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Structural stability of (1 → 3)-β-D-glucan macrocycles

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Abstract

The long-term stability of the distribution of macromolecular topologies of the alkaline treated (1 → 3)-β-D-glucan, scleroglucan (1.0 mg/ml), was investigated using HPLC and electron microscopy. The fraction of cyclic (C) and linear (L) topologies of the samples renatured from alkaline solution when [NaOH] was 0.35 M or higher, remained in most cases constant following storage in neutral aqueous solution for 18 months at room temperature. Electron microscopy revealed a reduction of C and increase of L following the long-term storage for scleroglucan renatured from [NaOH] less than 0.25 M. Annealing of renatured samples at temperature T_a equal 100°C gave rise to a stable distribution of 60% cyclic and 13% linear topologies. Increasing T_a above 100°C yielded a distribution of topologies that reduced in initial cyclic fraction from 17% to 5 ± 3% for $T_a = 133$°C following the 18 months storage. These changes in the distribution of topologies manifested itself also in changes in the HPLC elution profiles. In particular, the solvent history producing long-term structurally stable distribution of species of the annealed sample ($T_a = 100$°C) also yielded unchanged HPLC elution profiles. Our data suggest that rearrangements of quenched imperfections in a re-assembled partly triple-helical structure may explain the observed changes in the relative amounts of the linear and circular macromolecular topologies occurring during the storage. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Scleroglucan; Cyclic topology; Electron microscopy; HPLC; Annealing; Renaturation

1. Introduction

The polysaccharide scleroglucan, consisting of a (1 → 3)-β-D-glucose backbone substituted with (1 → 6)-β-D-glucopyranosyl residues at every third residue (Johnson, Kirkwood, Misaki, Nelson, Scaletti & Smith, 1963), adopts a triple helical conformation as determined by X-ray fibre diffraction (Bluhm, Deslandes, Marchessault, Perez & Rinaudo, 1982). Scleroglucan and polysaccharides with similar chemical structures, e.g. schizophyllan, lentinan, and cinerean, make up the (1 → 3)-β-D-glucan family (Stone & Clarke, 1992). These polysaccharides dissolve as a triple-helical structure in aqueous solution (Gawronski, Aguirre, Conrad, Springer & Stahmann, 1996; Norisuye, Yanaki & Fujita, 1980; Yanaki, Kojima & Norisuye, 1981). The scleroglucan triplex dissociates to random coils when heated above 135°C in aqueous solution (Kitamura & Kuge, 1989; Norisuye et al., 1980; Yanaki, Norisuye & Fujita, 1980; Yanaki, Tabata & Kojima, 1985), or dispersed in aqueous dimethylsulfoxide (DMSO) with water weight fraction, W_H, less than 0.13 (Kitamura & Kuge, 1989), or in aqueous alkaline solution with NaOH concentration larger than 0.25–0.35 M (Bo, Milas & Rinaudo, 1987; Kitamura et al., 1995; Tabata, Ito, Kojima, Kawabata & Misaki, 1981). Changing the solvent conditions from those where the (1 → 3)-β-D-glucan exists in random coil conformation to solutions favouring the triple-helical structure, have been reported to yield a blend of linear and cyclic topologies (McIntire & Brant, 1998; McIntire, Penner & Brant, 1995; Stokke, Elgsaeter, Brant & Kitamura, 1991; Stokke, Elgsaeter, Brant & Kuge, 1993; Stokke, Elgsaeter & Kitamura, 1993). The relative content of the additionally observed microgel clusters increased when the polymer concentration was raised above 2 mg/ml for a sample with weight average molecular weight, M_w, equal 43.7×10^4 g/mol (Kitamura et al., 1995). The triple-helical motif of the (1 → 3)-β-D-glucans was assumed to be the driving mechanism in the reassociation to these structures (Brant & McIntire, 1996). The chain stiffness of the reconstituted linear species was qualitatively similar to that of the starting material, but the apparent height above the mica surface was reduced by 10% as determined by non-contact atomic force microscopy (McIntire & Brant, 1998).

Most earlier works on the reconstituted (1 → 3)-β-D-glucans focus on the distribution of the various type of macromolecular species, and their dependence on

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parameters such as chain length, polymer concentration during reconstitution, and duration of the different steps in the solvent histories. The aim of the present work was to determine the structural stability of the different topologies, which is less investigated than factors influencing the initial formation of the individual topologies. The kinetics of triplex strand-separation and re-association have previously been quantified using light scattering, rheology and conformation dependent assays either based on limulus amebocyte lysate (LAL) or a fluorescent probe. Gawronski and coworkers reported that the triplex strand-separation for curdlan with $M_w = 15 \times 10^4$ g/mol occurred by a major disentanglement 12–18 h after dissolving the polymer in 0.5 M NaOH at 25°C, and that the reaction was completed after about one day (Gawronski, Donkai, Fukuda, Miyamoto, Conrad & Springer, 1997). The parameter $(\ln \eta_r)/C_p$, where η_r is the relative viscosity and C_p equals the polymer concentration, depended on time up to 30 h after adding water to a schizophyllan sample initially dissolved in DMSO to W_H larger than 0.14 (Yanaki et al., 1985). The data indicated both a C_p and a W_H dependent change in $(\ln \eta_r)/C_p$ over this period. The time-dependent increase in $(\ln \eta_r)/C_p$ at 25°C after quenching a thermally denatured (150°C, 10 min duration) schizophyllan ($C_p < 5$ mg/ml, $M_w = 43.7 \cdot 10^4$ g/mol) in aqueous solution showed that the viscosity increases with time up to the maximum reported time of 100 h. Higher schizophyllan concentrations yielded gels.

