Available online at www.sciencedirect.com

SCIENCE DIRECT®

Carbohydrate Research 340 (2005) 971–979

Carbohydrate
RESEARCH

Probing macromolecular architectures of nanosized cyclic structures of (1→3)-β-D-glucans by AFM and SEC-MALLS

Marit Sletmoen,[a] Bjørn E. Christensen[b] and Bjørn T. Stokke[a,*]

[a]*Biophysics and Medical Technology, Department of Physics, The Norwegian University of Science and Technology, NTNU, NO-7491 Trondheim, Norway*

[b]*Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology, The Norwegian University of Science and Technology, NTNU, NO-7491 Trondheim, Norway*

Received 16 September 2004; accepted 16 January 2005

In honour of Professor David A. Brant

Abstract—Comb-like branched (1→3)-β-D-glucans dissolve in water as stiff triple-helical structures. Dissociation followed by reassociation leads to the formation of a blend of various macromolecular topologies, where the cyclic species make up a significant fraction. In this study, the molecular properties of these nanosized cyclic structures of (1→3)-β-D-glucans were probed using a combination of AFM and SEC-MALLS. The cyclic structures were obtained by subjecting linear triple-helical molecules of (1→3)-β-D-glucans to a denaturation–renaturation cycle, and the fraction of cyclic structures in the renatured sample was determined by AFM. Samples containing different known fractions of linear and circular molecules were studied by SEC with online multi-angle laser-light scattering and viscometric detectors. The molecular weight and the radius of gyration of the molecules eluting from the SEC column, as well as the concentration and the intrinsic viscosity, were determined simultaneously. By extrapolating the results to a situation of only circular species, the results allowed to determine the linear mass per unit length (M_L) of not only the linear but also the circular morphologies of the (1→3)-β-D-glucans. The values obtained were $M_L = 2140 \pm 180$ g mol^{-1} nm^{-1} for the circular species and 2045 ± 80 g mol^{-1} nm^{-1} for the linear species. This is the first direct determination of the M_L parameter of the circular topology, and the results indicate that the reassociation of the individual chains yield a triplex structure also for the circular morphology, similar to the initial triple helix.

Keywords: Scleroglucan; Cyclic topology; AFM; SEC-MALLS

1. Introduction

Scleroglucan is an extracellular, high molecular weight neutral, water-soluble polysaccharide produced by the fungus *Sclerotium rolfsii*. It has a main chain composed of (1→3)-β-linked D-glucopyranosyl residues, and single (1→6)-linked D-glucopyranosyl residues attached as side chains to every third sugar residue of the main chain.[1] (1→3)-β-D-Glucans are only produced by a few prokaryotic microorganisms, but are widely distributed in plants, especially algae, and in fungi, where they have storage, structural or protective roles.[2] Commercially available (1→3)-β-D-glucans are often named after their biological sources. The compounds lentinan, laminaran, schizophyllan, and scleroglucan are all examples of (1→6) branched (1→3)-β-D-glucans, extracted from different sources. Solution studies of schizophyllan using light scattering and viscometry[3] identified a rod-like limiting behavior at molar mass less than 3×10^5 g/mol. These data allowed the persistence length of the structures to be calculated, and the results indicated a persistence length of 150 nm and a mass per unit length (M_L) equal to 2170 g mol^{-1} nm^{-1}. The expected value of M_L for single, double, and triple helixes of scleroglucan,

---

* Corresponding author. Fax: +47 73 59 77 10; e-mail: bjorn.stokke@phys.ntnu.no

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.01.026

calculated based on X-ray diffraction studies[4] are predicted to be 715, 1430, and 2150 g mol^{-1} nm^{-1}. In this calculation it is assumed that the pitch equal that of the triple helix irrespective of the number of chains included. The value of M_L for schizophyllan determined by Kashiwagi et al.[3] is therefore consistent with other studies concluding that in aqueous solution (1→6) branched (1→3)-β-D-glucans adopts a stiff, triple stranded structure. The side chains are exposed toward the exterior.[5]

Exposing aqueous, linear, triple-helical (1→3)-β-D-glucan molecules to denaturation conditions, destabilizes the intramolecular hydrogen bonds of the triplexes. A conformational transition will then take place where the triple-helical molecules dissociate into single stranded random coils.[6,7] Denaturation of triplexes occurs in alkaline solutions (>0.25 M NaOH)[5,8,9] or in dimethyl sulfoxide (Me_2SO) (water weight fraction $W_H < 0.13$),[10–13] or by increasing the temperature above the triple-helix melting temperature, $T_m = 135$ °C.[7,10] The single stranded random coils are much more flexible than the triple helixes, giving, for high molecular weight samples, lower viscosity compared to solutions containing triple helixes. When denaturated samples are restored to thermodynamic conditions favoring the triple-helical structure, circular structures can be observed in the 'renatured samples' by ultramicroscopic techniques.[14,15] The existence of the circular structures was unexpected given the stiff nature of the linear triple helix. Linear-, hairpin-, and aggregated structures are also observed in the mixture of renatured molecules. The proportion of circular and other morphologies in denaturated–renaturated (1→3)-β-D-glucan samples is a function of molecular weight, polymer concentration, as well as the duration of annealing of the denaturated samples.[14,16]

Some attempts have been made in order to conclude about the molecular architecture of the circular scleroglucan structures. The denatured (1→3)-β-D-glucans must be returned to the thermodynamic conditions favoring triple helix stability in order to generate the cyclic species. This fact has led to the assumption that the circles are stabilized by the same interchain interactions that stabilize the authentic linear triple helices.[15,17] This interpretation is strengthened by electron micrographs showing circular species with a partial separation of the three single strands of the helix.[14] The twisting of circular triple helices and the local partial unwinding of the triple helix was by the authors interpreted as modes of compensation for torsional stress arising from a linking number different from that of the relaxed circular topology. These observations of twisted or supercoiled forms arising from torsional stress in the backbone are similar to previous observations on supercoiling within DNA. Based on the M_w in aqueous solution and the weight average contour length (L_w) measured in these electron micrographs, a linear mass density (M_L) equal to 2080 ± 200 g mol^{-1} nm^{-1} was estimated for the linear molecules.[14,18] This value of M_L is consistent with the proposed triple-helical structure.

