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Triple Helix of β-D-Glucan from *Lentinus Edodes* in 0.5 M NaCl Aqueous Solution Characterized by Light Scattering

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ABSTRACT: β-(1→3)-D-glucan with (1→6) branching (L-FV-I) from *Lentinus edodes* in water was degraded into seven fractions of different molecular weights by ultrasonic irradiation. Weight-average molecular weight M_w, radius of gyration $<s^2>_z^{1/2}$ and intrinsic viscosity $[\eta]$ of the β-D-glucan and its fractions in 0.5 M NaCl aqueous solution and dimethylsulfoxide (DMSO) were studied by multi-angle laser light scattering (MALLS), GPC combined with MALLS, and viscometry. M_w dependence of $[\eta]$ for the glucan in 0.5 M NaCl aqueous solution was represented approximately by $[\eta] = 7.69\times10^{-6} M_w^{1.32}$ ($cm^3\ g^{-1}$) at M_w from 1.87×10^5 to 1.20×10^6 at 25℃. GPC chromatograms of the glucans in aqueous solution contained two peaks, a main peak corresponding to triple-stranded chains with molecular weight $M_{w,m}$, and small second peak corresponding to fragments of single chains with $M_{w,s}$ (about 20±5% content). Analysis of $M_{w,m}$ and $<s^2>_{z,m}^{1/2}$ in term of the known theory for wormlike chains yielded 2180±100 nm^{-1}, 120±10 nm and 0.31 nm for molar mass per unit contour length M_L, persistence length q, and contour length h per main-chain glucose residue, respectively, which agree closely with theory data of triple-helical chains and reported parameters for triple-helix schizophyllan in 0.01 M NaOH aqueous solution. The ratios of $M_{w,m}$ in 0.5 M NaCl to M_w in DMSO were calculated to be roughly 3. The predominant species of the glucan in 0.5 M NaCl aqueous solution exist as triple-helical chains with high rigidity, and in DMSO as single-flexible chains.

KEY WORDS Lentinan / β-D-Glucan / Molecular Weight / Intrinsic Viscosity / Conformation / Triple-Helix Chain / Light Scattering / Gel Permeation Chromatography /

The importance of polysaccharides have provide a major impetus for increasing attention, particularly mushroom polysaccharide as functional food and development of new drugs.[1] β-(1→3)-D-glucan, named *Lentinan* as an antitumor polysaccharide, was first isolated from *Lentinus edodes* by Chihara *et al.*[2,3] Many polysaccharides were isolated from *Lentinus edodes*, and extensively studied for biological activity, such as prominent antitumor activity[4−9] and immune-accelerator[10−13] mainly with T-cell-mediated response.[3] The molecular weight of the β-(1→3)-D-glucan was first reported by light scattering measurement as 9.5×10^5 to 10.5×10^5.[4] However, Suzuki reported average molecular weight of 3×10^5 to 8×10^5 according gel permeation chromatography (GPC) and quaselastic light scattering measurement.[14] Conformation analysis from X-Ray diffraction result[15] predicted five models, one single helix, two double helices, and two triple helices for the crystalline structure of β-(1→3)-D-glucan *Lentinan*, and a right-hand triple helix structure was given a pitch of 0.29 nm. In addition, the order structures of the β-(1→3)-D-glucan main chain and β-(1→6)-D-glucan linked side chain were identified as a single helix conformation, which forms multiple helixes as junction zones for gel structure.[16] Saito *et al.*[17−19] reported conformations readily converted to triple-helix form by lyophilization after dissolving in 8 M urea solution and dialysis against distilled water. Interestingly, a network structure of the branched β-(1→3)-D-glucan in the gel state arose mainly from the triple-helix conformation.[19] However, it has long been suspected to be a triple helix of *Lentinan* in aqueous solution without strong evidence because of the experimental difficulty of fractionation due to aggregation. Molecular weight, conformation, and solubility of the polysaccharides significantly affect antitumor and immunomodulatory activity.[20−22] Therefore, investigating the solution properties of the β-(1→3)-D-glucan is very essential for clarifying the conformation and correlation of second structure to bioactivity of *Lentinan*.

