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Branching Structure and Chain Conformation of Water-Soluble Glucan Extracted from *Auricularia auricula-judae*

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ABSTRACT: A water-soluble neutral polysaccharide (AF1) was extracted from *Auricularia* (*A.*) *auricula-judae* with 0.15 M aqueous NaCl at 80−100 °C. Its chemical components and structure were analyzed by GC, GC−MS, and NMR. AF1 was identified as a β-(1→3)-D-glucan with two β-(1→6)-D-glucosyl residues for every three main chain glucose residues, showing a comb-branched structure. The M_w values of AF1 in both aqueous solution and DMSO determined by LLS and SEC-LLS were in the narrow range of 2.07−2.15 × 10^6, indicating AF1 existed as single chains in the two solvents. The high intrinsic viscosity [η] of 1753 mL/g and the structure-sensitive parameter ρ ($\equiv R_g/R_h$) value of 2.3 in water revealed that AF1 existed as stiff chain conformation. Moreover, we directly observed the extended stiff chain conformation by AFM. The branching structure led to the water solubility of AF1, and the intramolecular hydrogen bonds sustained the stiff chain conformation. The rheological results showed that this polysaccharide aqueous solution had higher viscosity than even xanthan, a pronounced thickening agent. This work provided important information for developing new thickeners in food fields, and how neutral polysaccharides can be used as good candidates.

KEYWORDS: *Auricularia auricula-judae, water-soluble glucan, branched polysaccharide, stiff chain, viscosity*

INTRODUCTION

Polysaccharides displaying physicochemical and biological features of potential applications in the food and pharmaceutical industries as functional ingredients have been attracting great interest in recent years.[1−3] In the food field, polysaccharides used as food additives are mainly classified into two major kinds including charged and neutral polysaccharides, and show increasing applications.[4] All of these polysaccharides have complex chemical composition and chain structures, with the result that the structure−function correlation has not been completely understood. The common food thickening agents have charged structures such as xanthan,[3,5] carboxymethyl cellulose, carrageenan,[6] etc. or complex composition such as galactomannans.[7] In spite of their wide acceptance for food formulation, these polysaccharides still exhibit some disadvantages that they experience the equilibrium between the ordered and disordered states in aqueous solution controlled by the ionic strength of the medium.[4] For example, xanthan chains tend to assume helix conformations under high ionic strength, whereas more flexible structures are exhibited under low ionic strength. *k*-carrageenan has an extended flexible coil conformation where the electrostatic repulsions of the sulfated groups result in chain expansion. It can form a thermoreversible gel with conformational transition from a random coil to a helix. Based on these mentioned above, it is necessary to develop an alternative kind of polysaccharide with simple chain structure in the food industry.

β-(1,3)-Glucans are the simplest β-glucans with β-(1→3)-D-glucan as the main chain, and have been well-known to show immunomodulatory, antitumor, and anti-inflammatory activities.[8,9] Moreover, β-(1,3)-glucans have a strong nature to form a helical structure giving high viscosity and gelling properties. For example, curdlan,[10] scleroglucan,[8,11] schizophyllan,[9] and lentinan[12] belong to the classical β-(1,3)-glucans with triple helical structures, all of which show high viscosity stable over a wide range of pH even in the presence of electrolytes.[8−12] So they have the potential to be used as food additives. However, they have not been really used in the food field because the triple helical structures have limited their solubility in water at moderate temperature. Consequently, to find a β-(1,3)-glucan with good water solubility would be an especially important topic for its application in food systems.

Many β-(1→3)-D-glucans occurring in fungi, bacteria, algae, and annual plants have branches by β-(1→6) linkages, which play an important role in its water solubility.[8,13] The triple helical schizophyllan and lentinan with degree branching (DB) values of 1/3 and 2/5, respectively, have water solubility, while the linear curdlan with the same main chain is water-insoluble.[10] We have found that, even though they have low molecular weight, the linear β-(1→3)-D-glucans, such as PCS3-II isolated from sclerotium of *Poria cocos* with lower M_w (7.68 × 10^4−1.23 × 10^5)[14] and GL−IV-I extracted from the fruit body of *Ganoderma lucidum* with M_w (1.24 × 10^5),[15] are shown water-insoluble. Moreover, a water-soluble polysaccharide (TM3b) with much high molecular weight (M_w) 3.14 × 10^6, extracted from sclerotia of *Pleurotus tuber-rigium* has been determined to be a hyperbranched β-(1,3)-D-glucan.[16] It thus gives us a strong proof that the hydroxyl of the side chain associates easily with water clusters, leading to good dissolution in aqueous solution.[17,18] Usually, the hydroxyl groups of C6 in side chains exhibit more activity to associate with water than that in the main chains due to the less steric hindrance. Thus, a

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basic understanding of the branching structure of polysaccharides is essential for the successful interpretation of the correlation of their structure to functions.

The *Auricularia* mushroom species is the fourth most important cultivated mushroom used by humans throughout the world.[19] *Auricularia* (*A.*) *auricula-judae*, compared with other *Tremella* (*T.*) species such as *T. mesenterica*, *T. fuciformis*, and *T. aurantia*, which belongs to the jelly mushroom group of organisms that form gelatinous fruiting bodies, is an edible basidiomycetous fungus, and is well-known as a tonic and health food in East Asian countries.[20,21] In our laboratory, several polysaccharides have been extracted from *A. auricula-judae*, and their structures have been identified to be different. For example, a main chain of (1→4)-linked D-glucopyranosyl with glucopyranosyl side groups at O6, coded as AAG, has about 19% content of glucuronic acid.[22] The AAG sample has biological activities and can be considered as a candidate for possible antitumor drugs.[23] Another water-soluble acidic heteropolysaccharide (WAF) has also been isolated from *A. auricula-judae* by extracting with 0.9% aqueous NaCl solution. WAF is composed of a backbone of α-(1→3)-linked D-mannopyranose residues with pendant side groups of β-D-xylose, β-D-glucose, or β-D-glucuronic acid at position O6 or O2, showing a semirigid character typical of polysaccharides.[24] Meanwhile, various structures of acidic heteropolysaccharide were extracted from *Tremella* species. Most of them are composed mainly of xylose, mannose, and glucuronic acid in different sugar ratio. For example, heteropolysaccharide (TAPA1) extracted from *Tremella aurantialba* consisted of mannose, xylose, and glucuronic acid in the ratio of ca. 5:4:1, with a α-(1→3)-linked mannopyranosyl backbone, partially substituted at positions O4 and O2.[25,26] Moreover, two major fractions (TMPA and TMPB) extracted from *Tremella mesenterica* were composed of xylose, mannose, and glucose in molar ratios of 1.00:4.74:0.91 and 1.00:6.63:2.34, respectively.[27] Those studies have been published concerning heteropolysaccharides from gelly mushroom, which focused on the pharmacological properties.[25−28] However, reports on water-soluble β-(1→3)-D-glucans from *A. auricula-judae* has been very rare. We are interested in the branching structure and chain conformation as well as viscosity properties of water-soluble neutral β-glucan from *A. auricula-judae*. In the present work, we tried a new extraction condition and purification method to obtain the neutral β-glucan having water solubility. The chemical structure of the extract was determined by using carboxyl reduction, deacetylation, and methylation combined with gas chromatography (GC), gas chromatography−mass spectrometry (GC−MS), and nuclear magnetic resonance spectroscopy (NMR). Furthermore, static and dynamic light scattering (SLS/DLS), size exclusion chromatography combined with static laser light scattering (SEC-LLS), atomic force microscopy (AFM), and viscosity measurements were carried out to analyze the molecular weight and chain conformation of the β-D-glucan. The rheological behavior of the glucan aqueous solution was also investigated to evaluate the potential application in food industry.

