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Chain length distribution and aggregation of branched (1→3)-β-D-glucans from *Saccharomyces cerevisae*

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ABSTRACT

Water-soluble (1→3)-β-D-glucans with 1,6-linked branches (SBG), originally isolated from the cell walls of *Saccharomyces cerevisiae* and partially depolymerised for optimal performance in wound healing applications, were studied by size exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) detector and a viscosity detector at both high and ambient column temperatures. The strongly aggregating materials could be dispersed as single chains in water following partial carboxymethylation (degree of substitution (DS) 0.51 or higher). Lower DS (0.23) also dispersed as single chains provided a column temperature of 80 °C was applied. Reduction of reducing ends prior to carboxymethylation was required to avoid alkaline peeling and hence to obtain correct molecular weight distributions of the native material. DS was determined using ^{13}C NMR and potentiometric titration (range 0.23–0.91). Further analysis of CM-SBG in the single chain state suggested a randomly coiled behaviour with marginal influence of the branches in terms of macromolecular dimensions, which were close to those of CM-curdlan. The result of the investigation is a simple and reliable protocol for preparing undegraded and un-aggregated SBG derivatives, which are well suited as a standard analysis of the molecular weight distribution of SBG-like molecules.

1. Introduction

The cell wall of Baker's yeast (*Saccharomyces cerevisiae*) contains about 30% β-glucan (Nguyen, Fleet, & Rogers, 1998). Glucan preparations seem to share a (1→3)-β-D-glucan backbone with various amounts of side chains attached to the main chain through β-1,6 linkages. The detailed chemical structure (branching pattern) and physical properties of β-glucans depend on not only the extraction methods but also physical and chemical treatments (Hromádková et al., 2003; Williams et al., 1991).

There is currently large interest in fungal (1→3)-β-D-glucans, particularly because they may interact with the immune system (Breivik et al., 2005; Engstad, Engstad, Olsen, & Østerud, 2002) which can be stimulated against viral (Jung et al., 2004; Wang, Shao, Guo, & Yuan, 2008), and fungal infections (Pelizon, Kaneno, Soares, Meira, & Sartori, 2005). The host defence system has receptors for (1→3)-β-D-glucans in order to recognise and eliminate fungal cells which contain the glucans (Adachi et al., 2004). Dectin-1 seems to play a key role in recognising (1→3)-β-D-glucans (Palma et al., 2006; Taylor et al., 2002), although alternative explanations have been forwarded (Goodridge, Wolf, & Underhill, 2009). The ability of (1→3)-β-D-glucans to accelerate wound healing (Berdal et al., 2007) has recently prompted the development of glucan-based medical devices and pharmaceuticals.

The detailed physical and chemical properties of β-glucans determining biological effects remain somewhat unclear (Adams et al., 2008). Interactions with receptors will necessarily depend on the branching pattern, as well the chain length distribution and the extent and mode of chain aggregation. It has indeed been demonstrated that interaction between dectin-1 and β-1,3-linked glucose oligomers requires a DP (degree of polymerisation or, equivalently, number of sugar monomers per chain) of 10–11, and that oligomer clustering on liposomes mimic macromolecular glucan preparations such as zymosan (Palma et al., 2006). Hence, the chain length distribution as well as the aggregation behaviour, must be accurately known, both from a fundamental perspective and for regulatory approval.

A (1→3)-β-D-glucan preparation (SBG) isolated from *S. cerevisiae*, developed as a possible medical device and drug, has recently been studied in detail in our laboratory. Early methylation analyses of SBG (Engstad & Robertsen, 1993) suggest that it contains on average about 6% non-reducing ends, 83% β-1,3 linked glucosyl residues with about 6% β-1,6 links, and on average 5 branch points per 100 units in the (1→3)-β-D-glucan main chain (Engstad & Robertsen, 1993). Reducing end assays suggest that DP_n (number average degree of polymerisation) may be in the range 70–250. Although the DP_n values may be inaccurate due to well-known

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limitations in the assay (Horn & Eijsink, 2004), it is clear that SBG is fundamentally different from scleroglucan, lentinan and schizophyllan, which are fungal (1→3)-β-D-glucans having extremely high molecular weights, regular branching patterns (1 branch per 3 sugars of the main chain) and a tendency to form triple helices. SBG tends to aggregate strongly in aqueous solution, especially at low temperatures. Initial experiments using aqueous size-exclusion chromatography with multi-angle laser light scattering detection (SEC–MALLS) resulted in molecular weight estimates in the range 10^5–10^6 Da, whereas conventional end group analysis suggested values 10–100 times lower (see also Results section). A SEC–MALLS method employing post-column addition of alkali has been shown to provide both chain length distribution and aggregation numbers of (1→3)-β-D-glucans structurally similar to SBG (Gawronski, Park, Magee, & Conrad, 1999). However, extensive use of alkali is detrimental to the detector system, and we therefore sought for alternative methods, which can be applied as a routine method.

