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Free-radical degradation of triple-stranded scleroglucan by hydrogen peroxide and ferrous ions

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Abstract

The triple-stranded polysaccharide scleroglucan was subjected to free-radical depolymerisation with Fenton's reagent (H_2O_2/Fe^{2+}), and partially degraded samples in the molecular weight (M_w) range from 8 200 000 to 5200 were studied. Analyses of the chemical composition of partially degraded samples by ^{13}C nuclear magnetic resonance spectroscopy and by determination of reducing end-groups suggested that the degradation was random. The decrease in M_w followed the kinetics expected for a multiple-stranded polymer. Following an apparently initial stable regime, degradation occurred with an exponential decrease (exponent (ν) ~3.6) in M_w. Partially degraded samples displayed bimodal molecular weight distributions. This was attributed to the release of single-stranded oligomers having a degree of polymerisation (*DP*) too low to remain incorporated in the triplex structure ($DP < DP_c$; the critical lower *DP* for triplex formation). The weight-average molecular weight of the low-M_w fraction was estimated to about 36 000 (DP_w~56 tetrasaccharide units). Following a heat treatment at 100°C (15 min) of the partially degraded samples, a reduction in M_w by a factor of 2 to 2.5 was observed. This was attributed to the formation of more perfectly matched triple-stranded molecules, without aggregation or ring formation, as shown by electron microscopy. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Scleroglucan; Free-radical degradation; Ferrous ions

1. Introduction

The conformational state, in particular the strandedness of the molecules, has a large influence on the degradation behaviour of polysaccharides (Thomas, 1956; Sharples, 1957; Rinaudo and Milas, 1980; Christensen and Smidsrød, 1991; Hjerde et al., 1994). In our previous work the degradation of both single- and multiple-stranded polysaccharides by a free-radical mechanism (Hjerde et al., 1994; Christensen et al., 1996a) or acid hydrolysis (Christensen et al., 1993; Hjerde et al., 1996 Hjerde et al., 1997) was investigated. The occurrence of a multiple-stranded structure may in some cases influence the rate of cleavage of glycosidic linkages (Sharples, 1957; Hjerde et al., 1996). Acid hydrolysis of the double-stranded polysaccharides xanthan (Christensen et al., 1996a) and κ-carrageenan (Hjerde et al., 1996) showed a three- and 10-fold decrease in the rate of bond cleavage upon conformational ordering, respectively. However, no conformation dependence on the rate of bond cleavage was observed for the acid hydrolysis of ι-carrageenan (Hjerde et al., 1996) and for free-radical depolymerisation of xanthan (Christensen et al., 1996a). The attacks of free radicals along the chains are non-specific and the reaction has a low activation energy. As a consequence, the rate of bond cleavage may not be influenced by the strandedness of the polymer.

By monitoring the decrease in molecular weight (M_w) or intrinsic viscosity ($[\eta]$) with degradation time, a deviation in the behaviour of multiple-stranded polymers from that of single-stranded polymers is expected. Following an initial, apparently stable regime, where the cleavages in the glycosidic linkages are hidden in the multiple-stranded structures, a regime with exponential decrease in M_w or $[\eta]$ is found ($M_w \propto t^{-\nu}$) for xanthan and scleroglucan (Christensen et al., 1993; Hjerde et al., 1994). A Monte Carlo simulation of the degradation of double- and triple-stranded polymers has been performed (Stokke et al., 1992). The calculated exponent (ν) was predicted to be 1 for a single-stranded polymer, whereas it increased to 1.7 and 2.3 for a double- or triple-stranded polymer, respectively (Stokke et al., 1992). Experimental results on xanthan and scleroglucan could not quantitatively verify this exponent, although many of the

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qualitative features of the model were observed (Christensen et al., 1993; Hjerde et al., 1994).

A partially degraded multiple-stranded structure may contain hidden chain cleavages, which is indicated by the rapid disintegration of these structures upon further degradation (Stokke et al., 1992). The hidden chain cleavages can be revealed by subjecting a partially degraded sample to a denaturation/renaturation cycle, resulting in a substantial reduction in M_w depending on DP_c (critical lower degree of polymerisation, DP, for the formation of a multiple-stranded structure) and the degree of degradation (Christensen et al., 1996b).

The accumulation of short, single-stranded fragments from the degradation of multiple-stranded molecules can readily be observed by size exclusion chromatography (SEC) as bimodal molecular weight distributions (*MWD*s). These fragments are too short to attain an ordered structure ($DP < DP_c$), and are released from the ordered structures throughout the degradation. This has been reported for the depolymerisation of the double-stranded polysaccharides xanthan (Christensen et al., 1993, 1996a), κ-carrageenan and ι-carrageenan (Hjerde et al., 1997).

