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Structure and Antitumor Activity of a β-1,3-Glucan Isolated from the Culture Filtrate of *Sclerotinia sclerotiorum* IFO 9395

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An antitumor glucan (SSG) was isolated from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395 by sequential use of ethanol precipitation, ion exchange chromatography on diethylaminoethyl-Sephadex A-25, and precipitation with ammonium sulfate. From the results of methylation analysis and carbon-13 nuclear magnetic resonance spectroscopy, SSG was concluded to be a β-1,3-D-glucan branched at position 6 of every two 3-substituted β-glucosyl units. SSG formed a complex with congo red in dilute alkali solution, and showed potent antitumor activity against the solid form of sarcoma 180 in ICR mice.

Keywords——*Sclerotinia sclerotiorum*; scleroglucan; β-1,3-glucan; antitumor activity; Ascomycotina; polysaccharide

Introduction

Sclerotinia sclerotiorum is a fungus belonging to Ascomycotina, Discomycetes, Helotiales. This fungus is known to produce "scleroglucan," which is a β-1,3-glucan possessing a branch at position 6 of every three main chain glucosyl units.[1,2] Recently, we have studies the antitumor activity of "grifolan" obtained from *Grifola frondosa*.[3] The structure of grifolan was found to be similar to those of lentinan,[4] schizophyllan,[5] and scleroglucan. In order to compare the antitumor activities and the structures of these glucans, we tried to isolate the scleroglucan from *S. sclerotiorum* IFO 9395. However, the glucan obtained from strain IFO 9395 showed different properties from scleroglucan. This paper is concerned with the structure and the antitumor activity of the glucan (SSG) obtained from *S. sclerotiorum* IFO 9395.

Materials and Methods

Cultivation of *Sclerotinia sclerotiorum*——Cultivation was performed on medium containing glucose (2%), yeast extract (0.3%), and polypeptone (1%). *S. sclerotiorum* IFO 9395 was grown in 1000 ml (100 ml portions) of the medium with reciprocal shaking at 27°C for 5 d. When the culture no longer bubbled, the mycelia and the broth were separated by filtration with a glass filter. The filtrate was boiled for 5 min to inactivate any enzymes. After concentration, 1 volume of ethanol was added, and then fibrous precipitates, which came to the surface of the mixture, were separated from the reaction mixture. The precipitate was dried by washing with acetone and then ether (crude SSG, yield 0.6 g/l of culture).

Purification of SSG——A portion of crude SSG (1.0 g) dissolved in 8 M urea (700 ml) was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (Cl^-) (10 × 3 cm). The column was eluted with 8 M urea (350 ml) and the eluate was dialyzed against tap water and distilled water. The fraction was concentrated and adjusted to 2 mg/ml, and polysaccharide was precipitated with 0, 50, 75 and 100% saturation of ammonium sulfate, successively. The precipitate formed at 50% saturation was named SSG.

Other Methods——Paper electrophoresis was performed on Toyo Roshi GF90 glass fiber filters in 0.1 M borate, pH 9.3, at 3 mA/cm. Other procedures such as quantitative determination, antitumor assay, carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, and physicochemical analyses were performed as described previously.[3]

TABLE I. Antitumor Activity of Fractions Obtained from Culture Filtrate of *Sclerotinia sclerotiorum* IFO 9395[a]

Fraction	Dose ($\mu g \times 5$)	Tumor weight[c] (g, mean ± S.D.)	Inhibition[b] (%)	Complete regression[b, e]
Experiment-1				
Crude	50	<0.1[d]	>99	7/10
	500	0.1 ± 0.2[d]	99	2/10
Control	—	9.3 ± 6.8	—	0/12
Experiment-2				
DEAE pass	8	4.8 ± 3.2[d]	60	0/10
	40	3.1 ± 5.2[d]	74	3/10
Control	—	12.0 ± 4.3	—	0/16
Experiment-3				
SSG	4	6.3 ± 3.5	11	0/10
	20	3.9 ± 5.2	44	1/10
	100	0.5 ± 1.4[d]	93	4/9
Control	—	7.1 ± 5.2	—	0/16

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously (day 0). Each fraction was administered as a saline solution by intraperitoneal injection on days 1, 3, 5, 7, and 9. *b*) Inhibition and complete regression were determined at day 35 after tumor inoculation. *c*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from control (*d*) $p < 0.001$). *e*) Number of complete regressions/mice used.

Results and Discussion

Sclerotinia sclerotiorum IFO 9395 was cultured in a liquid medium. After about 5 d of cultivation, a viscous culture broth was obtained without any bubbles. After filtration and concentration, crude polysaccharide was obtained from the culture broth by adding 1 volume of ethanol. The crude polysaccharide fraction (carbohydrate, 65%; protein, 16%) showed potent antitumor activity (Table I). The crude polysaccharide was composed of mannose and glucose in a molar ratio of about 1 : 3 as judged by gas liquid chromatography of the alditol acetate derivatives. The mannose/glucose ratio was variable from lot to lot. It is assumed that the mannose originated from the broth because of the addition of the yeast extract to the medium. When the cultivation of the fungi was prolonged, the viscosity of the culture and the yield of the glucan decreased gradually, presumably because of the production of hydrolytic enzymes (the mannose content of the crude polysaccharide was increased; data not shown).