Young and Jacobs have recently reported the changes in fluorescence intensity of aniline blue bound to schizophyllan after denaturation—renaturation using a NaOH solution (pH 13) (Young & Jacobs, 1998). Aniline blue did not bind to the native triple helical conformation whereas the newly renatured schizophyllan bound aniline blue. This binding of aniline blue decreased on the consecutive days after the alkaline treatment. Schizophyllan (Sonifilan) reconstituted from alkaline solution was also reported to show a decrease in the LAL activity as a function of time after neutralization. It have been reported that about 84% of the LAL activity observed just after neutralization was lost four days later (Young & Jacobs, 1998), or nearly completely suppressed following 7 days of storage after the alkaline treatment (Nagi et al., 1993). Both these groups concluded that the observed changes were due to the changes from a putative stimulatory single helix conformation just following the renaturation to an inactive triple helix in the LAL assay. These reports all indicate that slow structural changes may occur in (1→3)-β-D-glucans, but none of these addressed possible changes in relative fraction of cyclic and linear topologies concomitant with changes in LAL activity. The aim of the present re-investigation of previous alkaline denatured and thermally annealed scleroglucan samples was therefore to investigate if the relative fractions of the cyclic and linear topology changed over time.

2. Materials and methods

2.1. Samples

The purified and ultrasonically depolymerized scleroglucan samples SCX_0 and the alkaline denatured and annealed samples (SCX_YA_Z) described previously (Falch, Elgsaeter & Stokke, 1999) were reinvestigated. Additionally, freshly prepared samples using the same procedure were included. The average molecular weights (M_w) of $SC0_0$ (native, non-sonicated sample), $SC3_0$ (3 min. sonication), $SC8_0$ (8 min.) and $SC15_0$ (15 min.) were estimated to be 220×10^4, 110×10^4, 67×10^4 and 49×10^4 g/mol, respectively (Falch et al., 1999).

2.2. Alkaline denaturation

The alkaline denaturation—renaturation procedure of the freshly prepared samples was carried out at scleroglucan concentration $C_p = 1.0$ mg/ml (Falch et al., 1999). Most of the samples were analysed after the dialysis step against distilled water and mQ-water following the neutralization. Aliquots of selected samples were analysed before this dialyses step and for the given times after the neutralization. All samples were filtered (0.22 μm Millex-GV) before characterisation. These de- and renatured samples were denoted by SCX_Y where Y is the maximum molar concentration of NaOH, or KOH used in the first alkaline dialyzing step (0.01–1.0 M).

2.3. Thermal annealing

Aliquots of 2–3 ml aq. selected scleroglucan samples, were heated for two hours at a temperature between 90 and 135°C, and mostly cooled by being left standing in air as detailed previously (Falch et al., 1999). These samples were named $SCX_YA_{Z(t)}$ where A denotes annealing, Z equals the annealing temperature (in degree Celsius), and t equals the duration of the annealing (in hours). The duration of the annealing was 2 h in the cases where the time t is not explicitly denoted.

2.4. Long term storage

The different aqueous scleroglucan samples, non-alkaline or alkaline treated which were annealed or not, were kept in small plastic tubes (2 ml) at room temperature and most of the time in darkness, from some weeks/months and up to 3.5 years. The pH in the solutions was in the range 5.5–6.5, with no detectable changes during the storage. These samples are named $SCX_YA_ZS_T$ where S means storage and T denotes the duration of storage in months.

2.5. Analytical methods

Analyses of the distribution of the hydrodynamic volumes were carried out using aqueous HPLC as previously described (Falch et al., 1999). Transmission

