Resistance of Highly Branched (1→3)-β-D-Glucans to Formolysis

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Small molecular weight (MW) glucan derivatives could be a useful tool for studying the mechanisms of β-glucan mediated biological activity, especially as antagonists for a β-glucan receptor. This paper described the stability of various (1→6) branched (1→3)-β-D-glucans to formolysis in the preparation of small MW derivatives. The glucans used were curdlan (linear), pachyman (few branches), GRN (one branch in every third main chain unit; 2/6), SPG (2/6), SSG (3/6), and OL-2 (4/6). Curdlan and pachyman were easily degraded to oligosaccharides by degradation for 20 min at 100 °C by 90% formic acid. However, branched glucans, especially the highly branched glucans, SSG and OL-2, were significantly resistant to degradation, and the majority remained high MW. SSG required a longer period and/or a higher temperature (121 °C treatment) to produce small MW derivatives. Branched glucans were also resistant to zymolyase (an endo-(1→3)-β-D-glucan hydrolase) digestion. These facts suggest that the (1→6)-β-D-branched residues contribute to the glucans' resistance to formic acid degradation and zymolyase digestion.

Key words β-glucan; formolysis; oligosaccharide; antitumor glucan

(1→3)-β-D-Glucan shows a variety of biological activities useful for clinical use.[1,2] These activities relate to the modulation of the host defense mechanism. Various parameters are considered for the underlying basic-mechanism of the immunomodulation.[3-5] At least a part of the activation would be related to the interaction with a specific β-glucan receptor on phagocytes. The β-glucan receptor has been originally found, by functional assay, to inhibit the phagocytosis of β-glucan particles.[6] Contribution of a complement receptor type 3 (CR3) to the recognition of the glucan was also proposed.[7] These β-glucans possess at least three conformations, triple helix, single helix and random coil. Immunopharmacological activity has been found to be significantly dependent on the conformation.[8,9] These activities are also influenced by the primary structure and the molecular weight.[5,10] We have previously reported the molecular weight dependency of the biological activities of a β-glucan, grifolan [GRN (one branch in every three main chain units)] obtained from *Grifola frondosa* by preparing small molecular weight (MW) derivatives by formolysis and heat degradation methods.[11,12]

There are many kinds of (1→3)-β-D-glucans with varying degrees of branching (DB). DB is also an important contributing factor for specifying biological activities.[13] It is important to prepare small MW (1→3)-β-D-glucans in order for molecular understanding of glucan mediated biological activities. To prepare small MW derivatives of various glucans, we planned to use formolysis. As a preliminary investigation, we prepared small MW products of SSG (one branch in every second main chain unit), a highly branched glucan obtained from the culture filtrate of *Sclerotinia sclerotiorum*, by formolysis under the condition of 90% formic acid at 100 °C for 12 h. However, unfortunately, only 8% of SSG was recovered from the dialysable fraction. These facts strongly suggested that the primary structure of the glucan significantly affects the stability of the glucosidic linkage to formolysis. It is interesting to precisely elucidate the stability of various glucans to formolysis to clarify the conditions required to prepare the oligosaccharides of these glucans. This paper deals with the physicochemical properties of branched (1→3)-β-D-glucans deduced from the information gained during the preparation of small MW derivatives of the glucans by formolysis.

Materials and Methods

Materials Curdlan was purchased from Wako Co. Pachyman was from Calbiochem Co. GRN and SSG were prepared in our laboratory as described previously.[14,15] SPG and OL-2[16] were generously provided from Kaken Pharmaceutical Co. and Dr. K. Saito (Tokyo Metropolitan Research Laboratory of Public Health). Standard dextrans were from Pharmacia Fine Chemicals. Toyopearl HW-50, -55 and -60 were from Toyo Soda Mfg. Co., Ltd. Ultrafilter UK-10 and -200 were from Toyo Roshi Kaisha, Ltd. Zymolyase 20T was from Seikagaku Corp.

Formolysis Formolysis was performed following the procedure in literature with slight modifications.[12] The representative experimental protocol was as follows: Each glucan (10 mg) was dissolved in 2.5 ml of 90% formic acid and boiled for 20 min or 3 h. After evaporation to dryness *in vacuo*, the resulting reaction mixture was dissolved in 2.5 ml of distilled water, boiled for 3 h, and then evaporated to dryness. The products were dissolved in distilled water and used for further experiments.