MATERIALS AND METHODS

Isolation and Fractionation. Crushed dried fruit bodies of *A. auricula-judae* as shown in Figure 1a, a commercial product cultivated in Fangxian (Hubei, China), were purchased from a market in China. The sample was defatted with hot ethyl acetate and methanol for 4 h and then homogenized with a homogenizer. Subsequently, it was

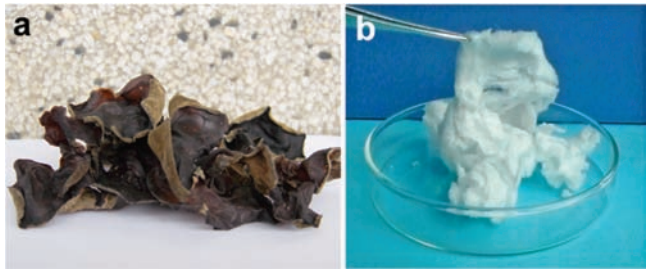

Figure 1. Pictures of dried *Auricularia auricula-judae* (a) and the AF1 sample (b).

immersed in 70% ethanol/water solution at room temperature for 24 h to remove the supernatant. The residue was extracted with 0.15 M aqueous NaCl at 80−100 °C for 2 h, and then the solution was stirred overnight. This procedure was repeated three times. The resultant solution was centrifuged to obtain a supernatant, named as crude product AF, which was further purified by precipitating with 70% ethanol/water at 25 °C. The precipitate was dissolved in water again and dialyzed using a generated cellulose tube (M_w cutoff 8000, Union Carbide USA) against tap water for 5 days, and then against distilled water for 3 days. The resultant solution was finally lyophilized to give the white sample, coded as AF1, which exhibited an absorbent cotton appearance, as shown in Figure 1b.

Characterizations of Structure. An infrared spectrum (IR) of the AF1 sample was recorded with a Nicolet 170SX FT-IR spectrometer (Spectrum One, PerkinElmer Co., Madison, WI) in the range of 4000−400 cm^{-1} using the KBr-disk method. One- and two-dimensional ^1H and ^{13}C NMR measurements of AF1 in DMSO-d_6 were taken on a Mercury 600 NMR spectrometer (Varian Inc., Palo, Alto, CA) at 20 °C. To prepare a concentrated solution as much as possible, 0.015 g AF1 was dissolved in 0.5 mL of DMSO-d_6 at 60 °C for 24 h.

Monosaccharide standards including galactose, glucose, mannose, xylose, arabinose, and rhamnose monohydrate were used in this experiment. AF1 and its monosaccharides were analyzed as their alditol acetates by GC after hydrolysis and derivatization.[22] The polysaccharides were hydrolyzed with 12 M H_2SO_4 (25 °C, 1 h) and 2 M H_2SO_4 (90 °C, 2 h), reduced with $NaBH_4$, and neutralized with acetic acid.[29] Gas chromatography was performed on an Agilent model 6820 instrument equipped with a FID detector using a DB-5 capillary column (30 m × 0.32 mm × 0.25 μm) programmed from 180 to 220 at 4 °C/min and held at 220 °C for 30 min. Two microliters of CH_2Cl_2 solution of each sample was injected, and helium was used as the carrier gas.

Gas chromatography−mass spectrometry (GC−MS) was carried out on a GCMS-QP2010 Plus (Shimadzn, Japan) equipped with a capillary split injector system and a FID detector on an Rxi-5MS capillary column (30 m × 0.32 mm × 0.25 μm) and a mass spectrometer (5973N, Agilent, Palo Alto, CA). To determine the sugar composition and linkage, the polysaccharides were permethylated three times by using CH_3I in DMSO,[29] then extracted by $CHCl_3$, and finally dialyzed in distilled water and freeze-dried. The methylated polysaccharides were subsequently hydrolyzed with 12 M H_2SO_4 (25 °C, 1 h) and 2 M H_2SO_4 (90 °C, 2 h), then reduced with $NaBH_4$, and neutralized with acetic acid. The mixture of methylated alditols was acetylated with acetic anhydride (Ac_2O) at 120 °C for 3 h. The oven temperature was set at 150 °C and was increased by 2 °C/min to 220 °C. The detector temperature was set at 280 °C, and nitrogen was used as the carrier gas.

Laser Light Scattering, SEC-LLS, and Viscometry Measurements. The scattering light intensities of AF1 in water and in DMSO were measured on a modified commercial light scattering spectrometer (ALV/SP-125, ALV, Germany) equipped with an ALV-5000/E multi-τ digital time correlator and a He−Ne laser (at λ = 632.8 nm). The angular and concentration dependences of the scattered intensities were analyzed by using Berry's square root plots to determine the weight-average molecular weight (M_w) and the radius of gyration (R_g)

of the AF1 sample in water and in DMSO. The basic light scattering equation is as follows:[30,31]

$$(Kc/R_\theta)^{1/2} = (1/M_w{}^{1/2})[1 + (8\pi^2 n_0{}^2/3\lambda_0{}^2)\langle R_g{}^2\rangle \sin^2(\theta/2) + A_2 M_w c] \quad (1)$$

$$K = 4\pi^2 n_0{}^2 (dn/dc)^2/(\lambda_0{}^4 N_A) \quad (2)$$

where A_2, N_A, n_0, λ_0, and R_θ are the second virial coefficient, Avogadro's number, the solvent refractive index, the wavelength of laser in vacuum, and the excess reduced scattering intensity at a certain scattering angle, θ. The polysaccharide solution with the desired concentration was prepared, and the optical clarification of the solution was carried out by filtration through a 0.45 μm Millipore filter into a scattering cell.

