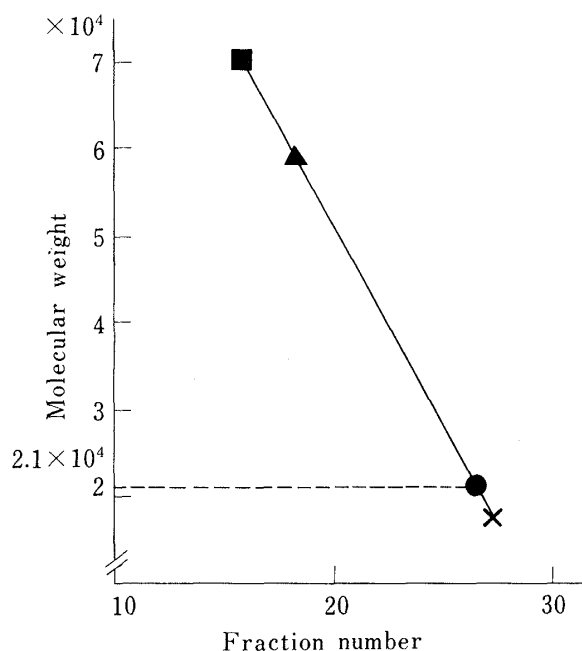


Fig. 2. Estimation of the Molecular Weight of Fraction A by Column Chromatography

Fraction A (●) and dextran [(■, mol. wt. 7×10^4), (▲, mol. wt. 5.9×10^4), (×, mol. wt. 1.75×10^4)] were placed on a Sephacryl 200 column (2.5×37 cm). Eluted compounds were detected by the anthrone method.

shoulder. The contents of tube Nos. 15–24 were pooled and concentrated, then 3 volumes of EtOH was added. The precipitate collected by centrifugation was designated as frac. A and subjected to further analyses. Frac. A (I) was a water-soluble colorless amorphous powder, and its optical rotation was $[\alpha]_D^{20} -76^\circ$ ($c=1$, water). This polysaccharide was homogeneous on high-voltage glass fiber electrophoresis and did not contain nitrogen, sulfur or phosphorus. To estimate the molecular weight of (I), Sephacryl 200 column chromatography was performed with some standard compounds. On the basis of the results shown in Fig. 2, the molecular weight of (I) is estimated at 2.1×10^4 . To examine the sugar compositions, (I) was hydrolyzed with 4N HCl for 17 h under reflux. Since the hydrolysate gave only glucose, which was characterized by PPC or GLC analysis, this polysaccharide is regarded as a “glucan.” In order to determine whether the linkage type of this glucan was α or β , the susceptibility of (I) to various glucosidases was examined. On incubation of (I) with β -glucosidase or β -1,3-glucanase, much released glucose was detected from the reaction mixture while no glucose was detected after incubation with α -glucosidase. Furthermore, the IR absorption spectrum of (I) in a KBr tablet and the $^1\text{H-NMR}$ spectrum of the permethylated (I) in CDCl_3 exhibited