Zymolyase Digestion Each glucan (2 mg) dissolved in 200 μl of distilled water was mixed with 3 N NaOH (25 μl) and immediately neutralized by adding 3 N HCl (25 μl). Acetate buffer (50 mM, pH 6.0) (2 ml) was added and 200 μg of zymolyase 20T was added. After overnight incubation at 37 °C, the reaction mixture was boiled for 3 min to inactivate the enzyme. The resulting supernatant was mixed with 200 μl of 3 N NaOH and applied to Toyopearl HW-50. The elution profile of each glucan was monitored by the phenol–H_2SO_4 method.

Antitumor Activity Antitumor activity against the solid form of Sarcoma 180 tumor was measured by the method described previously using 6-week-old (*ca.* 25 g) male ICR mice (Japan SLC, Hamamatsu) under specific pathogen-free conditions.[14,15]

Results

Sensitivity of Various Glucans to Formolysis To compare the stability of several glucans to formolysis, we used curdlan (linear), pachyman (few branches), GRN (2/6), SPG (2/6), SSG (3/6), and OL-2 (4/6), and each was

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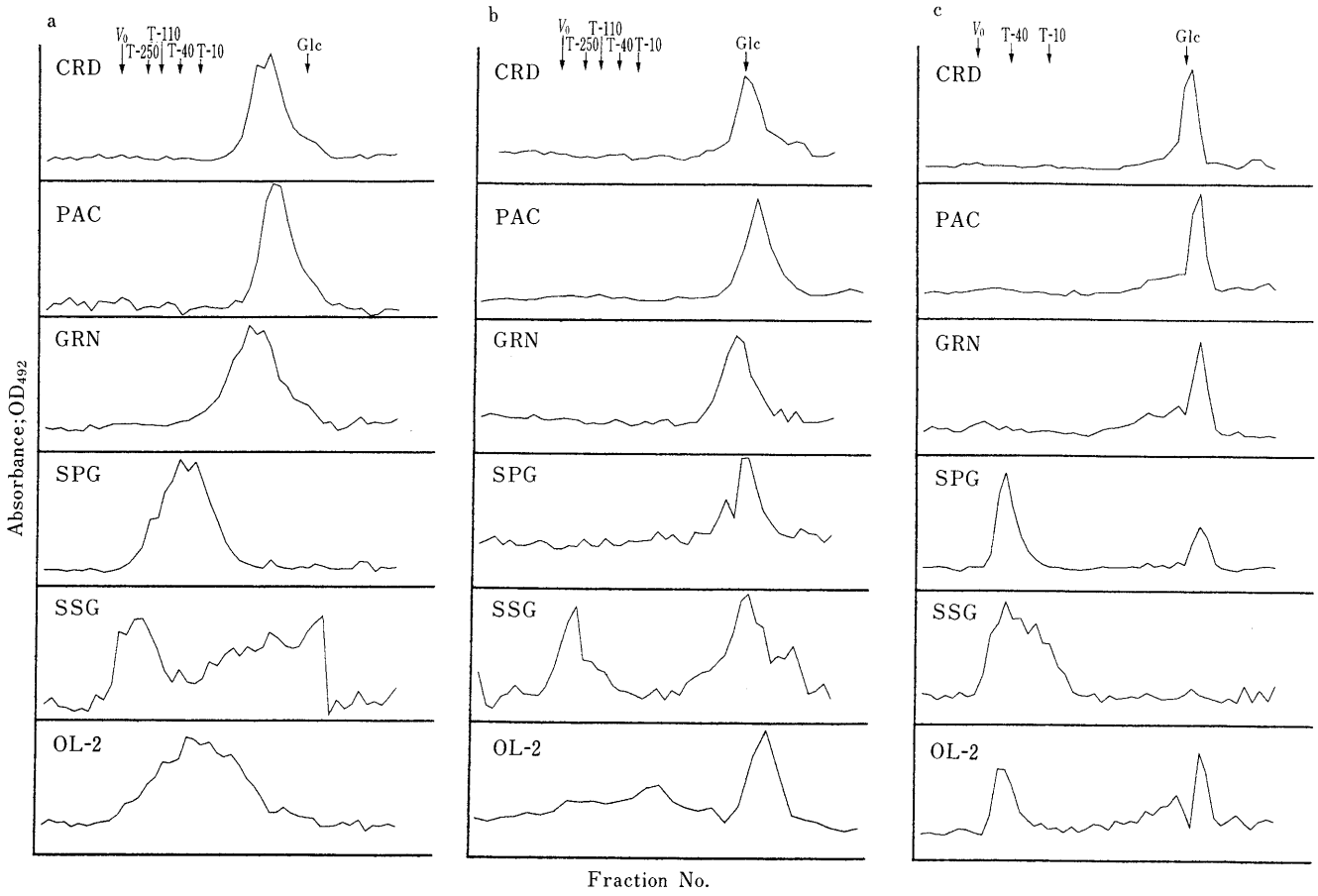

