[Chem. Pharm. Bull. 36(3)1016—1025(1988)]

Physicochemical Properties and Antitumor Activities of Carboxymethylated Derivatives of Glucan from *Sclerotinia sclerotiorum*

NAOHITO OHNO, KAZUYA KURACHI, and TOSHIRO YADOMAE*

Tokyo College of Pharmacy, Horinouchi, Hachioji, Tokyo 192-03, Japan

(Received July 17, 1987)

A highly branched antitumor (1→3)-β-D-glucan, SSG, obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, is hardly soluble in water. To increase the solubility of SSG, carboxymethylated derivatives were prepared by reaction with monochloroacetic acid, and the antitumor activity and physicochemical properties of the products were compared. Carboxymethylated derivatives of SSG (CM-SSG) having DS 0.04—0.49 (DS: degree of substitution with carboxymethyl groups per anhydro glucose unit) and CM-curdlan having DS 0.04—0.38 were obtained. As assessed by viscosity, optical rotation and carbon-13 nuclear magnetic resonance (NMR) measurements, CM-SSG and CM-curdlan having DS below *ca.* 0.1 and *ca.* 0.2, respectively, formed a gel in neutral aqueous media, but derivatives having higher DS values did not. CM-SSG having DS 0—0.14 showed potent antitumor activity, but higher DS derivatives did not show the activity. On the other hand, CM-curdlan having DS 0.08—0.38 showed antitumor activity. It is suggested that 1) the DS values corresponding to the gel-to-sol transition and the antitumor activity were affected by the existence of side chains and 2) the critical DS values for antitumor activity were higher than those for gel formation.

Keywords——*Sclerotinia sclerotiorum*; carboxymethylated *Sclerotinia sclerotiorum* glucan, β-1,3-glucan; antitumor activity; Sarcoma 180

Introduction

Recently, the fruit body and the liquid-cultured mycelium of fungi belonging to Basidiomycotina have been reported to contain useful antitumor polysaccharides. One such type of antitumor polysaccharides is (1→3)-β-D-glucans, and the antitumor activity is thought to be due to the activation of immune systems. Some of these glucans (*e.g.*, lentinan, PSK and schizophyllan) are now used clinically for cancer therapy in Japan. We have isolated an antitumor glucan (SSG) from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395. SSG is a (1→3)-β-D-glucan possessing a branch at position 6 of every other main chain glucosyl unit.[1a)]

Generally, (1→3)-β-D-glucan is hardly solubilized in water. So, it is important to prepare a water-soluble derivative, *e.g.*, by carboxymethylation (–OCH_2COOH), sulfation (–$OHSO_3$), or hydroxyethylation (–OCH_2CH_2OH). Previously, carboxymethylated pachymaran (CM-pachymaran),[2)] hydroxyethylated pachyman (HE-pachyman),[2)] and carboxymethylated curdlan (CM-curdlan)[3,4)] were synthesized and the antitumor and immunomodulating activities of these derivatives were examined. CM-curdlan having less than 1 carboxymethyl substitution per glucosyl residue showed antitumor activity similar to that of the unsubstituted curdlan,[3)] but highly substituted derivatives showed less antitumor activity. It is known that macrophages are activated by carboxymethylated chitin.[5)] We were interested in preparing carboxymethyl derivatives of SSG and comparing the antitumor activity of these derivatives. In this study, carboxymethyl derivatives of SSG having various degrees of substitution were

prepared and the physicochemical properties and antitumor activity of these derivatives were examined.

Materials and Methods

Materials——Curdlan, and monochloroacetic acid, were purchased from Wako Chemical Co., Ltd., and zymolyase 100T was purchased from Seikagaku Kogyo Co., Ltd.

Carboxymethylation——Preparation in 2-Propanol: A suspension of 2 g of the glucan (curdlan or SSG) in 150 ml of 2-propanol was stirred at room temperature for 60 min. Then, 10 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, 3 g of monochloroacetic acid was added and the mixture was stirred at 60 °C for 5 h. The product was recovered by filtration and thoroughly washed with a mixture of methanol and acetic acid (7 : 3). The precipitate was collected by filtration, washed successively with 80% aqueous methanol, methanol and acetone, and dried under reduced pressure.

Preparation of Carboxymethylated SSG(CM-SSG) in Aqueous Solution: A solution of SSG (150 mg in 10 ml) in aqueous sodium hydroxide (0.1, 0.2, 0.3, 0.5, 1.0 or 3.0 N) was treated with monochloroacetic acid (1.5 g), and the mixture was kept for 4 h at 60 °C with vigorous stirring, then neutralized with acetic acid and dialyzed against water for 3 d. The non-dialyzable fraction was concentrated to dryness.

Preparation of Carboxymethylated Curdlan (CM-Curdlan) in Aqueous Solution: A solution of curdlan (1 g in 50 ml) in aqueous sodium hydroxide (0.2, 0.3, 0.5, 1.0, 3.0 or 7.5 N) was treated with monochloroacetic acid (5 g) and kept for 3 h at 60 °C with vigorous stirring. The resulting mixture was neutralized with acetic acid and dialyzed against water for 3 d. Each non-dialyzable fraction was concentrated to dryness.

Colorimetric Determination of Carboxymethyl Group[6]——A 250 μl aliquot of sample solution (500 μg/ml) was taken in Pyrex test tube, and 250 μl of concentrated sulfuric acid was added. The tube was heated at 125 °C for 3.5 h. Then, 2 ml of dihydroxynaphthalene solution (100 μg/ml) was added and the tube was heated in a boiling water bath for 20 min. After cooling of the tube in a container of cold tap water, 2 ml of water was added and the optical density at 520 nm was measured.