Calculations based on qualitative equilibrium arguments[17] have afforded a possible explanation for the unexpected existence of the circular structures. The calculations showed that the apparent stability of the circular morphology could be explained in terms of the higher density of circular states at temperatures below the triple helix to random-coil transition temperature. The energetic penalty, if any, associated with bending the triple helix into a circle could be compensated by configurational degeneracy within the observed circles. This degeneracy arises from the absence of any requirement for the cyclic structures that the single strands be kept in register to achieve maximal energetic stabilization. An important issue addressed by such calculations is the possible formation of cyclic structures containing one or two unimeric chains. These are potentially capable of forming domains of triple-helical structure by coiling on themselves. Such unimeric and dimeric circles were considered of interest since they afford a possible explanation to the observations of cyclic structures having a relatively small contour length that might be expected for triple-helical structures composed of fewer than three chains. Based on these calculations and considerations, it was concluded that the observed population of cyclic structures in renatured samples of (1→3)-β-D-glucans might contain not only cyclic trimers. A substantial proportion of the molecules might exist as cyclic unimers and dimers, some or all of which are not fully zipped into a triple-helical structure.

Another observation in favor of the triple-helix structure of the circular species comes from the observed thickness of renatured linear and cyclic scleroglucan using noncontact AFM. The mean thickness measured normal to the mica surface was found to be almost identical for the two morphologies.[14] Although this thickness was only 45% of the anticipated cross-sectional diameter of the scleroglucan triple helix,[5] the results were taken to indicate that the re-associated species also have a triple-helical like structure. The discrepancy in the expected chain thickness is typical of many systems investigated with AFM, and is also observed in later studies of different (1→3)-β-D-glucans.[16] Possible reasons for the discrepancy in measured thickness include partial immersion of the polymer helix in a layer of water adhering to the mica surface and distortions and flattening of the helix by the interaction with the substrate.

Early studies on (1→3)-β-D-glucans revealed their high resistance to hydrolysis, temperature, and electrolytes. These properties are probably connected to their native, linear, noncharged, triple-helical structure, and make the molecules interesting for applications in high-temperature oil reservoirs.[19,20] The structure–function relation(s) and application of (1→3)-β-D-glucans as biological response modifiers based on their immunological properties,[21] namely their ability to induce leukocyte activation and the production of inflammatory mediators, such as tumor necrosis factor (TNF)-α. Identifying the receptors involved in generating the responses is important in order to understand how the polysaccharides exert their immunomodulatory effect. Macrophage mannose receptors (MR) and complement receptor 3 (CR3) have historically been considered the major macrophage lectins involved in the nonspecific recognition of fungi and yeast-derived particles, or β-glucans extracted from these.[21] But recently a specific β-glucan receptor was identified as the predominant receptor involved in the recognition of (1→3)-β-D-glucans.[22,23] The functional activity of these polysaccharides is known to depend on conformation, molecular weight and degree of branching, and their observed functional activity therefore also stimulates further investigation of molecular structure. The reported interaction of schizophyllan with polyribonucleotides is another motivation for further studies of the structure and function of these polysaccharides.[24,25]

In this work we used SEC-MALLS with online multiangle laser-light scattering and viscometric detectors to study the molecular architecture of the nanosized cyclic structures of (1→3)-β-D-glucans. In combination with observations obtained with AFM, the results afford new evidence for a triple-helical structure of not only the reassociated linear scleroglucan, but also of the cyclic topology, based on the calculated mass per unit length (M_L) of the molecules.

2. Materials and methods

2.1. Sample preparation

2.1.1. Linear scleroglucan. The scleroglucan used was Actigum CS11 provided by Sanofi Bioindustries, France. The scleroglucan powder was dissolved in MQ water to a concentration of 10 g/L, left under stirring at 55 °C for ~4 h then at room temperature for 3 days. The resulting solution was ultrasonically depolymerized (Braun Labsonic 1510, power 50 W, total 4 mL) for a duration of 15 min in order to obtain a chain length within the interval yielding a large fraction of cyclic topology upon denaturation–renaturation. This sample is in the following referred to as linear scleroglucan.

2.1.2. Renatured scleroglucan. A sample containing circular molecules of scleroglucan was prepared using experimental conditions that earlier has shown to give a low fraction of aggregates and a high fraction of circles in the renatured sample.[26] The solution of linear scleroglucan was diluted to a concentration equal to 1 g/L, transferred to a dialysis tubing (Medicell International, M_w cutoff (for proteins) 12,000–14,000 g/mol) and dialyzed against 0.5 M NaOH for 2 h at room temperature. The alkaline treated sample was renatured by exposing it to a gradual decrease in pH obtained by transferring the dialysis tubing to a bath containing 0.1 M NaOH, pH to 11.9 heated to 55 °C. The pH of the solution was adjusted by adding HCl. The tubing was first dialysed against this solution for 3 h, then against MQ water (pH = 6.3) at room temperature overnight. After re-establishing the neutral conditions the sample was subjected to a thermal treatment, which has shown to increase the stability of the circular species formed.[26,27] More precisely, the renatured sample was heated to 95 °C for 2 h, and cooled by being left standing in air.

