Physicochemical Properties and Antitumor Activities of Chemically Modified Derivatives of Antitumor Glucan "Grifolan LE" from *Grifola frondosa*

Yoshiyuki ADACHI,[a] Naohito OHNO,[a] Masumi OHSAWA,[b] Kichiro SATO (deceased),[b] Shozo OIKAWA[b] and Toshiro YADOMAE*,[a]

Tokyo College of Pharmacy,[a] Horinouchi, Hachioji, Tokyo 192-03, Japan and Nippon Beet Sugar Mfg. Co., Ltd.,[b] Kyobashi, Chuo-ku, Tokyo 104, Japan. *Received October 31, 1988*

Antitumor glucan, grifolan LE (GRN LE), from *Grifola frondosa* was chemically modified to examine the structure-function relationship of the products. Modification by periodate, borohydride and acid hydrolysis of side chains of GRN LE did not alter properties such as helical conformation and antitumor activity of GRN LE. Introduction of carboxylic acid groups into the side chains by oxidation with periodate and with sodium chlorite (GRN LE-PC), and substitution with carboxymethyl (CM) or hydroxyethyl (HE) groups abolished the gel-forming ability of GRN LE. Significant antitumor activity was observed in all of the derivtives having gel-forming ability as well as some derivatives having no such ability. These results suggested that essential factors required for antitumor activity were (1→3)-β-D-glucosyl linkages and high molecular weight, and that accessory groups could be linked to the main chain without loss of antitumor activity in a higher ratio than that of gel-forming ability.

Keywords *Grifola frondosa*; (1→3)-β-D-glucan; antitumor activity; grifolan; conformation; chemical modification

Introduction

It is generally accepted that antitumor (1→3)-β-D-glucans possess an ordered ultrastructure including a triple helix.[1] Many methods have been applied to elucidate the ultrastructure of these glucans, such as x-ray analysis in crystalline state,[2] cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance (CP/MAS ^{13}C-NMR) in the gel[3] or solid state,[4] ^{13}C-NMR analysis to assess molecular movement during gel to sol transition,[5] viscosity measurement,[6] and utilizing dyes (*e.g.* congo red[7] or aniline blue[8]). Chemical modifications involving substitution of hydrophilic groups such as carboxymethyl (CM), hydroxyethyl (HE) or sulfate at hydroxyl groups of the glucan chain have been made in attempts to solubilize the poorly soluble helical glucans. For example, CM-curdlan,[9] CM-pachymaran,[10] and HE-pachyman[11] are typical derivatives. In the case of CM-curdlan, the degree of substitution of CM groups was important for antitumor activity, and highly substituted curdlan did not show any significant antitumor effect.[9] It would be very interesting to investigate whether the physicochemical properties and antitumor activity of (1→3)-β-D-glucans change concomitantly with modification or not.

We have reported the antitumor activities and the structure of grifolan (GRN) from *Grifola frondosa*[12]; GRN is a (1→6)-branched (1→3)-β-D-glucan and the primary structure is very similar to those of schizophyllan[13] and scleroglucan.[14] GRN has a helix structure similar to that of curdlan as assessed by CP/MAS ^{13}C-NMR in the solid state.[15] In the present paper, we have investigated the relationship between the physicochemical properties and the antitumor effect of chemically modified derivatives of GRN.

Materials and Methods

Materials Sodium metaperiodate, sodium borohydride, sodium monochloroacetate, and monochloroacetic acid were purchased from Wako Chemical Co., Ltd. (Osaka, Japan).

Preparation of GRN LE-I, GRN LE-I/B, and SD-GRN LE-I/B GRN LE (1 mg/ml, 1000 ml) was oxidized with 10 mM sodium metaperiodate at 4 °C in the dark; periodate consumption was monitored by the method of Avigad.[16] To terminate the reaction, excess ethylene glycol was added, and the mixture was dialyzed against tap water for 2 d and distilled water for 1 d. The non-dialyzable fraction was named GRN LE-I (yield; 89.7%). A part of GRN LE-I was reduced with sodium borohydride at 4 °C for 48 h. After acidification with acetic acid, the mixture was dialyzed and lyophilized to give GRN LE-I/B (yield; 85.0%). A portion of GRN LE-I/B was partially hydrolyzed with 0.05 M sulfuric acid at 25 °C for 72 h. The mixture, after neutralization, was dialyzed against distilled water for 2 d. The non-dialyzable fraction was concentrated, and lyophilized (SD-GRN LE-I/B, yield; 84.3%).

Preparation of GRN LE-PC Poly-carboxylated GRN LE (GRN LE-PC) was prepared by the method Hofreiter *et al.*[17] as follows: GRN LE (1000 mg) was oxidized under the same conditions as GRN LE-I/B (170 h), and further oxidized with 40 mM sodium chlorite at pH 4.0 (adjusted with acetic acid) for 12 h. The mixture was dialyzed against distilled water for 3 d and lyophilized to obtain GRN LE-PC (yield 93.3%).

