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A study of bioactive, branched (1 → 3)-β-D-glucans in dimethylacetamide/LiCl and dimethyl sulphoxide/LiCl using size-exclusion chromatography with multi-angle light scattering detection

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ABSTRACT

Water-soluble (1 → 3)-β-D-glucans with 1,6-linked branches (SBG) isolated from the cell walls of *Saccharomyces cerevisiae* were studied by size exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) detector using dimethylacetamide (DMAc) containing 0.5% (0.12 M) LiCl, or dimethyl sulphoxide (DMSO) in the absence and presence of 0.25 M LiCl, respectively, as eluents. The aggregating glucan could be dispersed as single chains in both solvents, with chain length distributions in reasonable agreement with results obtained previously with carboxymethylated glucans in aqueous solvent. However, DMAc is preferred over DMSO because of higher sensitivity in MALLS, and also because the latter produces SEC anomalies. SBG dissolves slowly in DMAc/LICl at room temperature, but heating accelerates the process. The rate of depolymerisation of SBG in DMAc/LiCl at high temperatures (70–105 °C) was determined as a basis for defining dissolution procedures at elevated temperatures with a minimum of degradation. The result of the investigation is a simple and reliable protocol for preparing unaggregated, fully dissolved and undegraded SBG in DMAc/LiCl, which is well suited as a standard analysis of the molecular weight distribution of SBG-like molecules without chemical derivatization.

1. Introduction

The cell wall of baker's yeast, *Saccharomyces cerevisiae* contains extractable (1 → 3)-β-D-glucans [1–3]. NMR and methylation analyses show that β-glucan polymer is 83% β-1,3 glucan chains with 6% β-1,6 links [4]. It has approximately 5 branch points per 100 units in the β-1,3 glucan main chains. SBG (abbreviation for 'soluble beta glucan') refers to water-soluble preparations of the glucan that are optimized for biological activity and are currently developed as for uses as wound healing dressings.

The chain length or degree of polymerization (DP) distribution is a crucial parameter since the interaction of (1 → 3)-β-D-glucans with their receptors may be strongly dependent on DP [5]. Simple and robust methods for the determination of the chain length distribution are also critical from a regulatory point of view. SBG tend to aggregate strongly in aqueous solutions, even at elevated temperatures. This prevents accurate determination of the chain length distribution using SEC. Partial carboxymethylation (DS > 0.5) may be used as a basis for such analyses using SEC-MALLS [6]. However, methods avoiding chemical derivatives are sometimes more beneficial, and we therefore turned to non-aqueous solvents.

In recent years, DMAc containing 0.5–0.9% LiCl has become the most widespread solvent for studying celluloses using SEC-MALLS [7,8]. A few studies related to neutral glucans (celluloses, amyloses, amylopectins, dextrans, pullulans, curdlan and a mixed-linkage (1 → 3),(1 → 4)-β-D-glucan from oat have been reported [7,9,10]. However, few studies of polysaccharides having exclusively β-(1 → 3) linkages in the main chain in this solvent, including proper determination of $(dn/dn)_\mu$ have to our knowledge been published.

Initial experiments in our laboratories suggested that SEC-MALLS in DMAc/LiCl (cellulose protocol) could well be adapted to SBG. We here provide a full description of a protocol for SEC-MALLS of SBG-like glucans, including assessment of $(dn/dc)_\mu$ and identification of an improved dissolution procedure involving heating up to 105 °C based on assessment of the rate of depolymerisation.