2.2. Atomic force microscopy

2.2.1. Specimen preparation for atomic force microscopy. Aliquots of the samples were mixed with 60% aqueous glycerol to a final polysaccharide concentration of 2–4 μg/mL and a final weight fraction of glycerol equal to 50%. A small volume of these solutions were sprayed on freshly cleaved mica discs and vacuum dried at 10^{-6} Torr for at least 2 h.

2.2.2. Determination of AFM topographs. AFM topographs of the scleroglucan samples were obtained using a Digital Instruments Multimode IIIa atomic force microscope equipped with an E-scanner. Tapping mode silicon cantilevers TESP (Digital Instruments, Santa Barbara, CA) with nominal spring constant of 20–100 N/m and nominal resonance frequencies of 200–400 kHz were employed. The instrument was operated in tapping mode with topograph scan size in the range 1 μm × 1 μm to 5 μm × 5 μm.

2.2.3. Image analysis of AFM topographs. The fraction of linear and circular species visualized on the AFM topographs was determined employing a user-interactive software developed in the IDL language (Research System, Inc., Boulder, CO). A more detailed description of the analysis is given elsewhere.[28] In brief, the height topographs were thresholded and bileveled to identify regions of interest (ROI), each containing one scleroglucan molecule. An asphericity index, A, is calculated for each of the structures and used as a shape factor for subsequent collection of species into ensembles of linear or circular species. The theoretical values of the asphericity index are $A = 0$, 0.25, and 1.0 for a sphere, an infinitely thin circle, and a rigid rod, respectively.[29] Apparent branched species, aggregates, and structures touching the edges of the images were excluded from the analysis. Pretzel-formed structures were counted as circular and hairpin-formed structures were counted as either linear or circular depending on their shape (reflected in the asphericity index).

3. SEC-MALLS

The molecular weight and the radius of gyration of the scleroglucan samples were determined by size-exclusion chromatography (SEC) coupled with a refractive index (RI)-, a viscometric- and a multi-angle laser light-scattering detector (MALLS). The static laser light-scattering detectors measure excess Rayleigh scattering $R_{\theta i}$ at angle θ for each retention volume V_i of the SEC chromatogram. This scattering intensity is related to the concentration at each retention volume, C_i, which is measured by a separate concentration-sensitive (on-line) refractive index detector:

$$\frac{KC_i}{R_{\theta i}} = \frac{1}{M_{wi}P(\theta)_i} + 2A_{2i}C_i + \cdots \quad (1)$$

The particle scattering function $P(\theta)_i$ defines the angular variation of scattering intensity. $M_{w,i}$ is the molecular weight at retention volume V_i and is a weight-average since each slice due to column dispersion contains molecules of more than one molecular weight. However, in subsequent treatment of data each slice is considered effectively monodisperse. A_{2i} is the second virial coefficient and K is an optical constant. The second and higher concentration terms of Eq. 1 are negligible at the low polymer concentrations employed in SEC. In this case,

$$M_{wi} \approx \frac{R_{\theta i}}{KC_iP(\theta)_i} \quad (2)$$

The scattering data are presented in Zimm plots, which allow extrapolation to zero angle and determination of M_{wi} and R_{gi}. This information can be used to evaluate the mass per unit length (M_L) for structures adopting a well-defined geometry. The relevant expressions for rigid structures having circular or linear shape is given in Eqs. 3 and 4, respectively:

$$M_{L,c} = \frac{M_w}{R_{g,c} \times 2\pi} \quad (3)$$

$$M_{L,l} = \frac{M_w}{R_{g,l} \times \sqrt{12}} \quad (4)$$

$M_{L,c}$ and $M_{L,l}$ is the mass per unit length for the circular and linear molecules, respectively, and $R_{g,c}$ and $R_{g,l}$ their radius of gyration. In this study, a viscometric detector was also mounted on line and enabled determination of intrinsic viscosity $[\eta]$ as a function of the retention volume V to be collected.

The scleroglucan (approx. 1 mg/mL) was diluted 1:2 to a solution containing 0.05 M Na_2SO_4/0.01 M EDTA adjusted to pH 6, which also was the eluent in the SEC analysis, and subjected to filtration (pore-size = 800 nm, Millex-AA) prior to injection. The injected volume was 250 μL. Light scattering was monitored employing a DAWN DSP multi-angle laser light-scattering detector. The RI-detector used was a DAWN Optilab DSP, and the viscosity was measured using a Viscotek Model TDA 301. The intrinsic viscosity $[\eta]$ was estimated as the value of η_{sp}/c at the actual concentration in the elution profile. This assumption introduces only a marginal difference (<0.6%) compared to $[\eta]$ over the whole range of elution volumes due to the very low concentration compared to the overlap concentration. The refractive index increment (dn/dc) was taken to be of 0.15 mL/g.[30] The system was eluted at a flow rate of 0.4 mL/min when the viscosity detector was attached, otherwise 0.5 mL/min was used. Three serially connected HPLC-SEC columns TSK 6000, 5000, and 4000 PWXL were used. This characterization was carried out at room temperature. The inter-laboratory comparison employing similar procedures for molecular weight determination[31] indicate that the results obtained by our experimental set-up yields results in good agreement with other laboratories.

