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Solution properties of an α-(1→3)-D-glucan from *Lentinus edodes* and its sulfated derivatives

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Abstract

A water-insoluble α-(1→3)-D-glucan (**A**) from *Lentinus edodes* was fractionated into 13 fractions in dimethyl sulfoxide containing 0.25 M lithium chloride (0.25 M LiCl–Me_2SO). Five fractions were treated with sulfur trioxide–pyridine complex at 25 °C to synthesize water-soluble sulfated derivatives (**S-A**). The weight-average molecular weights, M_w, and intrinsic viscosities $[\eta]$, of the samples **A** and **S-A** were determined by multi-angler laser light scattering (MALLS), and viscosity. The M_w dependence of $[\eta]$ and of the radius of gyration $\langle S^2\rangle_z^{1/2}$, was found to be represented approximately by $[\eta] = 4.9 \times 10^{-2}\ M_w^{0.67}$ ($cm^3\ g^{-1}$), and $\langle S^2\rangle_z^{1/2} = 4.8 \times 10^{-2}\ M_w^{0.54}$ (nm) for the α-glucan in 0.25 M LiCl–Me_2SO in the M_w range from 7.24×10^4 to 4.21×10^5, and by $[\eta] = 6.8 \times 10^{-4}\ M_w{}^{1.06}$ ($cm^3\ g^{-1}$), and $\langle S^2\rangle_z^{1/2} = 9.4 \times 10^{-4}\ M_w^{0.92}$ (nm) for the sulfated α-glucan in aqueous 0.5 M NaCl in the M_w range from 5.92×10^4 to 1.42×10^5 at 25 °C. The results indicate that the α-(1→3)-D-glucan exists as a flexible chain in 0.25 M LiCl–Me_2SO, and its sulfated derivative in 0.5 M NaCl aqueous has stiffer chains than the original. ^{13}C NMR indicated that intramolecular hydrogen bonding occurred in the sulfated α-glucan, causing the observed chain stiffness.

Keywords: *Lentinus edodes*; α-(1→3)-D-glucan; Sulfated derivatives; Molecular weight; Intrinsic viscosity; Conformation; Solution properties

1. Introduction

The existing and potential commercial importance of polysaccharides has focused attention on such materials as mushroom polysaccharides as a functional food and a source for the development of drugs.[1] Most antitumor polysaccharides have a basic β-(1→3)-glucan structure, as in the β-(1→3)-D-glucan *Lentinan*, from *Lentinus edodes*.[2] Other antitumor polysaccharides include an α-glucan.[3] Interestingly, some sulfated polysaccharide derivatives exhibit antiviral action.[4,5] Sulfates of the β-(1→3)-D-glucan from *L. edodes* showing considerable anti-HIV activity as well as low anti-coagulant activities had been synthesized.[6] Introduction of a charged group at hydroxyl groups on the glucan chain improves the solubility and the antitumor activity.[7] In addition, a water-soluble product obtained by *O*-carboxymethylation of a linear α-(1→3)-D-glucan (molecular weight, 5.6×10^5) showed potent antitumor activity against Sarcoma 180 in mice.[8] Dextran sulfate shows an inhibitory effect on HIV infection.[9] It has been reported that the molecular weight, conformation, chemical modification, and solubility of the polysaccharides significantly affect their antitumor and immunomodulatory activities.[8,10,11] Therefore, investigating the solution properties of polysaccharides and of their derivatives is important for elucidating the correlation between physicochemical properties and bioactivities.

Our previous work[12] on a polysaccharide **A** (earlier designated L-FV-II), isolated from fruiting bodies of *L. edodes* demonstrated, by high-performance liquid chromatography (HPLC), infrared (IR), ^{13}C NMR, and gel-permeation chromatography (GPC), it to be an α-(1→3)-D-glucan, having purity 99.8%. Strong intermolecular hydrogen bonds and relatively weak glycosidic linkages exist in **A**, resulting in water-insolubility and ease of degradation during storage.[13] Good solvents for the α-glucan are 0.5 M urea–0.5 M NaOH and 0.25 M LiCl–Me_2SO. A molecular conformational transition in aqueous 0.5 M NaOH containing urea