Preparation of CM-GRN LE CM-GRN LE (HDS): A suspension of 0.6 g of the glucan in 16 ml of 2-propanol was stirred at room temperature for 30 min. Then, 1.6 ml of a 30% solution of sodium hydroxide was slowly added with stirring over a period of about 15 min. Vigorous stirring was continued at room temperature for about 90 min to prevent gel formation. Then, 0.72 g of monochloroacetic acid was added and the mixture was stirred at 50 °C for 150 min. The product was diluted with 16 ml of water, dialyzed and lyophilized. (yield; 94.0%)

CM-GRN LE (LDS): A solution of GRN LE (700 mg) in 1 N sodium hydroxide was treated with sodium monochloroacetate, and the mixture was stirred at 50 °C for 3 h. The reaction was terminated by adding acetic acid until neutral pH, and the non-dialyzable fraction was lyophilized. (yield; 92.1%).

Preparation of HE-GRN LE GRN LE was dissolved in 10 ml of 0.5 N sodium hydroxide (50 mg/ml), and 4 ml of 30% sodium hydroxide solution was added at 0 °C. The mixture was kept at −10 °C in a dry ice bath, 10 ml of ethylene oxide was added, and the whole was stirred for 2 h, further stirred at 4 °C overnight, and neutralized with acetic acid. The non-dialyzable fraction was concentrated and lyophilized to obtain HE-GRN LE (yield; 119.2%).

^{13}C-NMR Spectra ^{13}C-NMR spectra were measured with JEOL FX-200 instruments. The NMR spectra were measured using 10 i.d. sampling tubes at room temperature for aqueous solution and at 60 °C for dimethyl sulfoxide-d_6 (DMSO-d_6) solution. All spectra were obtained from 5000 to 100000 scans.

Fluorescence Measurement of Glucan–Aniline Blue Complex The glucans were dissolved in aniline blue solution (10 μg/ml; 0.1 M sodium hydroxide), and the intensity of fluorescence was measured at excitation at 400 nm and emission at 500 nm using a Hitachi 650-40 fluorescence spectrophotometer.

Viscosity Measurement Glucan samples were dissolved in distilled water (5 mg/ml, 15 ml) and the flow time of sample solution in an Ostwald-type viscometer was recorded at 25 °C in a water bath. All measurements were performed after equilibration of the solution at 25 °C. Under these

conditions, the influence of alkali on the molecular weight of the glucans was negligible.

Assay of Antitumor Activity Male ICR mice (6 weeks old and weighing 27—30 g) were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, and were bred under specific pathogen free (SPF) conditions. Sarcoma 180 cells were maintained serially in ascites form by weekly passage in ICR mice. Sarcoma 180 cells (5×10^6) were inoculated subcutaneously into the right groin of ICR mice (day 0). Each glucan sample was dissolved in saline and administered intraperitonealy on every other day from day 10 to 18 (5 times). After 5 weeks, the mice were killed and the tumors were excised. The inhibition ratio was calculated as follows:

$$\left[1 - \frac{\text{average tumor weight of the treated group}}{\text{average tumor weight of the control group}}\right] \times 100\ (\%)$$

General Methods Purification of GRN LE, methylation analysis, and other physicochemical methods were performed as described previously.[12]

Results

Preparation of Derivatives of GRN LE GRN LE was oxidized with sodium metaperiodate for various time (8, 18, and 170 h) to assess the contribution of degree of branching to antitumor activity. After reduction with $NaBH_4$ three type of samples named GRN LE-I/B (8 h), (18 h), and (170 h) were obtained. The polyol groups of GRN LE-I/B were hydrolyzed to prepare SD-GRN LE-I/B. The degree of branching of each derivative assessed by methylation analysis[18] is shown in Table I. The extents of oxidation of side chain glucosyl residues of GRN LE-I/B (8 h), (18 h), and (170 h) were 68.9%, 91.1%, and 100%, respectively. The degrees of branching of the SD-GRN LE-I/B (8 h), 18 h), and (170 h) were 0.13, 0.09 and 0.08, respectively, as estimated from the peak heights of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-dimethyl-glucitol. Glycerol was observed in the dialyzable fraction after the acid hydrolysis (data not shown). The structure of SD-GRN LE-I/B (170 h) was analyzed by ^{13}C-NMR (Fig. 1) in DMSO-d_6. The spectrum of SD-GRN LE-I/B (170 h) was similar to that of curdlan,[5] a linear (1→3)-β-D-glucan, and suggested that the side chain moiety of GRN LE had been almost wholly removed by the acid hydrolysis.

TABLE I. Gas Liquid Chromatography of Alditol Acetates Derived from the Methylated Derivative of GRN LE

Sample (Reaction time)	Alditol acetate of		
	2,3,4,6-Me_4-Glc	2,4,6-Me_3-Glc	2,4-Me_2-Glc
GRN LE-I/B			
(8 h)	0.14	1.00	0.45
(18 h)	0.04	1.00	0.36
(170 h)	0	1.00	0.37
SD-GRN LE-I/B			
(8 h)	0.08	1.00	0.19
(18 h)	0.04	1.00	0.14
(170 h)	0	1.00	0.12
GRN LE	0.45	1.00	0.50

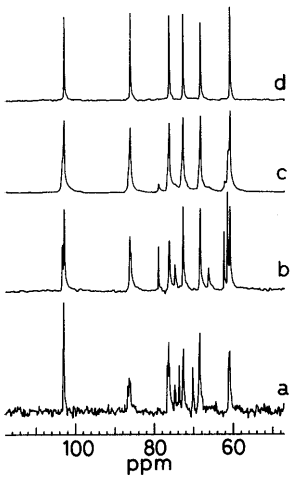

Fig. 1. ^{13}C-NMR Spectra of Side Chain Modified GRN LE in DMSO-d_6

Each glucan (40 mg) was dissolved in DMSO-d_6 (2 ml) and the ^{13}C-NMR spectrum was measured at 60 °C. a, GRN LE; b, GRN LE-I/B (170 h); c, SD-GRN LE-I/B (170 h); d, curdlan.