DMSO has generally been accepted as good solvent for branched and unbranched (1 → 3)-β-D-glucans. Examples include lentinan [11], curdlan [12], water-insoluble glucan from sclerotia of *Pleurotus tuberregium* [13], pachyman from *Poria cocos mycelia* [14], and a water-insoluble glucan from *S. cerevisiae* [15]. Early work suggested that DMSO could be used as eluent in SEC-MALLS studies of

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SBG-like glucans [15], especially in the presence of 0.25 M LiCl [16] or 0.1 M LiBr [17], whereas the presence of small amounts of water (in DMSO) seem to promote aggregation of β-1,3-glucans [14] and should thus be avoided. It may further be noted that despite the high (thermodynamic) solubility of (1 → 3)-β-D-glucans in DMSO, the rate of dissolution may be slow [18], requiring long dissolution times or heating. Another inherent limitation is the low $(dn/dc)_\mu$ values of polysaccharides in DMSO, which reduces the sensitivity in light scattering. In the second part of the present work we study the sample preparation, dissolution and SEC-MALLS analysis of SBG in DMSO in the absence or presence of 0.25 M LiCl. In both cases we compare results to polysaccharide standards such as pullulans and dextrans.

A critical parameter in light scattering is the refractive index increment at constant chemical potential $(dn/dc)_\mu$. The literature provides widely different values for glucans in general especially for DMAc/LiCl. Because of the requirement 'constant chemical potential' samples should ideally be dialysed against the SEC eluent to obtain true equilibrium. In the case of DMAc/LiCl this is almost impossible because dialysis bags, which are usually based on cellulose, dissolve in the solvent [19]. In the present case we have to rely on the '100% recovery' method based on the on-line refractive index detector [20], which requires accurately known concentrations and properly calibrated detectors, and no loss of material during chromatography.

2. Experimental

2.1. Materials

SBG batches were provided by Biotec Pharmacon ASA (Tromsø), either as 2% aqueous solutions, or as freeze-dried materials. Molecular weights (M_w and M_n) based on their reduced and carboxymethylated derivatives have recently been published [6]. Batch 221-7 was used for the development of the methods described below. Dextran analytical standards were obtained from Sigma–Aldrich. Pullulan standards were obtained from Hayashibara Biochemical Laboratories, Japan. All the samples were dried *in vacuo* over P_2O_5 before use. DMAc (Optigrade) was obtained from Promochem (Germany). DMSO (GC grade) was obtained from Merck. LiCl was obtained from VWR Int., Belgium.

2.2. Determination of the water content

The water content was determined by two methods. First, samples (triplicates of 2–4 mg each) were dried at 144 °C for 20 min in an oven. Following heating the samples were rapidly transferred to an analytical scale where mass was accurately determined at regular intervals (every 20 s up to 4 min). As water was absorbed during cooling the dry weight was obtained by back-extrapolation to zero time. The second method was based on a thermogravimetric balance (Netzsch STA 449C) using a 30–150 °C gradient. Both methods yielded consistent results, with an average water content of 1.95% for SBG.

2.3. Sample dissolution and SEC-MALLS in DMAc/LiCl

Dried samples were dissolved in the eluent (0.5% LiCl/DMAc) (typically at concentrations in the range 1–4 mg/ml). Due to slow dissolution, different heating procedures were applied (ambient temperature, 70 °C and 105 °C for up to 9 days). The samples were subsequently filtered (PTFE, 0.2 μm) and injected (100–250 μl) into the SEC-MALLS system, which consisted of Dawn DSP laser photometer (λ_0 = 632.8 nm) and an Optilab DSP refractive index detector (RI), or a Shodex RI SE-61 refractive index detector, and as described earlier [21]. The columns (operated at room temperature) were three serially connected PLgel Mixed-A LS columns (Polymer Laboratories, USA), and the flow rate was 1 ml/min. A second virial coefficient (A_2) of 1×10^{-4} (ml mol g^{-2}) was used for all the glucan samples in the processing of the data, although results were largely insensitive to A_2 due to the low concentrations used ($2A_2c \ll 1/M_w$). Parameters (dn/dc and A_2) for the dextran standards were taken from the literature. Data were processed using Astra software v. 5.3.14.