4. Results and discussion

4.1. Quantitative analysis of scleroglucan samples by AFM

Figure 1A shows an AFM topograph of scleroglucan dissolved in water to a concentration of 10 g/L, depolymerized by ultrasound for 8 min, further diluted to 2 μg/mL and sprayed onto mica. The AFM topograph reveals the stiff, linear structure of the scleroglucan molecules. From the SEC-MALLS experiments, the weight average molecular mass, M_w, of this sample was found to be equal to 7.9×10^5 g/mol. Figure 1B shows an AFM topograph of the same sample after exposing it to a denaturation–renaturation cycle. As a result of this

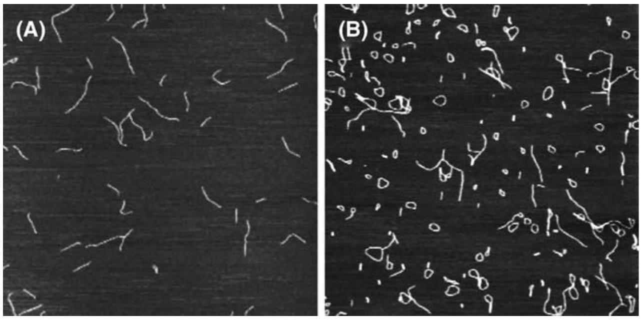

Figure 1. Tapping mode AFM height topographs of (A) native scleroglucan and (B) scleroglucan exposed to a denaturation–renaturation cycle. The scan sizes of the topographs are 1.4 × 1.4 μm.

treatment the molecular weight decreased to 4.5×10^5 g/mol, and the stiff linear morphology of the starting sample (Fig. 1A) is converted to a broad spectrum of structures, such as linear, circular, hairpin, and multichain (aggregated) structures (Fig. 1B). Even though a mixture of morphologies is observed, it is apparent that the dominating morphology formed as a result of this particular treatment is the circular one. This was confirmed by image analysis. The histograms in Figure 2 present the distributions of asphericity indexes obtained when analyzing AFM images of the starting sample (Fig. 2A) and on the renatured sample (Fig. 2B). The histogram characterizing the starting sample reveals a high probability for an asphericity index close to 1.0, consistent with the shape factor for stiff rods. A similar analysis of the structures observed after the exposure to alkaline conditions gave rise to a very different histogram (Fig. 2B). The peak in the distribution has changed to a value of A equal to 0.25–0.45, and a broad shoulder is extending up to $A = 1$. The region $A = 0.25$–0.3 is from theoretical analysis expected to contain only circular species. The extension of this peak up to $A = 0.45$ might be explained by imperfections in some of the circles in the sense that they are elongated, or it might be due to the existence of pretzel- or racket-formed structures. After manually counting circular and linear structures it was concluded that among the 594 species counted on the samples of the renatured sample, 70% fell into the category of circular structures and 30% were defined as linear structures.

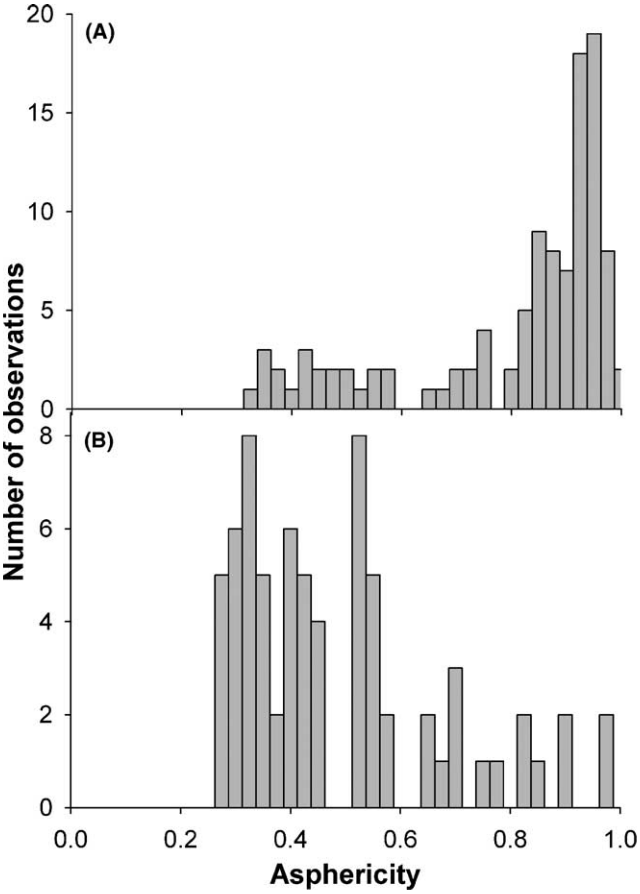

Figure 2. Distribution of asphericity indices based on a number of AFM topographs for the native (A) and the renatured–denatured (B) scleroglucan samples.

This fraction of circular structures is in agreement with earlier ultramicroscopic observations on samples subjected to the same denaturation–renaturation treatment. In a study of macrocyclization of (1→3)-β-D-glucans performed in our laboratory, the molecules contained in a similarly treated, renatured sample was separated using a GPC system. The elution volume from the column was divided in three fractions, and the molecules contained in each of them were visualized by electron microscopy.[32] The EM images revealed that high molecular weight clusters eluted closest to the void volume, whereas the intermediate elution volume was comrades of a blend of the linear topology and higher molecular weight clusters, with circular species occurring as a minor fraction. The fraction eluting closest to the total volume contained almost exclusively molecules with linear and circular topologies. In the continuation of these studies, the ratio between the fraction of circular, $[C]$, and linear, $[L]$, topologies was found to be $[C]/[L] = 1.75 \pm 0.15$, $n = 464$ molecules. The SD was calculated from the average within each of the six individual electron micrographs, comprised of at least 30 circular and linear molecules each. This corresponds to a percentage of linear molecules equal to 36 ± 2%, which is in the same range as the values determined by AFM. The slightly lower fraction of circular molecules present on the EM images might be due to slight differences in the preparation conditions, since these have been found to have large influences on the composition of the final sample. This AFM-based information about the distribution of morphologies in the sample was used in the analysis of the SEC-MALLS data.