Partial carboxymethylation of hydroxyl groups is commonly applied method to obtain polysaccharide derivatives that are soluble under neutral conditions (Horváthová et al., 1991; Kath & Kulicke, 1999; Sasaki, Abiko, & Nitta, 1979; Stojanovic, Jeremic, & Jovanovic, 2000; Wang, Zhang, Li, Hou, & Zeng, 2004; Xu et al., 2009), carboxymethyl cellulose (CMC) being the most prominent example. SEC–MALLS analysis of carboxymethylated fungal (1→3)-β-D-glucans has indeed been reported (Kath & Kulicke, 1999; Wang & Zhang, 2006; Williams et al., 1991). However, (1→3)-β-D-glucans can possibly be degraded in the process of carboxymethylation due to strongly alkaline conditions and high temperatures (Kath & Kulicke, 1999).

The purpose of the present work is to explore the possibility to use carboxymethylation as a simple tool to obtain un-aggregated SBG combined with determination of the chain length distribution. We show that SBG may be carboxymethylated (DS up to 0.91) completely without detectable degradation following borohydride reduction prior to carboxymethylation. The method provides perfectly dispersed chains under neutral conditions (for DS > 0.51) or at 80 °C (for DS > 0.23), allowing facile SEC–MALLS measurements suitable as a routine method, avoiding dependence on the Nelson and Somogyi method, which suffers from low sensitivity as well as dependence of the response on the chain length (Vlasenko, Ryan, Shoemaker, & Shoemaker, 1998). Inclusion of an on-line viscosity detector in the SEC–MALLS measurements further provide information of the shape and extension of both SBG and CM-SBG chains in solution, which we compare to several other well-characterised polysaccharide standards with different chain stiffness and geometries (dextran and pullulan (Nordmeier, 1993), mixed-linkage (1→3)(1→4)-β-D-glucans from barley (Christensen et al., 2001), carboxymethyl (CM) curdlan, triple-stranded scleroglucan (Sletmoen, Christensen, & Stokke, 2005) and 'sclerox', an oxidised/carboxylated sclergoglucan (Sletmoen et al., 2005)). We also describe some properties of semi-dilute SBG solutions (2%), corresponding to typical formulations intended for clinical uses, using small-strain oscillatory measurements and detection of particulate material.

2. Experimental

2.1. Materials

Different batches of SBG extracted from cell walls of *S. cerevisiae* and modified chemically and enzymatically were provided by Biotec Pharmacon ASA (Tromsø, Norway), either as 2% aqueous solutions, or as freeze-dried materials. The estimated DP_n ranged from 70 to 250 (Nelson and Somogyi method). The supplier reports a purity of 98%. No protein or mannoprotein could be detected. Batch 221-7 (DP_n = 117 by reducing end assay) was used for the development of the methods described below. Dextran analytical standards (M_w 1.2×10^4, 2.5×10^4, 5×10^4, 8×10^4, 1.5×10^5 and 2.7×10^5) were obtained from Sigma Aldrich. Pullulan standards P8, P16.2, P137 and P1390 (the number correspond to the molecular weight (kDa)) were obtained from Hayashibara Biochemical Laboratories, Japan (Vold, Kristiansen, & Christensen, 2006). Barley (1→3)(1→4)-β-D-glucan (mixed linkage) from barley and carboxymethyl curdlan (CM curdlan, DS = 0.4) were obtained from Megazyme International, Ireland. CM curdlans covering different molecular weight ranges were obtained by partial acid hydrolysis (0.1–100 mM H_2SO_4, 100 °C, 0–1 h, N_2 atmosphere), followed by neutralisation, dialysis and freeze-drying. Sodium carboxymethyl cellulose (CMC, DS = 0.7) was obtained from Wolff Cellulosics (Walocel CRT30). Scleroglucan (Actigum CS11) was originally obtained from Sanofi, France.

2.2. Small–strain oscillatory measurements

The small-strain oscillatory measurements were performed on a StressTech Rheometer from Reologica, Lund, Sweden. Measurements on different batches of SBG (concentration range 2–15%) were carried out on a 40 mm serrated plate/plate geometry with 1 mm gap and with the cone and plate geometry (d = 40 mm). The temperature gradient was 1 °C/min both on cooling and heating, while the frequency was 1 Hz. The shear stress varied depending on the test sample, and was chosen from stress sweep experiments. The start and end temperature was 70 °C, while the curing temperature was 10 °C. A sample of 2.1 mL was applied to the serrated plate/plate geometry, and 1.8 mL to the cone and plate geometry. The sample was covered with low viscosity silicone oil (BDH Silicone Products, KeboLab, 10cSt at 20 °C) to prevent evaporation.

2.3. Determination of solids

Solutions (20 mg/mL were heated to 50 °C, diluted to 0.4 mg/mL or 0.8 mg/mL), and kept at 50 °C for 40 min prior to centrifugation using a Beckman Coulter Avanti® J-30I High-performance centrifuge at 27,216 × *g* at 40 °C for up to 270 min. The supernatants were kept at 50 °C for further investigations by SEC–MALLS. The content of carbohydrate in the supernatant was measured by the phenol-sulphuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) method.