2.4. Sample dissolution and SEC-MALLS in DMSO

Samples were dissolved directly in DMSO or DMSO/LiCl and injected into the SEC-MALLS system as described above, except that a Dawn Heleos light scattering detector (Wyatt, USA) (λ_0 = 632.8 nm) was used to take advantage of the more powerful laser (due to weaker scattering in DMSO). Two different RI detectors were used, namely an Optilab T-rEX (Wyatt, USA) or a Shodex RI SE 61 detector. In this case TSK-GEL G1000-HHR and G4000 HHR columns (serially connected) were used. The flow rate was reduced to 0.35 ml/min due to the viscosity of DMSO leading to increased pressure in the system.

2.5. Determination of the refractive index increment

The refractive index increment at constant chemical potential $(dn/dc)_\mu$ of SBG was determined by SEC-MALLS using the 100% recovery method as described earlier [20]. In brief, the dried SBG samples were dissolved (1.0–4.0 mg/ml), filtered (PTFE, 0.2 μm) and injected (100 μl) into the SEC-MALLS system. By assuming 100% recovery from RI detector (operated at 40 °C), $(dn/dc)_\mu$ was calculated using the Astra software.

2.6. Intrinsic viscosity measurements

An Ubbelohde-type glass capillary tube suspended level viscometer (type 0a) (Schott-Geräte, Germany) was used for viscosity measurements. The viscometer was suspended in a water thermostat maintained at 24.8 °C with an uncertainty of ±0.01 °C. The Huggins and Hermann equations were used to estimate the value of the intrinsic viscosity.

3. Results and discussion

3.1. SEC-MALLS in DMAc/LiCl

The calculated molecular weights (or DPs) depend critically on the $(dn/dc)_\mu$ value for SBG in DMAc/LiCl. In the absence of adequate membrane or dialysis based methods we applied the 100% recovery method in SEC. This method has previously been applied to cellulose in the same solvent [20,22]. Our estimates for SBG and dextran (both 0.120 ml/g) are just slightly above that reported for cellulose based on the same method (0.117 ml/g) [20]. The difference may possibly differences in the interaction of 1,4- and 1,3- or 1,6-linked glucans with the chloride ions in the presence of DMAc. However, this topic seems not to be systematically studied in the literature.

The SEC-MALLS system, which is primarily adopted for analyses of celluloses [8,20] was first tested by injecting a series of dextran standards (5–270 kDa), which dissolve well in DMAc/LiCl. Results for M_w agreed well with the data certificates provided by the supplier, and also with independent SEC-MALLS analyses in aqueous solvent (0.05 M Na_2SO_4 containing 0.01 M EDTA, pH 6.0) (data not shown). Polydispersity indices (PI = M_w/M_n) were, on the other hand, systematically lower (typically in the range 1.06–1.23) compared to the certificates and also lower than those obtained by aqueous SEC. This is ascribed to the relatively poor selectivity of mixed bed columns, which instead favour a wide separation range.

