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Antitumor Activity and Structural Characterization of a (1→3)-β-D-Glucan Extracted with Cold Alkali from Sclerotia of *Sclerotinia sclerotiorum* IFO 9395

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An antitumor (1→3)-β-D-glucan (TS-CAG III) was purified from the cold aqueous sodium hydroxide extract of the residue of hot water extract of sclerotia of *Sclerotinia sclerotiorum* IFO 9395 by sequential use of ion exchange chromatography on diethylaminoethyl Sephadex A-25, ethanol precipitation, and gel filtration on Sepharose CL-2B. TS-CAG III showed $[\alpha]_D$ +18° (0.4 N NaOH). From the results of methylation analysis and carbon-13 nuclear magnetic resonance spectroscopy, TS-CAG III was concluded to be a (1→3)-β-D-glucan branched at position 6 of two in every five main chain glucosyl units. TS-CAG III 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*; sclerotia; (1→3)-β-D-glucan; antitumore activity; polysaccharide

Sclerotinia sclerotiorum is a fungus belonging to the Ascomycotina, Discomycetes, Helotiales. This fungus produces "sclerotia" when it is cultured on an agar plate. The hot water extract of the sclerotia, named TSHW, has been found previously to possess potent immunomodulating activities, such as B-cell mitogenic activity, polyclonal B-cell activation (PBA) activity, reticuloendothelial system (RES) activation activity, and antitumor activity.[1] TSHW is composed of protein 20—40%, carbohydrate 60—70%, and a trace of phosphate. It is speculated that the residue obtained after the extraction of TSHW also contains some kinds of immunomodulating materials.

Typical antitumor-active glucans applied clinically, such as lentinan (*Lentinus edodes*)[2] and schizophyllan (*Schizophyllum commune*),[3] are obtained from fungi. Previously, we have obtained two kinds of polysaccharides, which are (1→3)-β-D-glucans possessing different degrees of branching, from the fruit body of *Peziza vesiculosa* by extraction with cold sodium hydroxide, and found that both of them possessed potent antitumor activity.[4] We also obtained antitumor-active glucans from the cultured fruit bodies of *Grifola frondosa* by extraction with hot water and sodium hydroxide.[5] Including these cases, so-called antitumor-active glucans are almost always (1→3)-β-D-glucans. In this work, the polysaccharide in the cold sodium hydroxide extract from the hot water-extracted residue of sclerotia was examined.

Materials and Methods

Materials——Sepharose CL-2B and diethylaminoethyl (DEAE)-Sephadex A-25 were obtained from Pharmacia Fine Chemicals.

Extraction of Polysaccharide——The lyophilized sclerotia obtained from malt agar media (5 g) were boiled in water (*ca.* 200 ml, 3 times). The stored residue (10 g) was extracted with 10% sodium hydroxide containing 5% urea by stirring for 24 h at 4°C and centrifuged at 7000 rpm for 10 min at 4°C (*ca.*400 ml, 6 times). Then, the supernatant was

combined, neutralized with acetic acid and dialyzed. The non-dialyzable fraction was recovered by lyophilization and named TS-CAS.

Purification of (1→3)-β-D-Glucan from TS-CAS——TS-CAS (250 mg) was dissolved in 8 M urea (500 ml) and applied to a column of DEAE-Sephadex A-25 (Cl^-) ion exchange chromatography (50 ml) equilibrated with the same solution. The pass-through fraction was collected and dialyzed against distilled water. The non-dialyzable fraction was concentrated and precipitated with 2 volumes of ethanol. The precipitate was collected, and dried with acetone then ether. The resulting material was named TS-CAG (yield: 43%). A portion (40 mg) of TS-CAG was applied to a column of Sepharose CL-2B (2 × 90 cm) equilibrated with 8 M urea containing 0.2 N sodium hydroxide. The effluent was collected in 3.2 ml fractions, and fractionated into four fractions (fr. I—IV). The main peak (fr. III) was collected and further purified on the same column. The main peak (Fig. 1) was collected and neutralized with acetic acid, dialyzed against distilled water and then lyophilized. The resulting material was named TS-CAG III.

