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An antitumor, branched (1→3)-β-D-glucan from a water extract of fruiting bodies of *Cryptoporus volvatus*

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Abstract

A water-soluble, (1→6)-branched (1→3)-β-D-glucan (H-3-B) was isolated from a hot-water extract of the fruiting bodies of the fungus, *Cryptoporus volvatus* (Basidiomycetes). Enzymatic analysis using *exo*-(1→3)-β-D-glucanase and methylation analysis indicated that this polysaccharide has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, and single, β-(1→6)-linked D-glucopyranosyl residues attached as side chains to, on average, every fourth sugar residue of the main chain. This structure was confirmed by ^{13}C NMR spectra of the glucan in Me_2SO-d_6. The weight-average molecular weight (M_w) of H-3-B was determined to be 44.0×10^4 by gel permeation chromatography equipped with a low-angle laser-light-scattering photometer. The electron microscopic observations showed that H-3-B and its sonicated sample (S-H-3-B, $M_w = 13.7 \times 10^4$) can be described as linear worm-like chains. The mass per unit length for native and sonicated H-3-B was determined to be 1750 and 1780 g mol^{-1} nm^{-1}, respectively, from the contour lengths obtained by electron microscopy and the molecular weights. These values are in good agreement with that expected for the triple stranded structure. A sample denatured in 0.1 M NaOH and subsequently renatured by neutralization showed a mixture of linear and cyclic structures, and larger aggregates with less well-defined morphology. The H-3-B and S-H-3-B had antitumor activity against the Sarcoma 180 tumor.

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1. Introduction

A family of glucans which contain a main chain of (1→3)-linked β-D-glucopyranosyl units, substituted at 0-6 by single-unit β-D-glucopyranosyl side chains, have received considerable attention because of their antitumor activity (immunomodulating action) and their unique solution properties [1,2]. Lentinan [3,4], scleroglucan [5,6], and schizophyllan [7,8] are the most well-known members of this group of polysaccharides. It is reported that their antitumor activities are closely related to the triple helical structure of the (1→3)-β-D-glucan backbone chain [9,10]. These multi-stranded structures, which resist melting, also produce highly viscous aqueous solutions, thus making them potentially useful in petroleum drilling and water flooding operations [11,12].

In order to better understand the relationships between degree of branching of comb-like, branched (1→3)-β-D-glucans, and physical and biological properties, we have extended our studies to other glucans which have different degrees of side chain branching [2,13].

In this paper, we report on a new branched (1→3)-β-D-glucan, isolated from the fruiting bodies of *Cryptoporus volvatus*, which has an average of one side chain for every four main chain residues. The polysaccharide is characterized chemically with respect to sugar composition and linkage pattern and physically by determining the mass per unit length (M_L) of the glucan chains. The M_L was determined from the contour length of the glucan chains obtained by electron microscopic observations and the molecular weight by gel-permeation chromatography equipped with a low-angle laser-light-scattering photometer. Furthermore, data obtained on antitumor activity against Sarcoma 180 ascites cell are compared with those obtained for schizophyllan.

2. Experimental

Isolation of the polysaccharide.—Fruiting bodies of *Cryptoporus volvatus*, which is a bracket fungus (Aphyllophorales) and grows on recently killed conifer trees, especially Pine species, were collected in forests near Gifu, Japan. The finely ground fruiting bodies (100 g), after successive extraction with 1.2 L of hot acetone and 5 L of hot 70% aq EtOH, were extracted with 17 L of hot water at 85–90°C. Proteins in the water extract were digested by treatment with protease (Actinase E, 10×10^5 units/g: Kaken Seiyaku Co., Tokyo; 40°C, 72 h). The mixture, after inactivation of the enzymes by heating, was allowed to stand in a ice-bath for a minimum of 7 days. The gelatinous polysaccharides which precipitated were isolated by centrifugation and dissolved in hot water. The polysaccharides were reprecipitated, isolated by centrifugation, and redissolved in hot water as described above a total of four times. The materials thus obtained were dissolved in hot water, dialyzed, and the dialysate was passed through a Millipore filter (0.8 μm) to remove small amounts of insoluble impurities. The filtrate was then lyophilized to give the polysaccharide (H-3-B) as pale brownish flakes in ca. 10% yield.