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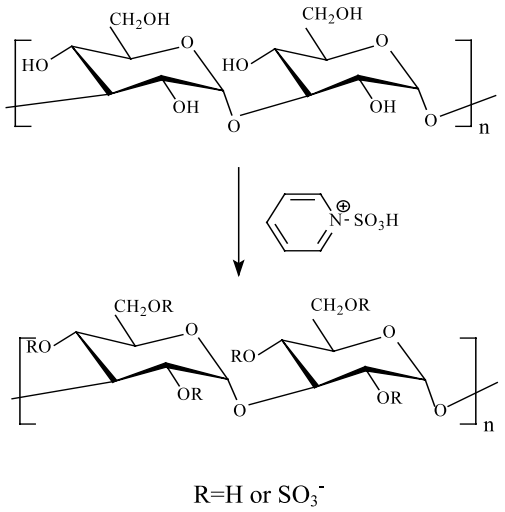
occurred in the range of 0.4–0.6 M, as indicated by specific rotation, viscosity, and fluorescence probe experiments.[13] However the solution properties of the α-(1→3)-D-glucan from *L. edodes* and of its sulfated derivatives have not been yet clarified. It is worth noting that two sulfated derivatives prepared from the α-(1→3)-D-glucan and β-(1→3)-D-glucan both exhibited significantly higher antitumor activity than the original polymers.[14] In the present work, fractions of the α-(1→3)-D-glucan having different molecular weights, and the corresponding sulfated derivatives, were prepared. Their solution properties were studied by viscometry and laser light scattering (LLS) in 0.25 M LiCl–Me_2SO and in aqueous 0.5 M NaCl to obtain conformational information.

2. Experimental

Fractionation.—The α-(1→3)-D-glucan **A** (earlier designated L-FV-II) used in this work was isolated from the fruiting bodies of *L. edodes* by extraction with 5% NaOH–0.05% $NaBH_4$, followed by precipitation with 1 M AcOH, then washing five times each with water and MeOH; yield; 3.2%.[12] Stocked in a silica-dried container for 15 months, its molecular weight decreased by 64% during storage.[13] All chemicals were of analytical grade.

The α-glucan **A** was fractionated by the non-solvent addition method. A 1% solution of **A** (5 g) in 0.25 M LiCl–Me_2SO and mixture of acetone and (4:1) 0.25 M LiCl–Me_2SO as precipitant was slowly added to the solution at 25 °C until the solution became turbid. The turbid solution was kept at 50 °C for 2 h, and then kept for 12 h at 25 °C; a separation into a liquid phase and a gel phase then occurred. The gel was removed by centrifugation and the liquid was subjected to the next fractionation. In this way, **A** was divided into 15 fractions. These fractions were precipitated from 0.25 M LiCl–Me_2SO solutions using 80% aq acetone, washed three times with 50% aq acetone and six times with anhyd acetone, and vacuum-dried for 7 days. Thirteen fractions (the first and last ones were discarded), coded **A**-01, -03, -04, -05, -07, -09, -10, and -11 were obtained and used directly for studying the solution properties.



Scheme 1. Synthetic route for sulfated α-(1→3)-D-glucan S-**A**.

Sulfation.—Five of the fractions (**A**-04, -06, -08, -10, -11, 100 mg each) were sulfated (Scheme 1). The samples were dissolved in 0.25 M LiCl–Me_2SO to form homogeneous solutions and 593 mg of sulfur trioxide–pyridine complex (2.0 equiv to hydroxyl groups) was added to the solution below 0 °C with vigorous stirring, followed by stirring for 8 h at 25 °C. After cooling, the solution was brought to pH 7.5–8.0 with aq 0.2 M NaOH and dialyzed against running water for 3 days, and then against distilled water for 2 days. The crude product was purified by dissolving it in a small amount of water, precipitating it by MeOH, and then drying in a vacuum for 5 days to give the sulfated polysaccharides as white powders, yield ~80 mg. These sulfated fractions were coded as S-**A**-04, -06, -08, -10 and -11.

Characterization.—Infrared (IR) spectra of the samples **A** and sulfates S-**A** were recorded by using a Nicolet FT-IR spectrometer, using the KBr-disk method. Elemental analyses were performed with a Heraeus Co. (Germany) elemental analyzer. ^{13}C NMR spectra were recorded at 300 MHz with a Bruker ARX-300 instrument, using dimethyl sulfoxide-d_6 (Me_2SO-d_6) as solvent at 320 K.

