Preparation and Antitumor Activity of Hydroxyethylated Derivatives of 6-Branched (1→3)-β-D-Glucan, SSG, Obtained from the Culture Filtrate of *Sclerotinia sclerotiorum* IFO 9395

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SSG is an antitumor branched (1→3)-β-D-glucan obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395. Hydroxyethylation of SSG higher than MS 0.45 (MS value represents molar ratio of hydroxyethyl group *vs.* glucosyl group) by ethyleneoxide in aqueous sodium hydroxide lose the antitumor activity. Degradation of branching point of hydroxyethylated SSG (HE-SSG) by the sequential treatments of periodate oxidation, borohydride reduction, and mild acid hydrolysis of these derivatives regenerated the antitumor activity. These results directly demonstrated that the branching point covered, at least a part of, the dormant active site of SSG.

Keywords hydroxyethylation; β-glucan; antitumor activity; branching point; SSG; *Sclerotinia sclerotiorum*

Introduction

(1→3)-β-D-Glucan is a biological response modifier having various biological activities such as, antitumor activity, radioprotective activity, antiviral activity, and immunomodulating activities. Some of these glucans have been used clinically as antitumor drugs in Japan. SSG is an antitumor (1→3)-β-D-glucan having branches at C-6 of every other main chain glucosyl unit, obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO9395.[1a] Previously, we have made a series of derivatives of SSG having substituents of acetyl, carboxymethyl, hydroxyethyl, or periodate oxidized-borohydride reduced, *etc.* and addition of substituents have easily lost the antitumor activity of SSG.[1b] Functional importance of branches in (1→3)-β-D-glucan has long been discussed but a clear explanation has not been given yet. In the previous paper, we discussed the preparation of carboxymethylated SSG and curdlan, a linear (1→3)-β-D-glucan, having various degrees of substitution (DS) and compared their physicochemical properties and antitumor activity.[1c] It was demonstrated that 1) the DS values corresponding to gel-to-sol transition and the antitumor activity were affected by the existence of side chains; DS value to lose antitumor activity of SSG was significantly lower than that of curdlan and 2) the critical DS values for antitumor activity were higher than those for gel formation. These results supported the concept that side chains and carboxymethylated groups did not exhibit antitumor activity directly but covered the active site of the antitumor activity. Therefore, the functioning of these groups are thought to modulate the environments of active moiety, (1→3)-β-D-glucosyl residues. One problem in using carboxymethylated derivatives is the presence of anionic groups in the glucan derivatives. Carboxyl groups bury a larger area than the neutral groups. The purpose of this paper is to obtain evidence to support the modulatory effect of the branched residues on antitumor activity by using a hydroxyethyl group which produces neutral derivatives.

Materials and Methods

Preparation of SSG, Hydroxyethylated SSG (HE-SSG) and Carboxymethylated SSG (CM-SSG) SSG was obtaind from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395 by ethanol precipitation and then chromatography on diethylaminoethyl (DEAE)-Sephadex A-25 as described in the previous publication.[1a] Series of HE-SSG derivatives having different MS values (MS value represents molar ratio of hydroxyethyl group *vs.* glucosyl group) were prepared by incubating SSG (150 mg) with ethyleneoxide (2.0 ml) in aqueous hydroxide (10 ml, 0.1—7.5 N).[1b] CM-SSG having a DS value of 0.3 (DS value represents molar ratio of carboxymethyl *vs.* glucosyl group) was prepared by incubating SSG with monochloroacetic acid in 3 N aqueous hydroxide at 60 °C as described previously.[1c] MS values of HE-SSGs and DS values of CM-SSGs were determined using procedures in the literature.[1c,2]

Removal of Side Chain Glucosyl Units Side chain glucosyl units were oxidized by sodium metaperiodate, reduced by sodium borohydride, and then hydrolyzed with 50 mM sulfuric acid at 37 °C for 72 h as described previously.[1b]

Evaluation of Antitumor Activity Antitumor activity was evaluated against the solid and the ascites forms of sarcoma 180 tumor cells. (Solid form) tumor cells (5×10^6) were inoculated subcutaneously into the right groin of ICR mice (day 0). Each sample was administered five times intraperitoneally at 7, 9, 11, 13, and 15 d as a saline solution. Five weeks after tumor inoculation mice were sacrificed and the weight of each tumor compared.[1] (Ascites form) tumor cells (1×10^6) were inoculated intraperitoneally into ICR mice (day 0). Each sample was administered three times intraperitoneally at −5, −3, and −1 d as a saline solution. Survival days of each group were compared.