2.4. Carbonyl reduction

Carbonyl-reduced SBG (reSBG) was obtained by reduction with sodium borohydride (Henry & Blakeney, 1988; Painter, 1988). Briefly, SBG was dried *in vacuo* over P_2O_5 and added to freshly prepared sodium borohydride solution (20 g/L $NaBH_4$ in 0.25 M NaOH). The reaction was carried out for 1 h at 40 °C, and subsequently stopped by cooling on an ice bath, adding a drop of ethanol (antifoaming), followed by neutralisation by adding acetic acid (final pH 3–4). Solutions were subsequently dialysed and freeze dried.

2.5. Carboxymethylation

Carboxymethylated SBG and reSBG (CM-SBG and CM-reSBG) were prepared according to established methods (Sasaki et al., 1979; Stojanovic et al., 2000; Wang & Zhang, 2006) with some modifications. In brief, 600 mg of SBG or reSBG were suspended in 25 mL of isopropanol at 0 °C (ice bath) and stirred for 10 min. 10 mL of 20% degassed (nitrogen bubbling) ice cold NaOH was added while stirring on ice bath for 1 h. Then, a solution of 5.25 g monochloroacetic acid (MCA) dissolved in 10 mL of 20% NaOH (freshly prepared) was

slowly added upon stirring for 10 min at room temperature. 25 mL isopropanol was further added upon stirring at room temperature for 2 h 50 min. The temperature was then increased to 58 °C (water bath) and the reaction was carried out for different times to obtain different degrees of substitutions (DS). The reactions were terminated by rapidly cooling the flask on an ice bath. The pH was adjusted to 7 by adding 0.5 M HCl. Solutions were extensively dialysed (M_w cut-off 8000) and freeze-dried. The final products were in the Na^+ form. The degree of substitution (DS) was determined by potentiometric titration (Stojanovic, Jeremic, Jovanovic, & Lechner, 2005) and ^{13}C NMR.

2.6. NMR spectroscopy (determination of DS)

The samples (50–60 mg) were dissolved in 99.9% D_2O (600 μL) and NMR spectra were recorded at 313 K on a BRUKER Avance 600 MHz spectrometer equipped with a 5 mm cryogenic CP-TCI z-gradient probe. The recorded 1D carbon spectrum (using inverse gated proton decoupling) contained 64 K data points, had a spectral width of 180 ppm, record with 4–12 K scans at a flip angle of 30°, and relaxation delay of 30 s. The NMR data were processed and analysed with Bruker TopSpin 2.1 and TopSpin 3.0 software. DS was calculated as the intensity for the carboxyl carbon signal (~177 ppm) relative to the intensity for the anomeric carbon signal (~103 ppm).

2.7. Multidetector SEC

The samples were dissolved in the eluent (0.05 M Na_2SO_4/0.01 M EDTA (pH 6) (typically 0.5–3 mg/mL, injection volume 100–300 μL)), and heated at 100 °C for 15 min. The solutions were cooled to room temperature, filtered (0.45 μm) and analysed by SEC–MALLS (including online viscometry) as described earlier (Vold et al., 2006). In brief, the detector system consisted of an Optilab DSP refractive index detector (λ_0 = 690 nm) (Wyatt, US), a Dawn DSP light scattering detector HELEOS-II (λ_0 = 658 nm) (Wyatt, US) and a ViscoStar viscometer (Wyatt, US). The columns were TSK G4000PWXL and G3000PWXL serially connected eluted at a flow rate of 0.5 mL/min. The system was operated either at ambient temperature (RT) or with a column temperature of 80 °C and the injector sample tray 70 °C (maximum). The MALLS detector was maintained at 30 °C (eluted material cooled effectively before reaching the detectors). The RI detector and the viscosity detector were both set to 40 °C in all cases. A refractive index increment (dn/dc) of 0.148 mL/g and a second virial coefficient (A_2) of 1×10^{-4} mL mol g^{-2} were used for all the glucan samples in the processing of the data. Parameters for the standards were taken from the literature. Data were processed using Astra software v. 5.3.14. The accuracy and reproducibility of the system was determined on the basis of dextran and pullulan standards, as well as for the non-aggregating CM-SBG. RSD values for the latter are included in Table 1.

2.8. End-group analysis

The number of reducing ends was determined by the classical method of Nelson and Somogyi (Nelson, 1944; Somogyi, 1952), from which the number average degree of polymerisation (DP_n) and the number average molecular weight (M_n) were directly obtained.

3. Results and discussion

3.1. Rheology of 2% SBG solutions

A typical formulation intended for clinical uses will consist of 2% SBG. Such solutions are gel-like and turbid, and consist of a mixture of dissolved and aggregated chains, as well as particulate material (see below). The rheological properties depend on various treatments that influence on the aggregation/disaggregation behaviour of SBG chains. We briefly explored the rheology (cone and plate geometry, 1 Hz) of SBG solutions (2–15%) through a temperature cycle (70–10 °C at 1 °C/min, 10 °C for 2 h, then 10–70 °C at 1 °C/min), where solutions undergo a setting/melting process. The gelling and melting temperatures were taken as the temperature where the phase angle was at its transition point (δ = 45°). Fig. 1 shows the temperature dependence of the storage modulus (G') for 2% and 5% solutions. Higher concentrations (10% and 15%) showed qualitatively similar data (not shown). The 2% solutions gelled at 16 °C (T_g) and melted at 31 °C (T_m). Both values increased with increasing concentrations, reaching 28 °C and 51 °C, respectively, at 15%. When kept at 10 °C the G'-curve showed the characteristic increasing slope of a non-equilibrium gel. This is probably due to chain reorganisations or growth of connections in the SBG solution. The maximum elastic modulus increased with increasing concentration, covering the range 250–8000 Pa.