Partial depolymerizaion of the polysaccharide.—A partially degraded sample (S-H-3-B) was prepared by sonication of 30 mL of an aqueous native H-3-B solution (3.3 mg/mL) containing 1 vol% acetone with a sonicator (Ultrasonic, Ltd., USV-V-4A) for 5 h at 180 W and 25 kHz. The temperature was maintained below 25°C during sonication. The sonicated sample was repurified by the method of Paradossi et al. [14] to remove metal eroded from the sonicator probe.

Sugar analysis.—The polysaccharide was hydrolyzed with 90% formic acid (100°C, 6h), and then with 1.0 M CF_3CO_2H (100°C, 20 h). The resulting sugars were analyzed by paper chromatography (PC), and by gas–liquid chromatography (GLC) as the corresponding alditol acetate derivatives [15]. The PC was performed on Advantec Toyo No. 51A filter paper with a solvent system of 6:4:3 1-butanol–pyridine–water by the double ascending method, and the sugars were detected with an alkaline silver nitrate reagent. The GLC was performed using a Shimadzu GC-8A chromatograph equipped with a flame-ionization detector and glass column (3 mm i.d. × 2 m) packed with 3% of Silicone OV-225 on Chromosorb W (80–100 mesh) at 193°C.

Methylation analysis.—The polysaccharide H-3-B was methylated twice by the Hakomori method [16]. The permethylated polysaccharide was hydrolyzed and converted to the partially methylated alditol acetates (PMAA) using the same procedures as described for the sugar analysis of the polysaccharide. The PMAA were analyzed and quantified using GLC. The instrument used was the same as that used for sugar analysis. The oven temperature was held constant at 187°C. Peak areas were measured with a Shimadzu C-R3A Chromatopac. The sample was also analyzed by GLC–MS using a Hewlett–Packard 5890 gas chromatograph equipped with a capillary column (Hicap-CBP10, 0.25 mm i.d. × 25 m, GL Science Co. Ltd., Tokyo) and interfaced with a Jeol mass spectrometer (SX-102A) operating in the electron impact mode. Identification of each of the PMAA was made by comparison of the mass spectra obtained with those of known standards reported by Jansson et. al [17].

Enzymatic analysis.—The degree of branching of the glucan was determined from the molar ratio of glucose and gentiobiose released on hydrolysis of the glucan with *exo*-(1→3)-β-D-glucanase from *Basidiomycete* QM-806 [18]. A solution of the glucan in 0.1 M MES buffer (pH 6.5) containing the enzyme was incubated at 40°C for 12 h. After filtration through a Millipore filter (Ultra free C3GC; molecular weight cutoff 10 000), the glucose and gentiobiose present in the digest were analyzed by high performance liquid chromatography (HPLC) on a Polyamine II column (4.6 i.d.x25 cm, YMC Co. Ltd., Japan). The HPLC was performed on a Jasco HPLC equipped with a refractive-index detector. The eluent was 65:35 MeCN–water with a flow rate of 1.2 mL/min, and the temperature of column was maintained at 35°C.