Evaluation of Antitumor Activity——The antitumor activity was evaluated against the solid form of sarcoma 180 tumor cells. Tumor cells (5×10^6) were inoculated subcutaneously into the right groin of mice. Each sample was administered at 7, 9, 11, 13, and 15 d. Five weeks after tumor inoculation, the mice were sacrified.

Other Methods——Other methods such as cultivation of *Sclerotinia sclelotiorum* IFO 9395, purification of SSG, quantitative determination, carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, and physicochemical analysis, were performed as described previously.[1]

Results

Preparation of CM-SSG and CM-Curdlan

In the case of (1→3)-β-D-glucan, carboxymethylated derivatives are usually prepared by the addition of sodium monochloroacetic acid to the glucan suspended in 2-propanol under basic conditions.[7] It was difficult to control the degree of substitution (DS) under these conditions. Thus, the carboxymethyl SSG and curdlan having various DS values were prepared in several molarities of aqueous sodium hydroxide (0.1—7.5 M). As shown in Table I, the DS value of derivatives increased gradually as the sodium hydroxide concentration was increased. DS values of carboxymethylated SSG(CM-SSG) were 0.36—0.04 and those of CM-curdlan were 0.38—0.04.

Determination of the Carboxymethylated Sites

The DS value can be determined by a simple colorimetric method,[6] but this does not give information about the homogeneity of distribution of the carboxymethylated sites. To establish this, diethylaminoethyl (DEAE)-Sephadex chromatography, enzyme digestion and structural determination of the carboxymethylated glucosyl unit were performed on CM-curdlan, as an example.

Figure 1 shows the elution profile of the CM-curdlan (DS 0.2) from a column of DEAE-Sephadex A25 on linear gradient elution from 0 to 1 M sodium chloride. The glucan was eluted as a single symmetrical peak, suggesting a homogeneous substitution of carboxymethylated

TABLE I. Summary of Properties of Carboxymethylated Curdlan and SSG

		DS[a]	Yield[b] (%)	Viscosity in H_2O[c]	$[\alpha]_D$[d]	^{13}C-NMR[e] C3 (ppm)	Antitumor activity[f]
CM-curdlan[g]	A	0.27	67	Low	n.d.	85	Strong
	B	0.38	94	Low	Negative	85	Strong
	C	0.34	103	Low	Negative	85	Strong
	D	0.28	104	Low	Negative	85	Strong
	E	0.20	97	High	Negative	85	Strong
	F	0.08	97	High	Positive	87	Strong
	G	0.04	91	High	Positive	87	Strong
	H	n.d.					
CM-SSG	A	0.49	73	Low	n.d.	85	Nil
	B	0.29	81	Low	n.d.	85	Weak
	C	n.d.					
	D	0.35	69	Low	Negative	85	Weak
	E	0.36	76	Low	Negative	85	Weak
	F	0.14	68	Low	Positive	85	Strong
	G	0.09	62	Low	Positive	87	Strong
	H	0.05	19	High	Positive	Nil	Strong
	I	0.04	39	High	n.d.	Nil	Strong

a) DS means degree of carboxymethyl group substitution per glucose unit. *b*) Weight of carboxymethylated glucan per glucan used. *c*) Summary of results in Fig. 4. *d*) $[\alpha]_D$ value in H_2O compared with the value after the sol transition. *e*) Chemical shift of C-3 in distilled water. *f*) Summary of results in Tables II and III. *g*) Conditions of carboxymethylation: A, in 2-propanol; B, in 2-propanol and an excess of $ClCH_2COONa$; C, in 7.5 M sodium hydroxide; D, in 3.0 M sodium hydroxide; E, in 1.0 M sodium hydroxide; F, in 0.5 M sodium hydroxide; G, in 0.3 M sodium hydroxide; H, in 0.2 M sodium hydroxide; I, in 0.1 M sodium hydroxide. n.d. = not detectable.

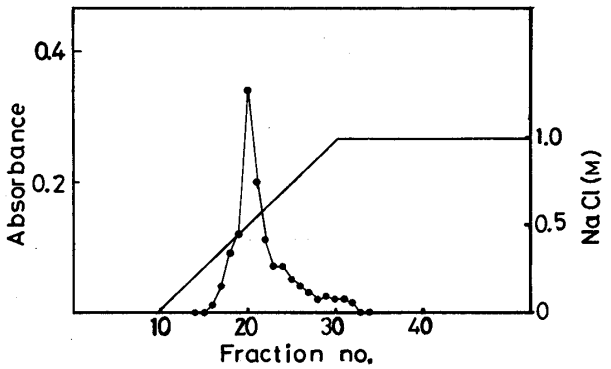

Fig. 1. Elution Profile of CM-Curdlan from a Column DEAE-Sephadex A-25 (Cl^-)

The column (7 ml) was equilibrated with H_2O, and 5 mg of CM-curdlan was applied. Fractions of 2 ml were collected, and carbohydrate was assayed by the phenol–H_2SO_4 method.

groups on the glucan. Next, the glucan was digested with zymolyase-100T, which is an endo-(1→3)-β-D-glucanase that does not cleave highly substituted sites, because it can not degrade branched (1→3)-β-D-glucans, such as grifolan and SSG (unpublished results). The glucan was digested with zymolyase and then the product was passed through a column of DEAE-Sephadex A25. The absorbed fraction, which contained the carboxymethylated fragments, was collected and applied to a column of Bio gel P2. The main peak was collected and the ^{13}C-NMR spectrum was measured (Fig. 2b). This spectrum showed the anomeric carbon at about 104 ppm, suggesting that the degree of polymerization was more than 2. A part of this oligosaccharide was reduced with sodium hydroxide and the carbohydrate content was determined by the phenol–sulfuric acid method. The carbohydrate content was decreased to 53% by the reduction treatment, suggesting that the oligosaccharide was the disaccharide. These results suggest that most of the carboxymethyl groups are distributed throughout the chain.