Table 1
Summary of SEC–MALLS results for SBG batch 221-7 obtained after various pre-treatments and column temperatures. M_w and M_n were calculated using a 1st order exponential fit in Astra except for samples marked with an asterisk (*), where no realistic fitting could be performed.

DS	Pre-treatment of solutions prior to injection	SEC–MALLS column temperature	M_w		M_n		M_w/M_n (PI)	n (# injections)
			Avg.	RSD (%)	Avg.	RSD (%)		
0	None	RT	5,270,000*	17.7	896,000*	41.1	6.33	6
	Heated (100 °C, 15 min)	80 °C	5,100,000*	65.8	913,000*	62.3	5.76	6
	Centrifuged at 27,216 g	RT	7,370,000*	0.3	794,000*	11.4	9.34	2
	Centrifuged at 27,216 g	80 °C	383,000*	0.4	38,400*	27.6	10.37	2
0.23	None	RT	39,500	15.3	17,600	5.3	2.23	5
	Stored 4 months at 4 °C, then heated (100 °C, 15 min)	RT	27,800	1.9	14,800	3.5	1.90	7
	Heated (100 °C, 15 min)	80 °C	26,300	2.4	16,600	3.4	1.59	2
0.51	None	RT	34,900	5.1	21,000	6.7	1.66	5
	Stored 4 months at 4 °C, then heated (100 °C, 15 min)	RT	30,100	3.4	18,200	3.1	1.66	5
	Heated (100 °C, 15 min)	80 °C	29,500	0.6	19,400	0.4	1.52	2
0.91	None	RT	34,200	4.3	21,700	4.2	1.57	5
	Stored 4 months at 4 °C, then heated (100 °C, 15 min)	RT	32,600	2.1	19,900	2.2	1.64	2
	Heated (100 °C, 15 min)	80 °C	32,500	0.3	21,200	2.7	1.53	2

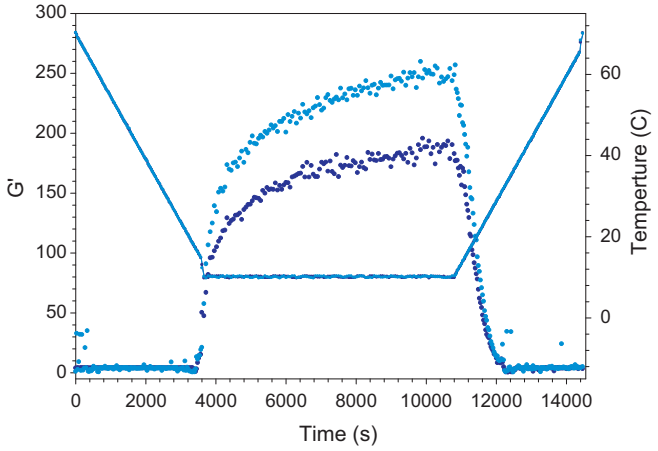

Fig. 1. Small-strain oscillatory measurements of 2 (w/w)% SBG (dark blue) and 5 (w/w)% SBG (light blue) on cone and plate geometry showing difference in G' at gelling and melting temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

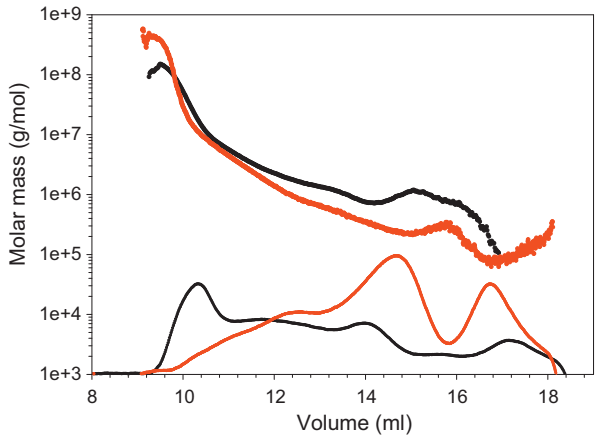

Fig. 2. SEC–MALLS data for unmodified SBG analysed with columns at room temperature (black) and 80 °C (red). Top curves refer to the molar mass (left axis), whereas the bottom curves refer to the concentration profiles (RI detector response, right axis – arbitrary units). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

The reproducibility decreased with increasing concentrations. This was tentatively attributed to the presence of inhomogeneities due to incomplete dissolution or other non-equilibrium phenomena.

3.2. Detection of solids in 2% solutions

The possible presence of solids influencing the solution rheology prompted us attempt their removal and quantification following dilution and high-speed centrifugation. Such treatment readily demonstrated that typical SBG formulations (2%) contained about 10–15% insoluble carbohydrate. However, taking a formulation through an alkali–acid cycle, which dissolves the gel (at high pH) and is followed by setting into a more inhomogeneous structure upon neutralisation, increased the amount of solid (particulate) material to 80–90%. This behaviour, which is attributed to unspecific re-association and annealing, is a recurrent theme among various (1→3)-β-D-glucans. In fact, a wide range of re-association modes (rods, rings, microgels, *etc.*) has been observed after treatment at high pH or high temperature, followed by neutralisation or cooling (Falch, Espevik, Ryan, & Stokke, 2000; Kashiwagi, Norisuye, & Fujita, 1981; Sato, Norisuye, & Fujita, 1983; Sletmoen et al., 2005).