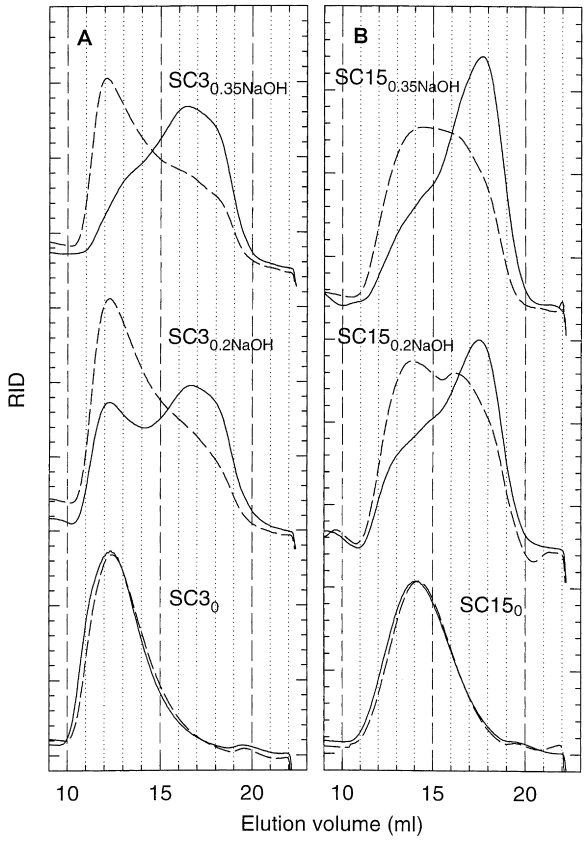

Fig. 1. (A) HPLC elution profiles of 3 min. ultrasonically depolymerized scleroglucan $SC3_0$ and sodium hydroxide denatured scleroglucan samples $SC3_{0.2NaOH}$ and $SC3_{0.35NaOH}$. (B) HPLC-elution profiles of 15 min. ultrasonically depolymerized scleroglucan $SC15_0$ and sodium hydroxide exposed scleroglucan samples $SC15_{0.2NaOH}$ and $SC15_{0.35NaOH}$. The elution profiles were obtained after neutralization and dialyzes to remove salt (—) and following storage in aqueous solution for 18 months at room temperature (– –).

electron micrographs of the scleroglucan samples were obtained as previously detailed (Falch et al., 1999; Stokke & Brant, 1990; Tyler & Branton, 1980). The relative content of the linear triplex and circular topology of the samples were estimated by counting these topologies in the micrographs (in the order of 1000 observations in each case). The results are presented as mean ±SD where the SD were obtained by using the average percentage observed within each electron micrograph containing at least 100 observed macromolecules, as the basis.

3. Results

Fig. 1 shows the HPLC elution profiles of scleroglucan samples sonicated for 3 and 15 min, ($SC3_0$ and $SC15_0$), and the same samples following alkaline denaturation in 0.2 and 0.35 M aqueous NaOH, and subsequently renatured by neutralization, ($SC3_{0.2NaOH}$, $SC15_{0.2NaOH}$, $SC3_{0.35NaOH}$ and $SC15_{0.35NaOH}$), respectively. The elution profiles were determined for samples following dialysis to remove excess salt after the neutralization (approximately one week after the neutralization), and after storage of the same solutions for 18 months at room temperature. The HPLC elution profiles showed that the non-denatured samples $SC3_0$ and $SC15_0$ were stable over this period, while the main part of the samples subjected to alkaline denaturation (≥0.25 M) eluted at smaller elution volume, V_{el}, after 18 months storage. A similar trend was observed for the reduction of V_{el} of the main part of the samples independent of initial M_w of the employed samples.

Fig. 2 shows the HPLC elution profiles of scleroglucan $SC15_{0.35NaOH}$ taken immediately after the neutralization, after the dialysis to remove salts, and the subsequent 3 months of the storage in salt-free solution. The elution profiles (Fig. 2) shifted towards smaller V_{el} for times after

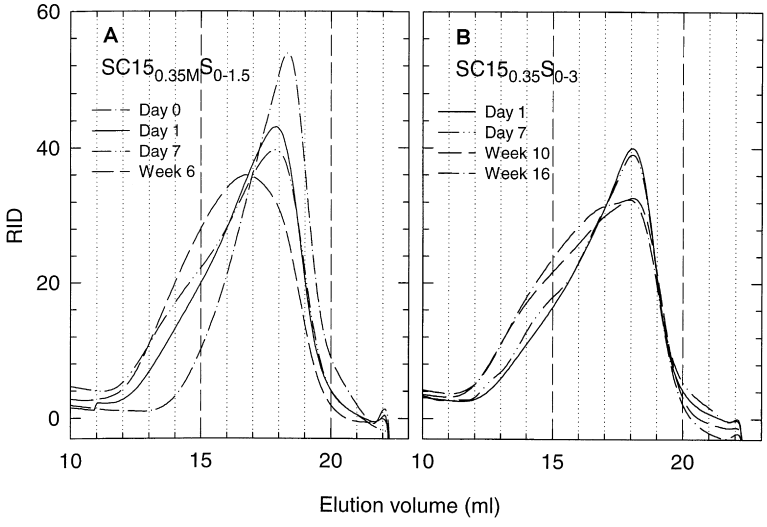

Fig. 2. HPLC elution profiles of 15 min. ultrasonically depolymerized and sodium hydroxide denatured scleroglucan sample $SC15_{0.35NaOH}$ immediately after neutralization and for various durations after neutralization.

Table 1
Linear and cyclic topologies in scleroglucan samples $SC3_Y$ and $SC15_Y$ renatured from alkaline solutions (Data from one experimental series using either NaOH or KOH as the denaturant, were obtained using scleroglucan samples prepared independently.)

Sample	Circles (%)	Circles after 18 months (%)	Linear (%)	Linear after 18 months (%)
$SC3_{0.1NaOH}$	5 ± 2	6 ± 4	71 ± 6	71 ± 6
$SC3_{0.2NaOH}$[a]	38 ± 4	12 ± 9	32 ± 2	65 ± 10
$SC3_{0.35NaOH}$[a]	40 ± 2	38 ± 16	20 ± 2	32 ± 12
$SC3_{1.0NaOH}$	38 ± 5	41 ± 4	18 ± 7	26 ± 9
$SC15_{0.2KOH}$	11 ± 3	6 ± 3	64 ± 2	78 ± 6
$SC15_{0.25KOH}$	53 ± 1	12 ± 1	19 ± 3	66 ± 3
$SC15_{0.35KOH}$	37 ± 2	44 ± 2	27 ± 2	22 ± 3
$SC15_{0.5KOH}$	42 ± 3	38 ± 10	24 ± 4	27 ± 1