an absorption band at 891 cm^{-1} (IR spectrum) and an anomeric proton signal at $\delta 4.80$ (d, $J=8.0\text{ Hz}$) (^1H -NMR spectrum), which indicate the β -configuration, while an absorption band at 844 cm^{-1} (IR spectrum), which indicates the α -configuration, was absent. Consequently, (I) was regarded as a " β -glucan." In specificity tests of the β -1,3-glucanase used in this study, no detectable amount of glucose was formed from any of the compounds tested, other than laminaran, as follows: amylose, nigeran, pustran, cellulose, laminaribiose, lactose, maltose, isomaltose, cellobiose, gentiobiose, *p*-nitrophenyl- β -glucoside and *p*-nitrophenyl- α -glucoside. Laminaran gave glucose and polysaccharide on incubation for 2 h and gave glucose and laminaribiose on further incubation for 20 h with this enzyme. These results show that this enzyme is an *exo*- β -1,3-glucanase with high specificity. Since the enzyme digestion tests with β -1,3-glucanase demonstrated the presence of some β -1,3-glucosyl linkages, at least at the non-reducing terminus, the fine chemical structure of the inner-chain glucan was studied by methylation analysis. Compound (I) was methylated repeatedly by the method of Hakomori. The progress of the reaction was monitored by checking the IR spectrum, and when methanolysis was complete, the methylated sugars were subjected to GLC-MS analysis. The following methylated sugars were prepared by the same procedure as authentic compounds: methyl pyranosides of 2,3,4,6-tetra-*O*-methyl glucose from D-glucose, 2,3,4-tri-*O*-methyl glucose from gentiobiose, 2,3,6-tri-*O*-methyl glucose from glycogen, 2,4,6-tri-*O*-methyl glucose from laminaran, 3,4,6-tri-*O*-methyl glucose from kojibiose and 2,3-di-*O*-methyl glucose from glycogen. On GLC analysis of glucose methyl ethers obtained from the permethylated (I), seven peaks appeared (Fig. 3). The methylated glucoses were identified by comparing the retention times and the relative intensities of main fragments in the mass spectra with those of authentic compounds or with the values in the literature,⁸⁾ and the molar ratio of each methyl ether of glucose was calculated from the peak area in the gas liquid chromatogram. The results of the methylation analysis are listed in Table III. From these results, it is established that methyl pyranosides of 2,3,4,6-tetra-*O*-methyl-, 2,3,4-tri-*O*-methyl-, 2,4,6-tri-*O*-methyl- and 2,4-di-*O*-methyl-glucose were obtained from I in the ratio of 1.0:1.4:3.5:1.0. The presence of these methylated derivatives indicates that I consists of 1,6-

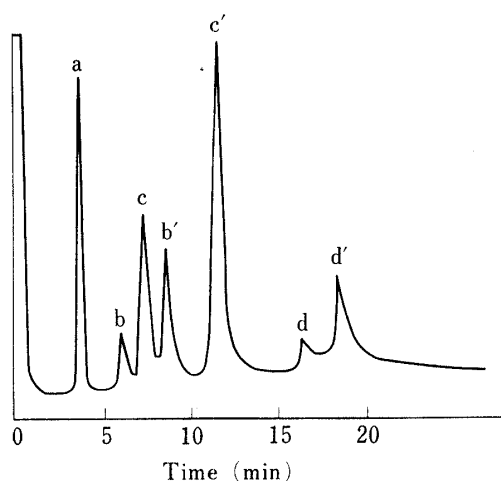


Fig. 3. Gas Chromatogram of Permethylated Fraction A

Conditions: as described in Table Ic.

Peaks: a, methyl 2,3,4,6-tetra-*O*-methyl glucoside; b, b', methyl 2,3,4-tri-*O*-methyl glucoside; c, c', methyl 2,4,6-tri-*O*-methyl glucoside; d, d', methyl 2,4-di-*O*-methyl glucoside.

Retention times (min): a, 3.85; b, 6.0; c, 7.35; b', 8.55; c', 11.4; d, 16.15; d', 17.9.

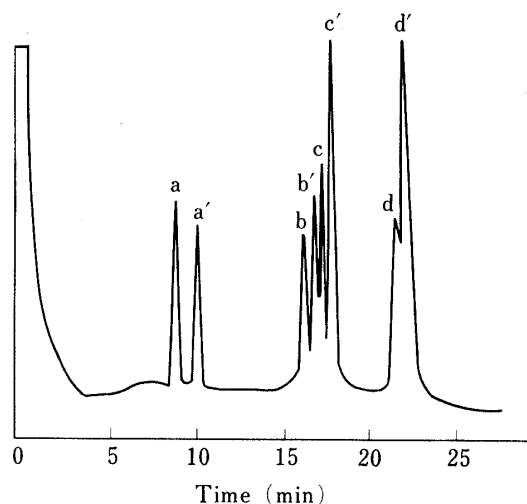


Fig. 4. Gas Chromatogram of Hydrolysate of the Triose with β -1,3-Glucanase

Conditions: as described in Table Iib.