Molecular weight and molecular weight distribution.—The number- and weight-average molecular weights (M_n, M_w), and molecular-weight distribution were estimated by high-performance gel permeation chromatography (GPC) using a low-angle laser-light-scattering photometer (, Tosoh Co. Ltd., LS-8000) and a differential refractometer (RI, Tosoh Co. Ltd., RI-8011) as detectors. Samples (1 mg/mL, 300 μL) dissolved in the eluent (0.25 M KCl) were injected on three sequentially connected columns of G3000PW, G5000PW, and G6000PW (Tosoh Co. Ltd.) which were kept at 40°C. The pressure in the column system was less than 50 kg/cm^2 and the flow rate was 0.7 mL/min. The LALLS

used in this system measures the scattering intensity at $\theta = 5°$, where the angular dependence can be ignored [19]. The RI and signals for each point of the chromatogram were analyzed as previously described [19] by using a software package provided by Tosoh Co. Ltd. to give M_n, M_w, and molecular-weight distribution curves. Pullulan fractions P-400 ($M_w = 34.8 \times 10^4$) and P-100 ($M_w = 10.0 \times 10^4$), manufactured by Showa Denko Co. Ltd., were used as standards with the assumption that these samples have the same specific refractive index increment as H-3-B and S-H-3-B.

Electron microscopy.—Preparation of replicas for electron microscopy (EM) was carried out by vacuum drying (10^{-6} Torr for 1 h) of 3–20 μg/mL H-3-B polysaccharide samples in 50% aq glycerol sprayed on freshly cleaved mica discs. Dried specimens were first rotary shadowed with a 0.7-nm thick film of 95% platinum–5% carbon deposited at an angle of 6°, and subsequently with a 7-nm thick carbon film from an angle of 90° [20,21]. The electron micrographs obtained using a Philips EM 400T were calibrated using a line grid (1200 lines/mm, Agar Aids). The contour length distributions of the S-H-3-B and H-3-B samples were obtained by digitalization of the visualized chains as described previously [21].

NMR spectroscopy.—For NMR spectra, 22.7 mg of H-3-B were dissolved in 0.7 mL of Me_2SO-d_6. Proton-decoupled ^{13}C NMR spectra at 75.46 MHz were recorded on a Varian VXR-300 spectrometer at 80°C. The spectral width was 4.9 kHz and pulse width 20.4 μs. Chemical shifts are expressed relative to that of tetramethylsilane.

Other physical measurements.—The intrinsic viscosity ([η]) of the samples in 0.25 M aq KCl was determined with a four-bulb, modified Ubbelode capillary viscometer at 25.0 ± 0.01°C. Optical rotations were measured using a Jasco DIP-4 spectropolarimeter with a 10-cm cell. The temperature was monitored by a calibrated thermistor probe immersed in the solution. Infrared (IR) spectra were recorded with a Horiba FT-300 spectrometer.

Assay of antitumor activity.—Antitumor activity of H-3-B and S-H-3-B was assayed as described previously [9]. A schizophyllan sample (SPG, $M_w = 45.0 \times 10^4$) was used in parallel tests. Sarcoma 180 ascites cells (ca. 2×10^6 cells) were implanted subcutaneously into the groin of ICR-JCL female mice (20–25 g). After 24 h, these polysaccharide samples were intramuscularly injected once into the hind paws of ten mice at a dose of 5 or 10 mg per kg of mouse. 33 Days after the tumor implantation, all the mice were sacrificed, and the tumors were excised and weighed. The tumor growth inhibition ratios (%) were calculated by $[(A - B)/A] \times 100$, where A is the average tumor weight of the control group and B is that of the test group.

3. Results and discussion

Chemical structure.—Chromatography (PC and GLC) clearly showed that the polysaccharide H-3-B is exclusively composed of glucose residues. H-3-B was almost completely digested with *exo*-(1→3)-β-D-glucanase to give glucose and gentiobiose in the molar ratio of 3.01:1.00 (data not shown). The GLC of the alditol acetates from fully methylated H-3,-B showed three peaks. These peaks were identified as the alditol acetates from 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl D-glucose (molar proportions 1.00:3.10:1.03 based on peak areas), and confirmed by GLC–MS spectrometry (Table 1). The H-3-B had a low