[a] HPLC elution profiles and electron micrographs of the scleroglucan samples of these experimental series are shown in Figs. 1A and 3.

neutralization larger than 24 h, with a concomitant increase in the material eluting at $V_{el} \in 12–15$ ml. The longest employed duration of the storage after neutralization was three months in these experiments. Partially denatured samples $SCX_{0.1NaOH}$ using 0.1 M NaOH before being renatured by neutralization revealed only minor changes in their elution profiles over time. Scleroglucan samples subjected to alkaline denaturation using KOH (Table 1) or LiOH (data not shown) showed similar effects as result of 18 months storage in aqueous solution.

Fig. 3 shows electron micrographs of samples before and after the long-term storage. The observed macromolecular species were classified either as linear, circular or other structures (hairpins and clusters of various sizes), and their relative amounts determined by counting. The electron micrographs of the untreated samples SCX_0 revealed no changes in relative amounts of the topologies (e.g. $SC3_0$, Fig. 3A and B). Electron micrographs of sample $SC3_{0.2NaOH}$ prepared after the dialysis step to remove salts and after 18 months storage (Fig. 3C and D) showed a reduction of the fraction of circular species from 38 ± 4 to 12 ± 9% and an increase of the linear topology from 32 ± 2 to 65 ± 10% (Table 1). Increase of the alkali concentration during the denaturation yielded a more stable distribution of the topologies. The percentage of topologies of sample $SC3_{0.35NaOH}$ (Fig. 3E and F) was observed to equal $C = 40 \pm 2\%$ and $L = 20 \pm 2\%$ at the start and $C = 38 \pm 16\%$ and $L = 32 \pm 12\%$ following 18 months storage (Table 1). The fraction of species that was difficult to classify within the two groups decreased after the storage. The long-term storage yielded conversion from C to L for samples renatured from alkaline solutions with alkaline concentration less and about 0.25 M (Table 1). The electron micrographs of denatured samples using [NaOH] > 0.25 M revealed in most cases no significant changes in distribution of topologies, although a shift of the elution profiles towards smaller V_{el} was observed (Fig. 1).

Fig. 4 shows the ratio between the relative fraction of cyclic topology to the linear topology, [C]/[L], versus the

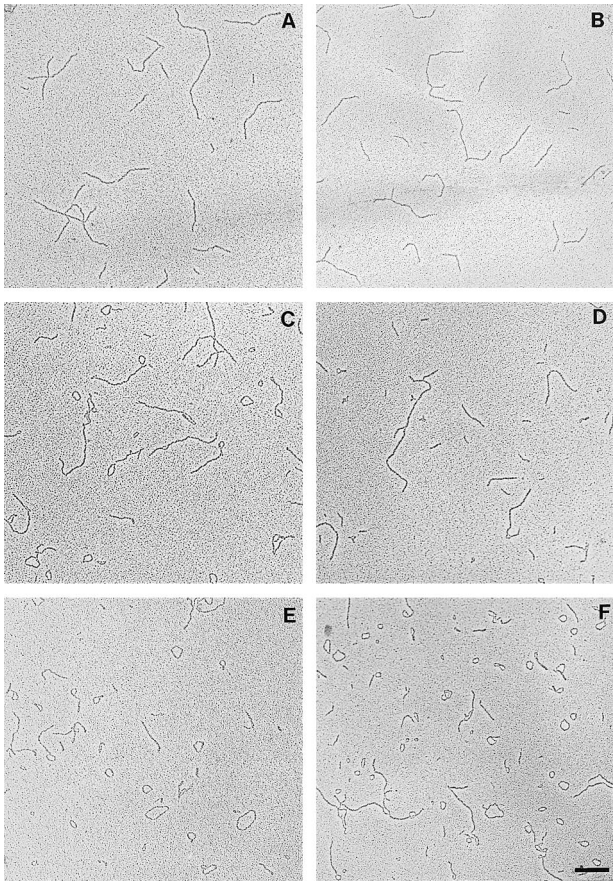

Fig. 3. Electron micrographs of the ultrasonically depolymerized and sodium hydroxide exposed scleroglucan samples: (A) $SC3_0$; (B) $SC3_0S_{18}$; (C) $SC3_{0.2NaOH}$; (D) $SC3_{0.2NaOH}S_{18}$; (E) $SC3_{0.35NaOH}$; and (F) $SC3_{0.35NaOH}S_{18}$. The scale bar is 200 nm.

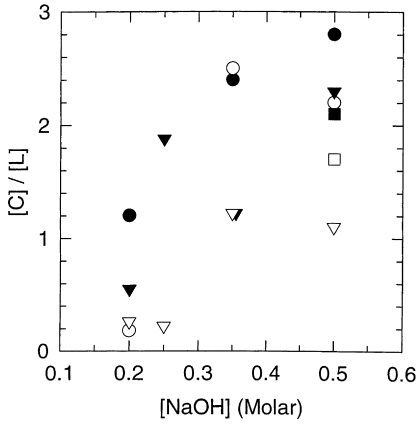

Fig. 4. Ratio between relative fraction of cyclic and linear topology, [C]/[L], versus sodium hydroxide concentration used for alkaline denaturation for 3 min. sonicated scleroglucans $SC3_{YNaOH}$ (○,●), 8 min sonicated scleroglucans $SC8_{YNaOH}$ (□, ■), and 15 min sonicated scleroglucans $SC15_{YNaOH}$ (▽, ▼). The ratios [C]/[L] were determined for freshly neutralized, desalted samples (filled symbols) and after subsequent 18 month storage in aqueous solution (open symbols). The data for [C]/[L] represent the average values from 3–5 independent experiments.