Scleroglucan is a fungal polysaccharide (*Sclerotium* spp.) consisting of a (1 → 3)-β-D-glucan backbone with a (1 → 6)-β-D-Glc*p* residue on every third glucose in the main chain (degree of branching (*DB*) ~0.33). The physical properties of scleroglucan and very similar (1 → 3)-β-D-glucans isolated from other sources, e.g., schizophyllan and lentinan, are almost identical. These polysaccharides appear in a triple-helical conformation in aqueous solution when $M_w > 40\,000$ (Yanaki et al., 1983). The disordered, single-stranded conformation can be induced above 135°C, at pH > 13 or in > 90% dimethyl sulfoxide (DMSO) (Norisuye et al., 1980; Yanaki et al., 1981; Rinaudo and Vincendon, 1982; Kitamura and Kuge, 1989; Kitamura et al., 1996). The high viscosifying power and enhanced apparent stability of scleroglucan, due to the triple-stranded conformation, may be utilised in, for example, improved oil recovery (IOR) (Davison and Mentzer, 1982). Apart from being a strong and stable viscosifyer, scleroglucan and other (1 → 3)-β-D-glucans may function as biological response modifiers in certain biological systems (Bohn and BeMiller, 1995). The activity is apparently dependent on both M_w and the degree of branching, with long triple-stranded chains with a *DB* of 0.33 often being the most active. Also short, single-stranded chains ($M_w < 20\,000$) with lower degree of branching (< 0.25) appear to very active (Bohn and BeMiller, 1995). Hence, the study of the degradation of scleroglucan and analysis of partially degraded samples are important with respect to its potential both as a viscosifyer and as a biological response modifier.

In this work we investigate the degradation of scleroglucan by a free-radical mechanism with Fenton's reagent (H_2O_2 in the presence of Fe(II)). The undegraded and partially degraded samples are analysed by SEC combined with low-angle laser light scattering (SEC–LALLS), reducing end-groups determination, and ^{13}C nuclear magnetic resonance spectroscopy (NMR). In addition, the partially degraded samples are subjected to a heat treatment and subsequently analysed by SEC–LALLS and electron microscopy.

2. Experimental

2.1. Preparation of partially degraded scleroglucan samples

Scleroglucan was purified from a fermentation broth (Statoil, ScR76F2) as previously described (Hjerde et al., 1994). The degradation of scleroglucan (1 mg/ml) with Fenton's reagent (1 mM $FeSO_4$, 100 mM H_2O_2, 10 mM Na_2EDTA, 10 mM Na-acetate buffer (pH 5) (Hjerde et al., 1994)) was performed at 20°C in an acid-washed glass bottle. Samples were taken during the degradation and the reaction was stopped by adding thiourea (12.5 mM), Na_2SO_3 (25 mM) and n-propanol (25 μl/ml) (Wellington, 1983). The samples were dialysed at ambient temperature against 0.01 M HCl + 0.1 M NaCl in order to remove Fe ions and EDTA, and then extensively against Milli-Q water. The volume change in the dialysis step was measured in order to give an accurate mass balance. The polysaccharide concentrations and the number of reducing end-groups were measured with the phenol–sulfuric acid method (Dubois et al., 1956) and the Nelson–Somogyi method (Hodge and Hofreiter, 1962), respectively, using glucose as standard.

2.2. SEC–LALLS

The undegraded and partially degraded samples were analysed by size exclusion chromatography (SEC) (columns TSK G6000PWXL and G5000PWXL, serially connected) combined with low-angle laser light scattering (LALLS) (KMX-6, Chromatix), as previously described (Christensen et al., 1993). Three injections at ambient temperature were made from each sample.

2.3. ^{13}C-NMR

The undegraded and four partially degraded samples were freeze-dried and dissolved (30 mg/ml) in DMSO–d_6. The samples were subsequently analysed at 90°C by proton-decoupled ^{13}C-NMR (Bruker Avance DPX400) at 100.63 MHz. The peaks were assigned according to Rinaudo and Vincendon (1982).