Table 1
Methylation analysis of H-3-B by GLC and GLC–MS

Methylated sugar (as alditol acetate)	t_R [a]	Molar ratio	Major mass fragments (*m/z*)
2,3,4,6-Me_4-Glc	1.00	1.00	43, 45, 71, 87, 101, 129, 145, 161, 205
2,4,6-Me_3-Glc	1.71	3.10	43, 45, 87, 101, 117, 129, 161, 203, 233
2,4-Me_2-Glc	3.72	1.03	43, 87, 117, 129, 159, 189, 233

[a] Relative retention time with respect to that of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

positive specific rotation $[\alpha]^{30}_D$ +19.2° (*c* 0.107%, H_2O), and showed characteristic IR absorbance at 891 cm^{-1}, indicating the presence of β-D-glycosidic linkages [22]. The above results indicate that H-3-B is a (1→3)-linked β-D-glucan with single glucose residues branched at O-6 of the main chain, and a branch point occurs, on average, at each fourth glucose residue of the main chain. The repeating unit, based on the data collected thus far, is shown in Fig. 1. Structural heterogeneity, however, is possible, and is the subject of further investigations.

^{13}C-NMR spectrometry.—Fig. 2 shows the ^{13}C NMR spectra of H-3-B and schizophyllan in Me_2SO-d_6. The spectral features of H-3-B are very similar to those of schizophyllan, although there are differences in the relative intensities of some signals, reflecting the difference in the degree of comb-like branching. In particular the peak heights of the C-3 and C-6 signals due to the (b) and (e) units in Fig. 1 are relatively small, indicating less branching than schizophyllan. Table 2 summarizes the ^{13}C NMR signal assignments. These

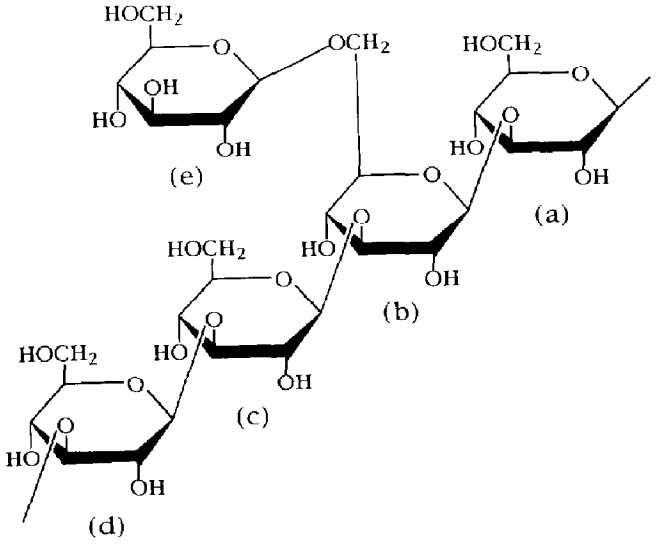

Fig. 1. A possible repeating unit for H-3-B glucan. The lower case letters denoting each glucose residue refer to NMR assignments in Table 2.