4.2. Characterization of the macromolecular cyclization of triple helical (1→6) branched (1→3)-β-D-glucans by SEC-MALLS

The SEC-MALLS elution profiles shown in Figure 3A show a shift toward higher elution volumes for the renatured sample compared to the linear one. This finding is in agreement with earlier observations.[26] As shown in Figure 3, analysis of the light-scattering data on-line in the elution profile reveals that several parameters characterizing the sample have changed significantly as a result of the exposure to alkaline conditions followed by renaturation. The data for the renatured sample showed a reduced R_g compared to the R_g of the subsequently eluting linear species (Fig. 3B and C). These observations reflect a change in the macromolecular architecture of the

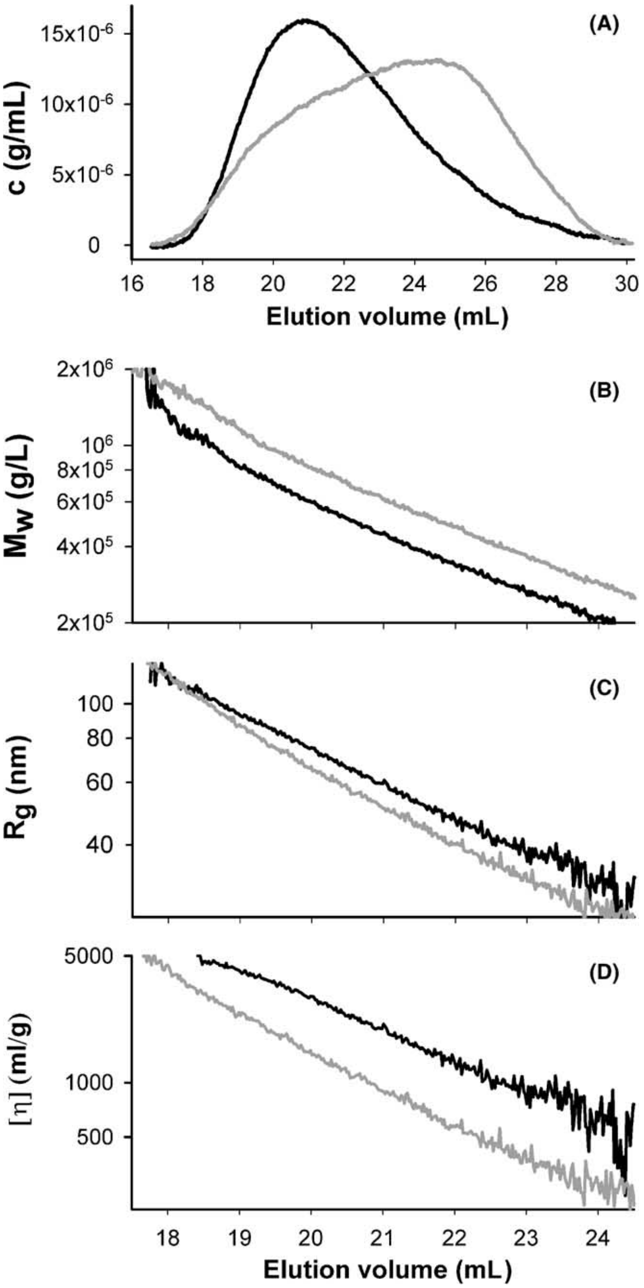

Figure 3. Elution profiles (A) as well as M_w (B), R_g (C), and [η] (D) as a function of elution volume for native, linear scleroglucan (black) and for the scleroglucan sample exposed to a denaturation–renaturation cycle (grey).

eluting structures, which also explain the decrease in intrinsic viscosity for the renatured sample compared to the native one (Fig. 3D). As illustrated in Figure 4, the R_g of the linear sample conforms to the expected behavior of stiff rod-like species at the low-M_w tail of the distribution. This was followed by a relative reduction due to the manifestation of chain flexibility at increasing M_w (Fig. 4).

A possible approach to obtain information about the strandedness of helical molecules is to estimate the mass per unit length based on experimentally accessible parameters. The experimentally determined R_g and M_w in the rod-like limit can be used in such a context. This approach is based on the fact that the radius of gyration of rigid rods of length L is given by the expression $R_g = L \times 12^{-0.5}$. If dividing this R_g by M_w, the inverse of M_L is obtained. This ratio, that is, R_g/M_w is presented in Figure 4 as a function of M_w. In the figure, our results are compared to earlier results obtained by Kashiwagi et al.[3] using light scattering and viscometry. Using this approach, the rod-like limiting behavior is evident as a plateau at low M_w, and the persistence length can be calculated directly from the value of M_w at half the plateau value.[3] As shown in Figure 4 our observations of the behavior of the linear scleroglucan are consistent with the results of Kashiwagi et al.,[3] which were taken to indicate a persistence length of 150 nm and a M_L equal to 2170 g mol^{-1} nm^{-1}. This value of M_L is close to the predicted value for triple helical linear scleroglucan.

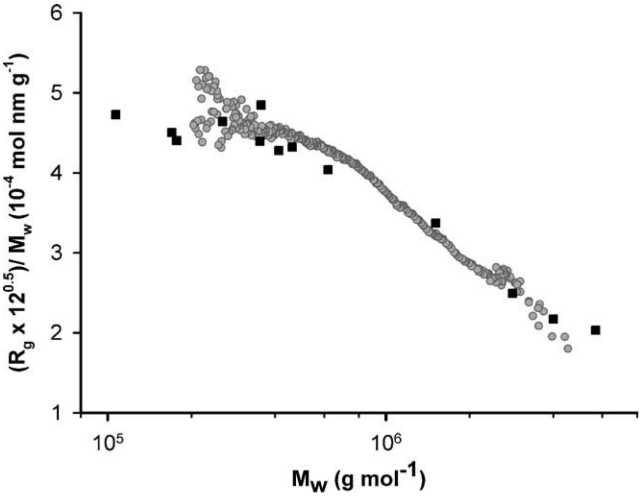

Figure 4. Ratio between the inverse of the mass per unit length (calculated as the experimentally determined radius of gyration multiplied by $12^{1/2}$ and divided by the molecular weight) versus molecular weight as determined for samples of native linear (1→6) branched (1→3)-β-D-glucans. Our data (circles) is compared to previously published data (squares).[3]