Peaks: a, a', trimethylsilylated glucose; b, b', trimethylsilylated laminaribiose; c, c', trimethylsilylated gentiobiose; d, d', trimethylsilylated undigestible triose.

TABLE III. Relative Intensity of Main Fragment Ions in GLC-Mass Spectra

Compound	t_{R} (min)	m/e									
		45	71	73	74	75	87	88	89	101	102
		Relative intensity									
Peak a	3.8	17	13	12	2	51	1	100	10	45	5
Peak b	6.0	19	8	18	8	44	6	100	11	51	6
Peak b'	8.6	13	8	15	10	52	6	100	11	59	10
Peak c	7.35	33	100	6	59	23	12	18	14	57	27
Peak c'	11.4	39	199	5	62	24	15	26	13	48	28
Peak d	16.15	52	92	17	100	35	28	55	8	58	30
Peak d'	17.9	41	86	20	100	35	31	52	6	73	28
Methyl 2,4-di- <i>O</i> -methyl-glucopyranoside		51	87	19	100	23	34	42	6	54	25
Methyl 2,3-di- <i>O</i> -methyl-glucopyranoside		39	—	39	21	72	36	100	13	32	—

TABLE IV. R_f Values on PPC of the Partial Hydrolysis Products of Fraction A obtained by Acetolysis or Acid Hydrolysis

Compound	R_f					
Glucose	0.41					
Laminaribiose		0.32				
Laminaritriose			0.21			
Laminaritetraose				0.16		
Laminaripentaose					0.11	
Acetolyzed fraction A	0.41	0.31	0.20		0.12	
Acid-hydrolyzed fraction A	0.41	0.32		0.16	0.12	

Conditions: filter paper, Whatman No. 1; solvent, *n*-BuOH-pyridine-H₂O (6:4:3); detector, aniline hydrogen phthalate.

disubstituted, 1,3-disubstituted and 1,3,6-trisubstituted glucose residues and non-reducing terminal glucose residues. Since the molecular weight of I is assumed to be about 2.1×10^4 (Fig. 2), one mol of I contains about 129 glucosyl residues, consisting of 19 mol of non-reducing terminal glucose, 26 mol of 1,6-disubstituted glucose, 66 mol of 1,3-disubstituted glucose and 18 mol of 1,3,6-trisubstituted glucose (branching points). As shown in Table IV, PPC analysis of the products of acetolysis or partial hydrolysis of I revealed five spots corresponding to mono-, di-, tri-, tetra- and penta-saccharides (glucose, laminaribiose, -triose, -tetraose and -pentaose were used as authentic compounds). The spot corresponding to tri-glucoside, which was a main product, was extracted with 0.05 M acetate buffer (pH 5.0) and incubated with the *exo*- β -1,3-glucanase at 50 °C for 2 h. After enzyme digestion, the reaction mixture was evaporated, trimethylsilylated and subjected to GLC. The GLC analysis indicated the presence of glucose, laminaribiose, gentiobiose and indigestible trisaccharide in the reaction mixture (Fig. 4), and the amount of released glucose was equivalent to 24% of the total glucosyl residues contained in the substrate. In another experiment, authentic laminaritriose was converted to glucose and laminaribiose completely by this enzyme under the same conditions and the amount of released glucose was equivalent to about 35% of the total glucosyl residues. From these results, we deduced that the enzyme digestible trisaccharides were a mixture of laminaritriose and β -D-glucopyranosyl-(1 \rightarrow 3)-*O*- β -D-glucopyranosyl-(1 \rightarrow 6)-D-glucopyranose or β -D-glucopyranosyl-(1 \rightarrow 3)-[*O*- β -D-glucopyranosyl-(1 \rightarrow 6)]-D-

glucopyranose, which are converted to glucose, laminaribiose and gentiobiose by this enzyme. Although the chemical structures of the enzyme-indigestible trisaccharides could not be clarified because the quantities were too small to permit methylation analyses, they are probably β -D-glucopyranosyl-(1 \rightarrow 6)-O- β -D-glucopyranosyl-(1 \rightarrow 6)-D-glucopyranose and/or β -D-glucopyranosyl-(1 \rightarrow 6)-O- β -D-glucopyranosyl-(1 \rightarrow 3)-D-glucopyranose.