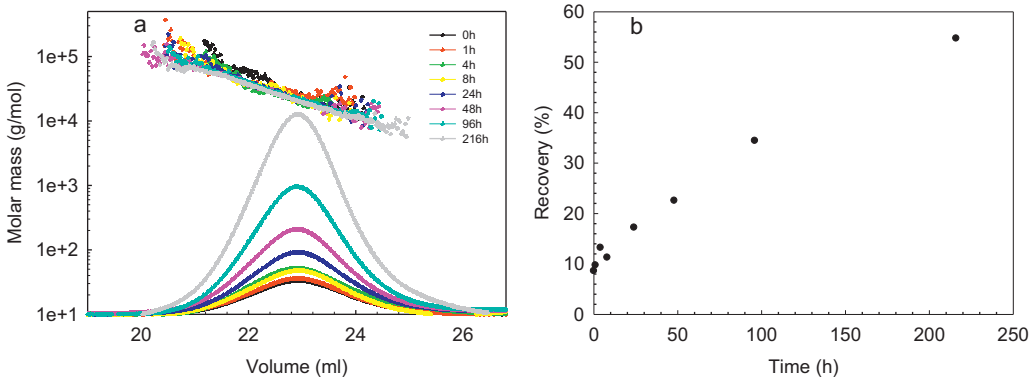

Fig. 1. (a) SEC-MALLS results (RI profile and calculated molecular weights across the profiles) of SBG (Batch 221-7) dissolved and analysed in 0.5% LiCl in DMAc at RT. Different colours represent different dissolution times at room temperature (0–216 h). (b) SEC-MALLS sample recoveries for various dissolution times (dissolution at ambient temperature).

Hence, M_n and DP_n values obtained in this system must be regarded as apparent values differing from those that can be obtained in other column systems.

We subsequently explored the behaviour of SBG (Batch 221-7). Fig. 1a shows the SEC-MALLS profiles obtained for different dissolution times at ambient temperature, whereas Fig. 2b shows the corresponding sample recoveries. The data clearly shows that SBG dissolves extremely slowly at ambient temperature, with a recovery of only 55% after 220 h. Nevertheless, the shape of the RI (refractive index detector) profile and the calculated molecular weights were essentially independent of the dissolution time. The RI peaks were quite symmetric, and eluted between the void and total volumes. The 'calibration plots' (plots of log M versus elution volume), were also linear, indicating that non-SEC effects were marginal or absent. Hence, the columns are suitable for (1 → 3)-β-D-glucans when DMAc/LiCl is the solvent. We applied in some cases a column temperature of 80 °C instead of room temperature. However, this had minimal influence on the results (data not shown).

Low sample recoveries may be acceptable in many cases, provided that no fractionation or selective extraction takes place, for example a more rapid dissolution of the shortest chains. However, as a general method maximum recovery is always preferable, and we therefore investigated the effect of heat treatment of SBG in DMAc/LiCl. Heating may accelerate the dissolution, but may possibly lead to depolymerisation, as reported for cellulose [23].

Fig. 2a shows SEC-MALLS data for SBG (Batch 221-7) heated at 70 °C and 105 °C for 0–216 h. In this case the sample dissolved rapidly. Maximum sample recovery at 105 °C was reached after 1–2 h. For longer heating times, depolymerisation was observed as the RI peak moved to higher elution volumes, with a concomitant decrease in M_w and M_n. The rate of depolymerisation was further analysed in terms of the theory of random depolymerisation [24]:

$$\frac{1}{DP_w} = \frac{1}{DP_{w,0}} + \frac{kt}{2} \quad (1)$$

Here, $DP_{w,0}$ is the weight average DP of the undegraded sample, whereas k is the pseudo first order rate constant of depolymerisation.

Fig. 2b shows plots of DP_w^{-1} versus time for data obtained at 70 °C and 105 °C. The plots become essentially linear after a few hours, with calculated rate constants of 3.1E−5 h^{-1} and 8.8E−6 h^{-1}, respectively. Applying the Arrhenius equation we obtain a rather low activation energy (E_A) of 39 kJ/mol for the degradation of SBG in DMAc/LiCl. The mechanism of degradation of (1 → 3)-β-D-glucans in DMAc/LiCl may well be the same as that reported for cellulose, involving reactive N,N-dimethylketeniminium ions [25].

Fig. 2b shows a downward curvature for very low degradation times. We attribute this to the presence of some aggregated or undissolved material, which disappeared after about 8 h at 70 °C and 2–4 h at 105 °C. For such short times the decrease in DP is less than 2%. As a routine method we therefore suggest a heat treatment of 2 h at 105 °C or 4 h at 70 °C prior to SEC.