Figure 5A shows the intrinsic viscosity, [η], and the radius of gyration (R_g), for two samples containing linear molecules and two renatured samples of scleroglucan, as a function of molecular weight (M_w). The samples were produced independently, but using the same experimental conditions during preparation. The overlap between the curves corresponding to linear or renatured samples, respectively, indicates that the sample preparation procedure used gives reproducible results. The samples containing linear molecules show a hydrodynamic behavior consistent with earlier studies on (1→6)-branched (1→3)-β-D-glucans.[3] When comparing the measured characteristics of these solutions of molecules having the same M but different molecular architecture, it can be concluded that the renatured samples have a lower

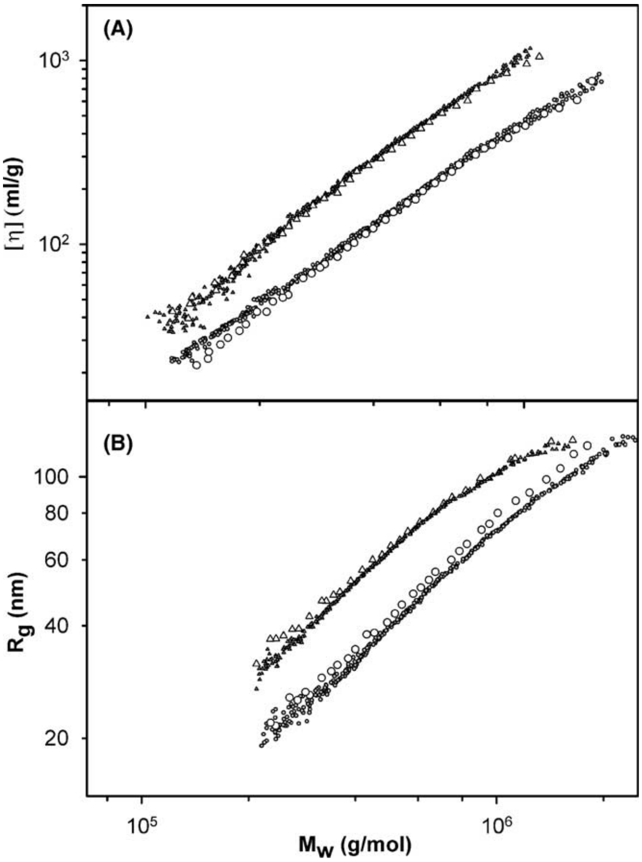

Figure 5. Double logarithmic plot of experimentally determined intrinsic viscosity, [η] (A) and radius of gyration, R_g (B) versus weight-averaged molecular weight, M_w, for two independent samples of native (triangles, every 10th datapoints for one series (large symbols)) and two independent samples of renatured (circles, every 10th datapoints for one series (large symbols)) scleroglucan in aqueous solution at room temperature.

intrinsic viscosity. This behavior is seen over the whole range of M_w studied (Fig. 5A). In Figure 5B the measured R_g as a function of M_w is shown for the linear and renatured sample. The observations of the linear molecules follow the expected behavior, whereas the renatured sample seems to contain more dense structures. The nearly identical slopes in the log–log plot of the [η] and R_g versus M_w (Fig. 5) are primarily a direct consequence of Eqs. 3 and 4, since R_g in both cases should be proportional to M_w. The observation further indicates that the fraction of circular species is nearly constant within this range, and possible contributions from deviating geometries such as pretzels or as apparent long chain branched species are marginal.

It has to our knowledge not yet been possible to produce a sample consisting exclusively of circular molecules of (1→6)-branched (1→3)-β-D-glucans. One reason is the inherent polydispersity of the native, linear scleroglucan sample combined with the fact that a certain molecular weight is required in order for the circular species to form. A molecular weight in the interval 5–7×10^5 g/mol is assumed to be the best for ring formation when using the procedures used in the present study.[26] Earlier attempts to produce a sample containing a higher fraction of circular structures that can be obtained by the denaturation–renaturation cycle followed by thermal annealing, has involved successive chromatographic fractionation of the renatured, annealed sample. Using this approach, the renatured sample was further enriched in circular species, but a situation of 100% circular structures was not attained.[32] In order to estimate M_w, η, and R_g for a sample consisting of only circular molecules, a dilution series was therefore prepared by mixing the native and the renatured samples. The polysaccharide concentration in the starting samples was 1 g/L. Figure 6 is the chromatograms obtained for different mixing ratios of the two samples. The number assigned to each of the chromatograms indicates the amount (in % of the total sample volume) of native, linear scleroglucan added to the mixture.

The fraction of linear molecules contained in a certain elution volume was as a first approximation taken to be equal to the amount added of the linear structures +30% of the amount added of renatured structures, since the AFM image analysis had shown that this sample contained 30% linear structures. In addition, due to the observed difference in elution profile of linear and circular molecules (Fig. 3A), the amount of linear molecules contained in a certain elution volume was corrected by a factor. This factor was obtained by calculating, using the SEC chromatograms, the area corresponding to a certain fraction of the elution volume. This area was calculated and compared for three different fractions of the elution volume: 21–22, 22–23, and 23–24 mL, for the chromatograms obtained when studying the linear and the renatured sample separately. Elution volumes smaller than 21 mL were not analysed, since multichain