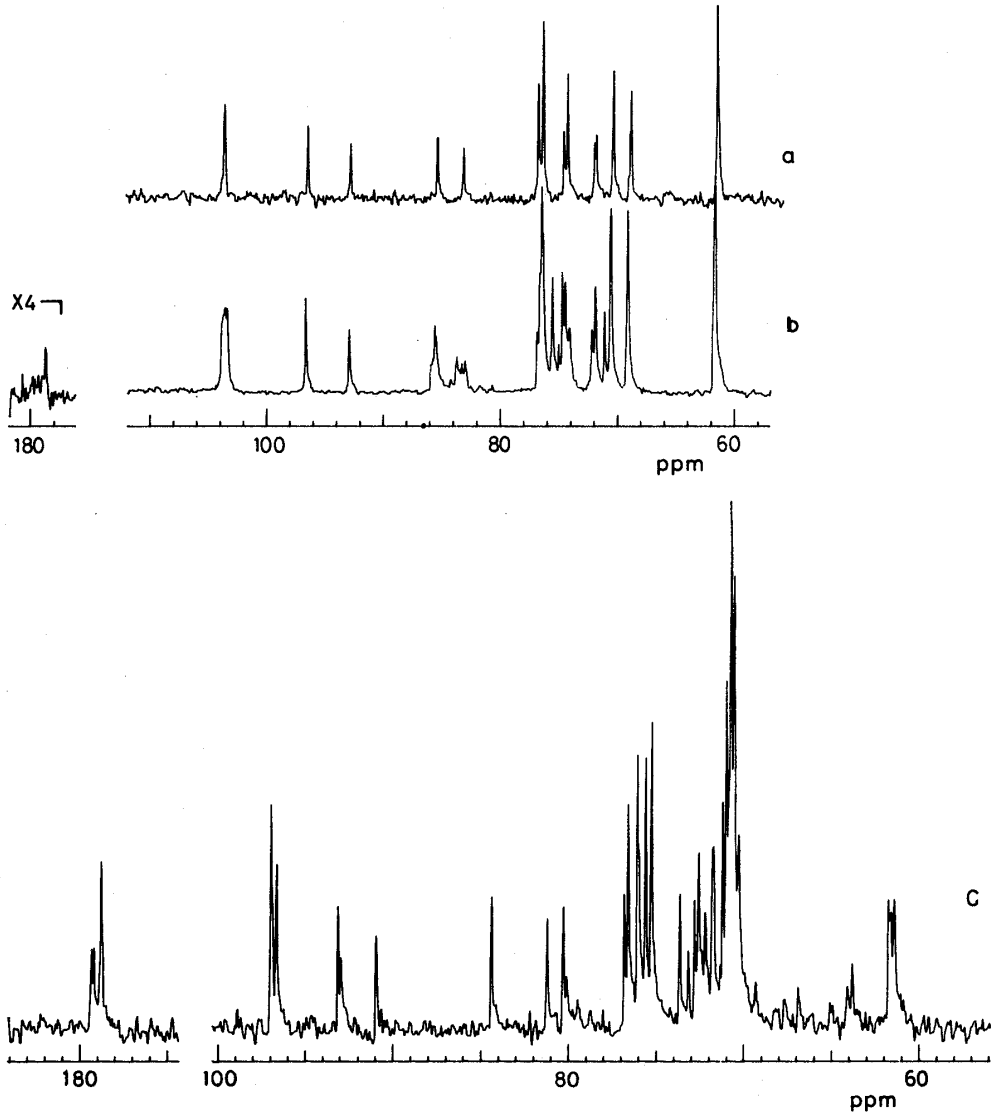

Fig. 2. ^{13}C-NMR Spectra of Carboxymethylated Fragments of CM-Curdlan in H_2O

a) Laminaribiose. b) Carboxymethylated oligosaccharide obtained by Zymolyase digestion of CM-curdlan. c) Carboxymethylated glucose obtained by acid hydrolysis of CM-curdlan.

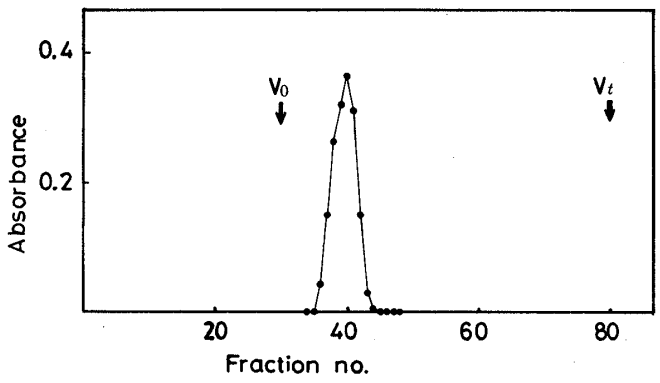

Fig. 3. Elution Profile of Carboxymethylated Fragments of CM-Curdlan from a Column of Big-Gel P2

The column (1.7 × 137.5 cm) was equilibrated with H_2O, and 30 mg of CM-curdlan was applied. The arrows indicate void and bed volumes, respectively.