Solution preparation.—Dry LiCl was added to distilled Me_2SO to afford a 0.25 M LiCl–Me_2SO solution. The solution was treated with molecular sieves for further dehydration. To minimized undesired effects on the determination of molecular weight and viscosity, the mode of preparation of the polysaccharide solutions was strictly the same in all steps. A relatively concentrated stock solution was carefully prepared by completely dissolving the appropriate amount of polysaccharide in the desired solvent for 24 h under stirring, and a series of concentrations were obtained by successive dilution. Finally, each solution was further filtered through a 0.45 μm filter (Whatman, UK) three times into the scintillation vial used for the parallel light scattering and viscosities measurements.

Laser light scattering (LLS).—In static LLS, the scattered light intensity, known as the Rayleigh ratio (R_θ), of a polymer solution at angle (θ) and concentration (c) is related to the weight-average molecular weight (M_w) by[15]

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c \quad (1)$$

Table 1
Sulfation of α-(1→3)-D-glucan with the sulfur trioxide–pyridine complex reacting for 8 h at 25 °C

Sample	Yield (mg)	S %w/w	DS [a]
S-**A**-04	78	11.6	1.0
S-**A**-06	84	12.7	1.1
S-**A**-08	77	13.9	1.3
S-**A**-10	85	15.7	1.6
S-**A**-11	77	16.5	1.7

[a] Degree of sulfation (DS): the number of sulfate groups per glucose residue.

where $K = 4\pi^2 n_0^2 (dn/dc)^2/(N_A \lambda_0^4)$, with N_A, n_0 and λ_0 being Avogadro's number, the refractive index of the solvent in a vacuum, and the wavelength of light in a vacuum, respectively. $P(\theta)$ is a function of the size, shape, and structure of the molecule.

The light-scattering intensities were measured with a multi-angle laser light scattering instrument (MALLS) equipped with a He–Ne laser ($\lambda = 632.8$ nm) (DAWN® DSP, Wyatt Technology Co.) over the angular range from 49 to 135° at 25 ± 1 °C. The refractive index of 0.25 M LiCl–Me_2SO, measured by an Abbé refractometer, was 1.4795. The refractive index increments (dn/dc) were measured with a double-beam differential refractometer (DRM-1020, Otsuka Electronics Co.) at 633 nm and 25 °C. The polysaccharide solutions were dialyzed against solvent for 72 h. The values of specific refractive index increment dn/dc in 0.25 M LiCl–Me_2SO and in aq 0.5 M NaCl were determined to be 0.058 and 0.133 $cm^3\ g^{-1}$, respectively. Astra software was utilized for data acquisition and analysis.

Viscosity measurement.—Viscosities of the fractions of **A** and of the sulfated derivatives S-**A** in 0.25 M LiCl–Me_2SO and aq 0.5 M NaCl were measured at 25 ± 0.1 °C by using a modified capillary viscometer, a gift from the Institute of Industrial Science, Tokyo University. The kinetic energy correction was always negligible. Huggins and Kraemers plots were used to determine the intrinsic viscosity [η].

3. Results and discussion

Structure of the sulfated derivative.—The IR spectra of the sulfated derivatives S-**A** show absorption peaks at 800–860 cm^{-1} and at 1240 cm^{-1}, characteristic of the sulfate acid group.[16] The degrees of substitution (DS) for the sulfated derivatives from elemental analysis are summarized in Table 1. Under the same sulfation conditions, the lower the molecular weight of the glucan, the higher the DS of the corresponding sulfated derivative. The water-solubility of the α-(1→3)-D-glucan was greatly enhanced by sulfation, enabling investigation of aqueous solution properties.

The ^{13}C NMR spectrum of S-**A** in Me_2SO-d_6 is shown in Fig. 1, and ^{13}C NMR peak assignments are summarized in Table 2. Compared with the ^{13}C NMR spectrum of the original α-glucan (**A**),[12] there are four new peaks for C-2s′ (87.5 ppm), C-2s (79.8 ppm), C-4s (77.4 ppm) and C-6s (61.3 ppm), resulting from sulfation at O-2, O-4, and O-6, with downfield shifts of the carbon atoms bearing sulfate groups of ~10 ppm.[17] The position of the C-1′ signal (103.8ppm) was influenced by the adjacent C-2s. These results indicate non-selective sulfation of the α-glucan. It is noteworthy that the downfield shift (16.4 ppm) of C-2s′ was much more that 10 ppm, and the C-4 peaks shifted to higher field