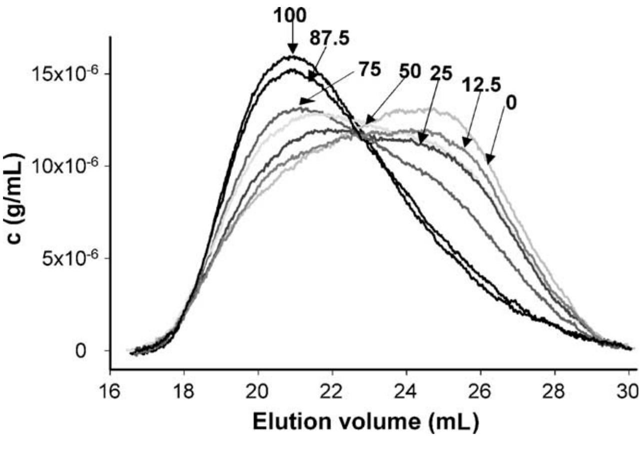

Figure 6. Elution profiles as a function of elution volume for native, linear scleroglucan and for the scleroglucan sample exposed to a denaturation–renaturation cycle as well as for mixtures of these two samples characterized by the content (%) of the native sample.

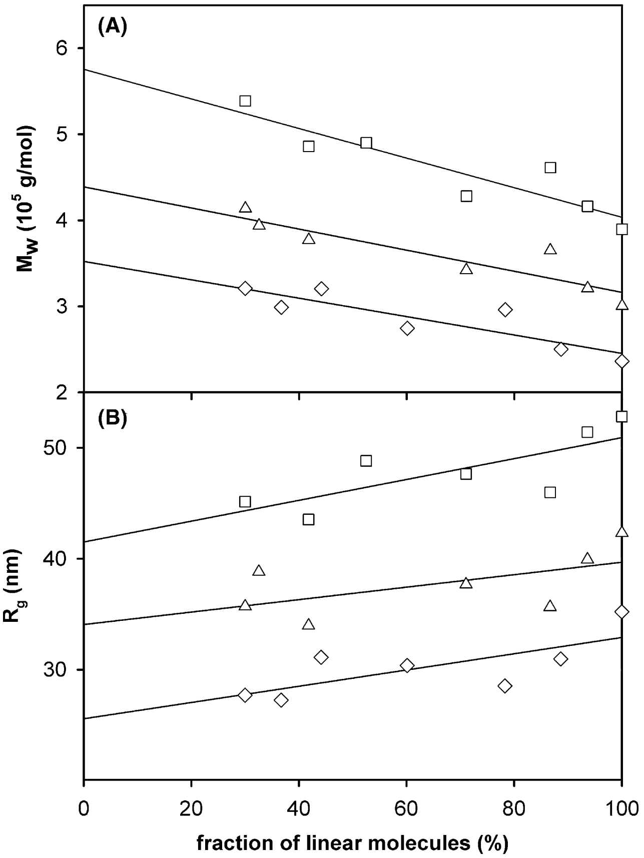

Figure 7. M_w (A) and R_g (B) plotted as a function of the fraction of linear molecules prepared by mixing the samples containing circular and linear scleroglucan molecules, extrapolated to 0% linear molecules. The data shown is based on the measured properties of the molecules contained in the volume eluting from the SEC column in the interval 21–22 mL (□), 22–23 mL (△), and 23–24 mL (◇). The lines are the extrapolations to the situation characterized by a fraction of circular molecules equal to 100%.

clusters, due to their relatively large size (see Fig. 1) are expected to elute at such small elution volumes. In Figure 7 the result of this approach is presented. This information about how M_w (Fig. 7A) and R_g (Fig. 7B) evolves with the fraction of circular species present in the sample allowed extrapolating the data to a situation with 100% circular species (Fig. 7A and B, continuous line).

The values obtained for M_w, R_g, and M_L when extrapolating the data to a situation of only circular or only linear species are presented in Table 1. As expected, the radius of gyration as well as the molecular weight decreases with increasing elution volume. The calculated mass per unit length, $M_L = 2190 \pm 150$, 2050 ± 170, and 2190 ± 210 g mol^{-1} nm^{-1} for three different fractions of circular molecules eluting from the column, obtained using Eqs. 3 and 4 and the measured M_w and R_g, indicate a triple-helical structure both for the linear and the circular species. The average mass per unit length of the circular species obtained from the analysis at the three different intervals of elution volume, $M_L = 2140 \pm 180$ g mol^{-1} nm^{-1} is not significantly different from the linear species with $M_L = 2045 \pm 80$ g mol^{-1} nm^{-1}. An image analysis of the AFM topographs giving the distribution of sizes of the circular structures, has not been carried out due to the lack of a analysis program capable of handling molecules of the observed small size. In addition to the circumference of the circular species that potentially can be estimated based on such image analysis approaches, the molar mass of the corresponding state is required to yield structural information in terms of $M_{L,c}$. Despite the limitation imposed by the image analysis, it can be concluded that the radius of gyration as obtained from the molecular weight determined by SEC-MALLS and assuming a triple-helical structure, is within the interval of sizes for the circular structures observed by AFM (Fig. 1). These results obtained by AFM and SEC-MALLS afford new structural details concerning a class of macrocyclic carbohydrates, of which the biological importance is yet to be known.

5. Conclusions

SEC-MALLS with online multi-angle laser light-scattering and viscometric detectors allowed determination of molecular weight (M_w) and radius of gyration (R_g) of molecules eluting from the SEC column. A series of samples containing different known fractions of linear and circular molecules of (1→3)-β-D-glucans, made an extrapolation to a situation of only circular species possible. Using this approach, the mass per unit length (M_L) of the linear and the circular morphologies of the (1→3)-β-D-glucans was determined. The values obtained, $M_L = 2140 \pm 180$ g mol^{-1} nm^{-1} for the reassociated nanosized cyclic structures and 2045 ± 80 g mol^{-1} nm^{-1} for the linear structures, indicate a triple-helical architecture for both the morphologies.