Fig. 1. Elution Profiles of Formolysis Products of β-Glucans

a, 20 min products from Toyopearl HW-60 (1.2×57 cm) eluted by 0.3 N NaOH. b, 3 h products from Toyopearl HW-60 (1.2×57 cm) eluted by 0.3 N NaOH. c, 3 h products from Toyopearl HW-50 (1.5×70 cm) eluted by 0.3 N NaOH. Void (V_0) and bed (Glc) volumes were determined by using dextran T-2000 and glucose, respectively. CRD, curdlan; PAC, pachyman.

formolyzed by 90% formic acid at 100 °C for 20 min or 3 h. The resulting products were analyzed by Toyopearl HW-50 and HW-60 chromatographies in 0.3 N NaOH as a solvent. The results are summarized in Figs. 1a—c. Figure 1a shows chromatograms of 100 °C, 20 min degraded products on HW-60. The average MWs of curdlan, pachyman, and GRN were less than 10 kDa. Of interest, SPG, SSG, and OL-2 gave larger products; those contained significant proportions of 100 kDa products. These facts strongly suggested that the stability of the glucan to formolysis was significantly dependent on the structure of the glucan, and higher branched glucans are probably be more resistant to formolysis.

In order to compare the stability of the glucan, formolysis was prolonged to 3 h and the products were analyzed by HW-60 and also HW-50. Products of curdlan, pachyman, GRN and SPG were eluted near the bed volume, but SSG and OL-2 still contained a significant proportion of larger MW products by HW-60 (Fig. 1b). In the case of HW-50 (Fig. 1c), curdlan, pachyman, and GRN did not elute at the void volume but SPG, SSG, and OL-2 showed a significant proportion of products eluted at the void volume. These facts strongly supported the above mentioned hypothesis.

Comparing the primary structure of these glucans, the degree of branching significantly influenced the stability of the glucan, such that highly branched glucans significantly resisted formolysis. Comparing the stability of GRN and SPG to formolysis, GRN was weaker than SPG. From the results of methylation and NMR analyses, we have shown that the unit structure of GRN and SPG are indistinguishable. However, the data shown in this paper clearly indicated a difference in sensitivity to formolysis; SPG is stronger than GRN. Considering the higher stability of SSG and OL-2, it is probable that SPG contained fewer branches than those, but a considerably higher number than GRN. To confirm the ratio of branches in these glucans, especially in GRN and SPG, these glucans were treated once with sodium hydroxide and digested with zymolyase. The elution profile of each digest was shown in Fig. 2. Curdlan and pachyman were completely digested to a low MW product, and SSG and OL-2 could not be digested at all. Of interest, GRN and SPG were partially degraded, and the ratio of the resistant part (ratio of high MW/total) was 56% in GRN and 68% in SPG. These results strongly suggested that the branching ratio was a little bit higher in SPG than GRN.