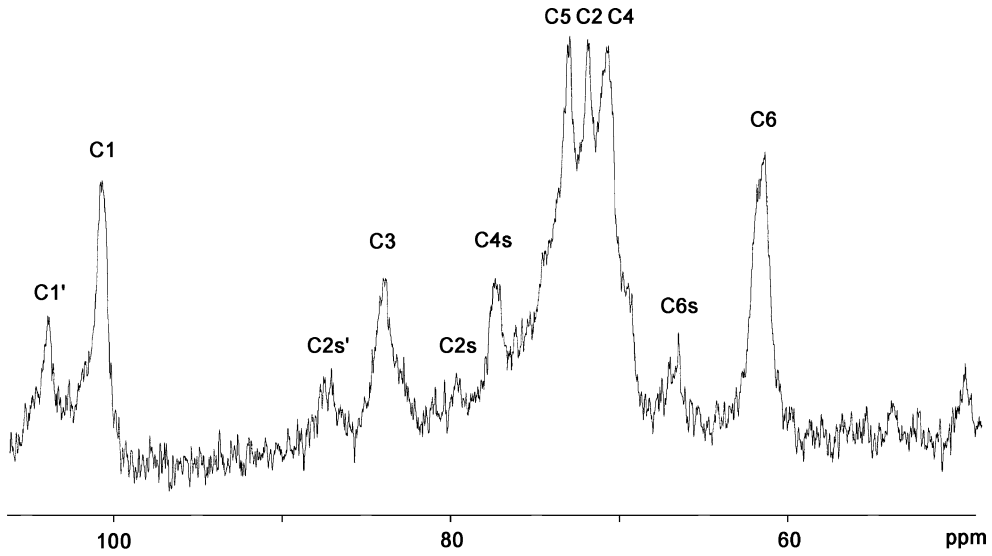


Fig. 1. ^{13}C NMR spectrum for the sulfated α-glucan (S-**A**) in Me_2SO-d_6 at 320 K.

Table 2
^{13}C NMR peak assignments (in ppm) of the α-glucan (**A**) and sulfated α-glucan (S-**A**) in Me_2SO-d_6 at 320 K

Sample	C1	C-1′	C-2	C-3	C-4	C-5	C-6	C-2s	C-2s′	C-4s	C-6s
A[12]	(101.7) 100.6		71.1	83.2	70.6	72.8	61.1				
S-**A**	100.7	103.8	71.9	84.0	70.4	70.3	61.3	79.8	87.5	77.4	66.5

for the sulfated α-glucan, as compared with the original α-glucan, suggesting that the electron density on C-2 is lower than that for normal sulfated C-2s atoms, and the electron density on C-4 was higher than that for C-2 and C-6 of the sulfated α-glucan. An intramolecular hydrogen bond between sulfated O-2 and OH-4 in the neighboring glucopyranose ring may cause cationation of C-2s′ and anionation of C-4, respectively. Kamide et al. reported[18] that when the intramolecular hydrogen bonding of cellulose in aqueous NaOH was broken down, the C-4 peak appeared at 79.7 ppm, and thus at higher field than that (87.9 ppm) for the original cellulose having intramolecular hydrogen bonds. Moreover, recent work has indicated[12] that intermolecular hydrogen bonding caused the water-insolubility of the α-glucan. Therefore, in the water-soluble sulfated glucan, intramolecular hydrogen bonding is caused by sulfate groups rather than intermolecular hydrogen bonding.

Molecular weight.—In order to minimize the electrostatic repulsion and Donnan effects caused by sulfate groups, 0.5 M aqueous NaCl was used as solvent rather than pure water. Fig. 2 shows the angular dependence of $(Kc/R_\theta)_{c=0}$ of the fractions of **A** in 0.25 M LiCl–Me_2SO at 25 °C. Three of the sulfated glucans were used for LS measurements, because of limited sample. Figs. 3 and 4 show Zimm plots of S-**A**-04 and S-**A**-08 in aqueous 0.5 M NaCl at 25 °C. The weight-average molecular weights M_w, radii of gyration $\langle S^2\rangle_z^{1/2}$ and second virial coefficient A_2 for the samples are listed in Table 3. The experimental error for A_2 and $\langle S^2\rangle_z^{1/2}$ was $< 10\%$. It is clear that the M_w values of sulfated derivatives were much lower than that of the original α-(1→3)-D-glucan, suggesting that the sulfation process causes the decrease of M_w, and also that the glycosidic linkage is comparatively weak.[13]