Analytical Methods——Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy was performed with a Bruker AM-400 instrument. Activity to stimulate the reticuloendothelial system (RES) was assessed by the method described previously.[1] Other methods used in this paper such as quantitative determination, antitumor activity, and physicochemical analyses were performed as described in the previous paper.[5]

Results and Discussion

The source of the fraction examined in this paper was the hot water-extracted residue of sclerotia of *Sclerotinia sclerotiorum* IFO 9395. The fractions were prepared by cold alkali extraction of the residue as described in Materials and Methods. Alkaline extracts were neutralized with acetic acid, dialyzed, concentrated, and then lyophilized. The resulting material was named TS-CAS (yield 54%). The major constituent of TS-CAS was carbohydrate (*ca.* 90%) and there was a small amount of protein (*ca.* 10%). Based on the result of component sugar analysis by using gas liquid chromatography, TS-CAS was composed of glucose and mannose in a molar ratio of 25 : 1. Furthermore, TS-CAS was separated into carbohydrate and protein completely when it was applied to a column of DEAE-Sephadex A-25 equilibrated with 8 M urea (data not shown). This result suggests that TS-CAS was not a carbohydrate-protein complex but was composed of carbohydrate and protein individually.

To obtain the purified glucan from TS-CAS, sequential purification was performed as described in Materials and Methods. The purified glucan, named TS-CAG III, gave a symmetrical peak on Sepharose CL-2B eluted with 8 M urea containing 0.2 N NaOH. The molecular weight was suggested to be more than 2×10^6 (Fig. 1). TS-CAG III was obtained in 13% yield from sclerotia and proved to consist solely of glucose as carbohydrate (>99%) and a trace of protein (<1%). The specific rotation of TS-CAG III was +18° (0.4 N NaOH). In methylation analysis, TS-CAG III gave 2,3,4,6-Me_4-Glc, 2,4,6-Me_3-Glc, and 2,4-Me_2-Glc in a molar ratio of 1.0 : 1.5 : 1.0. TS-CAG III showed metachromasy coupled with Congo Red (0.1 N NaOH, 510 nm), and showed the signal attributed to β-linkage (C-1: 103 ppm) in the

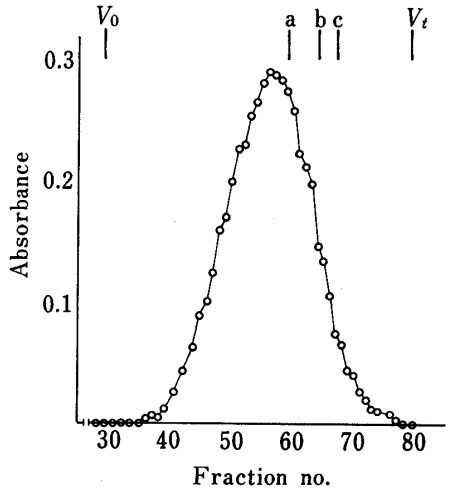

Fig. 1. Elution Profile of TS-CAG III from a Column of Sepharose CL-2B

The column (2 × 90 cm) was equilibrated with 8 M urea containing 0.2 N NaOH, and 20 mg of TS-CAG III was applied. Fractions of 3.5 ml were collected and carbohydrate was assayed by the phenol–sulfuric acid method (490 nm; —○—). a, T 2000; b, T 250; c, T 110.

