ANTITUMOR ACTIVITY OF A HIGHLY BRANCHED (1 → 3)- β -D-GLUCAN, SSG, OBTAINED FROM *SCLEROTINIA SCLEROTIORUM* IFO 9395

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(Received March 11, 1987)

The antitumor activity of a highly branched (1 → 3)- β -D-glucan, SSG, purified from the liquid culture filtrate of *Sclerotinia sclerotiorum* IFO 9395 and its several derivatives were treated in ICR mice bearing Sarcoma 180 cells. SSG was effective by both systemic (intraperitoneal and intravenous) and local (intratumoral) administrations on the solid form of Sarcoma 180 in ICR mice and the mice acquired resistance to subsequent inoculation of Sarcoma 180. However, SSG was not effective on the ascites form Sarcoma 180. The pretreatment of ICR mice with carrageenan suppressed the antitumor activity, suggesting the involvement of macrophages on the antitumor activity. Derivatives prepared from SSG by periodate oxidation/borohydride reduction showed antitumor activity, but those obtained after acetylation, carboxymethylation and hydroxyethylation were less active. From these results, it is suggested that SSG is a useful antitumor glucan which modifies biological responses and can be used as a source for some antitumor derivatives.

Keywords — *Sclerotinia sclerotiorum*; SSG; (1 → 3)- β -D-glucan; antitumor activity; Sarcoma 180

INTRODUCTION

Recently, some fungal glucans such as lentinan^{1,2)} and schizophyllan^{3,4)} have been used clinically as drugs for cancer therapy. These glucans showed no direct cytotoxicity on tumors but showed modifications of biological responses. These glucans are usually used with chemotherapy, surgery or physical therapy.¹⁻⁴⁾ For possible greater therapeutic effects, it would be important to learn the mode of antitumor action of these glucans. Thus, we have been studying the antitumor activity and the physicochemical properties of an antitumor glucan, grifolan, obtained from *Grifola frondosa*.^{5,6)} Grifolan is active on both allogeneic and syngeneic tumor systems and the periodate oxidized and borohydride reduced products also showed antitumor activities. Recently, we have found a highly branched (1 → 3)- β -D-glucan, SSG, obtained from the liquid culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, which possesses a branch at C-6 of almost every other main chain unit.⁷⁾ The precise examination of the highly branched (1 → 3)- β -D-glucan on antitumor and other biological activities and physicochemical properties were limited until now.⁸⁻¹⁰⁾ In this paper, we have examined the antitumor activity of SSG and some of its derivatives.

MATERIALS AND METHODS

Preparation of Periodate Oxidized/ Borohydride Reduced SSG (I/B-SSG) — SSG (1 g) was oxidized with 50 mM sodium metaperiodate (1000 ml) at 4 °C in the dark. When the reaction was complete (96 h; IO₄ consumption was 0.7 mol/anhydroglucose unit; formic acid produced was 0.3 mol/anhydroglucose unit), excess periodate was destroyed with ethylene glycol and the mixture was dialyzed against water. The non-dialyzable fraction was reduced with sodium borohydride (1 g) at 4 °C for 48 h. After acidification with acetic acid, the mixture was dialyzed and lyophilized to give I/B-SSG (820 mg).

A portion (250 mg) of I/B-SSG was partially hydrolyzed with 0.1 N sulfuric acid at 25 °C for 72 h. After neutralization, the reaction mixture was dialyzed against water. The non-dialyzable fraction was designated as I/B-H-SSG (225 mg).

Preparation of Carboxymethylated SSG (CM-SSG) — A suspension of 100 mg of SSG in 10 ml isopropanol was mixed with 0.5 ml of 30% aqueous sodium hydroxide and stirred vigorously. Monochloroacetic acid (150 mg) was then added to the mixture and the reaction was continued for 2 h at 50 °C with stirring. The reaction product CM-SSG was filtered and washed with 70% methanol-acetic acid solution, 80% aqueous methanol, 100% methanol and

acetone, successively. The product was dissolved in 10 ml of water and the soluble fraction was collected by centrifugation (yield; 33%). The degree of substitution (DS) was estimated as 0.4/glucosyl unit by colorimetric determination.¹¹⁾

Preparation of Hydroxyethylated SSG (HE-SSG) — A solution of SSG (100 mg) in 3 ml of 0.5 N sodium hydroxide was mixed with 40% sodium hydroxide (2 ml) and ethylene oxide (prepared with monochloroethanol, 4 ml, sodium hydroxide, 8 g, and tetrahydrofuran) and stirred vigorously at -10°C for 2 h and at room temperature for 12 h. The reaction mixture was neutralized by acetic acid and dialyzed against water for 3 d. The non-dialyzable fraction was lyophilized (HE-SSG; yield, 81%). The DS, which included polymerization of ethylene oxide were estimated as 0.5/glucosyl unit by the signal intensities in a carbon-13 magnetic resonance (^{13}C -NMR) spectrum.