Dynamic light scattering measurements were used to characterize the hydrodynamic radii (R_h) of the polysaccharide solution in water and in DMSO at 25 °C. The measurements were carried out on the modified commercial light scattering spectrometer mentioned above. The precisely measured intensity−intensity time correlation function $G^{(2)}(q,\tau)$ in the self-beating mode can be related to the normalized field−field autocorrelation function $g^{(1)}(q,\tau)$ via the Siegert relation as

$$G^{(2)}(q,\tau) = A[1 + \beta|g^{(1)}(q,\tau)^2|] \quad (3)$$

where A is the measured baseline and β is a constant related to the coherence of the detected optics. For a polydisperse system, $g^{(1)}(q,\tau)$ is related to the distribution of the characteristic line width $G(\Gamma)$ by

$$|g^{(1)}(q,\tau)| = \int_0^\infty G(\Gamma)e^{-\Gamma}\, d\Gamma \quad (4)$$

Thus, $g^{(1)}(q,\tau)$ can be converted to a line-width distribution $G(\Gamma)$ by the CONTIN Laplace inversion algorithm in the correlator according to eq 3. For a pure diffusive relaxation, Γ is related to the translational diffusion coefficient (D), and $G(\Gamma)$ can be converted to a translation diffusion coefficient distribution $G(D)$ by

$$\Gamma = Dq^2 \quad (5)$$

Thus, a hydrodynamic radius (R_h) can be calculated by using the Stokes−Einstein equation

$$R_h = \frac{k_B T}{6\pi\eta_0 D} \quad (6)$$

where k_B is Boltzmann's constant and η_0 is the solvent viscosity.

The M_w values of polysaccharides in 0.9% aqueous NaCl solution and in DMSO were determined by using size exclusion chromatography combined with static laser light scattering equipped with a He−Ne laser at λ = 633 nm (DAWNDSP, Wyatt Technology Co., USA). SEC-LLS measurements were carried out on the multiangle laser photometer mentioned above with a P100 pump (Thermo Separation Products, San Jose, CA, USA) equipped with columns of Shodex-OHpak SB-806 M HQ (8.0 mm × 300 mm) for aqueous solution and Shodex-GPC KF-806 L (8.0 mm × 300 mm) for DMSO at a flow rate of 0.5 mL/min at 25 °C and a scattering angle of θ = 90°, respectively. A differential refractive index detector (DAWN DSP, Wyatt Technology) was used. The Astra software was utilized for data acquisition and analysis. The specific refractive index increment (dn/dc) of AF1 was 0.136 mL/g in water and 0.068 mL/g in DMSO at 633 nm and 25 °C.[32] The polysaccharide solutions were prepared at concentrations of 0.3 mg/mL in 0.9% aqueous NaCl solution and 0.6 mg/mL in DMSO, purified by a 0.45 μm Millipore filter, and the injection volumes were 200 μL. The eluents were 0.9% aqueous NaCl solution and DMSO, respectively, which were purified by a 0.2 μm membrane and degassed before use.

The intrinsic viscosities ($[\eta]$) of AF1 in water, DMSO, and 0.9% aqueous NaCl solution were measured at 25 °C by using an Ubbelohde capillary viscometer with different original concentrations of 3 × 10^{-4} g/mL, 1.2 × 10^{-3} g/mL and 4 × 10^{-4} g/mL, respectively. The kinetic energy correlation was always found to be negligible. The Huggins and Kraemer equations were used to estimate the value of $[\eta]$.

$$\eta_{sp}/c = [\eta] + k[\eta]^2 c \quad (7)$$

$$\ln \eta_r/c = [\eta] - \beta[\eta]^2 c \quad (8)$$

where k and β are constants for a given polymer under the desired conditions, η_{sp}/c is the reduced viscosity, and $\ln \eta_r/c$ is the inherent viscosity.

Microscopy. AF1 was dissolved in ultrapure water to approximately 1.3 × 10^{-4} g/mL with vigorous stirring for 24 h, and then filtered through a 0.45 μm filter (NYL, 13 mm syringe filter, Whatman Inc., USA) and diluted to 5 μg/mL. A 10 μL drop was deposited onto freshly cleaved mica and allowed to dry in a small covered Petri dish prior to imaging. The sample was imaged under ambient conditions (relative humidity = 50−60%, T = ~20 °C) with a Picoscan AFM (Molecular Imaging, Agilent Technologies) in the magnetic alternating current (MAC) mode. Commercial MAC lever type II probes (spring constant = 0.95 N/m, Agilent Technologies) were used for all imaging. Typical scan rates were 1−3 Hz. The images were collected at a resolution of 256 × 256 pixels, unfiltered and flattened when needed.

Rheological Measurements. Dynamic viscoelastic behaviors were performed on ARES-RFS III rheometer (TA Instruments, USA). A double-concentric cylinder geometry with a gap of 2 mm was used to measure dynamic parameters such as the storage modulus (G'), loss modulus (G''), and complex viscosity (η^*) as functions of angular frequency (ω) at 25 °C . The values of the strain amplitude were checked to ensure that all measurements were set as 10%, which is within a linear viscoelastic regime. For the dynamic frequency sweep measurement, a fresh AF1 solution was prepared and poured into the Couette geometry instrument. Temperature control was established by a Julabo FS18 cooling/heating bath kept within ±0.4 °C over an extended time. The dynamic temperature sweep measurement was conducted from 5 to 65 °C at an angular frequency of 1 rad/s and heating rates of 1 °C/min. The solution was covered with a thin layer of low-viscosity silicone oil in order to prevent dehydration during rheological measurements.