TABLE I. Antitumor Activity[a] of TS-CAS and TS-CAG III against Sarcoma 180

Sample	Route	Dose (μg/mouse)	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression
TS-CAS	i.p.	20	0.34 ± 0.38[b]	96	0/10
		100	0.20 ± 0.32[b]	98	1/10
		250	0.10 ± 0.19[b]	99	6/10
	i.l.	20	2.66 ± 1.73[c]	65	0/ 9
		100	1.04 ± 1.29[b]	86	0/10
		250	0.69 ± 0.85[b]	91	0/10
TS-CAG III	i.p.	20	2.31 ± 3.06[c]	76	1/10
		100	0.20 ± 0.32[b]	98	4/10
		250	0.49 ± 0.83[b]	95	4/10
	i.l.	20	0.76 ± 1.03[b]	90	0/10
		100	0.83 ± 0.73[b]	89	0/10
		250	3.12 ± 2.05[c]	59	0/10
Nil	i.p.	—	9.49 ± 7.03	0	0/10
	i.l.	—	7.64 ± 5.83	0	0/10

a) Sarcoma 180 tumor cells (5×10^6 cells/mouse) were inoculated subcutaneously into the right groin of mice. Each sample was administered 5 times (days +7, +9, +11, +13, +15) as a saline solution by intraperitoneal (i.p.) or intralesional (i.l.) injection. Five weeks after tumor inoculation, the mice were sacrificed and tumor weights were compared. *b*) $p<0.01$, *c*) $p<0.05$.

^{13}C-NMR spectrum. These observations suggest that TS-CAG III is a (1→3)-β-D-glucan possessing a branch at position C-6 of two in every five main chain glucosyl units.

TS-CAG III showed potent antitumor activity on both intraperitoneal (i.p.) and intralesional (i.l.) injection against the solid form of sarcoma 180 in ICR mice (Table I). The optimum doses were about 100 μg and 20 μg/mouse/d in the case of i.p. and i.l. injection, respectively (Table I). These data suggest that TS-CAG III is effective on both systemic and local administrations against the solid form of sarcoma 180. Recently, we obtained an antitumor (1→3)-β-D-glucan, SSG, possessing a branch at C-6 of every other main chain glucosyl unit, from the culture broth of *S. sclerotiorum* IFO 9395,[6] and SSG showed antitumor activity against not only an allogeneic tumor (sarcoma 180-ICR mice) but also syngeneic tumors (Meth A fibrosarcoma-BALB/c mice; IMC carcinoma-CDF_1 mice) (submitted data). This observation suggests the possibility that TS-CAG III may also show antitumor activity against a syngeneic tumor. However, no antitumor activity was seen against the ascites form of sarcoma 180 administered at days +1—+9 (5 times) at a dose of 20, 100 or 500 μg/mouse/d (survival days: saline 15.6 ± 3.8; TS-CAG III 22.0 ± 15.2 (20 μg/mouse/d), 16.2 ± 2.9 (100 μg/mouse/d), 19.5 ± 8.0 (500 μg/mouse/d)). This result is consistent with the reports that antitumor glucans are less effective against ascites tumor.[7]

On the other hand, TS-CAS possessed antitumor activity against the ascites form of sarcoma 180 administered at days +1—+9 (5times) at a dose of 100 μg/mouse/d (survival days: saline 15.6 ± 3.8; TS-CAS 38.2 ± 24.8 ($p<0.05$)). A similar result was also obtained in the case of vesiculogen, which is a fungal immunomodulator extracted with hot water from the fruit bodies of *Peziza vesiculosa*.[8] Vesiculogen was composed of protein (*ca.* 60%), carbohydrate (*ca.* 30%), and a small amount of amino sugar, uronic acid, phosphate, and lipid,[9] and possessed antitumor effects against both solid- and ascites-form sarcoma 180.[10] Since vesiculogen does not seem to have a direct cytotoxic effect on cultured tumor cells *in vitro* (data not shown), we speculated that the antitumor effect of vesiculogen against ascites-form sarcoma 180 may be due to the cooperation of (1→3)-β-D-glucan and other immunomodulating substances from *P. vesiculosa*. TS-CAS showed mitogenic activity against ICR spleen