In our previous work,[23] the chemical structure of β-D-glucan isolated from fruiting bodies of *Lentinus edodes* was demonstrated by high-performance liquid chromatography (HPLC), infrared (IR), and ^{13}C NMR as a β-(1→3)-D-glucan with (1→6) branching. Because of gelatinization of the β-(1→3)-D-glucan in aqueous solution, the fractions were with difficultly prepared by nonsolvent addition. The present work used degradation of the β-(1→3)-D-glucan by ultrasonic irradiation, to obtain fractions of different molecular weights. Sonicated fragments of schizophyllan and xanthan still keep their triple- or double-helix structure.[24,25] Weight-average molecular M_w, radius of gyration $<s^2>_z^{1/2}$ and intrinsic viscosity $[\eta]$ of the fractions were studied by viscometry, laser light scattering (LLS) and GPC with LLS in 0.5 M NaCl aqueous solution and dimethylsulfoxide (DMSO). The conformation of the glucan was analyzed from $M_{w,m}$, and $<s^2>_{z,m}^{1/2}$ of the triple-helix component in GPC chromatograms by applying the theory for wormlike chains and Benoit–Doty expression.

EXPERIMENTAL

Preparation of Samples and Solutions

β-(1→3)-D-glucan (L-FV-I) was isolated from fruiting bodies of *Lentinus edodes*, a commercial product cultivated in Hubei of China, by extraction with 5% NaOH/

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0.05% $NaBH_4$ two times, and precipitation with 36% acetic acid to remove L-FV-II, α-(1→3)-D-glucan, according to previously reported method.[23] The supernatant was subjected to the Sevag method to remove proteins, and treated with 30% H_2O_2 to decolorize. Aqueous solution of the glucan was dialyzed against distilled water for 4 days, and concentrated by rotary evaporator at reduced pressure below 45℃, and lyophilized to obtain colorless flakes.

The β-(1→3)-D-glucan solution in distilled water (0.2 wt%, 300 mL) was exposed to 33 kHz ultrasonic irradiation by ultrasonic cleaner (MUS-1004, Shenzhen Modern Ultrasonic Industrial Co., Ltd., China) for 2 and 3.5 h to obtain the fractions coded as L-FV-I2, L-FV-I3. The jacket of the sonication vessel was maintained below 35℃. Sonicated solutions were concentrated by rotary evaporator at reduced pressure below 45℃, and lyophilized to obtain colorless flakes. The original unsonicated sample was coded as L-FV-I. A portion of glucan solution was prepared by ultrasonating the weighted sample L-FV-I in 0.5 M NaCl aqueous solution for 1 to 24 h, coded as L-FV-I1, L-FV-I4, L-FV-I5, L-FV-I6, L-FV-I7, to use directly for GPC-LLS and viscosity measurement.

Analytical grade NaCl was dissolved in distilled water to prepare 0.5 M NaCl aqueous solution. DMSO was distilled, and treated with a molecular sieve to further dehydrate. The preparation of polysaccharide solutions was done under the same conditions, such as stirring, dissolving and residence time. Relatively concentrated stock solution was carefully prepared by completely dissolving the proper amount of the glucan in solvent for over 24 h with stirring. A series of concentrations were obtained by successive dilution of clarified stock solution. Finally, each solution was filtered with sand filter for viscosity measurement, and further clarified with 0.45 μm filter (Whattman, England) two times into scintillation vial for light scattering and GPC-LLS measurement.

Laser Light Scattering (LLS)

In static LLS, scattering light intensity known as Rayleigh ratio (R_θ) of a polymer solution at angle (θ) and concentration (c) is related to weight-average molecular weight (M_w) by[27]

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2c \qquad (1)$$

where $K = 4\pi^2 n_0^2 (dn/dc)^2 / (N_A \lambda_0^4)$, with N_A, n_0 and λ_0 as Avogadro's number, refractive index of the solvent, and wavelength of light in vacuum, respectively. M_w is weight-average molecular weight. $P(\theta)$ is a function of particle scattering, radius of gyration $<s^2>_z^{1/2}$, angle θ, shape and structure. Appropriate representation in this case is

$$P(\theta)^{-1} = 1 + (1/3)(4\pi n_0/\lambda)^2 <s^2>_z \sin^2(\theta/2) \qquad (2)$$