To determine the site of carboxymethylation, the glucan was hydrolyzed with 1 M trifluoroacetic acid and the carboxymethylated glucose residue was collected by passing the hydrolysate through a column of DEAE-Sephadex A25. The absorbed fraction was collected

and desalted by passing it through a column of Bio Gel P2 (Fig. 3). The carboxymethylated glucose migrated as $R_{glucose}$ 0.79 on thin layer chromatography (TLC) (solvent, ethyl acetate–pyridine–acetic acid–water = 5 : 5 : 1 : 3). Figure 2c shows the ^{13}C-NMR spectrum of the CM-glucose. The signals at 178 and 179 ppm were characteristic of the carboxymethyl group, and those at 91.0, 84.4, 81.2, and 80.3 were characteristic of substitution at any hydroxyl group in the glucosyl unit. The signal at 91.0 would be attributable to the C-1 carbon of C-2 substituted glucosyl units. The signals at 84.4 and 81.2 would be attributable to the C-2 carbon of C-2 substituted glucosyl units, and the signal at 80.3 to the C-4 signal of C-4 substituted glucosyl units. It was difficult to observe representative signals attributable to C-6 substitution, but the fact that the signal intensity of C-6 carbon was lower than that of glucose suggests the occurrence of C-6 substitution as well as C-2 and C-4 substitutions. These data suggest that the carboxymethylated glucose residues can not be separated by the method used in this paper and that the carboxymethylated group was not incorporated at specific carbons.

Physicochemical Properties of CM-Curdlan and CM-SSG

Antitumor (1→3)-β-D-glucans are known to form gel structure under neutral and weakly alkaline conditions.[8] The conformational changes of (1→3)-β-D-glucan induced by PH change, urea, dimethyl sulfoxide (DMSO), temperature change, substituents, *etc.* influence the physicochemical properties of these glucans. In this section, the viscosity, specific optical rotation, and solution and solid-state ^{13}C-NMR spectra of the carboxymethylated derivatives are compared.

Figure 4 shows the viscosity of CM-curdlan and CM-SSG under neutral conditions. The viscosity of CM-curdlan decreased markedly between DS 0.20 and 0.27, and that of CM-SSG, between DS 0.04 and 0.09. The viscosity of lower DS derivatives were decreased by the addition of a final concentration of 0.05 M sodium hydroxide and became constant at 0.10 M (CM-curdlan: DS 0.04, 13.0 (0 N), 1.1 (0.05 N), 1.1 (0.1 N); DS 0.20, 14.9 (0 N), 1.1 (0.05 N), 1.2 (0.1 N); DS 0.38, 9.5 (0 N), 1.1 (0.05 N), 1.1 (0.1 N). CM-SSG: DS 0.09, 1.0 (0 N), 1.1 (0.05 N), 1.1 (0.1 N); DS 0.14, 1.7 (0 N), 1.1 (0.05 N), 1.1 (0.1 N); DS 0.35, 1.6 (0 N), 1.1 (0.05 N), 1.1 (0.1 N)). These results suggested that CM-SSG and CM-curdlan with lower DS (*ca.* 0.04 and *ca.* 0.20, respectively) formed a gel under neutral conditions.

Figure 5 shows the optical rotation values of these derivatives. CM-curdlan with lower DS (0.04, 0.08) showed positively shifted optical rotation values in relation to the higher DS derivatives. The optical rotation values of lower DS derivatives were decreased by the addition of a final concentration of 0.05 M sodium hydroxide and became constant at 0.2 M sodium hydroxide. A part of the positively shifted value appears to be due to gel formation. On the other hand, the value of higher DS derivatives was increased by the addition of sodium hydroxide and reached a constant value at about 0.05 M sodium hydroxide. At 0.2 M sodium hydroxide, all CM-curdlan preparations showed similar values. These results also suggest that

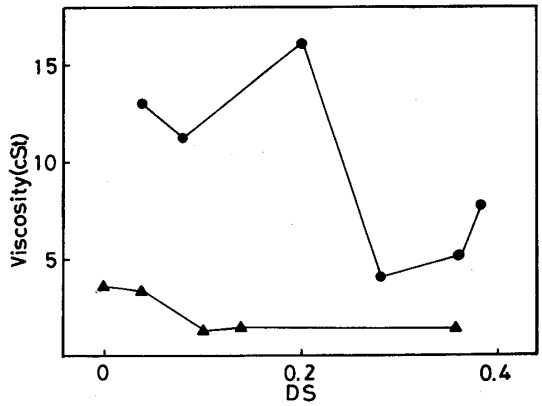

Fig. 4. Dependence of the Viscosity of CM-SSG and CM-Curdlan on the Degree of Carboxymethyl Group Substitution

The viscosity was measured by using an Ostwald type viscometer at the glucan concentration of 1 mg/ml. —▲—, CM-SSG; —●—, CM-curdlan.

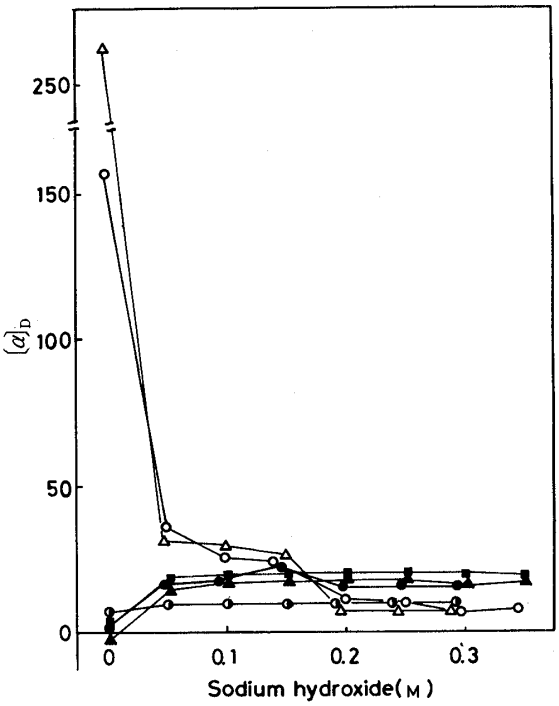

Fig. 5. The Optical Rotation Value of CM-Curdlan in Various Concentrations of Sodium Hydroxide

The glucan concentrations were 0.5 mg/ml. —△—, CM-curdlan (DS 0.04); —○—, CM-curdlan (DS 0.08); —◑—, CM-curdlan (DS 0.20); —■—, CM-curdlan (DS 0.28); —●—, CM-curdlan (DS 0.34); —▲—, CM-curdlan (DS 0.38).