RESULTS AND DISCUSSION

Chemical Structure. The FTIR spectra of the AF1 samples (Figure 2) exhibited an absorption peak at 890 cm^{-1}, which was

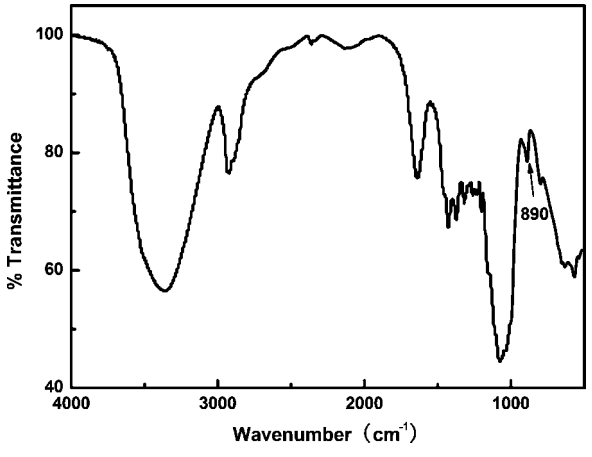

Figure 2. FT-IR spectrum of AF1.

the characteristic of the configuration of β-glucan.[33] Compared with the derivatives of monosaccharide standards, the GC trace of the alditol acetate derivatives of the hydrolyzed AF1 (Figure 3) indicated that AF1 consisted mainly of glucose. Analysis by GC−MS of its O-permethylated homopolymer suggested the presence of 2,3,4,6-tetra-*O*-methylglucose at 25.43 min, 2,4,6-

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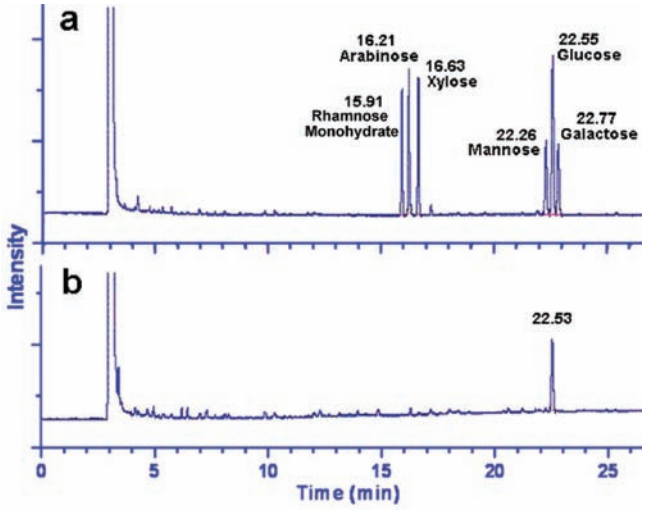

Figure 3. The GC traces of monosaccharide standards (a) and AF1(b).

tri-*O*-methylglucose at 26.17 min, and 2,4-di-*O*-methylglucose at 27.03 min, respectively (Table 1). This indicated that AF1

Table 1. Partially O-Methylated Alditol Acetates Formed from the Methylation Analysis of AF1

partially *O*-methylated alditol acetates	t_R [b]	% area of fragments	linkage type[a]
2,3,4,6-Me4-Glc	25.43	39.5	Glcp-(1→
2,4,6-Me3-Glc	26.17	21.0	3→)-Glcp-(1→
2,4-Me2-Glc	27.03	39.5	3,6→)-Glcp-(1→

[a]Based on derived *O*-methylalditol acetates. [b]Retention time (min).

consisted mainly of terminal glucose (t), 1,3-linked glucose (m), and 1,3,6-linked glucose (d) in the molar ratios of 1.9:1:1.9, suggesting a β-(1→3)-D-glucan with two β-(1→6)-D-glucosyl residues for every three main chain glucose residues.[34] The degree of branching (DB) was calculated to be 65.5% according to the molar ratios of branched glucose residue to glucose in the main chain.

To determine the chemical structure of AF1 and the sequences of the sugars, a detailed NMR study on AF1 has been made. Initially, AF1 was dissolved in D_2O at an elevated temperature and a concentration (0.01 g/mL) to obtain a gel-like solution. No information can be obtained, and the absence of ^{13}C signals might be attributed to the presence of ordered aggregates.[35] Then, DMSO-d_6 was chosen as the solvent. The 1D (^{13}C, ^{1}H) and 2D NMR spectra of AF1 in DMSO-d_6 are shown in Figures 4 and 5. All the signals were assigned based on the literature values for similar polysaccharides such as scleroglucan and lentinan,[34−37] and are summarized in Table 2. The signal characteristic of uronic acid at 176.5 ppm was not observed in the ^{13}C NMR spectrum, suggesting that the WAF component was not present in the AF1 sample. Namely, AF1 was a neutral polysaccharide. It indicated that the two water-soluble polysaccharides were separated successfully with ethanol/water precipitator on the basis of the dependence of their solubility on the isolation temperature.[22,24] The assignments of ^{1}H and ^{13}C resonances were obtained from two-dimensional correlation NMR spectra for AF1, as shown in Figure 5. Figure 5a presents the NMR spectra of heteronuclear

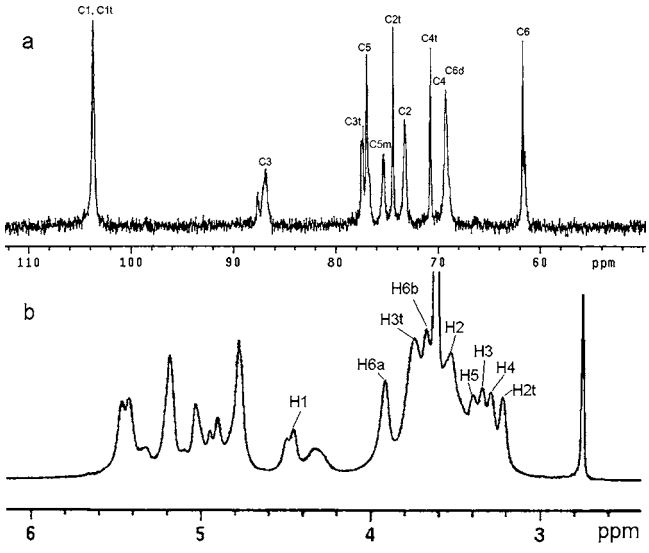

Figure 4. ^{13}C (a) and ^{1}H (b) NMR spectra for AF1 in DMSO-d_6 at 20 °C.