These values allow simple estimations of the extent of degradation of (1 → 3)-β-D-glucans in DMAc/LiCl, and are particularly relevant at higher DP values since, by rearranging Eq. (1), the relative change in DP depends on DP itself:

$$\frac{DP_w}{DP_{w,0}} = \left(1 - \frac{DP_{w,0}kt}{2}\right)^{-1} \quad (2)$$

Hence, larger (1 → 3)-β-D-glucans may in fact depolymerize to a significant extent under conditions where the molecular weight of SBG type β-glucans is only marginally affected.

Recently, Kivelä et al. [10] presented a related study on a mixed-linkage (1 → 4)(1 → 3)-β-D-glucan from oat (OBG), where dissolution in DMAc/LiCl was obtained without heating by wetting the dry glucan with ethanol. OBG is structurally closer related to cellulose than to SBG due to the strictly linear structure and presence of about 70% β(1 → 4) linkages in the former. OBG also lack the specific aggregation modes (triple helical structures) of pure (1 → 3)-β-D-glucans [26]. We thus maintain results from OBG cannot be directly applied to SBG without experimental support. However, the dissolution method based on ethanol wetting may be a viable alternative to heating. When applied to SBG we observed accelerated dissolution at ambient temperature but some insoluble, fibrous material remained in all cases, also after heating to 70 °C for a few hours. Hence, this method would need further optimization in the SBG case.

3.2. SEC-MALLS in DMSO

The refractive index increment $(dn/dc)_\mu$ of SBG in DMSO was first determined using the 100% recovery method described above. We obtained a value of 0.062 ml/g both in the absence and presence of LiCl, which is very close to values reported for several glucans, including amylose [27]. The much lower value compared to those obtained in aqueous solvents or DMAc/LiCl reflects less optical contrast compared to aqueous solvents, and results in a lower S/N ratio in light scattering. Strong lasers are therefore particularly useful in such cases.

SBG as well as the dextran standards dissolved completely in DMSO in about 4 h at ambient temperature, whereas pullulan standards dissolved slowly, both in DMSO and DMSO containing 0.25 M LiCl at ambient temperature. Pullulan could be readily dissolved

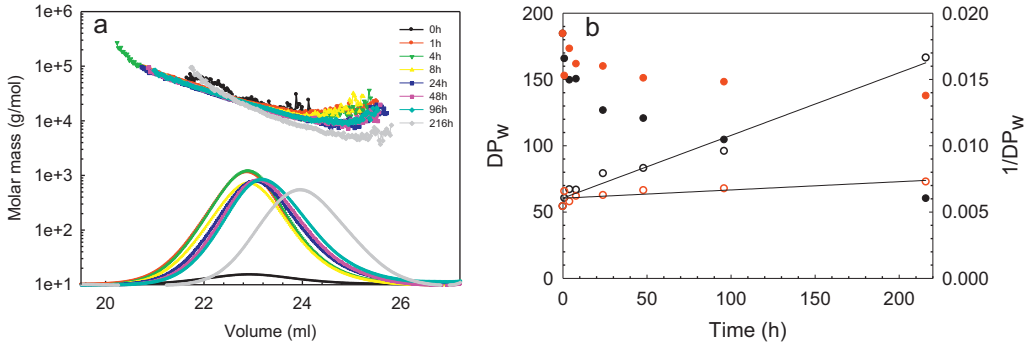

Fig. 2. (a) SEC-MALLS data of heat-treated SBG (105 °C, 0–216 h) in 0.5% LiCl in DMAc. (b) Changes in DP_w (filled circles) and $1/DP_w$ (open circles) obtained at 70 (red) and 105 °C (black). Solid lines refer to linear fits of the $1/DP_w$ data according to Eq. (1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

following a brief heat treatment (40 min at ambient temperature followed by heating at 110 °C for 15 min).