To obtain a less substituted CM-GRN LE (CM-GRN LE (LDS)), GRN LE was dissolved in 1 N NaOH and mixed with sodium monochloroacetate. In contrast, to prepare highly substituted CM-GRN LE (HDS), the reaction was performed in 2-propanol similarly to the case of CM-pachymaran.[10] The degrees of substitution of CM-GRN LE (LDS) and (HDS) were determined by Eyler's method[19] and were 0.25 and 0.51, respectively.

Hydroxyethyl GRN LE (HE-GRN LE) was prepared by the use of ethylene oxide in NaOH. The degree of substitution of HE-GRN LE was estimated to be 0.62 by gas chromatography according to Hodges *et al.*[20]

Polycarboxylated derivative of GRN LE (GRN LE-PC) was prepared by the methods of Hofreiter *et al.*[17] and Crescenzi *et al.*[21] The side chain glucosyl residues of GRN LE were oxidized with periodate and sodium chlorite to carboxylic acid (GRN LE-PC). Absorption of GRN LE-PC on DEAE-Sephadex A-25 (Cl^-) (Fig. 2) confirmed the introduction of polyanionic groups into the glucan by this procedure.

Physicochemical Properties of Derivatives Physicochemical properties of these derivatives were compared with those of GRN LE by using ^{13}C-NMR (Fig. 3, 4), aniline blue (Table II), and viscosity measurements (Fig. 5). Figure 3 shows the spectral changes of GRN LE-I (170 h), GRN LE-I/B (170 h) and SD-GRN LE-I/B (170 h) depending on the concentration of alkali. These derivatives did not show signals attributable to β-1,3-linkage in distilled water. However, in NaOH solution ranging from 0.05 N to 0.15 N these derivatives, except for GRN LE-I,

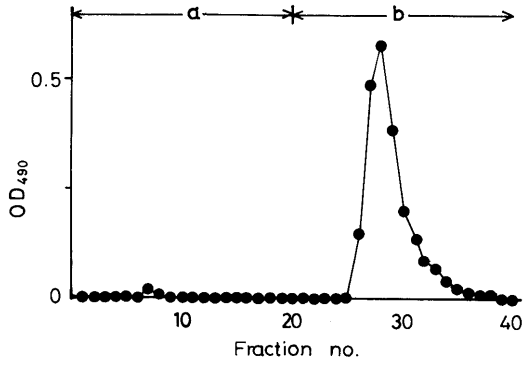

Fig. 2. Elution Profile of GRN LE-PC from a Column of DEAE-Sephadex A-25 (Cl^-)

The column (1.9 × 7.0 cm) was equilibrated with water, and 1.7 mg of GRN LE-PC was applied. After being washed with H_2O, the column was eluted with 2 M NaCl. Fractions of 1.72 ml were collected and carbohydrate was monitored by the phenol–H_2SO_4 method.

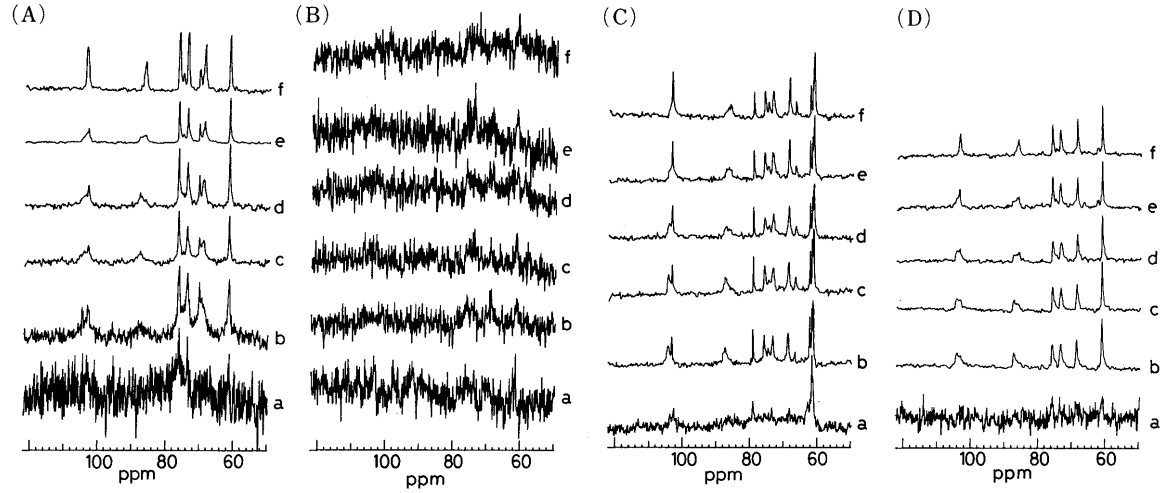

Fig. 3. Gel-to-Sol Transition of GRN LE(A), GRN LE-I(B), GRN LE-I/B(C) and SD-GRN LE-I/B(D) in Sodium Hydroxide Solution Measured by ^{13}C-NMR Spectroscopy