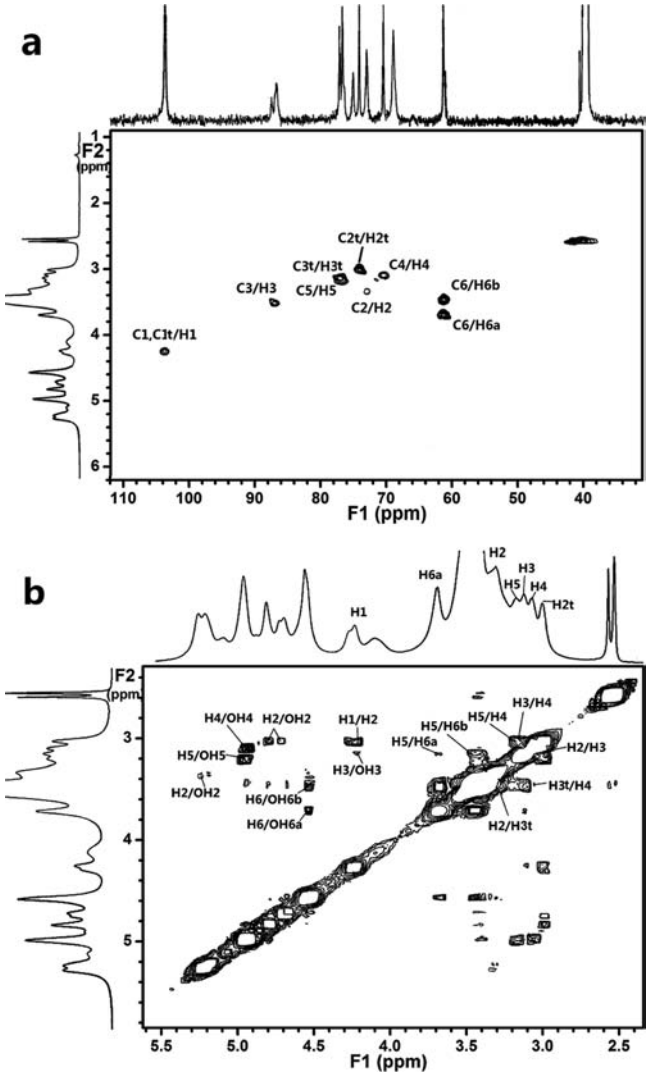

Figure 5. HMQC (a) and DQF (b) NMR spectra for AF1 in DMSO-d_6 at 20 °C.

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Table 2. ^{1}H and ^{13}C NMR Chemical Shifts of AF1 in DMSO-d_6[a]

		2		3		4		5		6	
resonance	1	2 m,d	2t	3 m,d	3t	4 m,d	4t	5	5m	6 m,t	6d
^{13}C ppm	103.7	73.3	74.4	86.9/87.6	77.4	69.2	70.7	77.0	75.3	61.5/61.7	69.2
^{1}H ppm	4.45	3.53	3.22	3.74	3.34	3.29		3.40		3.91a/3.67b	

[a]m, d, and t represent the 1,3-linked glucose, 1,3,6-linked glucose, and terminal glucose, respectively.

multiquantum coherence (HMQC), which map the correlation between the C atoms and the directly bonded H atoms. Figure 5b shows the 2D of a double-quantum filter (DQF) spectra, revealing the three-bond correlations among H atoms. AF1 was verified to be a linear (1→3)-linked β-glucan with typical C/H signals at δ 103.7/4.45, 77.4/3.34, 77.0/3.40, 74.4/3.22, 70.7/3.29, 61.7/3.91;3.67, arising from C1/H1, C3/H3, C5/H5, C2/H2, C4/H4, C6/H6a;b, respectively. The glycosidic linkages of the glucan containing (1→3)-linked and (1→6)-linked, β-D-glucopyranosyl were indicated by the presence of 3-O-substituted signal at δ 86.9/87.6, and O-substituted-CH_2-6 signal at δ 69.2 (in the form of an HMQC doublet at δ 86.9;87.6/3.74, δ 69.2;3.91/3.67), respectively. The signal at δ 77.4/3.34 was assigned to C3t/H3t, while the signal at δ 70.7 was assigned to C4 (signals at δ 70.7/3.29 for C4/H4 in Figure 5a and δ 3.07/4.93 for H4/OH4 in Figure 5b).

Moreover, the ratio of the integral value of C2t (side chain) at 74.4 ppm of glucan AF1 to that of C2 (main chain) at 73.3 ppm, corresponding to the ratio of the terminal units on side residue to the backbone units, was calculated to be 1:1.4. This supported the conclusion obtained by GC−MS, of two branching points for every three main glucosyl units. Therefore, AF1 was identified as a β-(1→3)-D-glucan with two β-(1→6)-D-glucosyl residues for every three main chain glucose residues, as shown in Figure 6a. As mentioned above, the water solubility

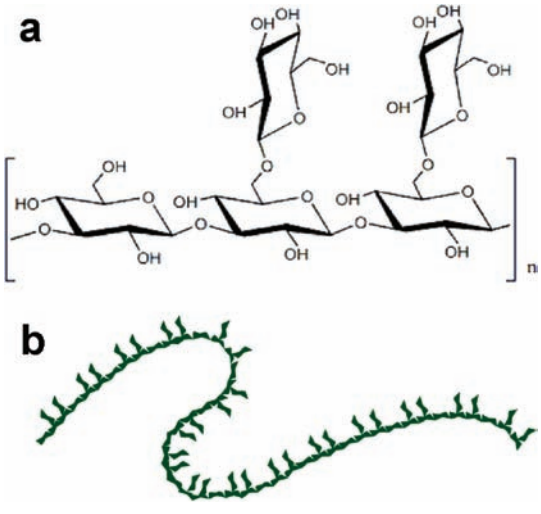

Figure 6. Chemical structure (a) and schematic model of AF1 (b).

of the polysaccharides leads to a relatively expanded chain, as a result of strong solvation between macromolecules and water. A model of comb-branched polysaccharide structure is proposed to describe the AF1 sample in water, as shown in Figure 6b.