SEC-MALLS in DMSO or DMSO/LiCl was performed using TSK-GEL G1000-HHR (pre-column) and G4000 HHR columns. Similar column systems have been described in several articles for various (1 → 3)-β-D-glucans without mention of any special problems in the analysis [14,28,29]. Samples (both unheated and heated) analysed in pure DMSO showed a separate RI peak or shoulder at low elution volumes (not shown), indicating the presence of small amounts of poorly dissolved materials. However, these peaks were reduced or disappeared in the presence of LiCl, which therefore is preferred over pure DMSO as SEC solvent for SBG.

Fig. 3 shows results for obtained for injections of batch 221-7 at different sample concentrations, corresponding to 75 μg, 150 μg, and 310 μg injected sample. The mass recovery was essentially 100%, but an anomaly in form of negative peaks was consistently observed in the RI profile in the elution volume range 7.0–7.5 ml, especially for the highest injected amounts. This phenomenon appeared with two different RI detectors (Optilab T-rEX and Shodex RI SE-61), and was also seen with the dextran standards. The former RI detector also provided (reproducibly) a strongly non-linear response to difference injected amounts (both for dextran and SBG), suggesting it cannot be used for DMSO in all cases. It is further noted in Fig. 3 that the calculated molecular weight data ('calibration curve') shifted downwards with increasing injected amounts. In total, the calculated M_w decreased linearly with increasing injected amount. The same was observed for the dextran standards. This could in principle be an effect of a non-negligible second virial coefficient (A_2). However, values in the range 2.5E−2 ml mol g^{-2} were needed to obtain concentration-independent results, which is 1–3 orders of magnitude higher than values obtained for polysaccharides in DMSO including dextran [30] linear (synthetic) amylose [27], and a β-(1 → 3)-D-glucan (pachyman) [14]. We were unable to find relevant A_2 data for β-(1 → 3)-D-glucans in DMSO/LiCl in the literature.

We found, however, that the M_w values obtained by extrapolating the data to zero injected amount were with in 5–10% of the values found for dextran 25 in aqueous solvent and the value given by the supplier. SBG sample 221-7 provided a value of 23.9 kDa by the same approach, corresponding to a DP_w of 148, which is in good agreement with the value obtained in aqueous SEC-MALLS for the carboxymethyl derivative (143). The same trend was observed for batch 321-5 (>30 kDa), yielding DP_w = 197 in DMSO/LiCl and 212 for CM-SBG. Despite a reasonable good agreement between the two methods, and the fact that data obtained in DMSO/LiCl look reasonable at a first glance, and that a comparison of different samples follow the trends seen in aqueous solvents and DMAc/LiCl, we nevertheless conclude that SEC-MALLS in DMSO/LiCl is unsuited for the study of SBG-like polysaccharides because of the anomalies observed.

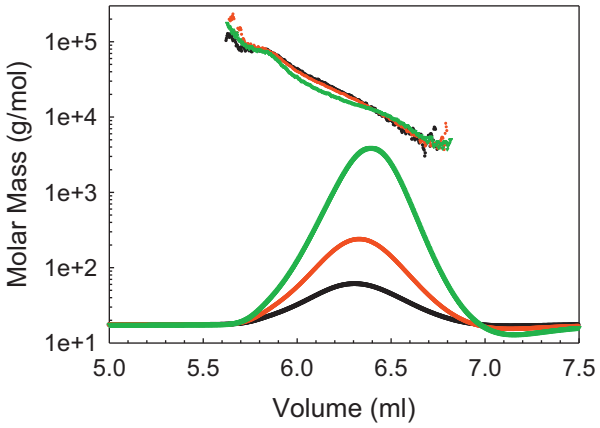

Fig. 3. SEC-MALLS results of SBG (Batch 221-7) in DMSO/LiCl at different sample concentrations, corresponding to 75 μg, 150 μg, and 310 μg injected sample.