Preparation of Acetylated SSG (Ac-SSG) — Each 100 mg of SSG dissolved in 3 ml of pyridine was mixed with 250 μl (Ac-1), 1250 μl (Ac-5), and 3 ml (Ac-A) of acetic anhydride and kept at 100°C for 2 h. The reaction mixture was evaporated, dialyzed against water for 3 d and then lyophilized (yield; Ac-1, 106%, Ac-5, 140%, Ac-A, 146%).

Other Methods — Other methods, such as cultivation of *S. sclerotiorum* IFO 9395, purification of SSG, quantitative determination, antitumor assay, ^{13}C -NMR spectrum, Congo red binding, and physicochemical analyses, were

performed as described previously.⁵⁻⁷⁾

RESULTS

Effect of SSG on Sarcoma 180 in ICR Mice

The ascites form of Sarcoma 180 (5×10^6 cells/0.05 ml) was inoculated into the right groin of mice subcutaneously (day 0). Several doses of SSG were administered intraperitoneally (i.p.) every other day for 3 d (day 7, 9, 11) from the 7th day after the tumor inoculation. As shown in Table I, administrations between 100 to 1000 μg were effective. Intravenous (i.v.) (Table II) and intratumoral (i.t.) (Table V) administrations also showed potent antitumor activities. I.t. administration was less active than other injection routes following this time schedule.

Next, the effect of administration timing on the antitumor activity of SSG was examined. Sarcoma 180 tumor cells were inoculated as described above. Doses of SSG were 200 and 500 $\mu\text{g}/\text{mouse}/\text{d}$ and injected i.p. on every other day as follows: (1) days $-5, -3, -1$, (2) days 1, 3, 5, (3) days 7, 9, 11, and (4) days 13, 15, 17. As shown in Table II, the pretreated group (days $-5, -3, -1$) exhibited only weak activity at doses of 200 and 500 μg . The later-administered groups (days 7, 9, 11 and days 13, 15, 17) showed greater effects than did the earlier administered groups. Antitumor activity of lentinan,¹²⁻¹⁴⁾ schizophyllan,¹⁵⁻¹⁹⁾ curdlan,²⁰⁾ and grifolan^{5,6,21)} against the solid form of Sarcoma 180 has previously been investigated. All of these glucans showed potent antitumor activity when injected either before or after tumor inocu-

TABLE I. Antitumor Activity of SSG against Sarcoma 180^{a)}

Treatment	Dose ($\mu\text{g} \times 3$)	Days	Tumor weight ^{c)} (g, mean $\pm$ S.D.)	Inhibition ratio (%) ^{b)}	Complete regression ^{b)}
SSG (i.p.)	100	7, 9, 11	$1.1 \pm 2.7^d)$	88	3/10
	500		$0.1 \pm 0.1^d)$	99	3/10
	1000		$0.8 \pm 2.1^d)$	91	5/10
SSG + carrageenan	100	7, 9, 11	9.1 ± 6.0	— 3	0/8
	500		7.6 ± 7.3	15	1/10
	1000		$2.6 \pm 5.9^e)$	70	1/9
Nil			8.9 ± 5.3	0	1/20

a) Sarcoma 180 tumor cell (5×10^6) were inoculated subcutaneously (day 0). Each sample in saline was administered by intraperitoneal injection. SSG was administered at days, 7, 9, and 11. Carrageenan (4 mg) was administered at days $-5, -3$, and -1 . b) Inhibition and complete regression were determined on 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) $p < 0.05$.