Other Methods Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, viscosity [η] (0.1%, 25 °C), and optical rotation $[\alpha]_D$ (0.1%, room temperature) were measured as described previously.[1] Molecular weight distribution was assessed by monitoring the quantity of a fraction passing through a 200 kilodalton (kDa) cut membrane filter (UK-200, Advantec, Tokyo, Japan).

Results

Preparation and Physicochemical Properties of HE-SSG Having Various MS Values HE-SSG was prepared by treatment with ethyleneoxide in various concentrations of aqueous sodium hydroxide. Physicochemical properties of the resulting products are summarized in Table I. Under the experimental conditions used in these experiments, a maximum MS value was obtained in 1.0 N sodium hydroxide solution. Lower as well as higher concentrations of sodium hydroxide reduced the MS value. Molecular weight of any of the products were not significantly reduced during the reaction, as assessed by the content of the retaintate from a ultrafilter (M.W. > 200 kDa). SSG as well as other (1→3)-β-glucans produce "gel" under physiological conditions. Gel-productivity of HE-SSGs were assessed by viscosity and optical rotation. Except for HE-SSG (0.2) (MS 0.45), HE-SSGs having higher MS derivatives showed none or less pH-dependent changes, suggesting no or only slight gel-productivity.

^{13}C-NMR spectroscopy is a powerful tool to assess the gel productivity of (1→3)-β-D-glucan. These gel producing

Table I. Physicochemical Properties of Hydroxyethylated Derivatives of SSG and Curdlan (CRD)

Name (Condition)[a]	MS	M.W. (%)[b]	Viscosity [η]		OR [α][c]	
			Neutral	0.25 N[d]	Neutral	0.25 N[d]
HE-SSG						
(0.1)	0.16	>200 kDa (94)	n.d.	n.d.	n.d.	n.d.
(0.2)	0.45	n.d.	2.6	1.7	42	19
(0.3)	0.80	n.d.	1.9	1.7	29	20
(0.5)	0.98	>200 kDa (97)	1.8	1.6	10	10
(1.0)	1.25	>200 kDa (99)	1.6	1.5	10	6
(3.0)	0.70	>200 kDa (99)	1.5	1.3	−10	−2
(7.5)	0.35	n.d.	n.d.	n.d.	n.d.	n.d.
HE-CRD						
(5)	0.37	>200 kDa (89)	1.1	1.1	8	8
(10)	0.42	>200 kDa (99)	1.3	1.3	8	8
(20)	1.27	>200 kDa (99)	1.5	1.3	−2	8
(50)	2.34	>200 kDa (99)	1.4	1.2	0	2

a) Experimental conditions to prepare HE-derivatives. Each number in HE-SSG and HE-CRD represent concentration (N) of sodium hydroxide and quantity (ml) of aqueous ethylene oxide, respectively. *b*) Numbers represent the percentage of the retaintate using a UK-200 membrane filter (200000 cut off). *c*) Optical rotation. *d*) Values obtained in 0.25 N sodium hydroxide.

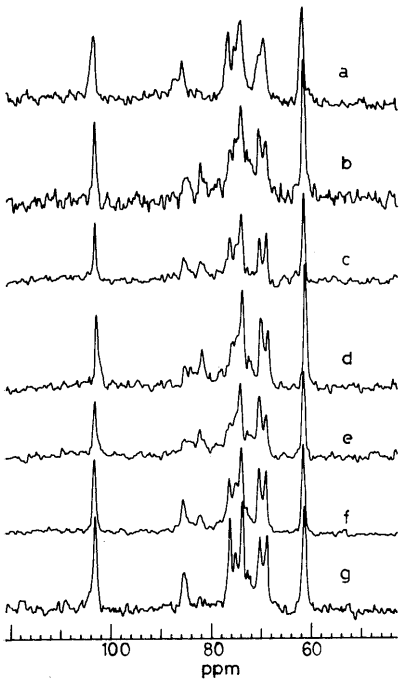

Fig. 1. ^{13}C-NMR Spectra of HE-SSGs

^{13}C-NMR spectra were measured in distilled water (add deuterium oxide for lock signal) with JEOL-FX 200 instruments as described in ref. 1. a, HE-SSG (0.1 N) (MS 0.16); b, HE-SSG (0.2 N) (MS 0.45); c, HE-SSG (0.3 N) (MS 0.80); d, HE-SSG (0.5 N) (MS 0.98); e, HE-SSG (1 N) (MS 1.27); f, HE-SSG (3 N) (MS 0.70); g, HE-SSG (7.5 N) (MS 0.35).