Scattering light intensity was measured with multi-angle laser light scattering instrument (MALLS) equipped with a He–Ne laser (λ = 632.8 nm) (DAWN® DSP, Wyatt Technology Co., USA) in the angles of 42°, 49°, 63°, 71°, 81°, 90°, 99°, 109°, 118°, and 127° at 25℃. Refractive indexes of DMSO and 0.5 M NaCl aqueous solution were measured by an Abbe refractometer as 1.478 and 1.338, respectively. Refractive index increments (dn/dc) were measured with a double-beam differential refractometer (DRM-1020, Otsuka Electronics Co.) at 632.8 nm and 25℃. Specific refractive index increment (dn/dc) for polysaccharide solution in DMSO and dialyzed solution in 0.5 M NaCl aqueous solution was 0.060 $cm^3\ g^{-1}$ and 0.1333 $cm^3\ g^{-1}$, respectively. Astra software was utilized for data acquisition and analysis.

GPC-MALLS Measurements

Gel permeation chromatography with mutil-angle static light scattering (GPC-MALLS) is convenient for determination of true molecular weight and distribution without standard samples. GPC-LLS measurement of the glucan samples was performed on a DAWN®DSP multi-angle laser photometer with a pump P100 (Thermo Separation Products, San Jose, USA) equipped with TSK-GEL G6000 PWXL with a G4000 PWXL column (7.8 mm × 300 mm) for aqueous solution and G4000-H8 with G3000H8 column for DMSO, and differential refractive index detector (RI-150) at 25℃. The eluent was 0.5 M NaCl aqueous solution and DMSO at a flow rate of 1.00 mL min^{-1}. All solutions were filtered with sand filter, and with 0.45 μm filter (CA, Puradisc™ 13 mm Syringe Filters, Whattman, England) for 0.5 M NaCl and a 0.45 μm filter (PTFE, Puradisc™ 13 mm Syringe Filters, Whattman, England) for DMSO. Astra software was utilized for data acquisition and analysis.

Viscosity Measurement

Viscosity of the glucan samples in 0.5 M NaCl aqueous solution and DMSO was measured at 25 ± 0.1℃ using a low-shear four-bulb capillary viscometer and Ubbelohde viscometer. Kinetic energy correction was always negligible. Huggins and Kraemer plots were used to get intrinsic viscosity [η]. From the dependence of intrinsic viscosity on shear rate $\dot{\gamma}$, zero shear-rate viscosity [η] for the samples in 0.5 M NaCl aqueous solution was determined.

RESULTS AND DISCUSSION

Molecular Weight and Intrinsic Viscosity

GPC chromatograms for fractions of the glucan in 0.5 M NaCl aqueous solution are shown in Figure 1. "LS, AUX (Volts)" represents an arbitrary unit of scattering intensity. Measured M_w and [η] for samples are listed in Table I. Error margin of measurement for M_w and $<s^2>_z$ was less than 5%. M_w and [η] for the fractions decreased with ultrasonic time, and polydispersity M_w/M_n of the fractions were 1.8—2.7. Degradation by ultrasonic irradiation was conducted to obtain fractions of different molecular weights, and was superior to nonsolvent addition for rigid polysaccharides.[26]

GPC chromatograms of the unultrasonic glucan in 0.5 M NaCl aqueous solution and DMSO at 25℃ are shown in Figure 2. Zimm plot of the fraction L-FV-I2 in DMSO at 25℃ is illustrated in Figure 3. M_w determined in DMSO by LLS and GPC-LLS are summarized in Table II. M_w dependence of [η] for the glucan (L-FV-I) in 0.5 M