Each glucan (40 mg) was dissolved in distilled water (2 ml) and sodium hydroxide (4 N) was added to the indicated normality, then the ^{13}C-NMR spectrum was measured. a, 0 N; b, 0.05 N; c, 0.1 N; d, 0.15 N; e, 0.2 N; f, 0.25 N.

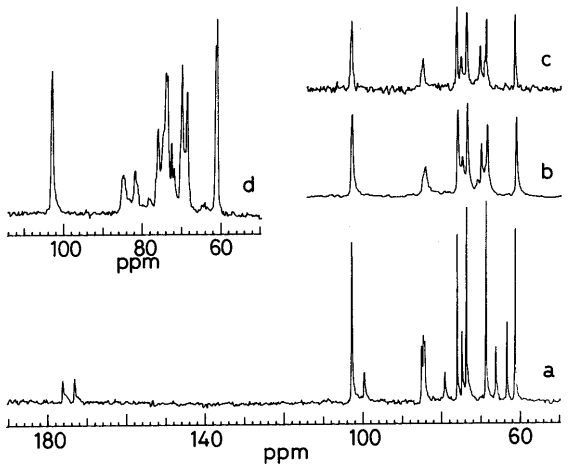

Fig. 4. ^{13}C-NMR Spectra of GRN LE Derivatives in H_2O

Each glucan (40 mg) was dissolved in distilled water (2 ml) and the ^{13}C-NMR spectrum was measured at 25 C. a GRN LE-PC; b, CM-GRN LE (LDS); c, CM-GRN LE (HDS); d, HE-GRN LE.

showed C-3 signal peak at 88 ppm, suggesting the presence of single-helical conformation.[10] At more than 0.2 N, the C-3 signal of GRN LE-I/B and SD-GRN LE-I/B shifted to 86 ppm, quite similar to that of GRN LE. From these results, GRN LE-I/B and SD-GRN LE-I/B were suggested to take a similar conformation to GRN LE. GRN LE-I was not soluble even in 0.25 N NaOH.

Figure 4 shows the ^{13}C-NMR spectra of CM-GRN LE, HE-GRN LE and GRN LE-PC. All the derivatives showed sharp signals in distilled water and gave C-3 signals at 85 ppm, suggesting the presence of a significant amount of random coil segment. Moreover, the C-3 signal of GRN LE-PC separated into a triplet which was the same as that of GRN LE dissolved in DMSO, also suggesting higher mobility of the glucan chain even in aqueous solution.[22]

The fluorescence intensity of glucan–aniline blue complex in dilute alkaline solution is closely related to the existence of single-helical conformation in (1→3)-β-D-glucans. Table II shows the fluorescence intensity of glucan–dye complex.

Table II. Fluorescence Intensity of Aniline Blue Admixed with Chemically Modified GRN LE[a]

Sample	Relative intensity (%)
GRN LE-I/B (170 h)	112.0
SD-GRN LE-I/B (170 h)	113.2
CM-GRN LE (LDS)	7.4
(HDS)	6.9
HE-GRN LE	12.5
GRN LE-PC	0.2
GRN LE	100.0

a) Three milliliter of aniline blue solution (10 μg/ml 0.1 N NaOH) was mixed with several derivatives (100 μg/ml) and the fluorescence intensity was recorded. Each value was calculated from the intensity of GRN LE, taken as 100%.

Each derivative was admixed with aniline blue solution and the fluorescence intensity was compared with GRN LE used as a standard. GRN LE-I/B (170 h) and SD-GRN LE-I/B (170 h) showed high fluorescence intensity comparable to that of GRN LE. Other derivatives, which were assessed as having random coil structure by ^{13}C-NMR, did not exhibit high intensity compared with GRN LE. These results also supported the results obtained from ^{13}C-NMR analyses, *i.e.*, that the conformation of derivatives modified by periodate oxidation was the same as that of GRN LE and the conformation of derivatives modified by substitution with carboxylic or hydroxyl groups was disordered.