glucans including SSG could not exhibit sharp signals under the physiological, neutral state. Solvent or temperature induced conformational change from gel to sol induced significant spectral changes and showed well resolved signals in the sol state. ^{13}C-NMR spectra of HE-SSGs in distilled water are shown in Fig. 1. As described above, parent SSG did not show any signals under these experimental conditions. On the contrary, all of the derivatives showed well resolved spectra even under physiological, neutral conditions. Comparing each spectrum, signal widths were wider in the cases of HE-SSG (0.1) and HE-SSG (0.2).

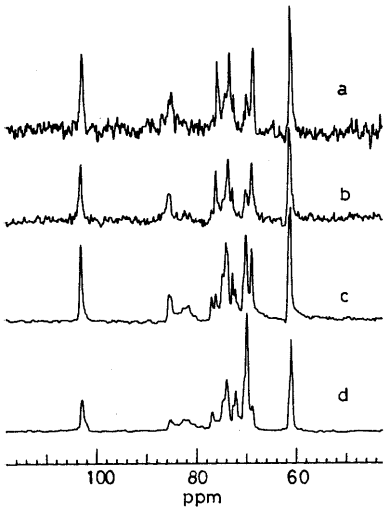

Fig. 2. ^{13}C-NMR Spectra of HE-Curdlans (CRDs)

^{13}C-NMR spectra were measured in distilled water (add deuterium oxide for lock signal) with JEOL-FX 200 instruments as described in ref. 1. a, HE-CRD (5) (MS 0.37); b, HE-CRD (10) (MS 0.42); c, HE-CRD (20) (MS 1.27); d, HE-CRD (50) (MS 2.34).

Their viscosity and optical rotation were changed dependent on pH. Spectra of HE-curdlan (Fig. 2) showed also well resolved signals, and parent curdlan showed a broad spectrum under these conditions, suggesting loss of the gel productivity even MS 0.37. These combined results suggest that HE-SSG having an MS lower than 0.45 contained a small proportion of gel-producing domain in addition to the sol-domain but other derivatives did not contain the gel producing domain. Assignment of the signals in HE-derivatives were not finished. Comparing the spectra of high and low MS value derivatives, signals around 60, 70, and 82 ppm would be closely related to the hydroxyethylation.

Antitumor Activities of HE-SSGs In the previous paper, we made HE-SSG having an MS of 0.5 and found disappearance of the antitumor activity. As shown in Table II, only the derivative having a MS 0.16 value showed antitumor activity. Even in the derivative, the activity was weaker than the parent SSG. Some of the derivatives showed relatively high percentages of inhibition, but were statistically not significant. On the contrary, HE-curdlan having an MS lower than 0.66 showed significant activity (Table III). These results support the fact that maximum substitution of HE as well as CM groups showing antitumor activity were higher in the case of curdlan.

Pretreatment of mice with SSG showed antitumor activity against the ascites form of S-180. As shown in Table IV, the activity of SSG against the ascites form of the tumor was also reduced by hydroxyethylation. In this case, the reduction of the activity was not as significant as the case of the solid form tumor. The optimum dose of SSG and derivatives to show activity were significantly higher than those against the solid form tumor. These differences would be due to some of the amplification processes of the antitumor mechanism against the solid and the ascites tumors.

Generation of Antitumor Activity from Highly Hydroxyethylated and Carboxymethylated Derivatives of SSG by Removal of the Side Chain Glucosyl Group by the Smith Type Degradation As described in the introduction, the

Table II. Antitumor Activity of Hydroxyethylated Derivatives of SSG against Solid Form of S-180[a]