TABLE II. *Effect of Administration Routes or Timings on Antitumor Activity of SSG against Sarcoma 180^{a)}*

Sample	Dose ($\mu\text{g} \times 3$)	Day	Tumor weight (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression
SSG	200 (i.p.)	-5, -3, -1	11.6 $\pm$ 7.2	19	0/10
		1, 3, 5	5.3 $\pm$ 4.4 ^{b)}	63	2/10
		7, 9, 11	0.3 $\pm$ 0.6 ^{b)}	98	5/10
		13, 15, 17	1.4 $\pm$ 2.7 ^{b)}	90	3/10
	200 (i.v.)	1, 3, 5	3.2 $\pm$ 4.2 ^{b)}	78	3/10
	500 (i.p.)	-5, -3, -1	8.7 $\pm$ 7.9	39	0/10
		1, 3, 5	4.0 $\pm$ 4.3 ^{b)}	72	2/10
		7, 9, 11	1.0 $\pm$ 2.5 ^{b)}	93	2/10
		13, 15, 17	3.2 $\pm$ 5.1 ^{b)}	78	2/10
	500 (i.v.)	1, 3, 5	2.1 $\pm$ 3.1 ^{b)}	85	6/10
Nil			14.3 $\pm$ 6.1	0	0/20

a) Experimental details were as described in Table I. b) Significant difference, $p < 0.001$.

TABLE III. *Effect of SSG against the Ascites Form of Sarcoma 180^{a)}*

Sample	Dose ($\mu\text{g} \times 3$)	Day	Survival days ^{b)} (d, mean $\pm$ S.D.)
Exp-1, 5×10^6 cells/mouse			
SSG	200	1, 3, 5	17.5 $\pm$ 6.6 ^{c)}
	500	1, 3, 5	20.4 $\pm$ 9.1 ^{c)}
Nil			16.6 $\pm$ 7.9
Exp-2, 5×10^5 cells/mouse			
SSG	100	-7, -4, -1	14.8 $\pm$ 8.6 ^{c)}
	500	-7, -4, -1	15.2 $\pm$ 3.0 ^{c)}
Nil			12.9 $\pm$ 3.9
Exp-3, 1×10^6 cells/mouse			
SSG	100	-7, -4, -1	13.9 $\pm$ 3.4 ^{c)}
	500	-7, -4, -1	16.3 $\pm$ 2.9 ^{c)}
Nil			14.4 $\pm$ 4.1

a) Sarcoma 180 tumor cell (5×10^6 , 5×10^5 , or 1×10^6 cells) were inoculated intraperitoneally (day 0). Each sample in saline was administered at the indicated days intraperitoneally. b) The significance of differences was evaluated according to Student's *t*-test and generalized Wilcoxon test. c) Not significant.

lation and when injected *via* i.p., i.v., or i.t. Antitumor activity of SSG was similar to these glucans except that SSG was ineffective when administered before tumor inoculation.

The effect of SSG on the ascites form of Sarcoma 180 was also examined but SSG was not very active against the ascites form of the tumor (Table III).

Effect of Blockade of Phagocytic System on Antitumor Activity of SSG

It is suggested that the phagocytic system plays an important role in the antitumor activity

of glucans. To clarify the participation of the phagocytic system in the antitumor activity of SSG, the effect of a blocking reagent for phagocytic system, carrageenan, was examined.²²⁻²⁴⁾ Mice were treated with carrageenan (4 mg/mouse) at days -5, -3, -1 from time of tumor inoculation and SSG was administered at days 7, 9, 11 after tumor inoculation. Antitumor activity was compared at 35 d from tumor inoculation. As shown in Table I, the antitumor activity of SSG was suppressed by the treatment with carrageenan. Marked suppression was observed at a

dose of 100 or 500 μg . However, the antitumor activity of higher doses of SSG (1000 μg) were less abrogated by this reagent. These facts suggest that the phagocytic system may be important for the antitumor activity of SSG, as it is for schizophyllan¹⁹⁾ and grifolan.²⁵⁾

Effect of Reinoculation of Sarcoma 180 into Tumor-Regressed Mice

Mice which showed complete regression of Sarcoma 180 on day 35 after tumor inoculation were reinoculated into their right groins with the same tumor cells (5×10^6 cells/mouse). All mice (11 mice) completely rejected the subsequent challenge by Sarcoma 180 tumor cells, whereas Sarcoma 180 cells in the control group grew (20 mice, 12.3 ± 7.5 g at 35 d after tumor inoculation). This result suggested that the antitumor activity of SSG was host mediated and SSG established an antitumor immunity against Sarcoma 180 in mice. This phenomenon was also observed in the case of grifolan.²¹⁾

Antitumor Activity of Derivatives of SSG

Until now, several derivatives of antitumor glucans have been prepared some of which showed stronger antitumor activities than the original glucans.^{6,8-10,26-32)} Accordingly, we prepared several derivatives of SSG, such as I/B-SSG, mild acid hydrolyzed I/B-SSG (I/B-H-SSG), Ac-SSG, CM-SSG and HE-SSG, and examined these derivatives for antitumor activity by both systemic (i.p.) and local (i.t.) administrations.