Comparing the stability of SSG and OL-2, OL-2 was not significantly stronger than SSG, though the degree of branching was higher in the case of OL-2. OL-2 was extracted from the sclerotia, but SSG was from a culture filtrate. It is probable that sclerotia is a kind of organ, and thus contained more complicated glucan synthetic

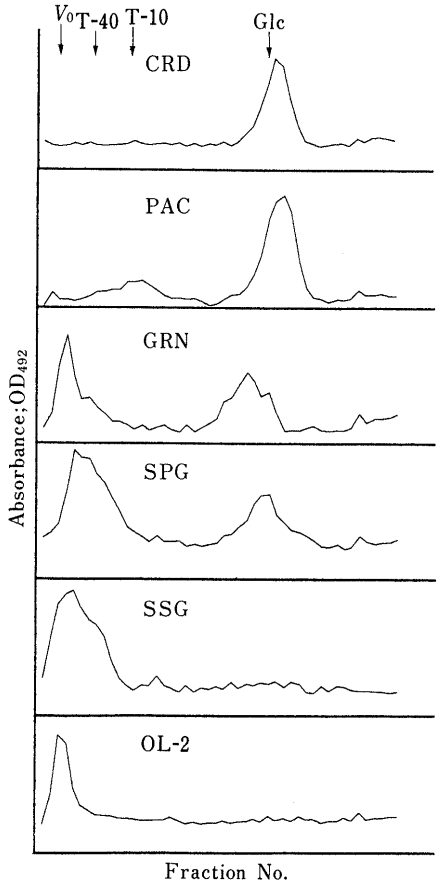

Fig. 2. Elution Profiles of Zymolyase Digests of β-Glucans from Toyopearl HW-50 (1.5 × 70 cm) Eluted by 0.3 N NaOH

Void (V_0) and bed (Glc) volumes were determined by using dextran T-2000 and glucose, respectively.

machinery than in liquid cultured mycelium. Considering these facts, OL-2 might contain both a higher branched and lesser branched part in whole glucan molecule, and the lesser branched part might produce a weaker domain.

In this report we did not compare the unit structure of the resulting products. However, if the side chain residue was not as stable as the main chain residue, a linear glucan segment might be produced during formolysis and thus, larger degradation products might not be present, especially after 3 h of formolysis.

Preparation of Small MW SSG (Small-SSG) The above results suggested that SSG is significantly resistant to formolysis. To find more effective conditions to prepare small MW SSG, SSG was treated by various concentrations of formic acid at 121 °C for 15 min. The molecular weight of the resulting products was estimated by passing through an ultrafiltration membrane, with cut-off MW of 200 and 10 kDa. As shown in Table 1, a proportion of the high MW fraction was gradually increased and the low MW decreased in relation to the concentration of formic acid.

To prepare a large quantity of small-SSG, SSG was formolysed by 70% or 20% of formic acid at 121 °C for 15 min, then fractionated by the stepwise addition of ethanol. The yield and MW of the resulting products are summarized in Table 2, and we were able to successfully prepare small-SSG by formic acid degradation.

Table 1. Preparation of Small-SSG by Various Concentrations of Formic Acid at 121 °C for 15 min

Concn. of formic acid (%)	% recovery		
	>200 kDa	200—10 kDa	<10 kDa
90	14.3	7.5	78.2
80	16.5	0	83.5
70	19.7	6.5	73.8
60	37.1	2.8	60.1
50	41.0	13.7	45.3
40	43.4	13.9	42.7
30	48.4	22.5	29.1
20	52.5	19.3	28.2
10	63.1	22.6	14.3

Each 5 mg of SSG was dissolved in 500 μl of distilled water and mixed with 5 ml of formic acid to make indicated % and heated at 121 °C for 15 min. Each hydrolysate was diluted ten-fold by distilled water and applied to ultrafiltration membranes having a MW cutoff of 200 and 10 kDa (Advantec). Carbohydrate concentration of both retentates and filtrates were determined to calculate recovery.

Table 2. Fractionation of Formolysis Product of SSG by Ethanol Precipitation

Formic acid concn. (%)	Ethanol concn.	Yield (%)	Average MW (kDa)
70	0 vol. ppt	16.2	
	1 vol. ppt	10.6	
	2 vol. ppt	6.0	>150
	8 vol. ppt	2.1	44, 2.4
	8 vol. ppt	32.8	<1
20	2 vol. ppt	21.3	
	4 vol. ppt	5.8	15
	8 vol. ppt	2.8	9

Each 2.0 g of SSG was dissolved in 90 ml (for 70%) or 240 ml (for 20%) of distilled water and mixed with 210 ml (for 70%) or 60 ml (for 20%) of formic acid. Each mixture was heated at 121 °C for 15 min. The resulting formolysate was evaporated to dryness, re-dissolved in distilled water, and fractionated by adding an aliquot of ethanol to precipitate products. MW was estimated by comparing the elution profile with dextran (Pharmacia) using Toyopearl HW-55 with 0.3 N NaOH as a solvent.