Name	Dose × 5 (μg)	Tumor weight (g, mean ± S.D.)	Inhibition (%)	CR/total
SSG	20	0.3 ± 0.4[b]	96	3/7
	100	0.2 ± 0.3[b]	98	3/7
	500	0.1 ± 0.2[b]	99	3/7
HE-SSG (0.1)	20	6.2 ± 3.7	14	0/7
(MS 0.16)	100	7.4 ± 3.3	−2	0/7
	500	2.0 ± 3.1[c]	72	1/7
HE-SSG (0.2)	20	9.8 ± 6.5	−34	0/7
(MS 0.45)	100	9.1 ± 3.6	−26	0/7
	500	3.3 ± 3.3	55	1/7
HE-SSG (3.0)	20	7.4 ± 5.1	−1	0/7
(MS 0.70)	100	8.0 ± 2.3	−10	0/7
	500	4.8 ± 2.5	34	0/7
HE-SSG (0.3)	20	8.2 ± 8.5	−13	0/7
(MS 0.80)	100	8.2 ± 5.5	−13	0/7
	500	9.9 ± 6.2	−36	0/7
HE-SSG (0.5)	20	5.9 ± 2.8	19	0/6
(MS 0.98)	100	5.3 ± 5.2	27	0/6
	500	9.1 ± 4.9	−25	0/6
HE-SSG (1.0)	20	6.3 ± 4.0	13	0/7
(MS 1.25)	100	7.2 ± 3.2	1	0/7
	500	8.7 ± 3.8	−19	0/7
Nil		7.3 ± 4.7	—	0/15

a) Sarcoma 180 tumor cells (5×10^6) were inoculated sucutaneously (day 0). Glucan derivatives at the indicated doses were intraperitoneally administered on days 7, 9, 11, 13 and 15 in 0.2 ml of saline solution. The significance was evaluated according to Student's *t*-test against the corresponding control group. *b*) $p < 0.001$, *c*) $p < 0.01$. CR, complete regression.

Table III. Antitumor Activity of Hydroxyethylated Derivatives of Curdlan (CRD) against Solid Form of S-180[a]

Name	Dose × 5 (μg)	Tumor weight (g, mean ± S.D.)	Inhibition (%)	CR/total
CRD	100	7.1 ± 6.7	3	0/7
	500	2.6 ± 5.8	65	2/6
HE-CRD (5)	20	3.7 ± 4.3	49	0/7
(MS 0.37)	100	1.2 ± 1.7[b]	84	2/7
	500	0.0 ± 0.0[c]	99	3/7
HE-CRD (20)	20	9.7 ± 7.4	−33	0/7
(MS 1.27)	100	9.0 ± 5.1	−24	0/7
	500	7.9 ± 4.1	−9	0/7
HE-CRD (50)	20	9.9 ± 6.5	−36	0/7
(MS 2.34)	100	11.2 ± 3.7	−54	0/7
	500	2.8 ± 3.1	62	2/7
Nil		7.3 ± 4.7	—	0/15
HE-CRD	100	0.6 ± 0.9	94	4/10
(MS 0.66)	500	0.1 ± 0.2	99	5/10
Nil		9.0 ± 5.0	—	0/20

a) See in Table II. *b*) $p < 0.01$. *c*) $p < 0.001$. CR, complete regression.

critical DS value to show antitumor activity of CM-SSG was significantly lower than that of CM-curdlan. Comparing the structure of CM-SSG and CM-curdlan, it is hypothesized that loss of the antitumor activity of CM-SSG is due to both the presence of carboxymethyl and side chain glucosyl groups. As described above, similar to the carboxymethylated derivatives, HE-SSG lost antitumor activity at a lower MS value than HE-curdlan. These facts suggested that carboxymethyl as well as hydroxyethyl groups and side chain glucosyl group covered the active site of SSG (induce dormant active site). If it is true, removal of side chain glucosyl group of HE-SSG as well as CM-SSG

Table IV. Antitumor Activity of HE-SSG against Ascites Form of Sarcoma 180[a]

Name	Dose × 3 (μg)	Survival time (day, mean ± S.D.)	Life span (%)	CR/total
SSG	500	30 ± 12	196	4/8
	1000	29 ± 12	192	3/8
	2000	31 ± 12	207	5/8
HE-SSG (0.1)	500	19 ± 10	128	1/8
(MS 0.16)	1000	24 ± 10	157	2/8
HE-SSG (0.2)	500	24 ± 11	157	2/8
(MS 0.45)	1000	26 ± 14	172	3/8
HE-SSG (0.3)	500	14 ± 4	92	0/8
(MS 0.80)	1000	16 ± 15	106	0/8
Nil		15 ± 4	100	0/8

a) Sarcoma 180 cells (1×10^6) were transplanted intraperitoneally on day 0. Each sample was administered three times intraperitoneally at day −5, −3, and −1 as saline solution.