Viscosity is also a sensitive parameter to assess the gel-to-sol transition of (1→3)-β-D-glucans. The change of viscosity induced by the addition of alkaline solution is consistent with the gel-to-sol transition. Each sample was dissolved in water at 5 mg/ml and the viscosity was measured at various concentrations of sodium hydroxide. As shown in Fig. 5, GRN LE exhibited high viscosity in the range of 0 to 0.15 N, and a marked decline of viscosity occurred at more than 0.2 N. This result is quite similar to other reports in which sol to gel transition of curdlan was observed around 0.2 N sodium hydroxide.[6] On the other hand, GRN LE-PC, CM-GRN LE (LDS) and (HDS), and

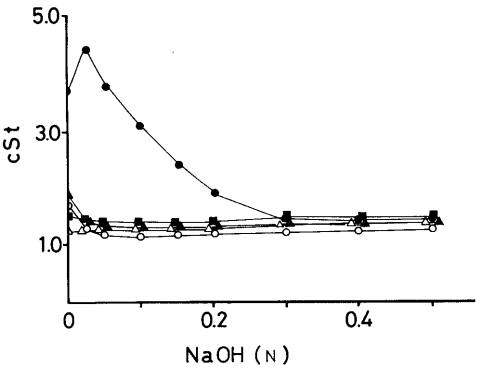

Fig. 5. Viscosity of Chemically Modified GRN LE Solutions in Various Concentrations of Sodium Hydroxide

Each glucan (75 mg) was dissolved in 15 ml of distilled water and sodium hydroxide (7.5 N) was added to the indicated normality. ●, GRN LE; ▲, CM-GRN LE (LDS); △, CM-GRN LE (HDS); ■, HE-GRN LE; ○, GRN LE-PC.

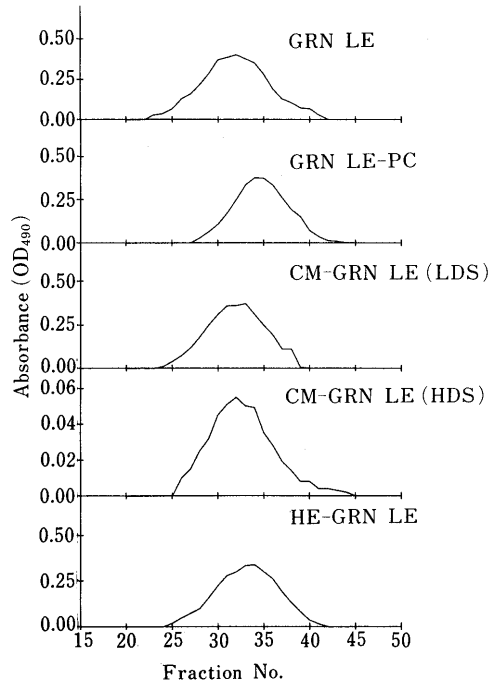

Fig. 6. Elution Profiles of GRN LE and Its Derivatives from a Column of TSK GEL HW-65F

Each derivative (2.5 mg) was dissolved in 1 ml of 0.3 N sodium hydroxide and eluted with the same solvent from a column of TSK GEL HW-65F (1.5 × 73 cm). Fractions (2.5 ml) were assayed by the phenol–sulfuric acid method: the void fraction and glucose was eluted at fraction No. 23 and No. 41 respectively.

HE-GRN LE showed very low viscosity even in neutral solution and thus did not exhibit gel-to-sol transition like GRN LE. This result was compatible with the observations obtained by ^{13}C-NMR and by using aniline blue.

In order to confirm the absence of degradation of glucan chain during chemical modification, the molecular weight of soluble derivatives was measured by using a gel filtration column equilibrated with 0.3 N sodium hydroxide. As shown in Fig. 6, the elution profiles were similar to each other. The molecular weights of derivatives were estimated to be as follows: GRN LE, 500000; GRN LE-PC, 240000; CM-GRN LE (LDS) and (HDS), 440000; HE-GRN LE, 240000. These results indicate that limited degradation occurred during chemical modifications. Because the minimum molecular weight of GRN LE required antitumor activity is 34000,[21] each derivative seemed to have sufficient molecular weight to show antitumor activity.

Table III. Antitumor Activity of Chemically Modified GRN LE against Sarcoma 180[a]

Sample	Dose × 5 (μg/mouse)	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)[b]	Complete regression
GRN LE	20	0.05 ± 0.06[e]	99	5/10
GRN LE-I	20	4.91 ± 2.80	27	0/10
(170 h)	100	6.47 ± 3.70	37	0/10
Nil	—	6.72 ± 5.22	—	0/14
GRN LE-I/B (170 h)	20	0.12 ± 0.11[c]	98	1/10
SD-GRN LE-I/B	20	0.01 ± 0.02[c]	>99	8/10
(8 h)	100	0.16 ± 0.36[c]	97	5/10
SD-GRN LE-I/B	20	0.18 ± 0.31[c]	97	6/10
(18 h)	100	0.11 ± 0.17[c]	98	1/10
SD-GRN LE-I/B	20	0.02 ± 0.05[c]	>99	8/10
(170 h)	100	0.02 ± 0.03[c]	>99	3/10
Nil	—	5.96 ± 3.10	—	0/20
CM-GRN LE	10	5.31 ± 3.16	12	0/10
(D.S. 0.25)	50	2.42 ± 2.67[d]	60	2/10
	250	1.95 ± 2.28[d]	68	3/10
(D.S. 0.51)	10	6.96 ± 3.28	−16	0/10
	50	6.14 ± 3.39	−2	0/10
	250	4.25 ± 2.85	29	0/10
HE-GRN LE	10	6.33 ± 2.82	−6	0/10
	50	3.98 ± 3.03	34	0/10
	250	4.36 ± 3.37	27	0/10
Nil	—	6.00 ± 4.00	—	0/26
GRN LE-PC	10	0.85 ± 2.18[d]	87	1/10
	50	0.16 ± 0.16[d]	98	2/10
	250	1.98 ± 1.57[c]	69	0/10
Nil	—	6.29 ± 4.64	—	0/10

a) Sarcoma 180 cells (5×10^6) were inoculated subcutaneously (day 0). A sample was administered as a saline solution by intraperitoneal injection on days 10, 12, 14, 16, and 18. *b*) Inhibition ratio 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, $p<0.05$. *d*) $p<0.01$. *e*) $p<0.001$.