Molecular Weight and Chain Conformation. To clarify the chain stiffness of polysaccharide (AF1), conformational parameters such as M_w, $[\eta]$, R_h, R_g, and the structure-sensitive parameter ρ ($\equiv R_g/R_h$) for AF1 in water and in DMSO were determined by static and dynamic LS and SEC combined with LLS at 25 °C, and the experimental results are summarized in Table 3. A linear relationship between $\eta_{sp}/c \sim c$ and $\ln \eta_r/c \sim c$

Table 3. Experimental Results of M_w, R_h, R_g, ρ, and $[\eta]$ for AF1 in Water and in DMSO at 25 °C

sample	$M_w \times 10^{-6}$	R_h (nm)	R_g (nm)	ρ ($\equiv R_g/R_h$)	$[\eta]$ (mL/g)
AF1 in water	2.15	90	203	2.3	1753
AF1 in DMSO	2.07	53	89	1.7	262

for AF1 in water (not shown) and 0.35 for Huggins constant k suggested that water is a good solvent of AF1 and it could be dispersed in water as single chains. The light intensity data obtained were analyzed by Zimm plots first. It was found that the (Kc/R_θ) vs q^2 plots did not give straight lines in the used angular range. Berry's square root plot was thus used instead (see Figure 7), and a reasonable determination of M_w and R_g

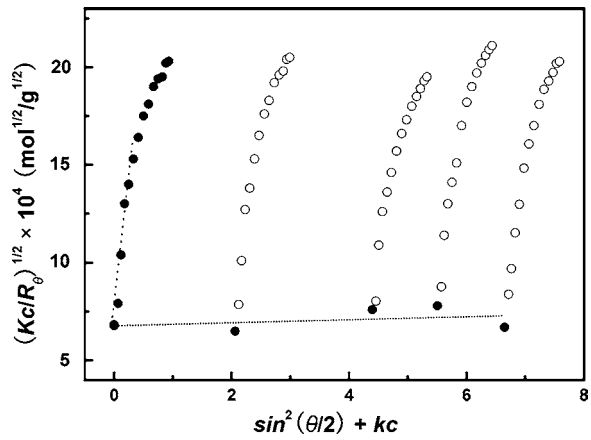

Figure 7. The Berry plots of the AF1 dilute solution in water with concentrations ranging from 1.0 to 3.2 × 10^{-4} g/mL at 25 °C. Filled data points are from extrapolations.

could be obtained from the linear extrapolation of the initial slopes at the low angular range to $q = 0$ and $c = 0$.[34] Consequently, the M_w of AF1 in water was obtained to be 2.15 × 10^6. Moreover, the molecular mass of AF1 was determined to be 2.07 × 10^6 in DMSO by SEC-LLS experiment as shown in Figure 8. Obviously, the values of M_w in different solvents were almost the same. Usually, triple- or double-stranded chains or aggregates of polysaccharides can be broken into a single one in a powerful solvent such as DMSO, leading to a significant difference in the M_w value from that in water.[17] The essentially identical M_w values indicated that AF1 existed mainly as a single-stranded chain in both water and DMSO.

The radius of gyration of (R_g) is regarded as the mean square of the distance between the segment and the mass center, whereas the hydrodynamic radius (R_h) is defined as the parameter to characterize the dimension of macromolecules in solution taking into account the hydrodynamic interactions.

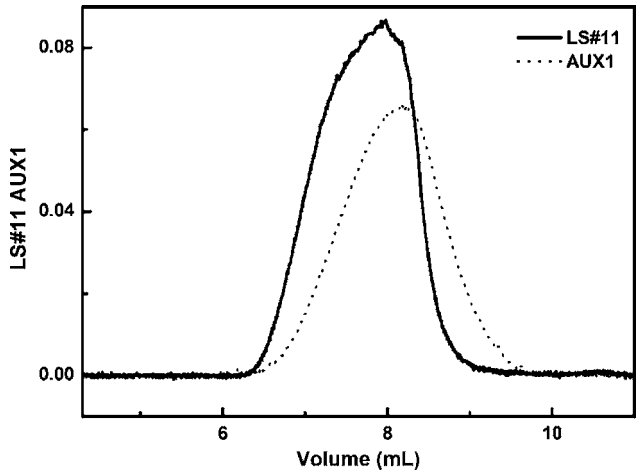

Figure 8. SEC-LLS chromatograms of AF1 in DMSO at 25 °C, as detected by LLS and differential refractometry. LS#11 and AUX1 represent signals from LLS at 90° and the refractive index detection, respectively.

The molecular shape and stiffness of polymers in dilute solution can be described from the value of ρ.[38] As shown in Table 3, the R_g values of AF1 in water and in DMSO were determined to be 203 and 89 nm, respectively, suggesting a more extended chain existed in water than in DMSO. Figure 9 shows the

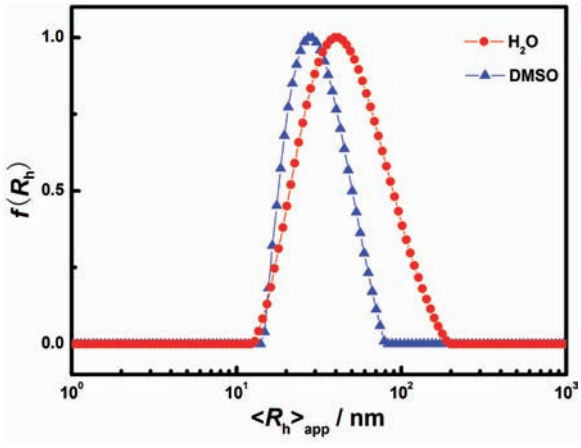

Figure 9. The corresponding hydrodynamic radius distributions of the AF1 dilute solutions (●, c = 1.6 × 10^{-4} g/mL in water; ▲, c = 6.0 × 10^{-4} g/mL in DMSO, scattering angle θ = 90°) at 25 °C.