The side chain moiety of SSG was oxidized with metaperiodate and then reduced with borohydride (I/B-SSG). A portion of I/B-SSG was partially hydrolyzed with dilute sulfuric acid to prepare debranched SSG (I/B-H-SSG). Analyzed by ^{13}C -NMR spectroscopy, the spectrum of I/B-H-SSG was similar to that of curdlan (Fig. 1), suggesting the production of linear (1 $\rightarrow$ 3)- β -D-glucan. Misaki *et al.* suggested that partial hydrolysis by 0.1 N sulfuric acid at 25 $^{\circ}\text{C}$ is not sufficient to hydrolyze the side chain polyol groups.^{8-10,31)} However, in the case of SSG, a majority of the side chain moiety is removed by the treatment used in this paper. Furthermore, it has been demonstrated that, in the case of grifolan, mild acid hydrolysis of the I/B-derivatives (SD(3): 0.2 N sulfuric acid, 90 $^{\circ}\text{C}$, 1 h) degraded a portion of the main chain moiety and the antitumor activity of the derivatives was lost. SD(3) produced no helical confor-

FIG. 1. ^{13}C -NMR Spectrum of I/B-H-SSG in Dimethylsulfoxide- d_6 at 60 $^{\circ}\text{C}$
a, I/B-H-SSG; b, curdlan; c, SSG.

mation in dilute sodium hydroxide solution as measured by the Congo red binding.⁶⁾ In the case of I/B-H-SSG, the production of the helical conformation remained, because the λ_{max} of Congo red was shifted to a longer wavelength at lower sodium hydroxide concentration and was returned at higher sodium hydroxide concentration (λ_{max} , 500 nm (0.1 N), 495 nm (0.2 N), 492 nm (0.3 N)).

Antitumor activity of various derivatives are shown in Tables IV and V. Both I/B- and I/B-H-SSG showed antitumor activity by i.p. as well as i.t. administrations and I/B-SSG was the most active by both i.p. and i.t. administrations. However, the activity varied for each derivative and administration route (I/B-H-SSG was less active than I/B-SSG by both i.p. and i.t. administrations, and I/B-SSG and I/B-H-SSG were more active than SSG by i.t. administration). Misaki *et al.* suggested that the antitumor activity of (1 $\rightarrow$ 3)- β -D-glucan was augmented by the substitution of polyols.⁸⁻¹⁰⁾ These observations suggested that the side chain moieties would positively produce an effect on the antitumor activity and the polyol substitution would be more effective than β -glucosyl substitution.

Carboxymethyl and hydroxyethyl derivatives are one of the most widely used prepara-

TABLE IV. *Antitumor Effect of Derivatives of SSG against Sarcoma 180^{a)}*

Sample	Dose ($\mu\text{g} \times 5$)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression
SSG	20	4.8 $\pm$ 7.8	48	1/10
	100	0.2 $\pm$ 0.3 ^{e)}	98	2/10
	500	0.1 $\pm$ 0.2 ^{e)}	99	4/10
I/B-SSG	20	0.1 $\pm$ 0.1 ^{e)}	99	3/10
	100	0.6 $\pm$ 0.6 ^{e)}	94	1/10
	500	1.2 $\pm$ 1.3 ^{e)}	87	0/10
I/B-H	20	7.5 $\pm$ 5.1	19	0/10
	100	3.5 $\pm$ 6.8 ^{c)}	63	1/10
	500	2.4 $\pm$ 4.1 ^{d)}	74	2/10
CM-SSG	20	9.8 $\pm$ 4.6	— 6	0/10
	100	10.9 $\pm$ 3.8	— 18	0/10
	500	8.9 $\pm$ 4.7	4	0/10
HE-SSG	20	7.1 $\pm$ 4.5	24	0/10
	100	8.4 $\pm$ 8.4	9	0/10
	500	8.1 $\pm$ 5.6	13	0/10
Ac-1	20	5.5 $\pm$ 3.2	42	0/10
	100	9.0 $\pm$ 3.8	3	0/10
	500	4.5 $\pm$ 2.3 ^{c)}	52	0/10
Ac-5	100	9.0 $\pm$ 7.4	3	0/10
	500	9.1 $\pm$ 4.7	2	0/10
Ac-A	100	9.6 $\pm$ 3.7	— 3	0/10
	500	6.9 $\pm$ 4.3	25	0/10
Nil		9.3 $\pm$ 6.9	0	0/22