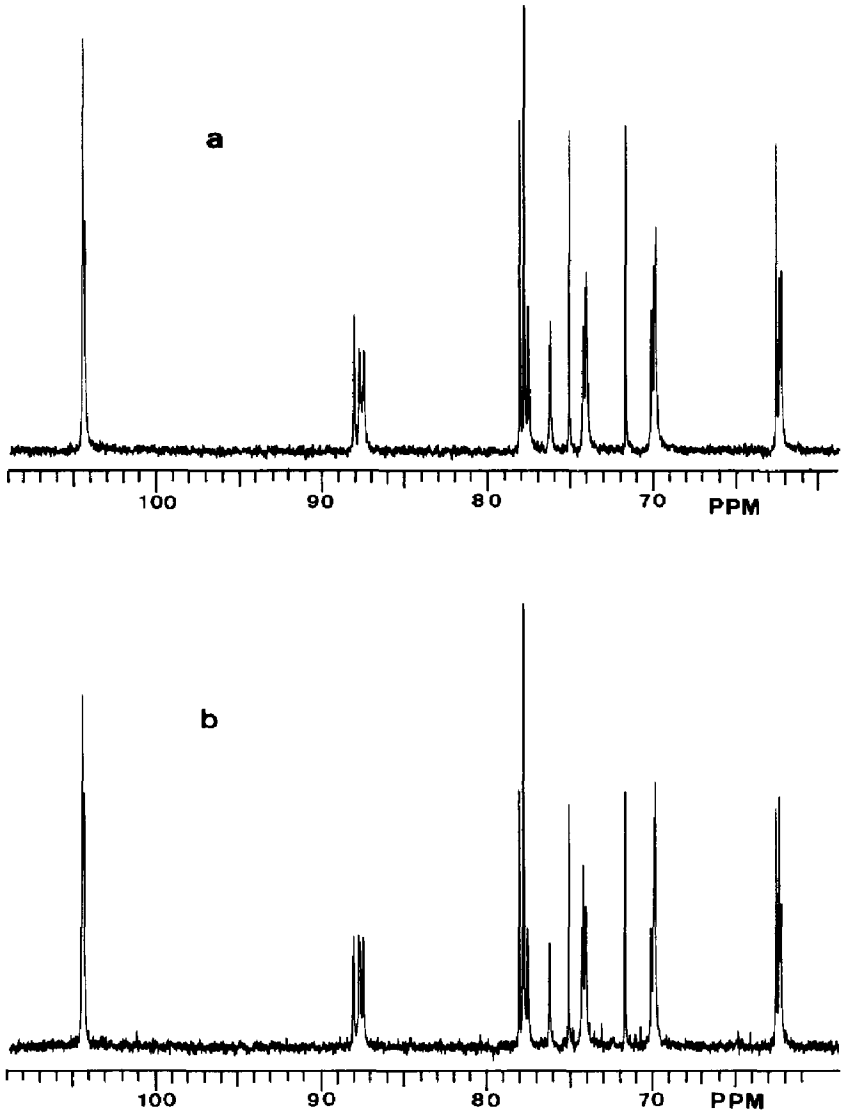

Fig. 2. ^{13}C NMR spectra of schizophyllan (a) and H-3-B (b) in Me_2SO-d_6 at 80°C.

assignments are based on the reported values obtained for scleroglucan [23], HA-β-glucan [24], and tylopilan [25].

Conformation and solution properties.—Fig. 3 shows RI and LALLS chromatograms of GPC for H-3-B and S-H-3-B in 0.25 M aqueous KCl. H-3-B has a broad distribution of molecular weight distributing from the void volume at $t_R = 45$ min, while S-H-3-B has a narrower molecular-weight distribution than S-H-3-B. The M_w and polydispersity index ($I_p = M_w/M_n$) of H-3-B and S-H-3-B determined from the chromatograms are shown in Table 3. Both values of M_w and I_p became smaller after sonication.

Fig. 4 shows the electron micrographs of H-3-B and S-H-3-B obtained from aqueous solution. The samples appear as uniformly thick and convoluted linear chains with polydispersity in length. Fig. 5 shows histograms of contour length distribution for H-3-B and S-

Table 2
^{13}C NMR chemical shifts [a] and signal assignments for H-3-B in Me_2SO-d_6

C-1	C-2	C-3	C-4	C-5	C-6
104.23	74.86(e) [b]	87.83(c)	71.45(e)	77.59(a,c,d)	69.66(b)
104.12	74.02(c)	87.52(a)	69.88(c)	77.32(e)	62.35(e)
	73.87	87.45(d)	69.73	76.00(b)	62.15(a,d)
	73.80	87.25(b)	69.66		62.04(c)
		77.85(e)			

[a] Given in ppm relative to tetramethylsilane.
[b] Corresponding to the sugar residues shown in Fig. 1.