3.3. SEC–MALLS analysis of SBG

SBG (batch 221-7) was analysed by SEC–MALLS both at ambient temperature and at 80 °C (column temperature). The RI profile at RT (Fig. 2) is distributed across the entire elution range, with multiple peaks. The corresponding molecular weight ranged from 10^7 to 10^5 across the profile. Increasing the column temperature to 80 °C, which is well above the melting temperatures of 2% SBG gels, resulted in a large shift in RI profile towards higher elution volumes. The profile also showed more pronounced splitting into distinct components, in particular a separate peak at $V = 17$ mL. Nevertheless, the molecular weight covered the same range as for the RT experiments. Pre-heating the samples above 80 °C (up to 105 °C for 30 min) had no detectable effect on the SEC behaviour. The calculated weight average (M_w) and number average (M_n) molecular weights were in the range 2×10^6 Da and 1×10^5 Da, respectively. The latter value corresponds to a DP_n of about 600, which is almost one order of magnitude above the corresponding estimate obtained by chemical analysis. The glucan isolated from the supernatants obtained after centrifugation, omitting any drying/dissolution, were also analysed by the same method. However, the results did not differ appreciably from those showed in Fig. 2. Including an ultracentrifugation step prior to SEC injection generally removed much of the very high molecular weight material (recovery decreased from 90 to 44%), but retained the main peaks at 15 mL and 17 mL (data not shown). However, M_w and M_n were still 6–7 times above those reported after carboxymethylation (see below). We therefore conclude that dilute solutions of SBG contain both particulate material and soluble aggregates of various sizes, both at RT and 80 °C, but the extent of aggregation is larger at room temperature. It may also be concluded that the melting observed in SBG gels upon heating (T_m in the range 16–28 °C for 2%) is not a result of complete disaggregation. Interestingly, the aggregates are stable even when subjected to the strong shear forces experienced in SEC. The aggregated or particulate nature of SBG is further corroborated by the markedly curved 'Debye plots' (Kc/R_θ *vs* $\sin^2(\theta/2)$) observed for individual elution slices. For the sample as a whole (average of all elution slices) we obtained weight average intrinsic viscosities ($[\eta]_w$, which corresponds to the intrinsic viscosity obtained by conventional 'batch mode' viscosity measurements) of 28 mL/g with columns at RT and 40 mL/g at 80 °C. These values are 1–2 orders of magnitude lower than those of both triple-stranded scleroglucan (Sletmoen et al., 2005) and single-stranded glucans from barley (Christensen et al., 2001) in the same molecular weight range (10^5 to 10^6). Hence, dilute solutions of SBG are undoubtedly aggregated or associated into a mixture of much more compact shapes, making SEC–MALLS unsuitable for determining the chain length distribution of underivatized SBG.

3.4. Carboxymethylation

SBG was carboxymethylated based on standard methods, *i.e.* reaction with MCA under strongly alkaline conditions in the presence of isopropanol, which preferentially leads to carboxymethylation at C-6, although some substitution at C-2 and C-4 has been reported (Wang & Zhang, 2006). DS was determined by ^{13}C NMR (Fig. 3a) and conventional potentiometric titration. Fig. 3b shows the degree of substitution (DS) as a function of reaction time. After heating the reaction mixture to 58 °C, DS increase from 0.23 to 0.91 after 90 min. DS clearly levels off with time, presumably due to the repulsive effect of increasing the charge density. More rapid and extensive carboxymethylation could be obtained using higher MCA concentrations and/or higher temperatures, but some depolymerisation was then observed. However, DS values higher than 0.91 were not needed in this study (see below).