a) Experimental details were as described in Table I. Sample solutions or suspensions were administered intraperitoneally at days 7, 9, 11, 13, and 15. *b)* The significance of differences was evaluated according to Student's *t*-test. Significant differences from control, *c)* $p < 0.05$, *d)* $p < 0.01$, *e)* $p < 0.001$.

TABLE V. *Antitumor Effect of Derivatives of SSG against Sarcoma 180 by Local Administration^{a)}*

Sample	Dose ($\mu\text{g} \times 5$)	Tumor weight ^{b)} (g, mean $\pm$ S.D.)	Inhibition ratio (%)	Complete regression
SSG	100	2.5 $\pm$ 4.3 ^{c)}	66	1/10
	500	2.4 $\pm$ 3.0 ^{d)}	67	1/10
I/B-SSG	100	0.7 $\pm$ 1.1 ^{e)}	90	1/10
	500	3.6 $\pm$ 2.5 ^{c)}	51	1/10
I/B-H-SSG	100	2.8 $\pm$ 3.6 ^{c)}	63	2/10
	500	0.9 $\pm$ 0.7 ^{d)}	89	2/10
CM-SSG	100	6.4 $\pm$ 5.7	14	0/10
	500	5.1 $\pm$ 4.0	32	0/10
HE-SSG	100	9.7 $\pm$ 4.4	— 30	0/10
	500	8.4 $\pm$ 4.5	— 13	0/10
Ac-1-SSG	100	7.3 $\pm$ 4.5	1	0/10
	500	2.1 $\pm$ 2.1 ^{d)}	72	0/10
Nil		7.5 $\pm$ 4.3	0	0/10

a) Experimental details were as described in Table I. Sample solutions (50 μl) were administered intratumorally at days 7, 9, 11, 13, and 15. *b)* The significance of differences was evaluated according to Student's *t*-test. Significant differences from control, *c)* $p < 0.05$, *d)* $p < 0.01$, *e)* $p < 0.001$.

tions.^{8,9,26-28)} CMC (carboxymethylcellulose) is used clinically as a purgative medicine, HE-chitin (hydroxyethylchitin) is used as a soluble substrate of lysozyme and CM-TAK (carboxymethylcurdlan) is reported to show antitumor activity.^{32,33)} Thus, CM- and HE-SSG were prepared by monochloro acetic acid and ethylene oxide. Both derivatives are more soluble in water than SSG and the DS value of CM-SSG was 0.4 and that of HE-SSG was 0.5. From the data described earlier (CM-curdlan, HE-pachymaran), the DS values were thought to be important in antitumor activity. Considering these facts, both CM- and HE-SSG might show antitumor activity. As shown in Tables IV and V, however, antitumor activity was not observed for both derivatives. Both derivatives suffered no significant degradation during the reactions as assessed by gel filtration chromatography and by NMR spectroscopy (data not shown). It is suggested that the DS values required for antitumor activity would be different from those of the linear (1→3)- β -D-glucans.

Some of the natural antitumor polysaccharides are known to contain the acetyl moiety.^{34,35)} Therefore, several acetyl derivatives were prepared and their antitumor activities were examined. Acetylation was controlled by adding a limited quantity of acetic anhydride to prepare 10% (Ac-1), 50% (Ac-5), and 100% (Ac-A) acetylation products. All of these derivatives were difficult to dissolve in saline. As shown in Tables IV and V, Ac-5- and Ac-A-SSG showed no antitumor activity by i.p. administration, however; Ac-1-SSG showed weak antitumor activity by i.t. and i.p. administrations. It is not clear why such differences were found. However, Ac-1-SSG which showed weak antitumor activity would partially soluble. Insolubilization which would include physical insolubilization and the reduction of helical segments would be properties disadvantageous for antitumor activity.