All the experimental results demonstrate that the polysaccharide acting as an endogenous substrate in the germ-tube elongation stages of the present fungus conidia is most probably one which consists of 129 β -linked glucosyl residues, comprising β -1,3- and β -1,6-linked glucosyl residues in the ratio of 5:2, and β -glucosyl branched units connected through C6 or C3 of the main-chain glucan and non-reducing termini in the ratio of one residue to seven glucosyl residues.

We have reported⁴⁾ that the polysaccharide in the mycelial cell walls of the present fungus are composed of β -1,3 linked glucan having branched units connected through C6 and C1 in the ratio of thirteen β -1,3-linked glucosyl residues to three branched units, and recently, several papers reported the presence of familiar types of polysaccharides in many kinds of fungal mycelia or fruit bodies. For instance, polysaccharides which are composed of β -1,3-glucan having some branched units in somewhat different ratios have been found as follows: lentinan from *Lentinus edodes*,⁹⁾ schizophylan from *Schizophyllum commune* FR,¹⁰⁾ scleroglucan from *Sclerotium glaucum*,¹¹⁾ etc. Furthermore, straight-chain glucans containing β -1,3- and β -1,6-linked glucosyl residues were found in *Neurospora*,¹²⁾ *Phytophthora*,¹³⁾ *Pythium*,¹⁴⁾ etc. These reports are compatible with the polysaccharide structure deduced in the present work. Further work is being undertaken to clarify the fine chemical structure of this polysaccharide.

References and Notes

- 1) Part V: M. Matsubara, S. Furuya, and H. Kuroda, *Chem. Pharm. Bull.*, **29**, 3723 (1981).
- 2) S. Bartnicki-Garcia, C. E. Bracker, E. Reyes, and J. Ruitz-Herrera, *Exp. Mycol.*, **2**, 173 (1978); V. Farkas, *Microbiol. Rev.*, **43**, 117 (1979); etc.
- 3) H. Oku, *Plant Cell Physiol.*, **1**, 231 (1960).
- 4) H. Namba and H. Kuroda, *Chem. Pharm. Bull.*, **19**, 448 (1971).
- 5) M. Matsubara and H. Kuroda, *Chem. Pharm. Bull.*, **28**, 1365 (1980).
- 6) S. Hakomori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
- 7) E. J. Corey and M. Chaykovsky, *J. Am. Chem. Soc.*, **84**, 866 (1962).
- 8) Y. Matsubara, F. Matsuura, and Y. Hayashi, *Shishitsu Seikagaku Kenkyu*, **15**, 57 (1973).
- 9) T. Sasaki and N. Takasuka, *Carbohydr. Res.*, **47**, 99 (1976).
- 10) S. Kikumoto, T. Miyajima, K. Kimura, S. Okubo, and N. Komatsu, *Nippon Nôgeikagaku Kaishi*, **45**, 162 (1971).
- 11) J. Johnson, S. Kirkwood, A. Misaki, J. E. Nelson, J. V. Scaletti, and F. Smith, *Chem. Ind. (London)*, **1963**, 820.
- 12) I. R. Johnson, *Biochem. J.*, **96**, 651 (1965).
- 13) S. Bartnicki-Garcia, *J. Gen. Microbiol.*, **42**, 57 (1966).
- 14) B. A. Cooper and J. M. Aronson, *Micologia*, **59**, 658 (1967).