corresponding hydrodynamic radius distributions profiles, $f(R_h)$, of the AF1 dilute solutions at 25 °C and scattering angle θ = 90°. There was only one symmetrical peak in $f(R_h)$ of AF1 in water and DMSO, further indicating that AF1 exhibited as a single chain in the very dilute solution. The dynamic Zimm plot of the AF1 dilute solution in water with concentrations ranging from 1.0 to 5.0 × 10^{-4} g/mL was obtained (not shown). The Zimm plot formalism allows consistent linear extrapolation of Γ/q^2 to θ = 0° at infinite dilution,[39] giving the R_h values of 90 nm in water and 53 nm in DMSO. Thus, the ρ value for AF1 in water was determined to be 2.3, further confirming that the comb-branched AF1 polysaccharide existed as a stiff chain conformation in water. It has been reported that the ρ value for an extended stiff chain (or rodlike chain) is higher than 2.0.[38] The result supported the stiff chain model of comb-branched structure for AF1 (Figure 6b). The $[\eta]$ value of 1753 mL/g for AF1 in water was much higher than that of common polymers[40] and polysaccharides[41] with similar M_w, and also higher than that of the triple helical lentinan.[17] Therefore, AF1 existed as adequately expanded chains in the aqueous solution. It was noted that the ρ value of AF1 in DMSO was 1.7, indicating a relatively flexible chain, as a possible result of destruction of the partial intrahydrogen bonding of the glucan. Moreover, the $[\eta]$ value of AF1 in DMSO decreased significantly, while the M_w value hardly changed, further confirming that intramolecular hydrogen-bonding occurred in AF1 chains in water and was broken in DMSO. It was not hard to imagine that the intrahydrogen bonds of AF1 sustained the chain stiffness, whereas their breaking led to the relatively flexible chain. It has been reported that strong intra- and intermolecular hydrogen bonds exist in lentinan to sustain the triple helix conformation.[17] In our findings, DMSO combined with the hydroxyl groups of AF1 to form new hydrogen bonds, leading to the destruction of the original hydrogen bonds of AF1.

The M_w and $[\eta]$ values of AF1 in 0.9% NaCl aqueous solution were 2.12 × 10^6 and 1420 mL/g, respectively, which were very similar to those obtained in water, indicating that AF1 is not a polyelectrolyte and hence not sensible to the ionic strength. The power law function $\langle S^2\rangle_z^{1/2} = kM_w^{\nu}$ of polymer in dilute solution can be estimated from many experimental points in the SEC-LLS chromatogram. Figure 10 shows the plots of $\langle S^2\rangle_z^{1/2}$ versus M_w for AF1 in aqueous solution and in DMSO in double logarithmic coordinates. The fitting trend of

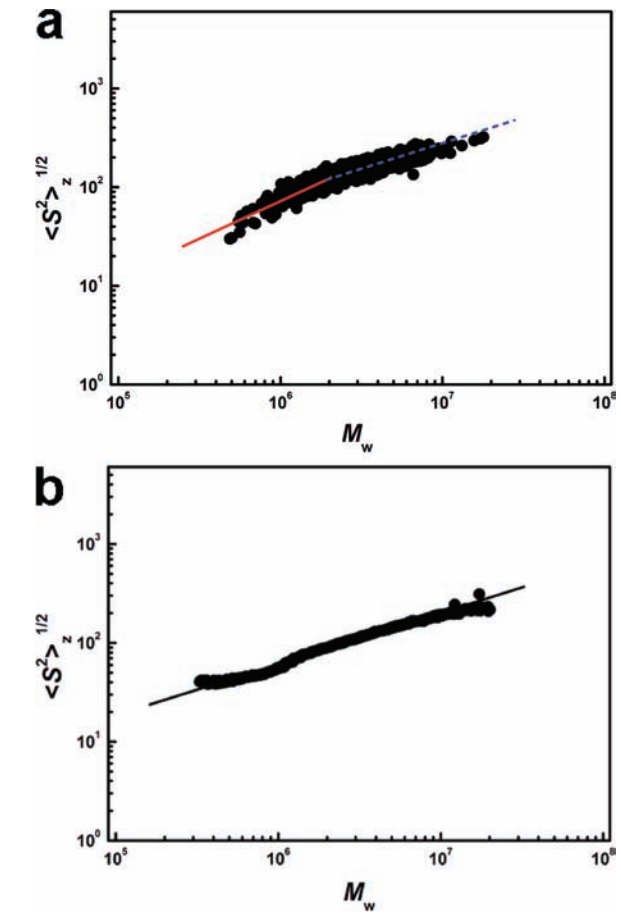

Figure 10. Dependence of $\langle S^2\rangle_z^{1/2}$ on M_w for AF1 in 0.9% aqueous NaCl solution (a) and in DMSO (b) at 25 °C.

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experimental points bends when the molecular weight is higher than 2.0×10^6, suggesting a decrease of the chain stiffness, as a result of the long chain (Figure 10a). The exponent (ν) values for AF1 in aqueous solution were calculated to be 0.76 for the experimental points in the M_w range from 4.7×10^5 to 2.0×10^6, and 0.58 for that from 2.0×10^6 to 1.8×10^7, respectively. Usually, the exponents of 0.33, 0.50−0.60, and 1.0 reflect the polymer molecular shape of sphere, random coil, and rigid rod, respectively.[42−44] The ν value of 0.76 for AF1 with M_w lower than 2.0×10^6 demonstrated further that the comb-branched polysaccharide existed as an extended chain conformation in the aqueous solution. However, the power law function for AF1 in DMSO was represented by $\langle S^2 \rangle^{1/2} = 3.7 \times 10^{-2} M_w^{0.54}$ (nm), as shown in Figure 10b. The ν value of 0.54 indicated that a relatively flexible chain existed in DMSO. The results were good agreement with the conformational parameter ρ and $[\eta]$.

AFM has become an invaluable metrological tool to characterize surface topology of macromolecules,[45] such as the linear and circular triple helix structures of β-(1→3)-D-glucans.[46] To provide additional direct evidence of the stiff chain for AF1 in water, AFM was used to observe their morphology. Figure 11 shows AFM images of AF1 deposited

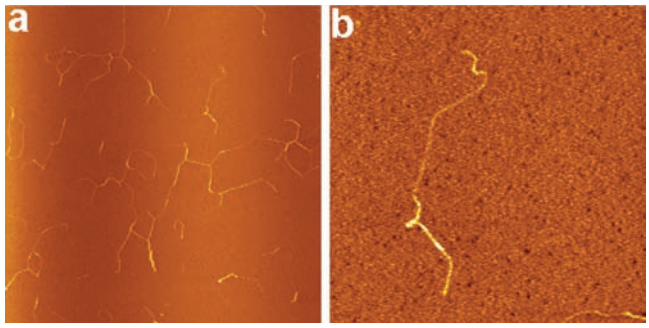

Figure 11. AFM images of AF1 in dilute aqueous solution. Images size are 5 μm × 5 μm (a) and 1.5 μm × 1.5 μm (b). Data scale =10 nm (a) and 5 nm (b), respectively.

onto the freshly cleaved mica surface from a dilute solution in air. As expected, a typical wormlike chain was displayed, further confirming that AF1 molecules existed as stiff chains in aqueous solution. Within the image, we could clearly observe perfect individual chains with an average height of 0.62 ± 0.17 nm (N = 50) and a length of over 1 μm. Compared with the height of triple-strand lentinan (1.21 nm),[46] AF1 would be a single chain in dilute aqueous solution, and more extended than lentinan chains, which was coherent with the results from laser light scattering.