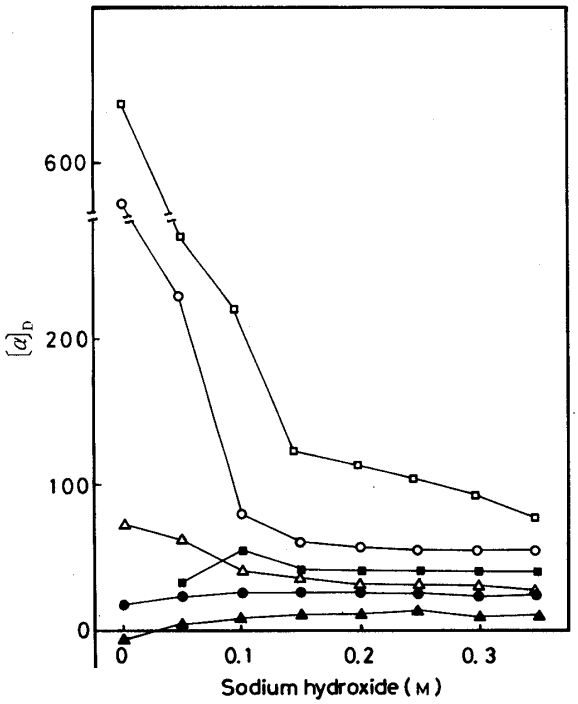

Fig. 6. The Optical Rotation Value of CM-SSG in Various Concentrations of Sodium Hydroxide

—△—, CM-SSG (DS 0.05); —○—, CM-SSG (DS 0.09); —□—, CM-SSG (DS 0.14); —▲—, CM-SSG (DS 0.35); —■—, CM-SSG (DS 0.36).

CM-curdlan possessing the high DS value (0.38) has a different ultrastructure from lower DS derivatives. As shown in Fig. 6, the optical rotation value of CM-SSG having lower DS (0.05, 0.09, 0.14) was decreased by the addition of sodium hydroxide. On the other hand, the optical rotation of CM-SSG having higher than DS 0.35 gradually increased in relation to the sodium hydroxide concentration and became constant at 0.10 M sodium hydroxide. These results suggest that CM-SSG with DS below 0.1 can also produce gel structure in neutral aqueous solution. The gel of (1→3)-β-D-glucan contained single helical segments, triple helical segments and junction zones which are produced by cross-linking of glucan segments. Saito *et al.* reported that ^{13}C-NMR analysis is applicable to these gels, though the triple helical segments and the junction zone could not be observed owing to limitated mobility.[9] They also reported that the signal intensities increased gradually as the gel-to-sol transition occurred upon addition of sodium hydroxide.[10] The C-3 signal of single helical (*ca.* 87 ppm) and random coiled segments (*ca.* 85 ppm) showed different chemical shifts. Figure 7 shows ^{13}C-NMR spectra of CM-curdlan in distilled water. The signals of curdlan and CM-curdlan having DS 0.04 and 0.08 were broad and the C-3 signal appeared at 87 ppm. However, the C-3 signal of CM-curdlan having higher DS values (0.20—0.38) was at about 85 ppm instead of 87 ppm. These results suggest that single helical regions exist in CM-curdlan having lower DS values in aqueous media. At DS 0.20, however, the C-3 signal appeared at 84.5 ppm, suggesting that few regions of multiple helical junction zones remain, because the peak heights of C1–C5 were lower and broader than that of C-6 (61 ppm). CM-SSG of lower DS (0.04, 0.05) showed no ^{13}C-signals, suggesting the production of a rigid gel similar to that of the parent SSG (Fig. 8). The broader signals attributable to the single helical segments were seen in the case of DS 0.09. At above DS 0.14, the C-3 signal appeared at 84.5 ppm. These results

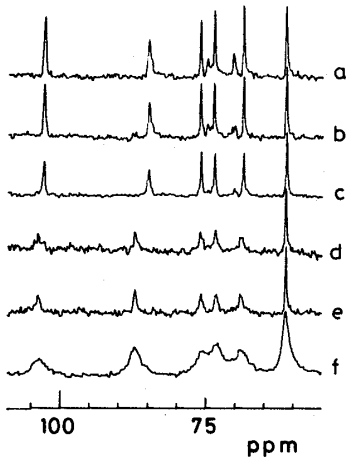

Fig. 7. ^{13}C-NMR Spectra of CM-Curdlan

a, CM-curdlan (DS 0.38); b, CM-curdlan (DS 0.34); c, CM-curdlan (DS 0.20); d, CM-curdlan (DS 0.08); e, CM-curdlan (DS 0.04); f, curdlan.

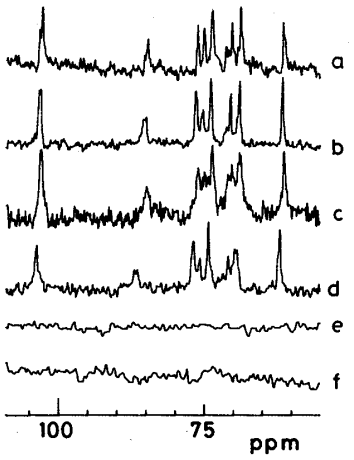

Fig. 8. ^{13}C-NMR Spectra of CM-SSG in H_2O

a, CM-SSG (DS 0.35); b, CM-SSG (DS 0.36); c, CM-SSG (DS 0.14); d, CM-SSG (DS 0.09); e, CM-SSG (DS 0.05); f, CM-SSG (DS 0.04).