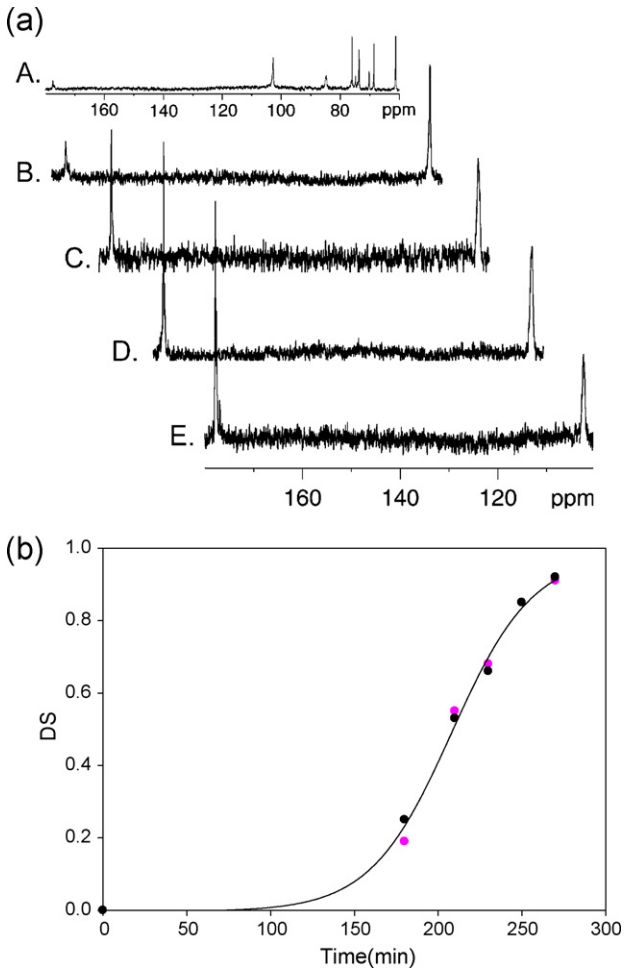

Fig. 3. (a) ^{13}C NMR spectra of SBG (A) and CM-SBG (100–180 ppm region) with DS = 0.19 (B), 0.55 (C), 0.68 (D) and 0.91 (E). (b) Time dependence of the degree of substitution (DS) for carboxymethylation of SBG at 58 °C following 3 h at room temperature. DS was obtained by ^{13}C NMR (red) and potentiometric titration (black). Experimental data were fitted to a simple saturation function to provide a basis for interpolation (DS larger than 0.2) and to provide a guide-to-the-eye. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.5. SEC–MALLS of CM-glucan (DS 0–0.91)

Fig. 4a shows SEC–MALLS data obtained at 80 °C (column temperature) for CM-reSBG with DS values ranging from 0 to 0.91. The introduction of carboxymethyl groups had a dramatic effect on the behaviour as the broad, multimodal RI peak of SBG gradually converged into unimodal peaks when DS exceeded 0.23. The sample recovery was typically 90–95%. Interestingly, the RI peaks for samples in the DS range 0.23–0.91 moved slightly to lower elution volumes with increasing DS, which we tentatively attribute to electrostatic chain expansion due to increased substitution and charge density. The molecular weight data (for elution slices) also converged into a single, straight line (log M *vs V*) for DS above 0.51, and with linear Debye plots throughout the peak (not shown). Following a fit of the log *M*–*V* line to a straight line (called 'Exponential fit 1' in the Astra software) the calculated DP_w values also converged to the same value (143 in this case), which is roughly 2 orders of magnitude lower than DP_w for underivatized SBG, and reasonably close to the range suggested by the Nelson–Somogyi method. Taken together these results indicate that CM-SBG disaggregates completely for DS > 0.23 when analysed by SEC–MALLS at a column temperature 80 °C.