DISCUSSION

SSG is a (1→3)- β -D-glucan branched at position 6 of every other 3-substituted β -glucosyl unit, obtained from liquid culture filtrates of *S. sclerotiorum* IFO 9395.⁷⁾ In this paper, the antitumor activity of SSG and several of its derivatives were examined and the results obtained indicated the following: (1) SSG was effective on

the solid form of tumor of Sarcoma 180 by i.p., i.v., or i.t. administration. (2) SSG was not effective on the ascites form of tumor. (3) The antitumor activity of SSG required a phagocytic system. (4) SSG established antitumor immunity against Sarcoma 180 in mice. (5) Modification of the side chain did not induce significant alterations of its antitumor activity but the addition of several functional groups reduced the antitumor activity significantly.

Many antitumor (1→3)- β -D-glucans form gels under certain physiological conditions and the gels are degraded into sol by the addition of sodium hydroxide. The gel to sol transition of curdlan, lentinan, schizophyllan, and grifolan were examined by ¹³C-NMR spectroscopy. Similarly, SSG also form a gel and the gel was degraded into sol by the addition of sodium hydroxide.³⁶⁾ Some antitumor (1→3)- β -D-glucans, especially glucans applied clinically, are effective against not only allogeneic but also syngeneic tumors. SSG is also effective against some syngeneic tumors by both systemic and local administrations.^{37,38)} Recently, we studied the antitumor glucan, grifolan, obtained from *G. frondosa*^{5,6,21)} and found SSG to be an interesting immunomodulating antitumor drug. Properties of SSG described above, such as (1) to (4), are similar to the properties of other antitumor glucans described previously, but some differences also exist. Lentinan, schizophyllan, curdlan, and grifolan are effective when administered before tumor inoculation whereas SSG was not. Furthermore, SSG was not so effective when administered at 1, 3, 5 d after tumor injection. This fact suggested that the effective timing of SSG was narrower than that of other antitumor glucans, which would be due to its having more branch points.

Periodate oxidation, borohydride reduction and then partial hydrolysis are well known modifications of branched (1→3)- β -D-glucan.^{6,8-10,31)} The antitumor activity of SSG was found to increase by I/B treatment and decrease by subsequent I/B-H treatment. This fact suggests that the side chain moiety positively produce an effect on the antitumor activity and that polyol substitution was more effective than β -glucosyl substitution. On the other hand, recently, Morikawa *et al.* reported that linear, not branched, (1→3)- β -D-glucan can activate polymorphonuclear leukocytes to show cytolytic

activity.^{39–42)} Considering these facts, it is probable that the side chain modified SSG mainly activates different sites of immune system than does SSG.

Some of antitumor glucans contain O-acetyl substitutions. GE-3 is a (1→6)- β -D-glucan containing about 2% acetyl groups at position 3.³⁵⁾ To compare the antitumor activity of acetylated derivatives with SSG, partially acetylated derivatives were prepared. However, none on the derivatives showed antitumor activity except for Ac-1-SSG which showed only weak activity. All of these derivatives were insoluble in water. Acetylation induced changes in the physicochemical properties of SSG, which included insolubilization and reduction of helical conformation. It is assumed that these alternations reduced the antitumor activity of SSG and that Ac-1-SSG contained some soluble parts which are essential for activating the immune system.

Carboxymethylation and hydroxyethylation of glucans yield derivatives such as CM-curdlan, CM-pachymaran and HE-pachymaran^{26,32)} and upon testing, neither carboxymethyl nor hydroxyethyl derivatives of SSG showed antitumor activity. The loss of the antitumor activity of the highly branched (1→3)- β -D-glucan by carboxymethylation or hydroxyethylation has also been reported by others.^{8,9)} It is assumed that the DS value required for exhibiting antitumor activity is different for linear and branched (1→3)- β -D-glucans. Many antitumor (1→3)- β -D-glucans produce gel in their neutral aqueous solutions. Therefore, such antitumor glucans showed slight ¹³C-NMR signals in neutral aqueous solution.^{43–45)} SSG showed no ¹³C-NMR signals in the neutral aqueous solution. However, both CM- and HE-SSG showed ¹³C-NMR signals in H₂O (data not shown). It is assumed that CM- and HE-substitution abolished the gel producing properties of SSG because substitution of the main chain moiety interfered with the physical crosslinking between (1→3)- β -D-glucan units. In the case of curdlan, the degree of carboxymethylation influenced biological activities, such as complement activation and antitumor activity.³³⁾ It is not clear what factors are important for SSG to show antitumor activity but certain physicochemical changes, such as gel formation induced by substitutions may be of great importance.

Acknowledgement The authors thank Mr. M. Minagawa for his technical assistance.

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