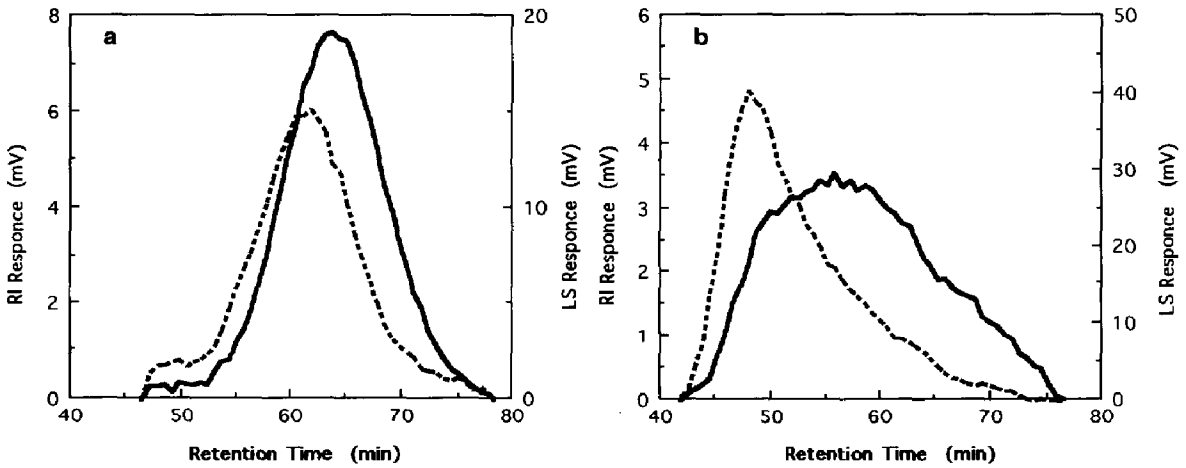

Fig. 3. RI (—) and LALLS (---) chromatograms of GPC for S-H-3-B (a) and H-3-B (b) in 0.25 M aqueous KCl.

Table 3
Molecular properties of H-3-B and S-H-3-B

Samples	M_w (10^4g/mol)	M_w/M_n	L_w (nm)	$M_L=M_w/L_w$ (g/mol nm)	$[\eta]$ [a] (dL/g)
H-3-B	44.0	2.30	252	1750	2.55
S-H-3-B	13.7	1.42	77	1780	0.67

[a] Determined in 0.25 M aqueous KCl at 25°C.

H-3-B. The weight-average contour lengths (L_w) for H-3-B and S-H-3-B are summarized in Table 3.

The values for M_w/L_w, which are equal to the mass per unit length (M_L), are 1750 and 1780 g mol^{-1} nm^{-1} for H-3-B and S-H-3-B, respectively. It should be noted that the corresponding average in the molecular weight and the contour length distribution are used in the calculation of M_L [2]. These M_L values are in good agreement with the value calculated for a triple-helical structure with helix pitch of 0.30, which was obtained for