Antitumor Activity of Small-SSG Molecular weight is important for the biological activity of (1→3)-β-D-glucan. To elucidate the MW dependency of the biological activity of SSG, we examined the antitumor activity of SSG to the solid form of Sarcoma-180 in this paper. As shown in Table 3, significant antitumor activity was observed at as high a MW as 15 kDa. The lower limit of MW in showing the antitumor activity was similar to that of GRN.

Discussion

The structure of (1→3)-β-D-glucan can be simply shown as the unit structure. However, its physicochemical properties are quite varied because of the presence of various DB, conformations, and MW.[3] Branching is a key factor in the structure–activity relationship of (1→3)-β-D-glucan.[13] Antitumor activity to the solid form tumor was high in the cases of GRN, SPG, and SSG and low in CRD and OL-2.[13-16] Priming activity to the lipopolysaccaride (LPS)-elicited tumor necrosis factor (TNF) synthesis was high in SSG and OL-2.[17] TNF production *in vitro* was only shown by GRN.[18] Reactivity to limulus factor G was inversely related to DB.[19] These facts suggested that the side chain is important for various

Table 3. Antitumor Activity of Small-SSG against Solid Form of Sarcoma 180

Sample	Dose ($\mu g \times 5$)	Tumor weight mean (g) $\pm$ S.D.	Inhibition (%)	Complete regression
SSG	20	5.6 $\pm$ 2.2	30.4	0/8
	100	0.8 $\pm$ 1.3***	89.6	5/8
70% Formolysis				
Small-SSG	20	2.5 $\pm$ 6.2*	68.7	4/8
0 Vol. ppt	100	0.1 $\pm$ 0.1***	99.4	6/8
Small-SSG	20	0.1 $\pm$ 0.3***	98.6	6/8
1 Vol. ppt	100	1.0 $\pm$ 1.4***	87.6	2/8
Small-SSG	20	0.1 $\pm$ 0.2***	99.3	6/8
2 Vol. ppt	100	0.9 $\pm$ 1.1***	89.1	3/8
Small-SSG	20	3.0 $\pm$ 7.1	62.9	4/8
8 Vol. ppt	100	0.8 $\pm$ 1.6***	90.4	4/8
Small-SSG	20	8.5 $\pm$ 6.5	−5.1	0/8
8 Vol. sup	100	10.1 $\pm$ 8.6	−26.0	0/8
Control	0	8.1 $\pm$ 4.3	0.0	0/13
20% Formolysis				
Small-SSG	20	1.6 $\pm$ 3.5**	79.9	2/7
4 Vol. ppt	100	6.6 $\pm$ 6.5	19.2	1/8
Small-SSG	20	6.3 $\pm$ 5.7	23.5	0/7
8 Vol. sup	100	6.3 $\pm$ 4.1	23.1	1/8
Control	0	8.2 $\pm$ 5.5	0.0	0/15

Sarcoma 180 cells ($5 \times 10^6/50\,\mu l$) were inoculated subcutaneously (day 0). Each sample was administered as a saline solution by intraperitoneal injection on days 7, 9, 11, 13, and 15. Inhibition ratio and complete regression were determined at day 35 after tumor inoculation. The significance of differences was evaluated according to Student's t-test. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

biological activities, but the structure–activity relationship varied.

Molecular understanding of the glucan-mediated biological activity and immunopharmacological activity is important in establishing clinical efficacy. Precise examination on the β-glucan receptor mediated biological activity might help to establish the mechanisms involved.[6] Phagocytosis of β-glucan particle was inhibited by an oligosaccharide prepared from yeast glucan. To examine the structure activity relationship of the glucan-mediated biological activity, use of a series of oligosaccharides, having various DB and MW, as antagonists would be fruitful. The evidence obtained in this paper would help to prepare oligosaccharides with various DB.

The data shown in this paper demonstrated that the side chain is important in resisting formolysis and the presence of a side chain makes glucan resistant to formolysis. The side chain is located outside of the triple helix chain, which is produced by an interaction of main chain unit. The stability of highly branched glucan to formolysis would be due to the restricted mobility of the side chain residue in between adjacent chains.

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