2.4. Ultrafiltration

A larger amount of a partially degraded sample (72 h) was ultrafiltered (Minitan system, Millipore) using filters (five Minitan plates, Millipore) with a cut-off value of 100 000 Da for globular proteins. The sample was

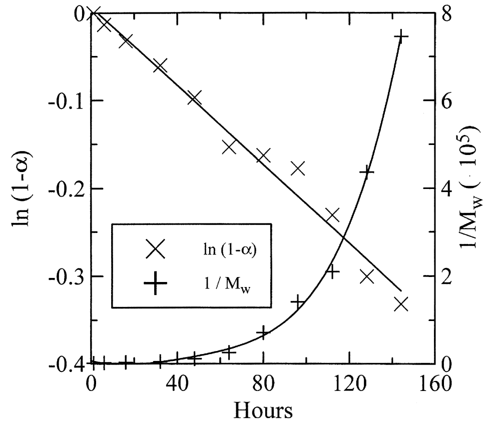

Fig. 1. Formation of reducing end-groups (ln(1 − α)) and change of the molecular weight (1/M_w) upon degradation of scleroglucan (1 mg/ml) with Fenton's reagent (1 mM $FeSO_4$ and 100 mM H_2O_2) at 20°C.

ultrafiltered for 2 h at 400 ml/min with a pressure over the filters of 103 kPa. The filtrate was concentrated by evaporation. Finally, the retentate sample and the filtrate were analysed by SEC–LALLS.

2.5. Monte Carlo simulations

The free-radical depolymerisation of scleroglucan was modelled employing the previous method for simulation of degradation of duplex and triplex polymers by random bond scissions in the individual chains (Stokke et al., 1992). This approach has later been extended to estimate explicitly the molecular weight distributions as well as the fraction of released single-stranded polymers during the depolymerisation (Christensen et al., 1993; Hjerde et al., 1997). The simulated depolymerisation of scleroglucan was carried out for an ensemble with initially 900 triple-stranded chains with degree of polymerisation (*DP*) of 2000. The rate constant for bond cleavage of the chains in the triplex state and that of the released single-stranded species were assumed to be identical. Following a random attack and cleavage of the bonds according to the bond cleavage factor, the average molecular weight of the triple-stranded chains and released oligomer fragments according to the triple-strand stability criterion, $DP \geq DP_c$, were calculated.

2.6. Heat treatment

The partially degraded samples (0.2–1 mg/ml) were subjected to a heat treatment at 100°C for 15 min, and then cooled slowly to ambient temperature. The samples were subsequently analysed by SEC–LALLS and compared with the untreated samples.

2.7. Electron microscopy

Preparation of replicas for electron microscopy was carried out as described previously (Tyler and Branton, 1980; Stokke and Elgsaeter, 1994). Aliquots of the aqueous scleroglucan samples were diluted with 1.0 M NH_4Ac and 60% glycerol to a final polymer concentration of 5–20 μg/ml in 0.1 M NH_4Ac and 50% glycerol. The volatile buffer was left out in some of the preparations. A portion (50 μl) of these diluted samples was sprayed onto freshly cleaved mica discs and vacuum-dried at 10^{-6} torr, $T = 20$°C, for 60 min. The vacuum-dried specimens were subsequently replicated while rotated with 0.6–0.8 nm Pt from an angle of 6° and 8–10 nm C from an angle of 90°. The electron micrographs were obtained with a Philips EM 400T electron microscope at a nominal electron optic magnification of 17 000 to 60 000 times. Calibration of the magnification of the final printed micrographs was carried with a line grid (1200 lines/mm, Agar Aids).

3. Results and discussion

3.1. Free-radical depolymerisation

Free-radical depolymerisation with Fenton's reagent was performed on intact, triple-stranded scleroglucan. Samples were taken at intervals during the degradation, dialysed and analysed with respect to the formation of reducing end-groups and decrease in molecular weight. The degree of chain scission (α) is defined as n_{red}/n_{tot}, where n_{red} is the number of reducing ends and n_{tot} is the total number of glucose residues in the main chain. The mechanisms of free-radical attack leading to depolymerisation of polysaccharides are complex and largely unknown, but it is assumed that n_{red} is at least proportional to the number of chain breaks. Uchiyama et al. (1990) have shown that reducing sugars correspond to about 75% of the reducing terminal residues in hyaluronic acid after depolymerisation with free radicals.

The observed linear decrease in ln(1 − α) with time (Fig. 1) suggests that the depolymerisation is random. A corresponding behaviour upon depolymerisation with Fenton's reagent has previously been observed in several other polysaccharides (Smidsrød et al., 1965; Herp et al., 1967; Hjerde et al., 1994; Christensen et al., 1996a). Since the degree of conformational order (i.e., fraction of triplex, high-M_w structures) decreases gradually throughout the degradation (see below), the degradation rate can therefore be presumed to be independent of the conformational state as previously shown for xanthan (Christensen et al., 1996a).