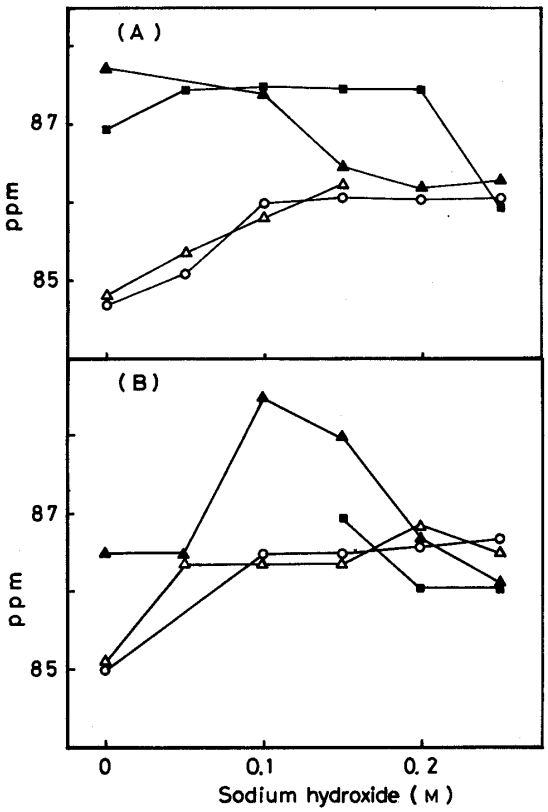

Fig. 9. Dependency of the C-3 Chemical Shift of CM-Curdlan and CM-SSG on Concentration of Sodium Hydroxide

(A) —■—, curdlan; —▲—, CM-curdlan (DS 0.08); —○—, CM-curdlan (DS 0.20); —△—, CM-curdlan (DS 0.38). (B) —■—, SSG; —▲—, CM-SSG (DS 0.09); —△—, CM-SSG (DS 0.14); —○—, CM-SSG (DS 0.35).

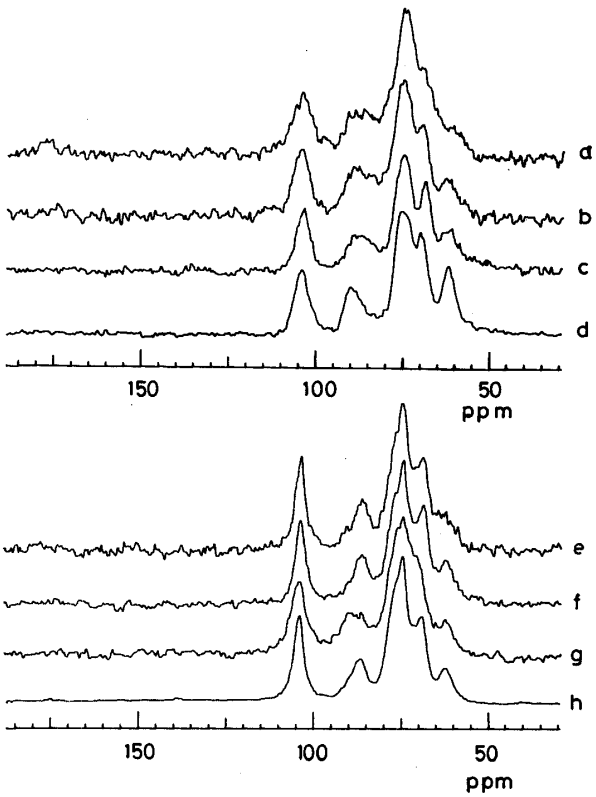

Fig. 10. ^{13}C-CP/MAS Spectra of CM-Curdlan and CM-SSG

a, CM-curdlan (DS 0.38); b, CM-curdlan (DS 0.20); c, CM-curdlan (DS 0.08); d, curdlan; e, CM-SSG (DS 0.14); f, CM-SSG (DS 0.09); g, SSG (helix form); h, SSG (native form).

also support the above observation that CM-SSG with DS higher than 0.14 could not form a gel. Figure 9 shows the chemical shift of C-3 signals of CM-curdlan and CM-SSG in various concentrations of sodium hydroxide. The signals that appeared at about 85 ppm in distilled

water were gradually shifted to about 86 ppm. These observations were similar to the case of laminaran, a random-coiled, small-molecular weight (1→3)-β-D-glucan. On the other hand, the signals at about 87 ppm in distilled water were gradually shifted to about 86 ppm. These observations were similar to those made with the parent glucan but the chemical shift changed at lower sodium hydroxide concentrations. These facts suggest that the carboxymethylated SSG having lower DS values produced gel structure in aqueous media, but the framework of the gel network of carboxymethylated glucans is weaker than that of the parent glucans.

In the solid state, (1→3)-β-D-glucan is known to take several conformations, such as form I (helix form) and form II (native form).[11,12] The differences were assessed by measuring the cross polarization-magic angle spinning (CP/MAS) ^{13}C-NMR spectra. Each conformation can be distinguished from the C-1 and C-3 chemical shifts. The CP-MAS NMR spectra obtained from CM-curdlan and CM-SSG are shown in Fig. 10. The C-3 signal of curdlan appeared at 90.4 ppm (curdlan type). In CM-curdlan having lower DS values (0.08, 0.20), C-3 showed broader signals at 89.6 ppm (still curdlan type). On the other hand, the derivative having DS 0.38 showed a quite broad C-3 signal, suggesting the transition state of the conformation between curdlan type and laminaran type. In the case of CM-SSG of DS 0.09 and 0.14, the C-3 signal appeared at 86.5 and 86.7 ppm, respectively. These results suggest that CM-SSG showed laminaran-type conformation. It appears that the ultrastructure of carboxymethylated derivatives in the solid state is different from that in aqueous media.