The purified glucan, called SSG, was obtained by the following procedures. The crude fraction was separated on a DEAE-Sephadex A-25 column into neutral (passed fraction) and acidic (NaCl-eluted fraction) fractions, in yields of 85% and 15%, respectively. The neutral fraction showed antitumor activity comparable to that of the crude polysaccharide (Table I). The neutral fraction was composed of glucan and a small amount of mannan (data not shown). The acidic fraction was composed of glucose and mannose in a molar ratio of 15 : 13. Furthermore, the neutral fraction was separated by precipitation with stepwise increases of ammonium sulfate concentration (0, 50, 75, 100% saturation). Polysaccharides were recovered in yields of 9.5% (0% saturation), 65% (50%), 6.8% (75%), and 19% (supernatant). The bulk of the polysaccharide (65%) was precipitated at 50% saturation of ammonium sulfate. The purified glucan (carbohydrate; 86.1%, protein; 1.3%) was composed of only glucose, and migrated as a single spot in paper electrophoresis on glass fiber (data not shown). This glucan (SSG) showed $[\alpha]_D -2^\circ$ (*c* 1%, 8 M urea–0.2 N NaOH). On Sepharose CL-4B column chromatography (0.2 N NaOH–8 M urea), most of this glucan was eluted at the void

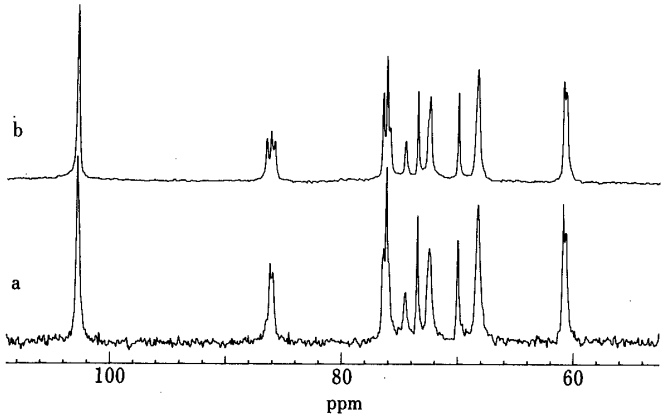

Fig. 1. ^{13}C-NMR Spectrum of SSG in DMSO-d_6 at 60 °C
a, SSG; b, grifolan NMF-5N (from the matted mycelia of *Grifola frondosa*).

TABLE II. ^{13}C-NMR Chemical Shifts of SSG and Grifolan in DMSO-d_6 Solution at 60 °C[a)]

Glucans[b,c)]	C-1	C-2	C-3	C-4	C-5	C-6
Grifolan A	102.80	72.44	86.57	68.29	76.17	60.70
Grifolan B	102.80	72.44	86.16	68.29	76.17	60.70
Grifolan C	102.80	72.44	85.81	68.29	74.54	68.29
Grifolan D	102.80	73.49	76.47	69.98	76.17	60.93
SSG I	102.86	72.50	86.22	68.29	76.17	60.70
SSG II	102.86	72.50	85.99	68.29	74.54	68.29
SSG III	102.86	73.49	76.41	70.04	76.17	60.93

a) Assignments were performed with reference to those in Ref. 9. *b*) A, B, C represents main chain glucosyl units and D represents a glucosyl unit at a branching point. D is present at C-6 of residue C. *c*) I, II represents main chain glucosyl units and III represents a glucosyl unit at a branching point. III is present at C-6 of residue II.

volume and the average molecular weight was suggested to be more than 5×10^6.

Upon methylation analysis, SSG gave 2,3,4,6-Me_4-, 2,4,6-Me_3-, and 2,4-Me_2-glucose in a molar ratio of 1.0 : 1.07 : 1.07. The ^{13}C-NMR spectrum of SSG is shown in Fig. 1, and the signal assignments are listed in Table II. This spectrum is similar to that of grifolan (Fig. 1b), except for the signal at 86 ppm. This signal is assigned to C-3 and appears as a triplet in the case of grifolan because a branching point is present at every three main chain glucosyl units. On the other hand, this signal is a doublet in the case of SSG. From the results of methylation and ^{13}C-NMR spectroscopy, it is suggested that SSG is a β-1,3-glucan possessing a branch at every two main chain glucosyl units at position C-6. SSG showed metachromasy coupled with congo red (0.1 N NaOH, 510 nm).

SSG showed potent antitumor activity against sarcoma 180 tumor in ICR mice (Table I). The optimum dose of antitumor activity is about 100 μg × 5/mouse. Unfortunately, the optimum dose did not decrease through the purification steps. Thus, it is assumed that more than one substance in the crude preparation showed antitumor activity. At least one other active substance probably originated from the medium, because yeast cell wall components, such as mannan and glucan, are known to show antitumor activity. However, the activity of the SSG was not solely due to contaminating medium components, because the optimum dose was consistent with those of other glucans.[3,6)]

Sclerotinia is known to produce "scleroglucan."[1,2)] Which is a β-1,3-glucan possessing

a branch at every three main chain glucosyl units. On the other hand, the strain IFO 9395 produces a different kind of β-1,3-glucan, SSG. Furthermore, this strain formed sclerotia, which is a characteristic property of *S. sclerotiorum* when cultured on agar plates. The hot water extract of the sclerotia contained a glucan similar to SSG (unpublished results). These observations suggest that IFO 9395 produces SSG instead of so-called "scleroglucan." It is interesting that fungi of the same type produce different kinds of glucans. *Monilinia fructigena*[7] and *Cordyceps ophioglossoides*,[8] belonging to Ascomycotina, Discomycetes or Pyrenomycetes, are known to produce glucans similar in structure to SSG. This suggests that highly branched β-1,3-glucan is also widely present in Ascomycotina.

Recently, we reported an antitumor glucan, "grifolan,"[3] which is a β-1,3-glucan possessing a branch at every three main chain glucosyl units, and PVG,[6] which is a β-1,3-glucan possessing a branch at every five main chain glycosyl units. It would be interesting to compare the antitumor activities of these glucans.

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