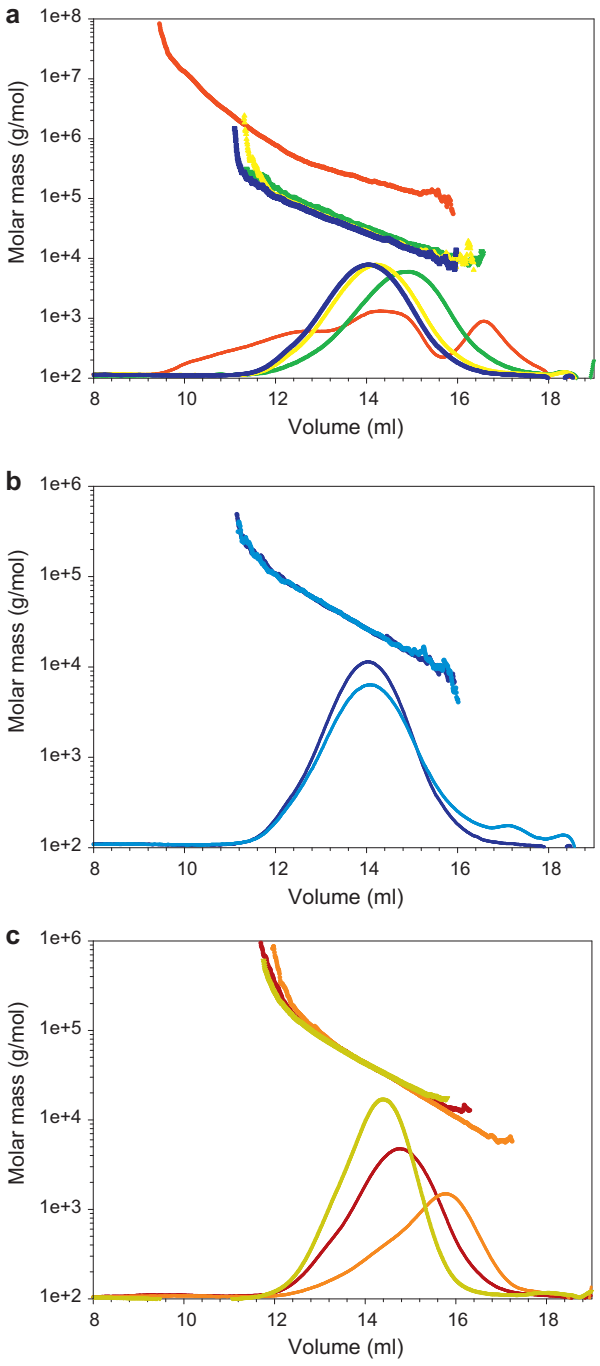

Fig. 4. (a) SEC–MALLS data for CM-re SBG (batch 221-7, reduced prior to carboxymethylation) (DS from 0 (red), 0.23 (green), 0.51 (yellow) and 0.91 (blue)) obtained at a column temperature of 80 °C. (b) SEC–MALLS data for CM-re SBG (batch 221-7) (dark blue) and CM-SBG (light blue), both with DS 0.91, obtained at a column temperature of 80 °C. (c) SEC–MALLS data (column at room temperature) for different batches of SBG (following reduction and carboxymethylation) SBG 321-5 HMW (dark yellow), SBG 221-7 (brown), and SBG 07-95 (orange). Top curves refer to the molar mass (left axis), whereas the bottom curves refer to the concentration profiles (RI detector response, right axis–arbitrary units). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Fig. 4b shows data for CM-SBG (DS = 0.91) obtained with and without reduction prior to carboxymethylation. In the latter case the chromatograms showed a small shoulder between 34 and 35 mL. The area of the peak increased with increasing DS (not shown), but the peak disappeared completely if the samples were

reduced prior to carboxymethylation. Thus, the shoulder most probably arises from alkaline peeling, and inclusion of the reduction step is therefore recommended if the method is used as routine measurements of chain length distributions.

It should be noted that A_2 of CM-SBG should be about one order of magnitude higher than for underivatised SBG due to the polyelectrolyte (Donnan) contribution. However, due to the low amounts injected the calculated molecular weights are essentially insensitive to A_2. For example, increasing A_2 from 0 to 5×10^{-3} changed the molecular weights only 2%.

In addition to batch 221-7 described above, two other batches were included to demonstrate that the approach works for a wider range of molecular weights. Data are summarised in Table 1. Batch 321-5 HMW is a sample where the low M tail had been removed using an ultrafiltration membrane with nominal cut-off 100 kDa (for proteins), and batch 07-95 is a somewhat more degraded β-glucan compared to the two other. Following reduction and carboxymethylation (DS = 0.51–0.91) SEC–MALLS could in this case be performed at RT. Results are shown in Fig. 4c, demonstrating excellent chromatographic behaviour, and illustrating clear differences in the chain length distribution. Removal of lower molecular weights by ultrafiltration is clearly seen. DP_n from SEC–MALLS were 93 (221-7), 157 (321-5 HMW) and 60 (07-95), for the three samples, respectively ($n = 3$ each). In comparison, DP_n values from the reducing end assay were 117 (221-7) and 46 (07-95), whereas DP_n for 321-5 HMW was above 200. The discrepancy at high DP is partly attributed to the inherent limitation of the reducing end assay for long chains, in addition to the limited solubility of the sample 321-5 HMW in the same assay. The difference at low DP (60 *vs* 46) could also be a systematic error in the reducing end assay, or the presence of small oligomers (in low amounts), which are not detected by SEC–MALLS due to elution in the salt peak. The issue may possibly be resolved by introducing a third method such as SEC–MALLS in solvents known to dissolve (1→3)-β-D-glucans, which is the subject of a forthcoming study in our laboratory.

Whereas reduction with borohydride prevents alkaline peeling during the carboxymethylation step, there exists a possibility for the introduction of internal (random) chain breaks (Kath & Kulicke, 1999; Wang et al., 2004). We therefore compared the DP_w and DP_n values obtained in the DS range 0.23–0.91. Following exponential fitting of the log M–V data, DP_w and DP_n were calculated. Results are given in Fig. 5. Within experimental error there is no dependence of DP on DS, and hence, reaction time during carboxymethylation. We therefore conclude the protocol does not lead to degradation of SBG, and may be adapted as a routine method to obtain the true chain length distribution of SBG.

SEC–MALLS of CM-SBG was also carried out at ambient column temperature. CM-SBG with DS of 0.51 or higher gave the same results as for high temperature. However, samples with lower DS remained aggregated, and could not be processed to obtain realistic chain length distributions.

3.6. Macromolecular properties

In addition to the molecular weight, multi-angle light scattering also provide the radius of gyration (R_G) based on the angular dependence of the scattering. When analysed in relation to the molecular weight R_G provides information about the shape and extension of the glucan chains. However, radii below approximately 20 nm ($\lambda_0/20$) are generally not accurately detected. This applies to all CM-glucans explored here. Instead, we took advantage of the intrinsic viscosity data obtained from the on-line viscosity detector, since the molecular weight dependence of the intrinsic viscosity (Mark–Houwink–Sakurada (MHS) plot) is readily obtained across the entire molecular weight distribution, and also discriminates better between different macromolecular geometries. The MHS plot for a CM-SBG (DS = 0.51) is shown in Fig. 6. Data for DS 0.91 were indistinguishable from DS 0.51, and are therefore not shown. The results are compared to corresponding data of a series of reference polysaccharides with different, but well described, chain geometries (dextran, pullulan, barley mixed linkage (1→3)(1→4)-β-D-glucan, CM-cellulose (DS 0.9), CM-curdlan (DS 0.4)). These were all analysed in the same experimental setup (same detectors, pH, ionic strength, temperature and flow rate).