3.3. Intrinsic viscosity

As a supplement to the SEC-MALLS studies we determined the intrinsic viscosities of SBG (Batch 221-7) by conventional capillary viscometry (at 24.8 °C) in both DMAc/LiCl, DMSO/LiCl and pure

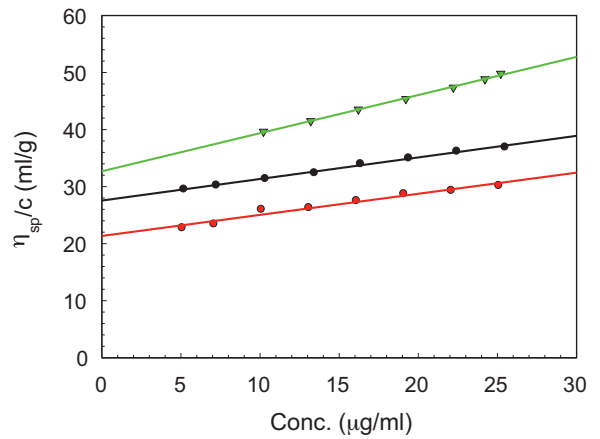

Fig. 4. Huggins plots (reduced viscosity (η_{sp}/c) as a function of concentration) of SBG (Batch 221-7) in DMAc/LiCl (upper curve), DMSO/LiCl (middle curve) and pure DMSO (lower curve). Data were obtained by capillary viscometry at 24.8 °C.

Table 1
Off-line measurements of the intrinsic viscosity of SBG (Batch 221-7) in the SEC solvents.

Solvent	Intrinsic viscosity (ml/g)
DMAc/LiCl (0.5%)	34
DMSO	22
DMSO/LiCl (0.25 M)	28

DMSO. Huggins' plots are given in Fig. 4, and the intrinsic viscosities are listed in Table 1.

The intrinsic viscosity ($[\eta]$) is the product of the specific hydrodynamic volume of the polymer (ν_h) and a shape factor (ν) [24]. Analysis of the $[\eta]$–M relationship of carboxymethylated SBG in aqueous solution revealed a randomly coiled structure [6]. The corresponding intrinsic viscosity obtained from SEC-MALLS including an online viscosity detector was 40 ml/g. It should be noted the presence of carboxylic substituents induces salt-dependent chain expansion due to intramolecular electrostatic repulsion, and cannot be directly compared to underivatized SBG. Nevertheless, SBG behaves differently in the three organic solvents listed in Table 1. For pure DMSO a low value of 22 ml/g indicates a compact structure. Adding LiCl to 0.25 M leads to a significant increase to 28 ml/g. This must be attributed to chain expansion, which again must reflect strong solvent interaction, possibly adsorption of LiCl analogous to that proposed for the cellulose-DMAc/LiCl system [7,31]. In 0.5% DMAc/LiCl an even higher intrinsic viscosity (34 m/g) indicates even stronger interaction. This also suggests DMAC/LiCl is a thermodynamically better solvent for SBG-like glucans than DMSO, and may possibly explain the difficulties in obtaining good SEC-MALLS results In DMSO.

4. Conclusions

Soluble, branched (1 → 3)-β-D-glucans (SBG) tend to aggregate strongly in aqueous solutions, especially at low temperatures, preventing the analysis of the chain length distribution by SEC-MALLS without chemical derivatization. Organic solvents (DMSO, DMSO/0.25 M LiCl, DMAc/0.5% LiCl), which can disrupt the hydrogen bonds of the polymer, can instead be used to dissolve SBG and carry out SEC-MALLS analyses without any need for derivatization. DMAc/LiCl seems to be a better solvent for SBG than DMSO as judged from intrinsic viscosity measurements, and provided also better and more consistent SEC-MALLS data. Since the rate of dissolution in DMAc/LiCl was rather slow, a heating regime was identified where complete dissolution with a minimum degree of depolymerisation was determined. We conclude that this protocol for dissolving (1 → 3)-β-D-glucans in DMAc/0.5% LiCl combined with SEC-MALLS is well suited as a standard analysis of the molecular weight distribution of SBG-like molecules.

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