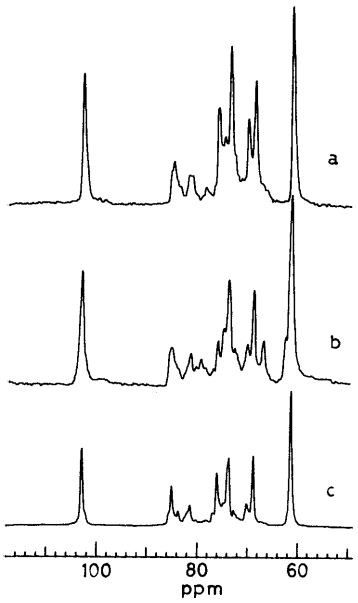

Fig. 3. ^{13}C-NMR Spectra of IBH-Derivatives of HE-SSG

^{13}C-NMR spectra were measured in distilled water (add deuterium oxide for lock signal) with JEOL-FX 200 instruments as described in ref. 1. a, HE-SSG; b, IB-HE-SSG; c, IBH-HE-SSG.

regenerate the active center of SSG and result in showing antitumor activity, because the structure of the resulting product would be similar to HE- and CM-curdlan, respectively.

HE-SSG having an MS value higher than about 0.2 lost its antitumor activity (Table II). The MS value of HE-SSG used in this experiment was 0.58. Physicochemical properties of derivatives obtained by periodate oxidation, borohydride reduction and mild acid hydrolysis were shown in Table V. Molecular weight as well as viscosity of derivatives were not changed by this treatment, suggesting only little breakage of main chain moiety during these treatments. ^{13}C-NMR spectra of these derivatives were shown in Fig. 3 and the appearance of a signal at *ca.* 67 ppm in oxidized/reduced HE-SSG and the disappearance of the signal after mild acid hydrolysis indicated the complete removal of oxidized residues. In contrast to the CM-derivatives as described later, MS value was reduced after this treatment, suggesting the higher distribution of HE-groups in the side chain. Antitumor activity of these

TABLE V. Physicochemical Properties of IBH-Derivatives

Name	MS or DS	M.W. (%)[a]	Viscosity [η]	OR $[\alpha]_D$[b] Neutral	0.25 N[c]
HE-SSG	0.58	>200 k Da (96)	1.17	13	10
IB-HE-SSG	0.46	>200 k Da (88)	1.05	21	20
IBH-HE-SSG	0.28	>200 k Da (82)	1.05	21	13
CM-SSG	0.31	>200 k Da (75)	1.02	2	6
IB-CM-SSG	0.32	>200 k Da (57)	1.07	6	14
IBH-CM-SSG	0.31	>200 k Da (56)	1.01	−4	n.d.

a—*c*) See in footnotes in Table I.

TABLE VI. Antitumor Activity of IBH-Derivatives against Solid Form of Sarcoma 180[a]

Name	Dose × 5 (g)	Tumor weight (g, mean ± S.D.)	Inhibition (%)	CR/total
SSG	20	1.2 ± 3.1[b]	85	1/8
	100	2.3 ± 3.8[c]	73	1/8
HE-SSG	100	8.9 ± 6.3	−9	0/8
	500	11.4 ± 5.7	−40	0/8
IBH-HE-SSG	100	1.7 ± 3.6[c]	80	0/7
	500	1.6 ± 2.6[b]	81	4/8
Nil		8.2 ± 5.5	—	0/15
SSG	100	0.6 ± 1.1[b]	90	5/10
	500	0.2 ± 0.5[b]	98	8/10
CM-SSG	100	4.4 ± 4.1	26	1/10
	500	4.0 ± 2.3	33	0/10
IBH-CM-SSG	100	0.2 ± 0.6[b]	97	7/10
	500	2.1 ± 3.5[b]	65	6/10
Nil		5.9 ± 5.2	—	0/20

a) See footnotes in Table I for the experimental conditions. The derivatives shown in Table V were used in this experiment. *b*) $p > 0.001$, *c*) $p > 0.01$.

derivatives are shown in Table VI. HE-SSG exhibited no antitumor activity but the activity was generated in the case of IBH-HE-SSG. These results support the above concept that a side chain of SSG covered the antitumor active site of SSG to produce dormant active sites.