Antitumor Activity of Soluble Derivatives Table III shows the antitumor activity of the above derivatives in a murine transplantable tumor system. GRN LE-I/B and SD-GRN LE-I/B showed remarkably high antitumor activities comparable to GRN LE. GRN LE-PC also showed significant antitumor activity, though it had different physicochemical properties from GRN LE. CM-GRN LE exhibited antitumor activity only in the case of CM-GRN LE (LDS) at a dose of 250 μg/mouse, for 5 times. HE-GRN LE showed no antitumor activity in this system.

Discussion

Various chemical modification studies of antitumor glucans have been carried out to elucidate structure–function relationships. Pachyman from *Poria cocos* is a (1→3)-β-D-glucan showing no antitumor activity in the native state, but removal of its side chains by a series of chemical modifications involving periodate oxidation, reduction by borohydride and acid hydrolysis, yielded an antitumor glucan termed pachymaran.[23] Misaki *et al.* also reported an alkali-insoluble glucan from *Auricularia auricula-judae* (glucan II), which had no antitumor activity, but became active after modification of its side chains.[24] These observations suggest that the side chain moiety of branched (1→3)-β-D-glucans is important for the antitumor activity.

Some investigators suggested that introduction of carboxyl groups into antitumor (1→3)-β-D-glucans (scleroglucan and schizophyllan) possessing triple helical conformation by oxidation of side chains or substitution with phthalic acid induced disordered structure.[21,25] Antitumor activity of pachyman and pachymaran could be induced by hydroxyethylation[11] or carboxymethylation.[10] Curdlan, a linear (1→3)-β-D-glucan, also has no antitumor activity,[26] but carboxymethylation of provided active derivatives.[26] These findings might be correlated to some change of the physicochemical properties of the parent glucan caused by the substitution with hydrophilic groups. Therefore, we prepared GRN LE-I/B, SD-GRN LE-I/B, GRN LE-PC, CM-GRN LE (LDS and HDS), and HE-GRN LE to examine whether modification of the side chains and substitution with hydrophilic groups causes conformational change and/or affects the antitumor activity of GRN LE. In order to assess the conformation of the derivatives at in the solution state, we used ^{13}C-NMR, aniline blue and viscosity measurements. Saito *et al.* reported that ^{13}C-NMR analyses could offer information on conformational changes of gel-forming antitumor (1→3)-β-D-glucans.[5] In their reports, antitumor glucans such as lentinan, curdlan, and schizophyllan showed broad signals suggesting physical cross-linking of glucosyl main chains in the gel state. In contrast, relatively sharp signals were observed in the case of randomly coiled (1→3)-β-D-glucans which were denatured by adding NaOH, DMSO, or urea, or which had low molecular weight.[5] This alteration of the signals, from broad to sharp, is considered to be a result of ultrastructural change in which ordered structure is transformed to a disordered state.

Wood and Fulcher reported that fluorescence was emitted by admixing of aniline blue and glucans having ordered conformation in dilute alkaline solution, and that the fluorescence intensity was diminished when ordered conformation was destroyed by higher concentrations of alkali.[8] In our previous paper, we reported that GRN LE showed strong fluorescence in the presence of aniline blue and the fluorescence intensity decreased with the reduction of molecular weight.[27] Therefore we thought that fluorescence measurement of aniline blue might be a suitable method to determine the conformation of the derivatives.

GRN LE-I/B and SD-GRN LE-I/B were suggested to have helical conformation as assessed by ^{13}C-NMR, and fluorometric analysis. The antitumor activity of both derivatives was significant, as was that of GRN LE. GRN LE-I had different physicochemical properties and activity from GRN LE. From the above results, it was suggested that the modification of the side chain moiety to polyalcohol or elimination of the side chain by mild acid hydrolysis did not significantly affect the conformation and activity of GRN LE, whereas the insoluble derivative (GRN LE-I) was not effective.

GRN LE-PC showed clear NMR signals suggesting that GRN LE-PC took a random coiled conformation even in water. GRN LE-PC exhibited significantly less fluorescence intensity with aniline blue as compared with GRN LE. No viscosity change of GRN LE PC was observed on adding of sodium hydroxide. These data suggested that the introduction of the carboxylic acid group on the side chain moiety of GRN LE effectively to changed the original helix conformation.