Based on the above analysis, AF1 is a neutral branched β-(1→3)-D-glucan with DB of 2/3, and adopts single stiff chains in water due to relatively high steric hindrance and intra-hydrogen bonding. Compared with branched scleroglucan, schizophyllan, and lentinan, AF1 exhibited similar physical properties including water solubility and stiff chain conformation in water. But the higher DB imparts AF1 better water solubility than scleroglucan, schizophyllan, and lentinan, which will be seen in the following rheological test. It is surprising that AF1 exists as single stiff chains in water, whereas other three branched β-(1→3)-D-glucans adopt a triple helical conformation. It is possible that the little higher DB results in the failure in association into a triple helix. It is thus tentatively proposed that DB is very important for the chain conformation of branched β-(1→3)-D-glucans, and higher DB is maybe not beneficial for triple helix formation in nature. Whether it is true or not will be further studied in our future work.

Rheological Behaviors. The rheological behaviors of AF1 aqueous solution was also studied. Figure 12a shows the storage

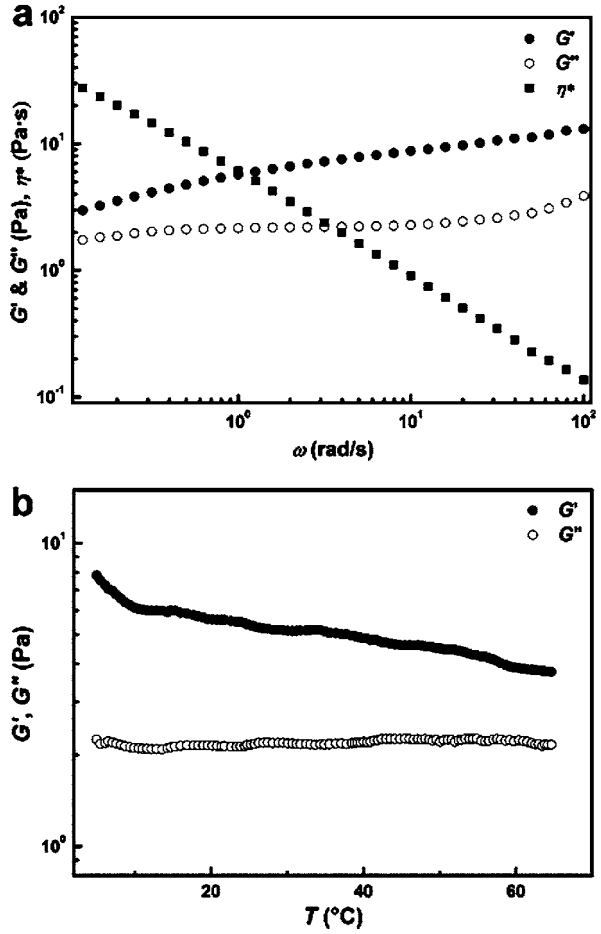

Figure 12. Storage modulus G' (solid symbols), loss modulus G'' (open symbols), and dynamic complex viscosity η^* (square symbols) as a function of angular frequency ω (a) and G' and G'' as a function of temperature at a heating rate of 1.0 °C/min and an angular frequency of 1 rad/s (b) for AF1 solutions with concentration of 4×10^{-3} mg/mL at 25 °C.

moduli (G'), loss moduli (G''), and dynamic complex viscosity (η^*) of AF1 in water with a concentration of 4×10^{-3} g/mL against frequency ω at 25 °C. The spectrum displayed an obvious weak gel-like character, showing that G' was higher than G'' over the entire frequency range and a frequency-dependence, and η^* decrease with increasing ω exhibited shear-thinning flow behavior.[12] The zero-shear viscosity η^* of AF1 was assessed to be 31 Pa·s, obtained from the viscosity curve at a shear rate of 0.1/s, which was compared with the reference values for the triple helical β-glucans and the charged polysaccharides. For example, scleroglucan having higher molecular mass of 5.03×10^6 showed a comparable viscosity at the given concentration.[11] The viscosity of AF1 also was comparable with that of the pronounced thickening agent xanthan with double helical structure and uronic acid (M_w = 2.2 × 10^6, 2 × 10^{-3} g/mL) in 0.1 N salt concentration,[47] Interestingly, AF1 exhibited high viscosity at low concentration (lower than 4×10^{-3} g/mL) at 25 °C, showing a solution

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appearance, whereas few general polymers show such a phenomenon. For example, xanthan and schizophyllan having such high viscosity form hydrocolloids without movability. Even though it showed gel character with $G' > G''$ in the whole frequency range as shown in Figure 12a, AF1 looked like liquid with movability, which might be due to its higher DB resulting in stronger binding to water molecules and thus better water solubility. This rheological feature may make it a specific application in food, pharmaceutical, and cosmetic products where high viscosity and good fluidity are needed. Figure 12b showed the effect of temperature on the viscoelastic property of AF1 which was investigated by dynamic temperature sweep experiment. The AF1 solution exhibited gel-like behavior with $G' > G''$ and thermal stability over the temperature range from 5 to 65 °C. The gradual decrease in G' with increasing temperature may be caused by the breaking of some weakly associated interactions between the β-glucan chains. The G'' showed high stability with almost the same value in the whole temperature range, suggesting a very steady viscose nature of AF1. It provided valuable and fundamental information for developing new thickeners in the food industry, and polysaccharide with comb-branched structure like AF1 from natural origin would be a potential candidate. To gain a greater insight into the rheological characteristics of this fraction, a systematic investigation would be required in our next work.

In conclusion, AF1 was identified as a water-soluble β-(1→3)-D-glucan with two β-(1→6)-D-glucosyl residues for every three main chain glucose residues, showing a comb-branched structure. It exhibited a rigid chain conformation in water showing high intrinsic viscosity and stable viscose nature in a wide temperature range of 5−65 °C. It gives us such a hint that the β-(1→3)-D-glucans with comb-branched structure like AF1 can be used as good candidates for thickening agents in the food field.

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Notes

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