Antitumor Activity of CM-SSG and CM-Curdlan

The DS of CM-curdlan is known to be closely related to the antitumor activity (compounds with a DS of about 1.0 per glucose residue having less activity). Tables II and III show the antitumor activity of CM derivatives of curdlan and SSG against the solid form of

TABLE II. Antitumor Activity towards Sarcoma 180

Sample	Dose (μg × 5)	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression
CM-curdlan	20	1.69 ± 3.26[a]	80.9	0/7
PrOH	100	0.75 ± 0.35[a]	91.5	0/6
(DS 0.27)	500	0.81 ± 0.43[a]	90.9	0/7
CM-curdlan	20	0.01 ± 0.01[a]	99.9	5/7
7.5 N NaOH	100	0.05 ± 0.05[a]	99.4	1/8
(DS 0.38)	500	0.28 ± 0.21[a]	96.8	1/7
CM-curdlan	20	4.90 ± 3.72[b]	44.7	0/7
1 N NaOH	100	0.13 ± 0.16[a]	98.5	1/7
(DS 0.28)	500	0.34 ± 0.50[a]	96.2	4/7
CM-curdlan	20	0.04 ± 0.03[a]	99.6	2/7
0.5 N NaOH	100	0.33 ± 0.41[a]	96.3	2/7
(DS 0.20)	500	0.51 ± 0.60[a]	94.2	0/7
CM-curdlan	20	8.39 ± 6.90	5.3	0/7
0.3 N NaOH	100	0.51 ± 0.73[a]	95.2	1/7
(DS 0.08)	500	0.08 ± 0.08[a]	99.1	2/7
CM-curdlan	20	0.04 ± 0.04[a]	99.6	2/6
0.1 N NaOH	100	0.01 ± 0.02[a]	99.9	5/7
(DS 0.21)	500	0.54 ± 0.44[a]	93.9	0/7
Curdlan	20	9.53 ± 9.93	−7.7	0/7
	100	9.84 ± 5.18	−11.1	0/7
	500	8.76 ± 3.33	1.1	0/7
Nil		8.86 ± 4.61		0/16

a) $p<0.001$. b) $p<0.05$.

Table III. Antitumor Activity towards Sarcoma 180

Sample	Dose ($\mu g \times 5$)	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression
CM-SSG	20	12.14 ± 5.73	−37.0	0/7
HDS	100	10.05 ± 2.63	−13.4	0/7
(DS 0.49)	500	9.44 ± 6.27	−6.6	0/7
CM-SSG	20	7.56 ± 3.01	14.7	0/7
Pro-OH	100	10.67 ± 7.00	−20.1	0/7
(DS 0.29)	500	2.60 ± 3.22[a]	70.65	0/7
CM-SSG	20	8.22 ± 5.42	7.2	0/7
1 N NaOH	100	5.33 ± 2.47[b]	39.8	0/7
(DS 0.36)	500	8.83 ± 5.52	0.3	0/7
CM-SSG	20	4.74 ± 5.06	46.5	0/7
0.5 N NaOH	100	1.51 ± 2.25[c]	83.0	0/7
(DS 0.14)	500	0.60 ± 1.07[c]	93.2	2/7
CM-SSG	20	0.44 ± 0.42[c]	95.0	0/7
0.3 N NaOH	100	0.27 ± 0.32[c]	97.0	0/7
(DS 0.09)	500	0.08 ± 0.11[c]	99.1	0/7
CM-SSG	20	0.16 ± 0.19[c]	98.2	1/7
0.1 N NaOH	100	0.07 ± 0.02[c]	99.2	0/7
(DS 0.04)	500	0.46 ± 0.26[c]	94.8	0/7
SSG	20	8.78 ± 3.56[a]	0.9	0/7
	100	1.27 ± 1.31[c]	85.7	1/7
	500	0.02 ± 0.04[c]	99.8	5/7
Nil		8.86 ± 4.61		0/16

a) $p<0.01$. *b*) $p<0.05$. *c*) $p<0.001$.

Sarcoma 180, respectively. Curdlan itself did not show significant antitumor activity. In the case of CM-curdlan, all derivatives showed significant activity. As described above, CM-curdlan of above DS *ca.* 0.20 did not produce gel structure (Fig. 4). On the other hand, CM-SSG of DS lower than 0.14 showed significant activity. These results suggest that the antitumor activity of curdlan and SSG is not related to the gel-to-sol transition.

Discussion

SSG is a highly branched antitumor (1→3)-β-D-glucan obtained from the culture filtrate of *Sclerotinia sclerotiorm* IFO 9395, and is poorly soluble in water. It is important to prepare highly soluble antitumor glucans for ease of administration. This paper deals with the preparation of carboxymethylated derivatives of SSG and with the biological and physicochemical properties of these derivatives assessed by ^{13}C-NMR, CP/MAS ^{13}C-NMR, viscosity and optical rotation measurements. Carboxymethylation of (1→3)-β-D-glucan was found to cause large changes in both physicochemical properties and antitumor activity.

In this paper, the ultrastructure of carboxymethylated glucans was assessed by several methods. As summarized in Table I, the critical point of the gel-to-sol transition was slightly different depending on the method used to detect it. For example, the viscosity of CM-curdlan (DS 0.2) was high but this derivative showed an NMR signal about 85 ppm. These differences would be due to the differences of the moieties observed: the total structure of the gel was observed by the viscosity measurement and the segmental structure was observed by the ^{13}C-NMR. Thus, carboxymethylation of SSG to higher than DS about 0.1 caused loss of gel formation, while the corresponding value for curdlan is about 0.2. The difference may depend on the branching of the glucans.