Chemical modification by substitution with CM and HE groups changed the helical conformation into a random coiled one assessed by using ^{13}C-NMR, aniline blue, and viscosity measurements. These substituted derivatives showed similar results to GRN LE-PC. It was assumed that substitution occurred at hydroxyl groups on both main chain glucosyl residue and side chains. Since the modifications were performed in sodium hydroxide solution in which glucan strands existed as random coils, the hydroxyl groups at C-2, C-4, C-6 of main chain glucose and C-2, C-3, C-4, C-6 of side chain glucose should be accessible for substitution with CM or HE. Our previous data using CM-curdlan also supported this possibility.[26] This is due to the fact that the helical conformation of (1→3)-β-D-glucans was stabilized by hydrogen bonds involving the hydroxyl groups at C-2.[2] It is reasonable to consider that a substituted hydroxyl group could no longer form such a cross-linking hydrogen bond.

GRN LE-PC and CM-GRN LE (LDS) showed significant activities. However, highly substituted derivatives, CM-GRN LE (HDS) and HE-GRN LE, showed no activity. Disappearance of the antitumor activity of CM- and HE-derivatives was not due to extensive degradation of the main chain during the reaction, because the molecular weights of the products were similar to that of the parent glucan (Fig. 6).[28] These observations also suggested that some of the randomly coiled (1→3)-β-D-glucan could exihibit antitumor activity. The degree of substitution of CM-curdlan is known to be closely related to the antitumor activity and the derivatives with a DS value of 1.0 per glucose residue lost the activity.[9] The reason why highly substituted CM-curdlan did not show significant activity remains to be elucidated. If there is a certain structural unit (including conformation) which is specifically recognized by host immune systems, it can be speculated that the derivatives could no longer interact with phagocytes or other components to induce antitumor activity. In the case of CM-GRN LE and HE-GRN LE, a similar explanation may be applicable, although the DS values of CM-GRN LE and HE-GRN LE resulting in loss of antitumor activity were less than that of CM-curdlan[9] or HE-pachyman.[11] This difference would be due to the presence of 6-branched side chain moieties of the glucans. GRN LE-PC has a disordered structure as measured by several analyses. We can not conclude at present that even a random coiled glucan can show antitumor activity, because we can not eliminate the possibility that the *in vivo* conformation of GRN LE-PC is different from that observed *in vitro*. Namely, some polyelectrolytic polymers are known to form ordered conformation when chelated with calcium ion.[21,29] We have demonstrated that GRN LE-PC could be insolubilized by admixing calcium chloride (data not shown). It is possible that GRN LE-PC, and also CM-GRN LE, may acquire changed into ordered conformation *in vivo*. Another possibility is that GRN LE-PC may have another pathway to activate host defense systems, which can be activated by a random-structured glucan. Hamuro *et al.* reported the inability of CM-pachymaran to stimulate peritoneal exudate cells[30] and to activate the complement pathway.[11] GRN LE-PC also could not activate macrophages or the alternative complement pathway *in vitro*

(data not shown). Further work is necessary to establish the activation mechanisms induced by GRN LE-PC.

In conclusion, polyhydroxy groups at 0—6 of (1→3)-linked D-glucosyl residues (GRN LE-I/B) and a decrease in the degree of branching (SD-GRN LE-I/B) did not affect the native conformation, whereas polycarboxyl residues (GRN LE-PC) altered the helical conformation to random coil. Substitution by CM and HE groups on GRN LE (CM-GRN LE and HE-GRN LE) also affected the original conformation. The essential factors for effective derivatives of GRN LE are solubility in water or alkali, high molecular weight, and intact (1→3)-β-D-glucosyl linkage.

Acknowledgment We wish to thank Mr. H. Tanaka, and Mr. M. Tsunogae for their technical assistance.