Data for CM-SBG fall on an essentially perfectly straight line with a slope of 0.84 (±0.01), in agreement with a randomly coiled structure. The data lie between the much more flexible dextran and the more extended CM-cellulose. Data for a un-carboxymethylated mixed-linkage barley glucan have a slightly lower slope, but are shifted upwards, whereas data for pullulan (also un-carboxymethylated) are shifted downwards compared to CM-SBG. Interestingly, there is no overlap whatsoever with curve for triple-stranded scleroglucan, which also has a much higher slope. The latter data, which were obtained by the same method in the same solvent, was reproduced from a previous publication (Sletmoen et al., 2005). Hence, we can safely conclude CM-SBG is by no means triple-stranded under these conditions.

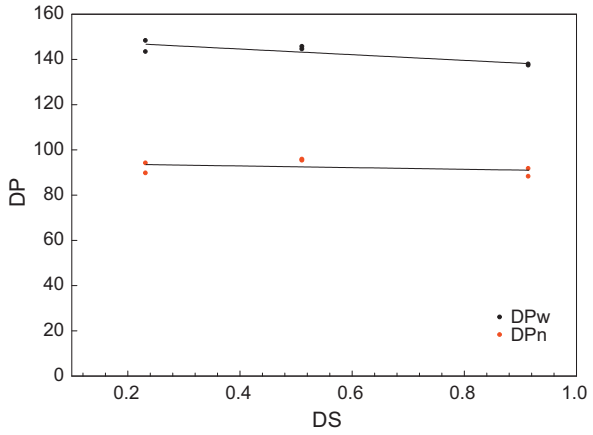

Fig. 5. Dependence of DS on DP_w (black) and DP_n (red) for CM-reSBG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

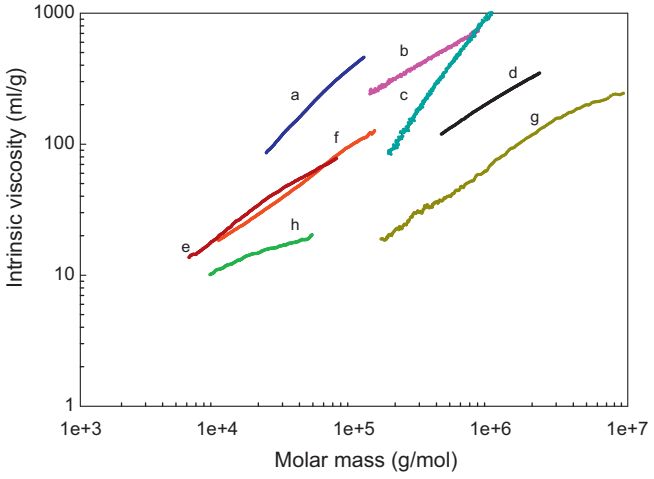

Fig. 6. Molecular weight dependence of the intrinsic viscosity (MHS plots) of CM-cellulose (DS = 0.9) (a), mixed linkage (β-1,4/1,3) glucan from barley (b), triple stranded scleroglucan (c) (reproduced from (Sletmoen et al., 2005)) pullulan (d), CM-curdlan (DS = 0.4) (e), CM-SBG (DS = 0.51) (f), dextran (h), and SBG (g).

A comparison between the branched CM-SBG and the unbranched CM-curdlan could in principle provide some information about the branching pattern of the former. The CM-SBG plot is close to, but does not overlap that of CM-curdlan, which is also slightly curved. The reason for the curvature is unclear, as CM-curdlan is supposedly unbranched. As a tentative explanation we suggest the carboxymethylation pattern may be somewhat heterogeneous. Significantly higher DS in some part of the population may increase the intrinsic viscosity due to more electrostatic expansion. Nevertheless, the overall similarity suggests that CM-SBG, and hence, SBG, have only a low number of short branches which do not influence the hydrodynamic volume to any large extent.

4. Conclusions

SBG tends to aggregate in aqueous solutions, especially at low temperature. Centrifugation of dilute SBG solutions at 40 °C, heating up to 80 °C, or ultracentrifugation can remove some the larger aggregates. However, these treatments are not sufficient to remove all the aggregates. In contrast, completely dispersed, single-stranded SBG was obtained in SEC–MALLS following partial carboxymethylation (DS 0.51–0.91). Lower DS resulted in partial aggregation at low temperatures, but even DS 0.23 may disperse completely provided SEC–MALLS is carried out with a column temperature of 80 °C. Reducing end protection was needed to avoid detectable alkaline peeling during carboxymethylation. The molecular weight dependence of the intrinsic viscosity demonstrated clearly that CM-SBG behaved as randomly coiled chains, with a chain extension close to CM-curdlan, but well above that of dextran and pullulan, and well below that of barley glucans with about 70% β-1,4 and 30% β-1,3 linkages. A triple-stranded structure of CM-SBG was completely ruled out by comparison to scleroglucan. We conclude the modified protocol for preparing un-degraded CM-SBG (DS = 0.23–0.91) combined with high temperature SEC–MALLS is well suited as a standard analysis of the molecular weight distribution of SBG-like molecules.

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