Mark–Houwink equation.—Although the DS values of the sulfates are different, the influence on the $[\eta]$–M relationship could be negligible over a small range of DS, because the electrostatic interactions are basically screened. Fig. 5 shows the molecular weight dependence of $[\eta]$, and the Mark–Houwink equations for the α-glucan **A** in 0.25 M LiCl–Me_2SO in the M_w range from 7.24×10^4 to 4.21×10^5, and the sulfated fractions in aqueous 0.5 M NaCl in the M_w range from 5.92×10^4 to 1.42×10^5 are represented by:

$$[\eta] = 4.9 \times 10^{-2} M_w^{0.67} \ (\mathrm{cm^3\ g^{-1}}) \quad (2)$$

$$[\eta] = 6.8 \times 10^{-4} M_w^{1.06} \ (\mathrm{cm^3\ g^{-1}}) \quad (3)$$

respectively. From analysis of Eq. (3), the α-glucan exists as a flexible chain in 0.25 M LiCl–Me_2SO. The high exponent (1.06) of the equation indicates that the sulfated α-glucan has its chains more extended than the original ($\alpha = 0.67$). The exponent $\alpha \geq 1$ in the Mark–Houwink equation can be explained by third-power

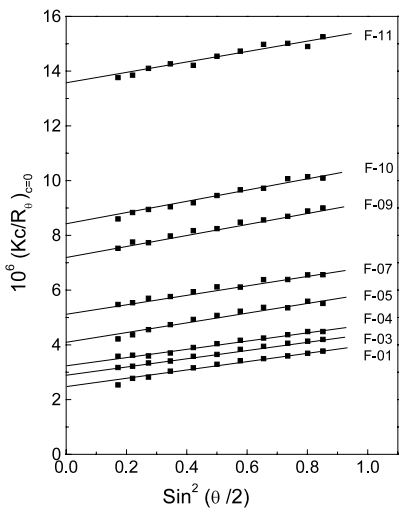


Fig. 2. Angular dependences of $(Kc/R_\theta)_{c=0}$ of the eight fractions of α-glucan **A** in 0.25 M LiCl–Me_2SO at 25 °C.

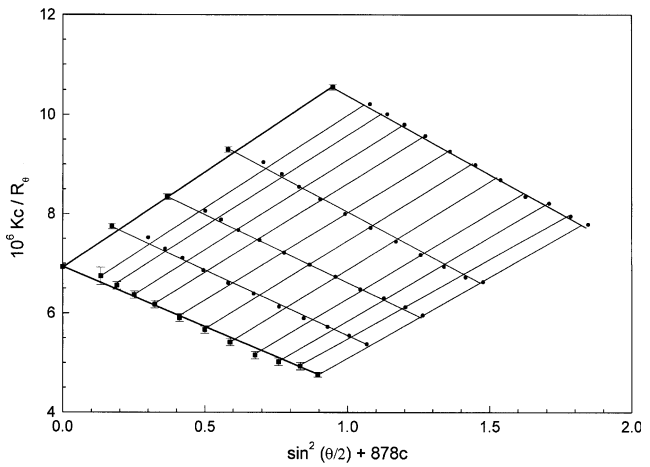


Fig. 3. Zimm plot of sulfated α-glucan S-**A**-04 in aqueous 0.5 M NaCl at 25 °C. [Polysaccharide concentration: 1.949×10^{-4} (g/g), 4.192×10^{-4} (g/g), 6.617×10^{-4} (g/g), 1.082×10^{-3} (g/g).]

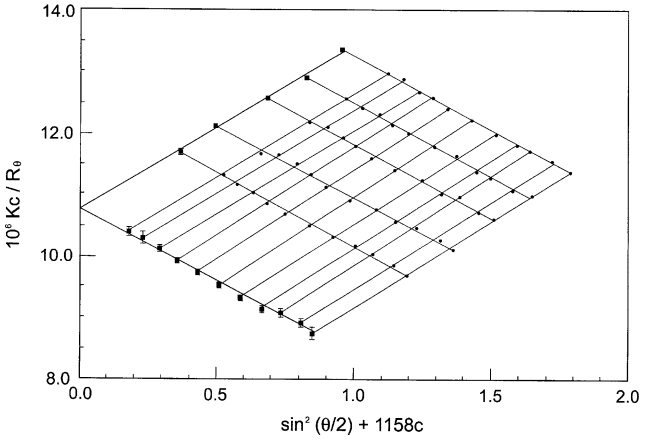


Fig. 4. Zimm plot of sulfated α-glucan S-**A**-08 in aqueous 0.5 M NaCl at 25 °C. [Polysaccharide concentration: 3.148×10^{-4} (g/g), 4.294×10^{-3} (g/g), 5.910×10^{-4} (g/g), 7.126×10^{-4} (g/g), 8.202×10^{-4} (g/g).]