Deslandes *et al.* reported that, in crystalline (1→3)-β-D-glucan, the three strands of the glucan chains lie in parallel.[13] Saito *et al.* reported that a gel is formed by cross-linking of the chains, producing some double- or triple-helical junction zones.[9] One pitch of branched SSG is composed of 9 glucose residues and that of curdlan is composed of 6 glucose residues. If one pitch of (1→3)-β-D-glucan contains about one carboxymethylated group, DS of CM-SSG and CM-curdlan would be 0.11 and 0.17, respectively. Thus, in both glucans, 1 substitution in 1 pitch of glucan segment seems to be enough to prevent gel formation.

CM-curdlan showed significant antitumor activity at any DS value used in this paper, but CM-SSG did not show activity at DS in excess of 0.14. The difference presumably arises from the branching of the glucans. Matsuzaki *et al.* reported that branched polysaccharides with degrees of branching of 39.0% and 57.4% synthesized from curdlan showed remarkably high antitumor activity, but that with a degree of branching of 63.7% showed lower antitumor activity.[14] Extensive branching may mean that the immune system would not be able to recognize the main chain moiety of (1→3)-β-D-glucan. The relation between the DS values and antitumor activity in the case of SSG may be explained similarly.

Acknowledgments The authors thank Miss K. Shimamura for technical assistance.

References

1) *a*) N. Ohno, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, **24**, 1362 (1986); N. Ohno, K. Kurachi, and T. Yadomae, *J. Pharmacobio-Dyn.*, **10**, 478 (1987); *b*) I. Suzuki, T. Itani, N. Ohno, S. Oikawa, T. Miyazaki, and T. Yadomae, *ibid.*, **7**, 492 (1984); N. Ohno, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, **32**, 1142 (1984); K. Iino, N. Ohno, I. Suzuki, T. Miyazaki, and T. Yadomae, *Carbohydr. Res.*, **141**, 111 (1985); N. Ohno, K. Iino, T. Takeyama, I. Suzuki, K. Sato, S. Oikawa, T. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, **33**, 3395 (1985); N. Ohno, K. Iino, I. Suzuki, and T. Yadomae, *ibid.*, **33**, 1557 (1985); N. Ohno, K. Iino, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *ibid.*, **33**, 1181 (1985); N. Ohno, I. Suzuki, K. Sato, S. Oikawa, T. Miyazaki, and T. Yadomae, *ibid.*, **33**, 4522 (1985); I. Suzuki, T. Itani, N. Ohno, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *J. Pharmacobio-Dyn.*, **8**, 217 (1985); N. Ohno, M. Hayashi, I. Suzuki, S. Oikawa, K. Oikawa, and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 4377 (1986); N. Ohno, K. Iino, S. Oikawa, K. Sato, M. Ohsawa, and T. Yadomae, *ibid.*, **34**, 3328 (1986); N. Ohno, Y. Adachi, I. Suzuki, S. Oikawa, K. Sato, M. Ohsawa, and T. Yadomae, *J. Pharmacobio-Dyn.*, **9**, 861 (1986); N. Ohno, M. Hayashi, K. Iino, I. Suzuki, S. Oikawa, K. Sato, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 2149 (1986); N. Ohno, Y. Adachi, M. Ohsawa, K. Sato, S. Oikawa, and T. Yadomae, *ibid.*, **35**, 2108 (1987).
2) J. Hamuro, M. Rollinghoff, and H. Wagner, *Immunol.*, **39**, 551 (1980); J. Hamuro, U. Handding, and D. Bitter-Suermann, *ibid.*, **34**, 695 (1978).
3) T. Sasaki, N. Abiko, K. Nitta, N. Takasuka, and Y. Sugino, *Europ. J. Cancer*, **15**, 211 (1979).
4) S. Honda, H. Sugino, T. Asano, and A. Kakinuma, *Immunopharmacology*, **11**, 29 (1986).
5) K. Nishimura, S. Nishimura, N. Nishi, I. Saiki, S. Tokura, and I. Azuma, *Vaccine*, **2**, 93 (1984); K. Nishimura, S. Nishimura, N. Nishi, F. Numata, Y. Tone, S. Tokura, and I. Azuma, *ibid.*, **3**, 379 (1985).
6) R. W. Eyler, E. D. Klung, and F. Diephuis, *Analyt. Chem.*, **19**, 24 (1947).
7) J. Hamuro, Y. Yamashita, Y. Ohsaka, Y. Maeda, and G. Chihara, *Nature* (London), **233**, 486 (1971).
8) N. Ohno and T. Yadomae, *Carbohydr. Res.*, **159**, 293 (1987).
9) H. Saito, T. Ohki, N. Takasuka, and T. Sasaki, *ibid.*, **58**, 293 (1977).
10) H. Saito, T. Ohki, and T. Sasaki, *ibid.*, **74**, 227 (1979).
11) N. Ohno, Y. Adachi, I. Suzuki, S. Oikawa, K. Sato, Y. Suzuki, M. Ohsawa, and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 2555 (1986).
12) H. Saito, R. Tabeta, T. Sasaki, and Y. Yoshioka, *Bull. Chem. Soc. Jpn.*, **59**, 2093 (1986).
13) Y. Deslandes, R. H. Marchessault, and A. Sarco, *Macromolecules*, **13**, 1466 (1980).
14) K. Matsuzaki, I. Yamamoto, and T. Sato, *Macromol. Chem.*, **187**, 317 (1986).