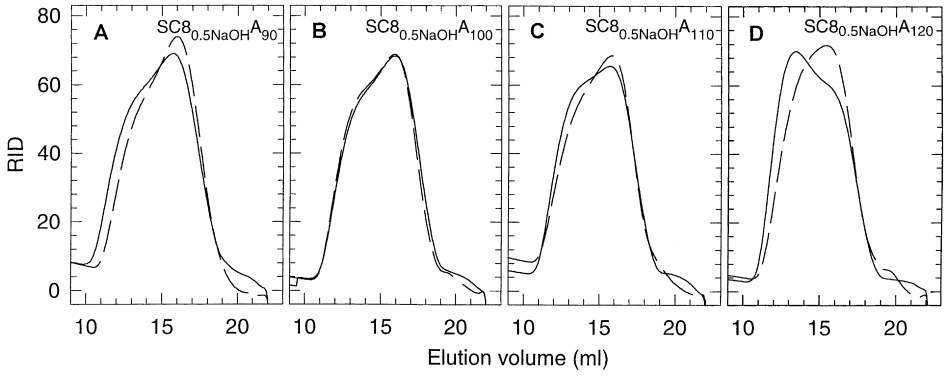

Fig. 5. HPLC elution profiles of 8 min. ultrasonically depolymerized scleroglucan, 0.5 M NaOH denatured, and annealed scleroglucan $SC8_{0.5NaOH}A_Z$ for annealing temperatures of 90, 100, 110 and 120°C. The elution profiles were determined immediately after the annealing (—), and following 18 months subsequent storage in aqueous solution at room temperature (– – –).

concentration of NaOH used for the alkaline denaturation. The largest change in [C]/[L] occurred for NaOH concentrations less than 0.35 M after the 18 months storage. For NaOH $\geq$ 0.35 M this ratio was less reduced due to the storage than at lower NaOH concentration and less dependent of the NaOH concentration than below this NaOH concentration. However, one additional series of experiments of sample $SC8_{1.0NaOH}$ (not shown), did not follow this general trend determined as the average of 3–5 independent experiments.

Thermally annealed samples SCX_0A_Z ($Z = 90 - 135$°C, duration from 2 to 72 h) were found by HPLC and electron microscopy to be stable during the 18 month storage at room temperature (data not shown). These results were similar to that obtained for samples $SC3_0$ (Figs. 1 and 3A and B) and $SC15_0$ (Fig. 1).

Fig. 5 shows the HPLC elution profiles of alkaline (0.5 M NaOH) denatured, renatured and annealed samples $SC8_{0.5NaOH}A_{90}$, $SC8_{0.5NaOH}A_{100}$, $SC8_{0.5NaOH}A_{110}$, and $SC8_{0.5NaOH}A_{120}$ at 90, 100, 110, and 120°C for 2 h, respectively. Notice, however, that the $SC8_{0.5NaOH}$ sample used as starting material for the annealing series, itself was structurally unstable during the long-term storage. The HPLC elution profiles revealed that the sample annealed at 100°C was least affected by the 18 months storage at room temperature, whereas the 90, 110, and 120°C annealed samples showed a relative decrease of the material eluting at 13–14 ml after the 18 months storage. Equivalent changes in the elution profile were observed for sample $SC8_{0.5NaOH}A_{135}S_{18}$. Increasing the annealing temperature (100–135°C), increased the fraction of material eluting at larger V_{el} after the long term storage. The relative abundance of the cyclic and linear topologies was most stable for samples annealed at 100°C (Figs. 6C and D, Table 2). Alkaline denatured samples annealed at 90 and at 110°C showed a 30% reduction in the cyclic topology, and 50% increase in the linear topology (Table 2). Increasing T_a further, to 135°C, yielded samples that lost most of their cyclic species during the storage, and ended up with most of the material in the form of a linear topology (Figs. 6A and B).

Fig. 7 shows the ratio [C]/[L], versus the annealing temperature for the $SC8_{0.5NaOH}A_Z$ sample series. The data show that [C]/[L] was least affected by the long-term storage for T_a around 100°C. The value of [C]/[L] of the 18 months stored samples appears to have an optimum around 100°C, and declines as result of annealing temperature above 100°C to nearly at 120°C (Fig. 7).