type theories, and usually reflects the extension of the polysaccharide chains, such as a semi-flexible chain or a stiff chain.[19]

Molecular dimensions.—The double-logarithmic plots of $\langle S^2\rangle_z^{1/2} - M_w$ for the α-glucan and for its sulfated derivative are shown in Fig. 6. The equations are the following:

$$\langle S^2\rangle_z^{1/2} = 4.8 \times 10^{-2} M_w^{0.54} \text{ (nm)} \quad (4)$$

$$\langle S^2\rangle_z^{1/2} = 9.4 \times 10^{-4} M_w^{0.92} \text{ (nm)} \quad (5)$$

The exponent of 0.54 indicates that this α-(1→3)-D-glucan exists as an random coil in 0.25 M LiCl–Me_2SO, and the exponent (0.92) for the sulfates S-**A** is larger than that of a normal flexible chain (0.5–0.6), and shows the character of a stiff chain in aqueous 0.5 M NaCl. The relatively high chain-stiffness of the sulfated polysaccharide is probably related to intramolecular hydrogen bonding caused by sulfate groups, and the ^{13}C NMR results support this conclusion. If all hydroxyl groups of

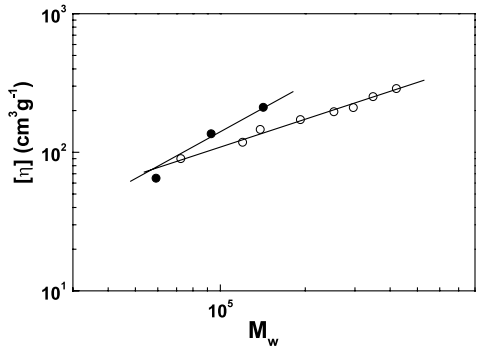


Fig. 5. Molecular weight dependence of [η] for α-glucan **A** in 0.25 M LiCl–Me_2SO (○) and S-**A** in aqueous 0.5 M NaCl (●) at 25 °C.

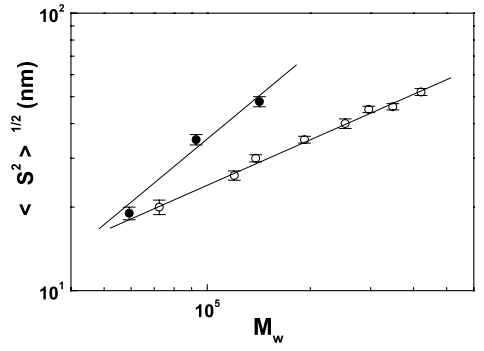


Fig. 6. Molecular weight dependence of $\langle S^2\rangle_z^{1/2}$ for α-glucan **A** in 0.25 M LiCl–Me_2SO (○) and sulfated α-glucan S-**A** in aqueous 0.5 M NaCl (●) at 25 °C.

polysaccharides contribute to intramolecular hydrogen bonding, the polymers are unusually water-soluble and stiff, as in the β-(1→3)-D-glucan from *Auricularia auricula-judae*, which is a water-soluble single helix.[19] The value of [η] and $\langle S^2\rangle^{1/2}$ reflect the extent of chain stiffness. The data in this work indicate the chain of the sulfated α-glucan to be less stiff than a single helix, but more than flexible polymers.

Table 3
Experimental results from laser light scattering, GPC-LLS and viscosity measurements for the fractions of **A** and S-**A** at 25 °C

Fractions	Solvent	[η] (cm³ g⁻¹)	k'	$M_w \times 10^{-4}$ (g mol⁻¹)	$\langle S^2\rangle_z^{1/2}$ (nm)	$A_2 \times 10^4$ (mol g⁻² cm³)
A-01	0.25 M LiCl–Me_2SO	288	0.38	42.1	52	1.0
A-03		252	0.38	34.8	46	2.8
A-04		210	0.39	29.6	45	1.6
A-05		196	0.40	25.3	40	3.1
A-07		172	0.40	19.2	35	3.5
A-09		146	0.42	13.9	30	4.4
A-10		118	0.42	12.0	26	6.4
A-11		90	0.44	7.24	20	12.0
S-**A**-04	0.5 M NaCl	211	0.45	14.2	48	16.1
S-**A**-08		136	0.46	9.28	35	15.4
S-**A**-11		65	0.49	5.92	21	42.2

Acknowledgements

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