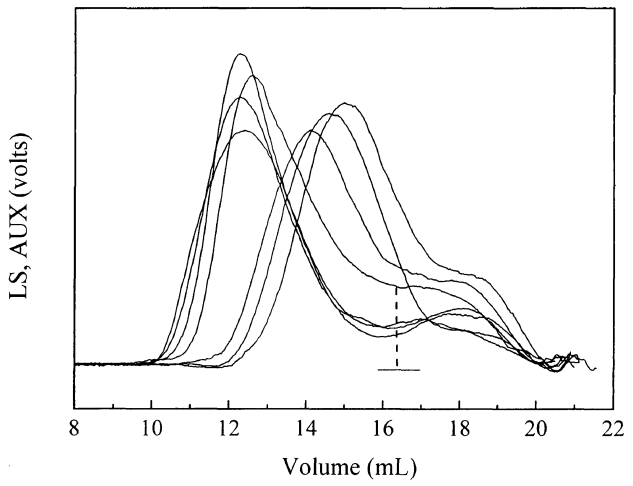

Figure 1. GPC chromatograms of glucan fractions from L-FV-I1 to L-FV-I7 (from left to right) in 0.5 M NaCl aqueous solution at 25℃ by GPC-MALLS. Dash line shows a dividing line of main peak and shoulder peak for L-FV-I4.

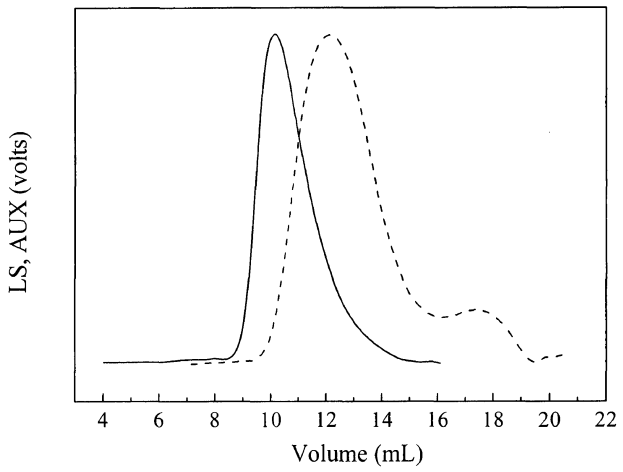

Figure 2. GPC chromatograms of the unultrasonic glucan in DMSO (—) by G4000H8 with G3000H8 column and in 0.5 M NaCl (····) by TSK-GEL G6000 PWXL with G4000 PWXL column at 25℃.

Table I. Experimental results from GPC-LLS, LLS, and viscometry for the glucan L-FV-I and fractions in 0.5 M NaCl at 25℃

Samples	$[\eta]\times10^{-2}$ / $g^{-1}\,cm^3$	k'	$M_w\times10^{-5}$	M_w/M_n
L-FV-I	8.14	0.51	12.0	2.4
L-FV-I1	7.66	0.53	11.4	2.5
L-FV-I2	7.34	0.49	10.6	2.6
			10.7[a]	
L-FV-I3	7.16	0.48	9.88	2.7
L-FV-I4	4.74	0.41	8.09	2.7
L-FV-I5	3.12	0.40	5.88	2.0
L-FV-I6	1.07	0.46	2.73	1.8
L-FV-I7	0.71	0.44	1.87	1.9

[a] Data from Zimm by LLS.

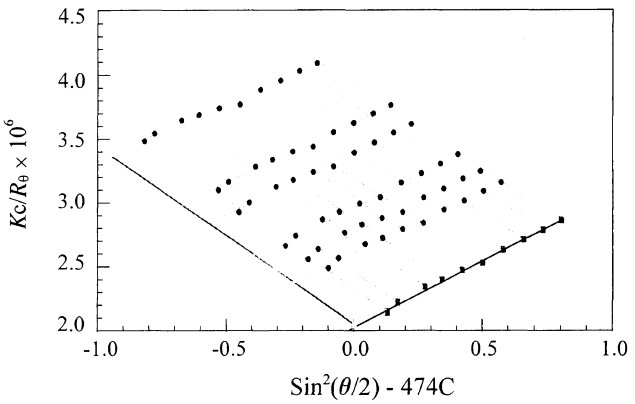

Figure 3. Zimm plot of the glucan L-FV-I2 in DMSO at 25℃.