References

1) T. Yanaki, W. Itoh, K. Tabata, T. Kojima, T. Norisuye, N. Takano and H. Fujita, *Biophys. Chem.*, **17**, 337 (1983).
2) Y. Deslandes, R. H. Marchessault and A. Sarko, *Macromolecules*, **13**, 1466 (1980); T. L. Bluhm and A. Sarko, *Can. J. Chem.*, **55**, 293 (1977).
3) A. J. Stipanovic, P. J. Giammatteo and S. B. Robie, *Biopolymers*, **24**, 2333 (1985).
4) C. A. Fyfe, P. J. Stephenson, M. G. Taylor, T. L. Bluhm, Y. Deslandes and R. H. Marchessault, *Macromolecules*, **17**, 501 (1984); H. Saito, R. Tabeta, T. Sasaki and Y. Yoshioka, *Bull. Chem. Soc. Jpn.*, **59**, 2093 (1986); H. Saito, R. Tabeta, M. Yokoi and T. Erata, *ibid.*, **60**, 4259 (1987); H. Saito, R. Tabeta, Y. Yoshioka, C. Hara, T. Kiho and S. Ukai, *ibid.*, **60**, 4267 (1987).
5) H. Saito, T. Ohki, Y. Yoshioka and F. Fukuoka, *FEBS Lett.*, **68**, 15 (1976); H. Saito, T. Ohki, N. Takasuka and T. Sasaki, *Carbohydr. Res.*, **58**, 293 (1977); H. Saito, T. Ohki and T. Sasaki, *Biochemistry*, **16**, 908 (1977).
6) K. Ogawa, T. Watanabe, J. Tsurugi and S. Ono, *Carbohydr. Res.*, **23**, 399 (1972); T. Norisuye, T. Yanaki and H. Fujita, *J. Polym. Sci., Polym. Phys. Ed.*, **18**, 547 (1980); T. Yanaki, T. Norisuye and H. Fujita, *Macromolecules*, **13**, 1462 (1980); T. Yanaki, W. Ito, K. Tabata, T. Kojima, T. Norisuye, N. Takano and H. Fujita, *Biophys. Chem.*, **17**, 337 (1983); T. Itou, A. Teramoto, T. Matsumoto and H. Suga, *Macromolecules*, **19**, 1234 (1986).
7) K. Ogawa, J. Tsurugi and T. Watanabe, *Chem. Lett.*, 689 (1972).
8) P. J. Wood and R. G. Fulcher, *Carbohydr. Polym.*, **4**, 49 (1984).
9) T. Sasaki, N. Abiko, K. Nitta, N. Takasuka and Y. Sugino, *Eur. J.*
10) J. Hamuro, Y. Yamashita, Y. Ohsawa, Y. Y. Maeda and G. Chihara, *Nature* (London), **233**, 486 (1971).
11) J. Hamuro, M. Rollinghoff and H. Wagner, *Cancer Res.*, **38**, 3080 (1978); J. Hamuro, U. Hadding and D. Bitter-Suermann, *Immunology*, **34**, 695 (1978).
12) N. Ohno, I. Suzuki, K. Sato, S. Oikawa, T. Miyazaki and T. Yadomae, *Chem. Pharm. Bull.*, **33**, 4522 (1985); N. Ohno, K. Iino, T. Takeyama, I. Suzuki, K. Sato, S. Oikawa, T. Miyazaki and T. Yadomae, *ibid.*, **33**, 3395 (1985); K. Iino, N. Ohno, I. Suzuki, T. Yadomae, S. Oikawa and K. Sato, *Carbohydr. Res.*, **141**, 111 (1985); N. Ohno, Y. Adachi, I. Suzuki, K. Sato, S. Oikawa and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 1709 (1986); N. Ohno, Y. Adachi, I. Suzuki, S. Oikawa, K. Sato, M. Ohsawa and T. Yadomae, *J. Pharmacobio-Dyn.*, **9**, 861 (1986).
13) K. Tabata, W. Ito, T. Kojima, S. Kawabata and A. Misaki, *Carbohydr. Res.*, **89**, 121 (1981).
14) M. Rinaud and M. Vincendon, *Carbohydr. Polym.*, **2**, 135 (1982).
15) N. Ohno, Y. Adachi, I. Suzuki, S. Oikawa, K. Sato, Y. Suzuki, M. Ohsawa and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 2555 (1986).
16) G. Avigad, *Carbohydr. Res.*, **11**, 119 (1969).
17) B. T. Hofreiter, I. A. Wolff and C. L. Mehltretter, *J. Am. Chem. Soc.*, **79**, 6454 (1957).
18) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
19) R. W. Eyler, E. D. Klug and F. Diephuis, *Anal. Chem.*, **19**, 24 (1947).
20) K. L. Hodges, W. E. Kester, D. L. Wiederrich and J. A. Grover, *Anal. Chem.*, **51**, 2172 (1979).
21) V. Crescenzi, A. Gamini and G. Pardossi, *Carbohydr. Polym.*, **6**, 177 (1986); A. Gamini, V. Crescenzi and R. Abruzzese, *ibid.*, **4**, 461 (1984).
22) N. Ohno, Y. Adachi, M. Ohsawa, K. Sato, S. Oikawa and T. Yadomae, *Chem. Pharm. Bull.*, **35**, 2108 (1987).
23) G. Chihara, J. Hamuro, Y. Maeda, Y. Arai and F. Fukuoka, *Nature* (London), **225**, 943 (1970).
24) A. Misaki, M. Kakuta, T. Sasaki, M. Tanaka and H. Miyaji, *Carbohydr. Res.*, **92**, 115 (1981).
25) G. Muller, *Carbohydr. Polym.*, **6**, 177 (1986).
26) N. Ohno, K. Kurachi and T. Yadomae, *Chem. Pharm. Bull.*, **36**, 1016 (1988).
27) Y. Adachi, N. Ohno, T. Yadomae, Y. Suzuki, K. Sato and S. Oikawa, *Carbohydr. Res.*, **177**, 91 (1988).
28) T. Sasaki, N. Takasuka, G. Chihara and Y. Y. Maeda, *Gann*, **67**, 191 (1976); T. Sasaki, N. Abiko, Y. Sugino and K. Nitta, *Cancer Res.*, **38**, 379 (1978); T. Kojima, K. Tabata, W. Itoh and T. Yanaki, *Agric. Biol. Chem.*, **50**, 231 (1986).
29) M. S. Nieuwenhuizen, A. P. G. Kieboom and H. van Bekkum, *Starch/Starke*, **37**, 192 (1985).
30) J. Hamuro, M. Rollinghoff and H. Wagner, *Immunology*, **39**, 551 (1980).