Table 1. R_g and M_L of circular and linear scleroglucan

Fraction (mL)	Circular structures[a]			Linear structures		
	M_w (10^3 g/mol)	R_g (nm)	M_L (g mol^{-1} nm^{-1})	M_w (10^3 g/mol)	R_g (nm)	M_L (g mol^{-1} nm^{-1})
23–24	350 ± 20	26 ± 2	2190 ± 150	240	35	1950
22–23	440 ± 20	34 ± 2	2050 ± 170	300	42	2060
21–22	575 ± 25	42 ± 1	2190 ± 210	390	53	2120

[a] Obtained from data extrapolated to only circular species (Fig. 7). The uncertainties represent the SD of the extrapolated values.

Acknowledgements

This work was supported by the Norwegian Research Council Grant No. 145523/432.

References

1. Johnson, J.; Kirkwood, S.; Misaki, A.; Nelson, T. E.; Scaletti, J. V.; Smith, F. *Chem. Ind.* **1963**, 820–822.
2. Stone, B. A.; Clarke, A. E. *Chemistry and Biology of (1→3)−β−Glucans*; La Trobe University Press: Melbourne, 1992.
3. Kashiwagi, Y.; Norisuye, T.; Fujita, H. *Macromolecules* **1981**, *14*, 1220–1225.
4. Chuah, C. T.; Sarko, A.; Deslandes, Y.; Marchessault, R. H. *Macromolecules* **1983**, *16*, 1375–1382.
5. Bluhm, T. L.; Deslandes, Y.; Marchessault, R. M.; Perez, S.; Rinaudo, M. *Carbohydr. Res.* **1982**, *100*, 117–130.
6. Yanaki, T.; Norisuye, T. *Polym. J.* **1983**, *15*, 389–396.
7. Yanaki, T.; Tabata, K.; Kojima, T. *Carbohydr. Polym.* **1985**, *5*, 275–283.
8. Tabata, K.; Ito, W.; Kojima, T.; Kawabata, T.; Misaki, A. *Carbohydr. Res.* **1981**, *89*, 121–135.
9. Bo, S.; Milas, M.; Rinaudo, M. *Int. J. Biol. Macromol.* **1987**, *9*, 153–157.
10. Norisuye, T.; Yanaki, T.; Fujita, H. *J. Polym. Sci. [Polym. Phys. Ed.]* **1980**, *18*, 547–558.
11. Yanaki, T.; Norisuye, T.; Fujita, H. *Macromolecules* **1980**, *13*, 1462–1466.
12. Sato, T.; Norisuye, T.; Fujita, H. *Carbohydr. Res.* **1981**, *95*, 195–204.
13. Kitamura, S.; Kuge, T. *Biopolymers* **1989**, *28*, 639–654.
14. Stokke, B. T.; Elgsaeter, A.; Brant, D. A.; Kitamura, S. *Macromolecules* **1991**, *24*, 6349–6351.
15. Stokke, B. T.; Elgsaeter, A.; Brant, D. A.; Kuge, T.; Kitamura, S. *Biopolymers* **1993**, *33*, 193–198.
16. McIntire, T. M.; Brant, D. A. *J. Am. Chem. Soc.* **1998**, *120*, 6909–6919.
17. Brant, D. A.; McIntire, T. M. Cyclic Polysaccharides. In *Large Ring Molecules*; Semlyen, J. A., Ed.; J. Wiley: New York, 1996; pp 113–154.
18. Stokke, B. T.; Elgsaeter, A.; Hara, C.; Kitamura, S.; Takeo, S. *Biopolymers* **1993**, *33*, 561–573.
19. Holzwarth, G. *Dev. Ind. Microbiol.* **1985**, *26*, 271–280.
20. Hjerde, T.; Stokke, B. T.; Smidsrød, O.; Christensen, B. E. *Carbohydr. Polym.* **1998**, *37*, 41–48.
21. Bohn, J. A.; BeMiller, J. N. *Carbohydr. Polym.* **1995**, *28*, 3–14.
22. Brown, G. D.; Taylor, P. R.; Reid, D. M.; Willment, J. A.; Williams, D. L.; Martinez-Pomarez, L.; Wong, S. Y. C.; Gordon, S. *J. Exp. Med.* **2002**, *196*, 407–412.
23. Herre, J.; Gordon, S.; Brown, G. D. *Mol. Immunol.* **2004**, *40*, 869–876.
24. Sakurai, K.; Shinkai, S. *J. Am. Chem. Soc.* **2000**, *122*, 4520–4521.
25. Sakurai, K.; Mizu, M.; Shinkai, S. *Biomacromolecules* **2001**, *2*, 641–650.
26. Falch, B.; Elgsaeter, A.; Stokke, B. T. *Biopolymers* **1999**, *50*, 496–512.
27. Falch, B. H.; Stokke, B. T. *Carbohydr. Polym.* **2001**, *44*, 113–121.
28. Maurstad, G.; Danielsen, S.; Stokke, B. T. *J. Phys. Chem. B* **2003**, *107*, 8172–8180.
29. Noguchi, H.; Yoshikawa, K. *J. Chem. Phys.* **1998**, *109*, 5070–5077.
30. Yanaki, T.; Kojima, T.; Norisuye, T. *Polym. J.* **1981**, *13*, 1135–1143.
31. Christensen, B. E.; Ulset, A.-S.; Beer, M. U.; Knuckles, B. E.; Williams, D. L.; Fishman, M. L.; Chau, H. K.; Wood, P. J. *Carbohydr. Polym.* **2001**, *45*, 11–22.
32. Stokke, B. T.; Elgsaeter, A.; Kitamura, S. *Int. J. Biol. Macromol.* **1993**, *15*, 63–68.