The HPLC elution profiles and the electron micrographs of scleroglucan $SCX_{0.5NaOH}A_{100}$ did not show any dependence on whether the sample was cooled by air or by quenched on ice (Table 3). This was observed for the $SC8_{0.5NaOH}A_{100}$ samples just following the preparation and

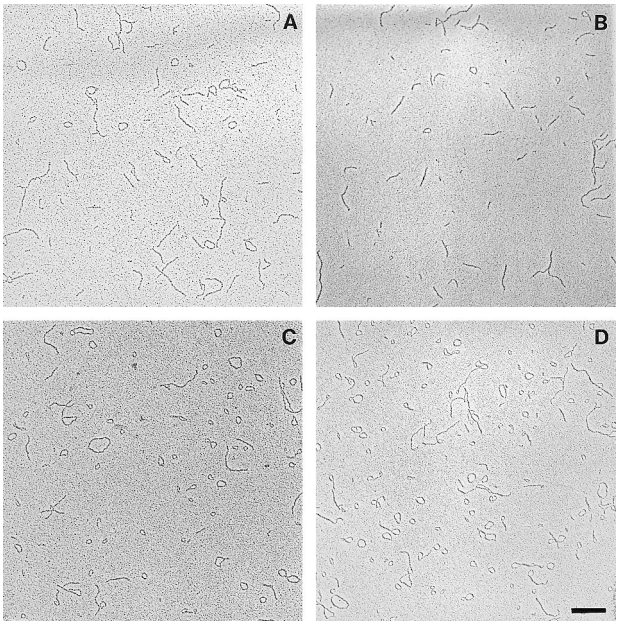

Fig. 6. Electron micrographs of the ultrasonically depolymerized, sodium hydroxide exposed and thermally annealed scleroglucan samples: (A) $SC8_{0.5NaOH}A_{135}$; (B) $SC8_{0.5NaOH}A_{135}S_{18}$; (C) $SC8_{0.5NaOH}A_{100}$; and (D) $SC8_{0.5NaOH}A_{100}S_{18}$. The scale bar is 200 nm.

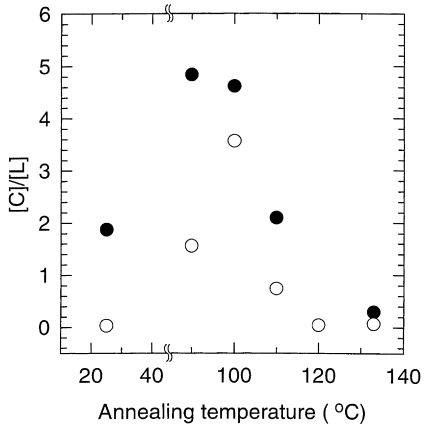

Fig. 7. Ratio between relative fraction of cyclic and linear topology, [C]/[L], versus annealing temperature for 8 min. sonicated, alkaline denatured and thermally annealed scleroglucans $SC8_{0.5NaOH}A_Z$ determined immediately after the annealing (●) and following 18 months subsequent storage in aqueous solution at room temperature (○).The data for [C]/[L] represent the average values from one series of experiments.

the $SC3_{0.5NaOH}A_{100}$ after the subsequent 18 months of storage.

4. Discussion

The aim of the present work was to investigate the long-term structural stability of scleroglucan samples employing HPLC and EM. Samples subjected to various alkaline denaturation treatments and thermal annealing were included alongside the only sonicated samples. The samples were stored at a polymer concentration of about $C_p = 1$ mg/ml in aqueous solution (pH 5.5–6.5) for up to 18 months (a few samples up to 3.5 years) at room temperature. Selected samples were also followed by more frequent characterization of their structure the first 1–3 months after the various solvents histories.

The results showed that the distribution of topologies and HPLC elution profiles remained unchanged during the long-term storage of the sonicated samples (SCX_0). This is in accordance with the reported thermal stability of scleroglucan at elevated temperatures (Davison & Mentzer, 1982; Kalpakci, Jeans, Magri & Padolewski, 1990). In the later study, it was reported that the viscosity, η, of the scleroglucan in aqueous solution remained practically constant up to 18 months at temperatures of 90 and 110°C, whereas further increase of T yielded a declining η with increased storage time (Kalpakci et al., 1990). The triple-helical conformation facilitating reversal of the transition states to a larger extent than single-chain polymers, and the ability to delay decrease in overall molar mass relative to that of the bond scission events, are possible molecular mechanisms contributing to the thermal stability of these structures.

Scleroglucan samples undergoing thermal annealing between 90 and 135°C for 2 h (SCX_0A_{90-135}) revealed no change in the distribution of topologies just after the treatment. The HPLC elution profiles of the $SCX_0A_{135(t)}S_{18}$ samples ($t = 6$–72 h), nearly coincided with those obtained prior to the long term storage (samples $SCX_0A_{90-135(t)}$). The electron micrographs of these stored samples confirmed an unchanged distribution of topologies. Thus, only samples subjected to denaturation—renaturation treatments at conditions inducing the cyclic topology or multichain clusters, show changes in distribution of topologies during subsequent long-term storage.

Possible molecular mechanisms that can account for the observations should allow for the constant total areas under the elution profiles, also for the samples revealing redistribution of topologies. A shift of the elution profiles to smaller V_{el}, without considering the redistribution of topologies, would in itself indicate an increase of chain length or aggregation. Thus, degradation appears not to be a major factor leading to the observed changes following the long-term storage.