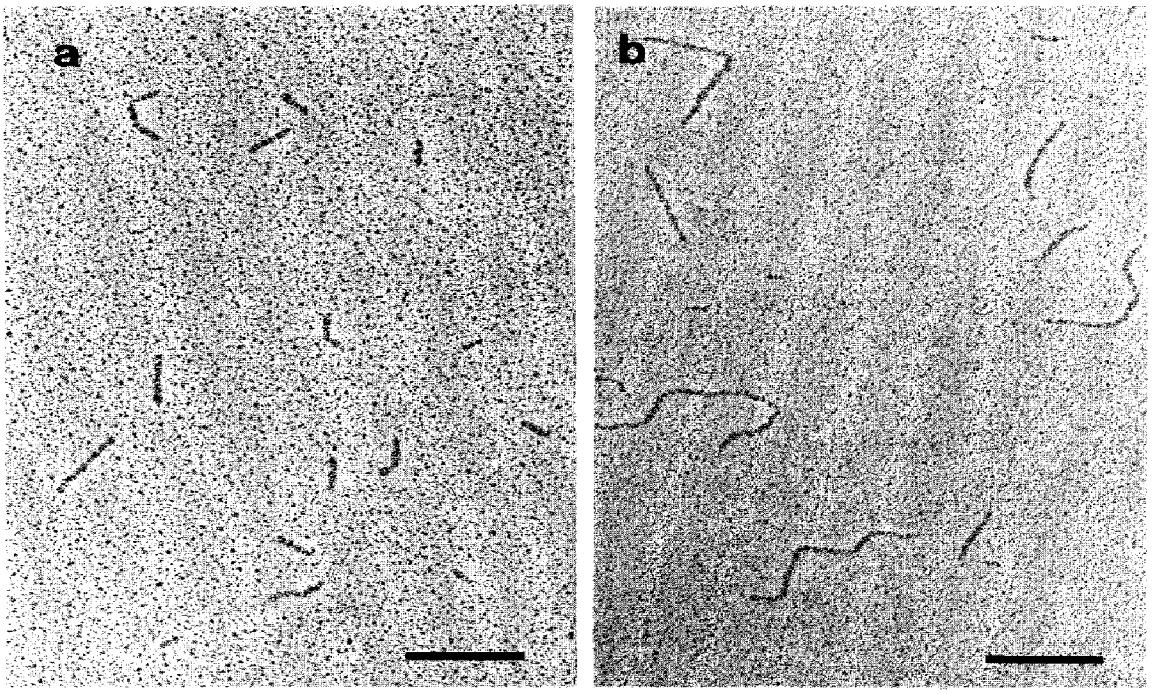

Fig. 4. Electron micrographs of S-H-3-B (a) and H-3-B (b). Scale bar, 200 nm.

schizophyllan and curdlan by X-ray fiber diffraction studies [26,27], and with a degree of branching (db) of 1/4. The db is defined as the mole of side chain glucose per mole of main chain glucose. The relative uncertainties of the experimentally determined M_L are in the order of 10%.

Fig. 6 shows a typical electron micrograph of the sample which had been subjected to a denaturation–renaturation cycle; that is, the sample was denatured in 0.1 M NaOH (0.1

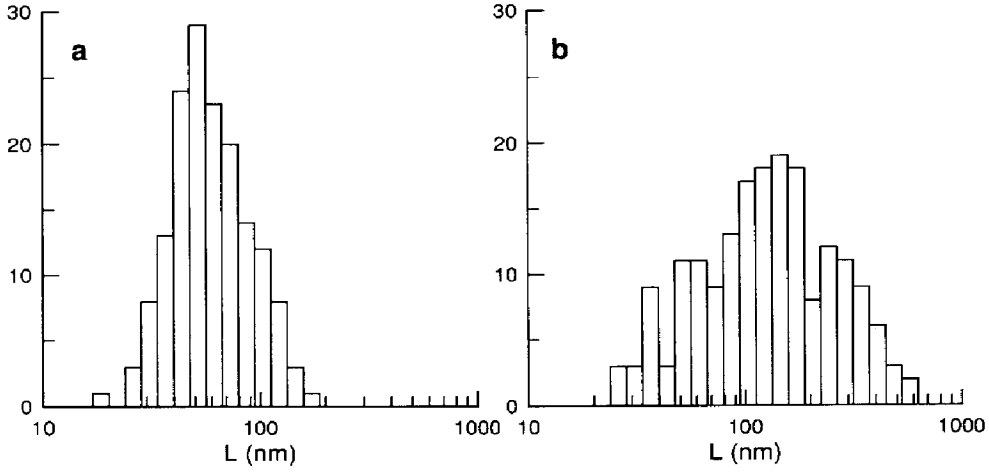

Fig. 5. Bar graphs of number of observations versus contour length obtained from electron micrographs of S-H-3-B (a) and H-3-B (b).