Table II. Data of molecular weights $M_{w,m}$ and radius of gyration $<s^2>_{z,m}$ for triple-helical chains, $M_{w,s}$ for fragments and M_w for single chain of the glucan and fractions in 0.5 M NaCl aqueous solution and DMSO, respectively, at 25℃ characterized by GPC-LLS and LLS

Samples	$M_{w,m}\times10^{-5}$ in 0.5 M NaCl	$<s^2>_{z,m}^{1/2}$/nm in 0.5 M NaCl	$M_{w,m}/M_{n,m}$	$M_{w,s}\times10^{-5}$ in 0.5 M NaCl	$M_{w,m}/M_{w,s}$ in 0.5 M NaCl	$M_w\times10^{-5}$ in DMSO	$M_{w,m}$(0.5 M NaCl)/ M_w(DMSO)
L-FV-I	16.32	137.1	1.3	3.26	5.0	5.58	2.9
L-FV-I1	14.68	129.3	1.7	2.81	5.2		
L-FV-I2	13.96	120.3	1.6	2.57	5.4	4.88[a]	2.9
L-FV-I3	12.92	114.9	1.4	1.92	6.7	4.64	3.0
L-FV-I4	10.22	101.3	1.3	2.51	4.1	4.14[a]	3.1
L-FV-I5	8.05	87.3	1.2	2.61	3.1		
L-FV-I6	4.35	49.8	1.3	1.37	3.2		
L-FV-I7	3.04	36.4	1.3	0.83	3.7		

[a] Data from Zimm plot by LLS.

NaCl aqueous solution and DMSO at 25℃ is illustrated in Figure 4. Mark–Houwink equation for the glucan in 0.5 M NaCl aqueous solution in the M_w from 1.87×10^5 to 1.20×10^6 is

$$[\eta]=7.69\times10^{-6}M_w^{1.32} \quad (cm^3\,g^{-1}) \qquad (3)$$

An approximated slope of $[\eta]$—M_w relationship was estimated from three samples in DMSO as 0.5. Usually, the exponent α of flexible polymer in good solvent is from 0.5 to 0.8, and that of stiff chain polymer, higher than 0.8. Obviously, α of the glucan in 0.5 M NaCl aqueous solution is far higher than in DMSO, and similar to triple-helix schizophyllan.[28,29] The rigidity of the glucan chain in 0.5 M NaCl aqueous solution may thus be higher that of in DMSO, in which it exists as a flexible chain.

GPC chromatograms of the glucans in 0.5 M NaCl aqueous solution contained a main peak of aggregated molecules and small second peak corresponding to frag-

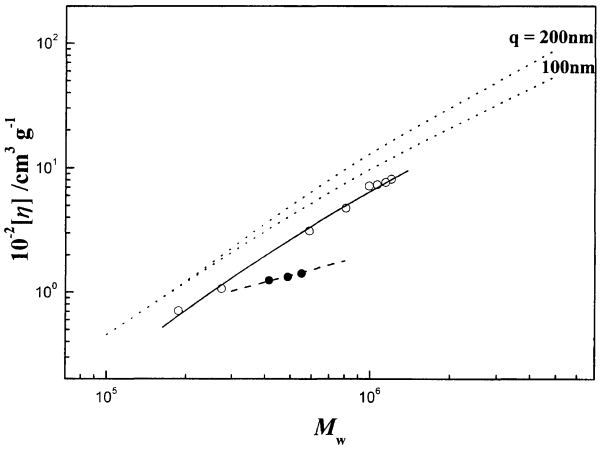

Figure 4. M_w dependence of $[\eta]$ for glucan fractions in 0.5 M NaCl aqueous solution (○) and DMSO (●) at 25℃. A dotted lines shows values calculated from Yamakawa–Yoshizaki theory for a wormlike cylinder[35] for $q=100$ and 200 nm with M_L and d fixed at 2150 nm^{-1} and 2.6 nm, respectively.