A similar approach was used for CM-SSG. As shown in the previous paper, a DS of more than 0.3 significantly reduce the antitumor activity of SSG.[1c] In this paper, CM-SSG having DS of 0.3/glucose unit were prepared. As shown in Tables V and VI, this preparation showed no gel-formability and also showed no antitumor activity. These properties corresponded to the previous publications. To obtain the direct evidences of the contribution of branching points on the antitumor activity of CM-SSG, periodate oxidation and borohydride reduction were performed and then the oxidized side chain groups were hydrolyzed by mild acid. The molecular weight of the derivative indicated the limited breakage of main chain during this procedure. Similar to the cases of HE-SSGs, ^{13}C-NMR spectra of these derivatives showed a signal at 67 ppm in oxidized/reduced CM-SSG and disappeared after mild acid hydrolysis, indicating the complete removal of oxidized residues (data not shown). DS values were nearly the same during this treatment (Table V). These results suggested that a majority of the side chain groups were removed during this treatment and that carboxymethyl groups were distributed equally in main chain and side chain units of SSG. Viscosity and optical density of these derivatives suggested that all of the products retained their "sol" property under the physiological conditions. The physicochemical properties of these derivatives were consistent with those of the CM-curdlan ("sol" property in this range of DS values). Table VI shows the antitumor activity of these derivatives. CM-SSG did not show any antitumor activity as expected and the activity was regenerated only in IBH-CM-SSG. This observation clearly demonstrated that removal of the side chain moiety, at least a part, changed the dormant active site of SSG to the active form.

Discussion

The data shown in this paper directly demonstrate the function of side chain moiety of SSG on antitumor activity, that side chain glucosyl groups covered the antitumor active site of SSG to produce a dormant active site. In the previous paper, we have demonstrated that the upper limit of DS values of carboxymethylation to maintain antitumor activity for SSG was much lower than that of curdlan. Theoretically, the structure of side chain-removed CM-SSG or HE-SSG would be quite similar to CM-curdlan or HE-curdlan, thus it would show antitumor activity. The concept leading from the data shown in this paper was also theoretically supported. Loss of the antitumor activity of (1→3)-β-D-glucan by extensive substitution was also shown by Matsuzaki *et al.*[3]

Commercially available curdlan, a linear (1→3)-β-D-glucan, did not shown antitumor activity in our assay system and was hardly soluble in water. Hydroxyethylated or carboxymethylated curdlan were readily soluble in water and showed significant antitumor activity.[1c] As shown in Table III, antitumor activity of HE-curdlan derivatives having various MS values confirmed that with significant substitution there is a loss in the antitumor activity. The reason was not examined in detail why the parent curdlan did not show the antitumor activity. However, solubility of curdlan might be one of the important reasons. Considering the fact that these derivatives did not produce "gel" under the physiological conditions, gel productivity would not strongly correlated with the antitumor activity. In the case of branched glucans such as SSG, and grifolan, DS value of the gel to sol transition and that of the loss of antitumor activity was similar. Considering these facts, the similarity of DS (MS) values to lose antitumor activity and the gel productivity would not correlate directly. These results suggested that one role of side chain group is increasing the solubility of (1→3)-β-D-glucan, but extensive substitution covered the active site of the glucan (making a dormant active site). The structural requirements of (1→3)-β-D-glucans to show antitumor activity is still hard to define. The concept shown in this paper that some of the side chain group produced a "dormant active site" do give a clearer explanation. However, (1→3)-β-D-glucan could not produce a certain specific ultrastructure like "enzyme protein," thus the concept still contains some ambiguity.

The hydroxyl group at C-2 of main chain glucosyl group would be quite important to produce hydrogen bonding between each chain. Substitution of any group at C-2 position would result in loss of the gel productivity. C-2 substitution seems not to be critical for the antitumor

activity, because CM-(HE-)curdlan having no gel productivity exhibited significant antitumor activity.

Antitumor activity of (1→3)-β-D-glucan is classified as an "indirect effect" *via* the modulation of the host immune systems. Many examinations regarding the biological mechanisms of these glucans have already been done, however, little is known about the direct effect (the very first reactions(s) between glucan and the host components) of these glucans to the host. *In vitro* study including reactions to blood constituents and coagulation systems would give helpful information.[4] Precise examination is still required to learn the action of (1→3)-β-D-glucan at the molecular level.

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