Fig. 6. Electron micrograph of a reconstituted H-3-B sample from 0.1 M NaOH aqueous solution. Scale bar, 200 nm.

mg/mL) and subsequently renatured by neutralization. A mixture of linear, cyclic structures, and larger aggregates with less well-defined morphology, is observed in the sample, in contrast to the linear chain structure observed for "native" H-3-B. Preliminary analysis of macrocycle formation in branched (1→3)-β-D-glucans has been reported elsewhere [28,29]. The distribution of macromolecular structures of the "renatured" sample thus prepared provides additional information about the influence of extraction conditions used in the isolation procedure, not only for the H-3-B sample presented here, but also for other members of the same family of polysaccharides. Analogous blends of linear and circular structures, as well as multichain clusters, were also observed for lentinan [28] and T-5-N glucan [2,30] which were extracted by strong alkali solution from the cell wall of fruiting bodies. Although the use of aqueous alkali at various concentrations for selectively extracting populations of glucans from cell walls of fungi is widely practiced, such conditions may well alter the three dimensional structure of the glucans.

The intrinsic viscosities for H-3-B and S-H-3-B, determined in 0.25 M aqueous KCl, were 2.55 and 0.67 dL/g which are consistent with the values calculated by Yamakawa's equation [31] derived for $[\eta]$ of long worm-like cylinders with $M_L = 2000$, diameter of cylinder = 2.0 nm, and persistence length = 140 nm [2]. However, it was found that the intrinsic viscosity increased with time. The aqueous solutions with more than 0.1% polymer concentration form a soft gel after one week at room temperature. This behavior is very different from that of aqueous schizophyllan solutions which are fairly stable for a long time even at 4°C. These results suggest that the degree of branching of the side chain is a determinant of the stability of the aqueous solution.

Antitumor activity.—The results on the antitumor activity of H-3-B and S-H-3-B against Sarcoma-180 are summarized in Table 4, which also contains the results obtained for schizophyllan (SPG) in parallel tests. These data indicate that H-3-B and S-H-3-B have antitumor activity similar to, but slightly less effective than, SPG.

Table 4
Antitumor activities of H-3-B, S-H-3-B, and SPG

Sample	Dose (mg/kg mouse)	W_T [a] (g)	Tumor growth inhibition (%)	Complete regression
Control		5.34 (0.81)		0/10
H-3-B	10	0.13 (0.07)	97.6	7/10
	5	0.47 (0.19)	91.2	5/10
S-H-3-B	10	0.59 (0.23)	89.0	5/10
	5	0.90 (0.42)	83.1	2/10
SPG	5	0.16 (0.08)	97.0	6/10

[a] Mean tumor weight of ten mice. The standard error is given in parentheses.

It has been shown that antitumor activities of branched (1→3)-β-D-glucans correlate with the degree of triple helical structure of the glucan backbone chain [9,10]. In this way the antitumor activity of H-3-B and S-H-3-B is consistent with previous findings. In addition to the conformation of the glucan backbone, the degree of sidechain branching [32,33] and the molecular weight [34] also appear to play an important role in antitumor action. These additional effects may account for the small differences in the antitumor activity between H-3-B, S-H-3-B, and SPG at the same dosage.

The discussion on structure–antitumor activity of this class of polysaccharides should be extended to include the effects of morphological features (ultrastructures) on activity. As shown in this study and our previous works [28,29], samples regenerated from alkaline or dimethylsulfoxide solutions consist of mixtures of aggregates, and linear and cyclic species. We have been examining the biological activities, including antitumor activity against Sarcoma 180, of purified samples having different morphologies [35]. These results may account for results [36–38] previously reported for ''denatured'' and ''renatured'' branched (1→3)-β-D-glucans.

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