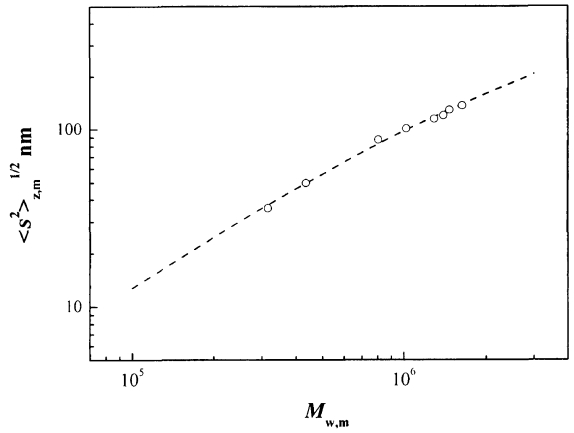

Figure 5. $M_{w,m}$ dependence of $<s^2>_{z,m}^{1/2}$ for the glucan in 0.5 M NaCl aqueous solution at 25℃. Dashed line presents theoretical values calculated from eq 4 with $M_L=2180$ nm^{-1} and $q=120$ nm.

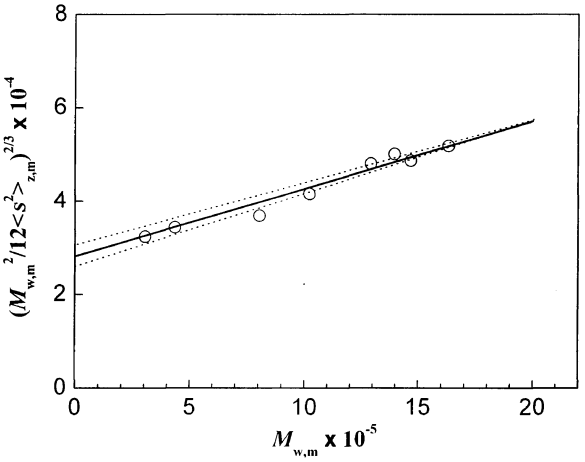

Figure 6. Plots of $(M_{w,m}^2/12<s^2>_{z,m})^{2/3}$ *vs.* M_w for the glucan and fractions in 0.5 M NaCl aqueous solution at 25℃.

ments of single chains with lower molecular weight. GPC chromatograms of the glucan in DMSO showed only a sharp peak, indicating aggregates broken into single chains. GPC curves divided into the main peak and the shoulder peak by dash line from the inflexion point of the curve perpendicular to the base line, and such as L-FV-I4 is shown in Figure 1. Molecular weights $M_{w,m}$ of the main peak and $M_{w,s}$ of the shoulder peak were determined with the Zimm fit method from light scattering signals.[30] The results are summarized in Table II. The weight fraction of the single-chain fragments (w_s) was estimated using the division principle of GPC chromatogram to be 0.20 ± 0.05. The main peak may represent multiple-stranded chains, and the second peak, fragments of single-stranded chains of the glucan in the 0.5 M NaCl aqueous solution. The data from main peak were thus used to estimate chain rigidity. Polydispersity $M_{w,m}/M_{n,m}$ lies in the range 1.2—1.7. $M_{w,m}$ dependence of $<s^2>_{z,m}^{1/2}$ of the glucan is shown in Figure 5. The slope of $<s^2>_{z,m}^{1/2}$—$M_{w,m}$ was 0.80. Usually, the exponent of stiff polymers in a good solvent is more than 0.6. The glucan in 0.5 M NaCl may thus be a rigid chain.

Rigidity of Chain

The rigidity of a wormlike chain is defined by the persistence length q. The Benoit–Doty expression[31] for $<s^2>$ of the Kratky–Porod wormlike chain is

$$<s^2>=(qM_w/3M_L)-q^2+(2q^3M_L/M_w)[1-(qM_L/M_w)(1-\exp(-M_w/qM_L)] \quad (4)$$

and can be approximated by[32]