Analysis of the average *DB* of undegraded and partially degraded samples may give additional information on the random depolymerisation process. A decrease in the degree of branching is expected, because a glucose residue in the side-chain is released upon cleavage of a single glycosidic linkage, whereas a main-chain residue requires two chain cleavages. If all linkages are cleaved at the same rate, the degree of branching should be about 0.23 when α is 0.3 (144 h). The ^{13}C-NMR spectra of the undegraded and four

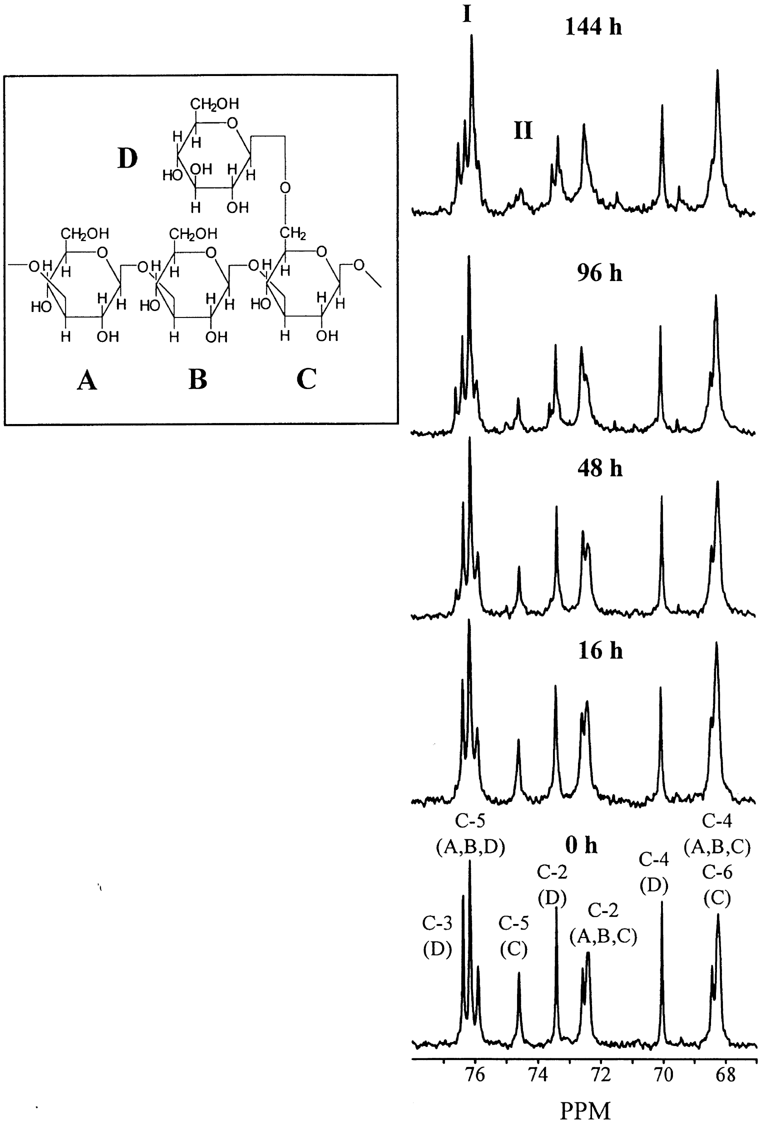

Fig. 2. Sections of proton-decoupled ^{13}C-NMR spectra (100.63 MHz at 90°C) of the undegraded and four partially degraded scleroglucan samples. The assignment of the peaks is explained in the figure.

partially degraded scleroglucan samples are shown in Fig. 2. The spectra reveal peaks corresponding to carbon atoms in the side-chain sugar (D; see figure legend) or in the branching point (C). The fraction of side-chains per repeating unit can be found by dividing the area of peak II, corresponding to C-5 in the C unit, and the area of peak I minus peak II, which corresponds to the total glucose residues in the main chain. For the undegraded sample *DB* is about 0.32 in accordance with previously reported results for scleroglucan (Yanaki et al., 1981). At prolonged degradation *DB* decreases slowly, and for the most degraded sample (144 h) it is about 0.20, which is in reasonable agreement with *DB* calculated for a random degradation. The results thus show that there is no preferential cleavage of side-chains, and supports the earlier observation of a totally random depolymerisation mechanism by Fenton's reagent (Smidsrød et al., 1965; Hjerde et al., 1994). The release of side-chains will give a significant contribution to the loss of mass in the dialysis step, i.e., 16% of the dialysable material after 144 h.