A thoroughly investigated biopolymer where long term effects are clearly identified, are collagen or gelatin (for a recent review, see te Nijenhuis, 1997). There are structural similarities between these and scleroglucan that, by analogy, may yield clues concerning the mechanism for the observed long-term effects reported here. Firstly, the starting structures are both triplexes. Secondly, the long-term processes are observed only when the triplex structures have been strand-separated and re-assembled. Thirdly, it is in both cases suggested that the triplex structural motif prevalent in each individual chain is strongly contributing to the assembly of the polymer chains occurring during the renaturation. The long term effects in gelatin have mainly

Table 2
Linear and cyclic topologies in scleroglucan samples $SC8_YA_Z$ renatured from alkaline solutions and annealed (one experimental series)

Sample	Circles (%)	Circles after 2 months (%)	Circles after 18 months (%)	Linear (%)	Linear after 2 months (%)	Linear after 18 months (%)
$SC8_{0.5KOH}$	47 ± 2	–	7 ± 6	18 ± 2	–	70 ± 11
$SC8_{0.5NaOH}$	43 ± 3	34 ± 1	2 ± 2	23 ± 4	31 ± 4	78 ± 10
$SC8_{0.5NaOH}A_{90C}$	58 ± 4	–	42 ± 13	12 ± 1	–	27 ± 7
$SC8_{0.5NaOH}A_{100C}$	60 ± 1	–	57 ± 8	13 ± 0	–	16 ± 4
$SC8_{0.5NaOH}A_{110C}$	46 ± 3	–	33 ± 1	22 ± 0	–	45 ± 3
$SC8_{0.5NaOH}A_{120C}$	–	–	3 ± 2	–	–	83 ± 3
$SC8_{0.5NaOH}A_{133C}$	17 ± 0	–	5 ± 3	59 ± 2	–	82 ± 9

Table 3
Linear and cyclic topologies in scleroglucan samples $SC3_YA_Z$ and $SC8_YA_Z$ renatured from alkaline solutions, annealed and quenched at different rates (one experimental series)

Sample	Circular (%)	Circular after 18 months (%)	Linear (%)	Linear after 18 months (%)
$SC3_{0.5NaOH}A_{100}$(air)	56 ± 4	56 ± 3	10 ± 4	15 ± 3
$SC3_{0.5NaOH}A_{100}$(ice)	–	62 ± 1	–	13 ± 1
$SC8_{0.5NaOH}A_{100}$(air)	51 ± 4	–	15 ± 4	–
$SC8_{0.5NaOH}A_{100}$(ice)	50 ± 2	52 ± 1	17 ± 0	19 ± 2

been observed for gels, whereas the present data for scleroglucan also include smaller assemblies. The main difference between these two cases is therefore considered to be the number of associated chains, and topological constraints due to this difference in assembly size. It is reported that the elastic moduli of gelatin gels changes slowly over time up to weeks, and that such long term effects depend on the quench depths (te Nijenhuis, 1997). By analogy, we therefore suggest that the initial reassociation for scleroglucan occur based on the triplex motif, and allowing for free single stranded ends with an overall linear topology. This may facilitate cyclization depending on the cyclization probability. Imperfections in the triplex structure, or possibly a duplex structure, that are allowed to rearrange during either annealing and or subsequent long-term storage are expected to be a general feature of such cooperatively associated structures.

Fig. 8 shows a schematic illustration of possible arrangements of an imperfectly assembled structure that may qualitatively account for the main observations. This illustration depicts triplex stretches interspersed with less perfectly organised sequences where the two possibilities are either a duplex structure while the third chain makes a loop that subsequently merge into the triplex, or all three chains are strand-separated in individual sequences (triplex loop, Fig. 8). The average length of the triplex parts corresponds to the co-operative length of the transition, which can be obtained from the partition function of the system (Poland, 1978; Schwarz & Poland, 1974; Shaw & Schurr, 1975). The loops may introduce imperfect matching in the structure because of the repeating primary structure. Kitamura and Kuge reported that the calorimetric enthalpy for the strand separation transition was less in the second thermal cycle than the initial (Kitamura & Kuge, 1989). This suggests partial regeneration of the physical bonds stabilizing the triplex. Although this study was carried out in a different solvent and using thermal denaturation, this finding nevertheless suggest that fractional recovery of interstrand bonds within the triplex can be expected to be a general feature. The calorimetric data also suggest the co-operative length to be on the order of 100 repeating units for the thermal driven transition (Kitamura & Kuge, 1989). Calorimetric determination of the alkali induced strand-separation transition was reported to be overshadowed by the large heat associated with depolymerization compared to that for the physical bonds stabilising the triplex structure. Thus, the cooperative length for this transition is not known at present. Such imperfectly assembled sequences may undergo structural changes towards equilibrium in the annealing or long-term storage. The length of the suggested loop fractions should be less than the resolution limit of the employed EM technique, because strand separated segments, either internally or at the terminals, have not been clearly identified. The average height of renatured linear species from thermal denaturation, found to be 90% of the non-treated linear species (McIntire & Brant, 1998) may indicate a rearrangement of the interstrand bonds stabilizing the triplex.