TABLE II. Effect of TS-CAG III on Carbon Clearance Activity of ICR Mice

Sample	Dose × times (μg/mouse)	Phagocytic index[a] K (mean ± S.D.)	Sample	Dose × times (μg/mouse)	Phagocytic index[a] K (mean ± S.D.)
TS-CAG III	50 × 5	0.1573 ± 0.0317[b]	*P. acnes*	350 × 1	0.1281 ± 0.0373[c]
	250 × 1	0.0762 ± 0.0072[c]	Nil	—	0.0599 ± 0.0100

a) Phagocytic index (K) was calculated by means of the following equation: $K=(\ln OD_1-\ln OD_2)/(t_2-t_1)$ where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. *b*) $p<0.01$, *c*) $p<0.05$.

cells and its activity disappeared after alkali treatment for 20 h at 37 °C (data not shown), suggesting that the protein moiety of TS-CAS may play a role in the mitogenic activity. Thus, the antitumor effect of TS-CAS against ascites-form sarcoma 180 may be due to the synergistic effects of the protein moiety and the carbohydrate moiety of TS-CAS.

Furthermore, we studied the stimulant activity of the RES of TS-CAG III in ICR mice. As a positive control, *Propionibacterium acnes* K-674 was used in ICR mice. TS-CAG III showed RES activation activity when it was administered both at days −10——2 (5 times) at a dose of 50 μg/mouse/d or at day −2 (one time) at a dose of 250 μg/mouse/d (Table II). In the case of *P. acnes*, Scott and Milas proposed that the ability to stimulate the RES was associated with resistance to intracellular degradation.[11] Further, we showed that the disappearance rate of grifolan (antitumor-active β-1,3-glucan) from the peritoneal cavity of ICR mice was slower than that of islandican (antitumor-inactive β-1,6-glucan) or starch (antitumor-inactive α-1,4-glucan).[12] Considering these points, it is speculated that TS-CAG III is probably resistant to intracellular degradation and this may contribute to the antitumor activity of TS-CAG III.

Generally, the ultrastructure is thought to be important for antitumor activity of (1→3)-β-D-glucans. Saito *et al.* have suggested that (1→3)-β-D-glucans possess at least 4 types of solid-state conformations, form I (curdlan-type), form II (laminaran-type), form III (laminaripentaose-type), and form IV (dimethyl sulfoxide (DMSO)-adduct) by using cross-polarization magic angle spinning nuclear magnetic resonance spectroscopy (CP/MAS NMR).[13] As judged from a comparison of the spectra with those reported by Saito *et al.*, SSG, as mentioned above, possessed the native form, corresponding to form II (laminaran type), and was degraded to the helix form, corresponding to form I (curdlan type), on treatment of urea or alkali. The CP-MAS NMR spectra of the sclerotia of *S. sclerotiorum* IFO 9395 and the hot water extract of the sclerotia, TSHW, showed a chemical shift (C-3: 86 ppm) similar to that of the native form of SSG.[14] On the other hand, the spectrum of TS-CAS is similar to that of the helix form of SSG.[14] A glucan fraction partially purified from TS-CAS by DEAE-Sephadex ion exchange chromatography eluted with 8M urea also had a spectrum similar to that of the helix form of SSG.[14] These results suggest that TS-CAG III probably had a spectrum similar to that of the helix form of SSG, and the conformation of the structural glucans from sclerotia was thought to be transformed from native to helix during the cold sodium hydroxide extraction. This is consistent with the fact that treatment with urea and/or sodium hydroxide changed the conformation of SSG from native to helix.[15]

Previously, Sietsma and Wessels have reported the presence of three glucans in the cell wall of *Schizophyllum commune*.[16] Furthermore, Shida *et al.* have reported the heterogeneity of the chemical structure of the alkali-insoluble skeletal glucan of the fruit body of *Lentinus edodes*.[17] These fungi are known to contain the antitumor glucans schizophyllan and lentinan, respectively. Based on these observations and our results described in this paper, it is suggested that the sclerotia of *S. sclerotiorum* IFO 9395 may contain several kinds of (1→3)(1→6)-β-D-glucans, and some of them possess potent antitumor effect. The differences include

ratio of (1→3) and (1→6) linkages, distribution of the branching points and the architecture of the framework. Further structural characterization of the component polysaccharides of sclerotia is in progress.

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