$$(M_w^2/12<s^2>)^{2/3}=M_L^{4/3}+(2/15)(M_L^{1/3}/q)M_w \quad (5)$$

where M_L is molar mass per unit contour length, and M_w is molecular weight of polymer. Equation 5 is suitable for $M_w/2qM_L<2$, and M_w should be the mean value from the main peak of the fractions used. Plots $(M_{w,m}^2/12<s^2>_{z,m})^{2/3}$ against $M_{w,m}$ constructed from the data in Table II are shown in Figure 6. The two dotted lines indicate the upper and lower bounds of uncertainty in determining the slope and intercept of the plots. M_L and q were calculated as 2180 ± 100 nm^{-1} and 120 ± 10 nm, respectively. M_L and q of the glucan were in agreement with theory data for triple helical chains, and close to triple-helix schizophyllan,[28,29] but significantly higher than double helical xanthan[25] and single-stranded helical β-(1→3)-D-glucan from *Auricularia auricula-judae*.[26] As shown in Figure 5, the dashed line represents values computed from eq 4 with $M_L=2180$ nm^{-1} and $q=120$ nm. Data points of $M_{w,m}$ and $<s^2>_{z,m}$ agree most satisfactorily with the theoretical curve at $M_{w,m}$ range 3×10^5 to 1.6×10^6, indicating eq 5 applicable to this case, although $M_w/2qM_L$ values of L-FV-I ~ L-FV-I3 were slightly higher than 2.

M_w and $[\eta]$ for the glucan in 0.5 M NaCl aqueous solution and DMSO, and the theoretical values for wormlike cylinders are shown in Figure 4. These data of M_w and $[\eta]$ for the glucan in 0.5 M NaCl aqueous solution deviated from the Yamakawa–Yoshizaki theory curve for triple-helix chain.[35] This is because M_w and $[\eta]$ in this case include the contribution from the component of lower molecular weight, namely fragments of single

chains with $M_{w,s}$, which may be caused by treating with 5% NaOH in preparation. $M_{w,m}/M_{w,s}$ of the glucan in the aqueous solution are summarized in Table II. The triple helix and *ca.* 20% single-flexible random coil coexist in glucan aqueous solution. These results support the explanation mentioned above.

Triple-Helical Structure

The ratio of $M_{w,m}$ for the glucan in 0.5 M NaCl aqueous solution to DMSO was approximately 3, indicating that the predominant species of the β-(1→3)-D-glucan L-FV-I in 0.5 M NaCl aqueous solution may be triple-stranded helix. Assuming the triple-helix model for the polysaccharide, contour length h per main-chain residue of triple helix glucan along the axis is related to M_L as[33]

$$h = (M_0/5)/(M_L/3) \quad (6)$$

with M_0 (1134 g mol^{-1}), molar mass of the glucan repeating unit, because the *Lentinan* has two branched residues for every five D-gluopyranosyl residues.[18] Each repeating unit of the main chain of glucan L-FV-I contains seven β-D-glucan residues. By substituting for M_L in eq 6, h was 0.31 nm. This value is close to 0.29 nm from X-Ray,[15] and in good agreement with other triple helixes such as schizophyllan and scheroglucan.[28,34] Therefore, predominant species of the glucan in 0.5 M NaCl aqueous solution exist as triple-helical chains, and in DMSO as single-flexible chains. The triple-helix structure of the glucan in 0.5 M NaCl aqueous solution may thus be sustained by inter- and intramolecular hydrogen bonds, and broken to form single random coil in DMSO.

CONCLUSIONS

Water-soluble β-(1→3)-D-glucan with (1→6) branching (L-FV-I) from *Lentinus edodes* was degraded into seven fractions by ultrasonic irradiation. $[\eta]$—M_w relationship of the glucan in 0.5 M NaCl aqueous solution at 25℃ was $[\eta] = 7.69 \times 10^{-6} M_w^{1.32}$ (cm^3 g^{-1}) at M_w from 1.87×10^5 to 1.20×10^6. GPC chromatograms of the glucan and fractions in the aqueous solution contained a main peak corresponding to triple-stranded chains and a small second peak (about 20%) corresponding to fragments of single-stranded chains. Analysis of $M_{w,m}$ and $<s^2>_{z,m}^{1/2}$ from the main peak of GPC by the theory for wormlike chain yielded M_L, q, and h, ascribed to the high rigidity of triple-helical chains. Predominant species of the β-(1→3)-D-glucan exist as triple-stranded helical chains in 0.5 M NaCl aqueous solution, and as single-flexible chains in DMSO.

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