Different denaturation conditions or annealing temperatures is therefore suggested to yield structures with various

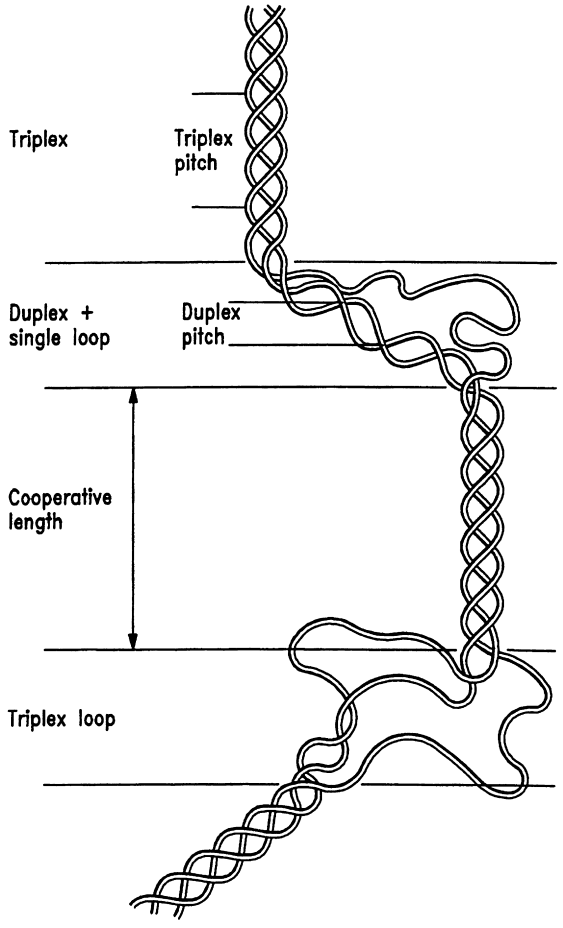

Fig. 8. Schematic illustration of possible structural imperfections in re-assembled triplex structure.

cooperative lengths and single stranded fractions (loops) as starting points for the subsequent long-term effects. In this regard, it is of interest to note that samples renatured from alkali, and annealed at 100°C (2 h) are the most stable during the subsequent storage. This is evident from both the unchanged HPLC profiles of the $SCX_{0.35-0.5NaOH}A_{100}$ samples (Fig. 5) and no change in distribution of topologies following the 18 months of storage (Fig. 6, Table 2). It should also be noted, as reported previously (Falch et al., 1999; Kitamura et al., 1995), that this annealing process yields an increase in the cyclic fraction with concomitant changes in the HPLC elution profile compared to the samples reconstituted from alkali. Increasing the annealing temperature to 110, 120 or 135°C yielded less stable structures during the long-term storage. A fraction of the material eluted at larger V_{el}, and concomitant reduction of circular species following the storage. These changes were more prominent the higher the annealing temperature was. It is suggested that this is due to different quench depths, with $T_a = 100$°C yielding propagation parameters producing triplex assemblies with a large fraction of the interstrand bonds being recovered and the chains in register. The starting structures obtained at the higher annealing temperatures, closer to the strand-separation midpoint transition, would have a smaller propagation parameter, and hence a smaller fraction of the potential interaction sites being active, resulting in more segments being involved in imperfectly assembled structures. Migration of loops, or inactive interacting sites due to unfavourable mutual localization, can be effective during the long-term storage. This is expected to be more important the larger the fraction of these structural elements are, i.e. when cooled from the higher annealing temperatures. Loss of cyclic topology in this process may occur because the ring-closure free energy penalty may increase with decreasing temperature due to both an increasing fraction of triplex segments as the structural rearrangements occur yielding an increased average persistence length, L_{pe}, and an increase in L_{pe} with decreasing temperature (Stokke, Elgsaeter, Bjørnestad & Lund, 1992).

Similar qualitative accounts of the structural redistribution in the samples prepared by renaturation from the alkaline solutions are more difficult. This is because the selected experimental starting conditions are at pH above that needed for the strand separation. Thus, the distribution of species at the beginning of the long-term storage appears to be influenced by the kinetics of the structural regeneration to a larger extent than for the thermally quenched samples. Additionally, the relation between the propagation parameter for the formation of an adjacent pair of interaction sites and the quench depth expressed in terms of the enthalpy and entropy is well established for the thermal driven transition. A similar relation depending on quench-depth in terms of the difference between the actual pH and that needed for the strand-separation is lacking at present. In some of the samples the observed relative amounts of rings and linear species remained constant, whereas the elution profiles showed that the material eluted at larger elution volumes. Reduction of imperfectly assembled segments without change in distribution of topology is considered to be a possible molecular mechanism for this. It is expected that the average L_{pe} of the structures would increase due to such a mechanism.

The above suggested mechanisms for the changes in the structures following annealing and long term storage in aqueous solution are not at variance with the reported slow changes in the binding of aniline blue to a renatured β-D-glucan (Young & Jacobs, 1998) or the effect of thermal annealing on the biological activity of grifolan (Adachi, Miura, Ohno, Tamura, Tanaka & Yadomae, 1999). Assuming that the binding of aniline blue occur only to the single-stranded segments, the above mechanism accounts, at least qualitatively, for the reported decrease in binding due to increasing saturation of non-bonded interactions during the long-term storage. Adachi et al. tested the reactivity of grifolan on a sandwich-type enzyme immuno-assay (EIA) system (Adachi et al., 1999). The 0.5 M alkaline treated grifolan showed less reactivity than the corresponding samples heated at a temperature between 40 and 100°C for 15 min. These authors suggested that EIA has higher reactivity to the triple helical structure of (1 → 6)-branched (1 → 3)-β-D-glucans.

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