The plot of $1/M_w$ against degradation time (Fig. 1) deviates from the linearity expected for a single-stranded polymer (Smidsrød et al., 1965; Herp et al., 1967; Hjerde et al., 1994) and displays an apparently stable regime at low α, as previously shown for multiple-stranded polymers (Christensen and Smidsrød, 1991; Hjerde et al., 1994). At prolonged depolymerisation the rate of degradation appears to increase. This is attributed to the gradual increase in the number of hidden chain cleavages in the triplex structures and the subsequent disintegration of these structures (Stokke et al., 1992; Christensen et al., 1993). Consequently, the rate constant calculated on the basis of the decrease in M_w or $[\eta]$ will increase with time.

The apparently stable regime (< 10 h) is followed by a

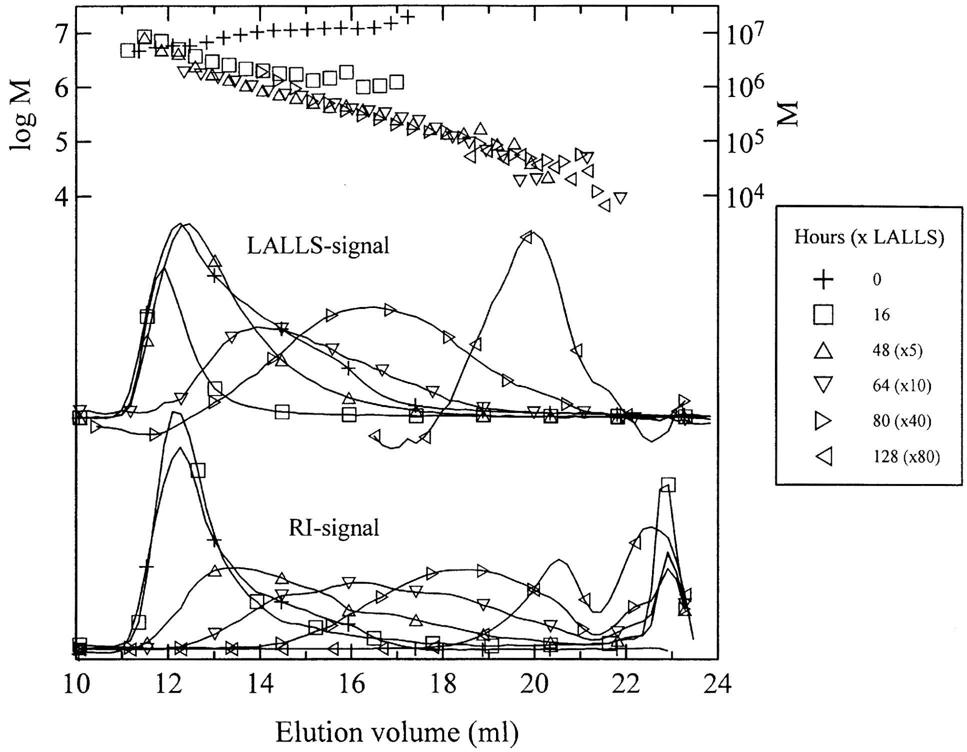

Fig. 3. SEC–LALLS profiles (RI and LALLS detectors) and calibration curves (log *M* versus elution volume) for partially degraded scleroglucans.

regime with an exponential decrease in M_w (data not shown). The observed exponent (ν) is 3.6, which is substantially higher than the value of 1 for the random depolymerisation of a single-stranded polymer. The exponent is also higher than the calculated value (2.3) from a Monte Carlo simulation of random degradation of homogeneous triplex structures (Stokke et al., 1992). Previous experimental data for xanthan also gave too high an exponent, suggesting that this method is not adequate to assess the number of strands which form the ordered conformation. One explanation may be that the simulation model does not consider kinetic effects or rearrangements (see below) which contribute to an additional reduction in M_w and therefore higher values of the exponent ν.

3.2. SEC–LALLS analysis

The undegraded and partially degraded samples were analysed by SEC–LALLS. The elution profiles and calibration curves (log *M* against elution volume, *V*) are shown in Fig. 3. The undegraded sample displays an apparent monomodal molecular weight distribution. At prolonged degradation the refractive index (RI) signal moves towards higher elution volumes and the LALLS signal decreases, both indicating a decrease in M_w. The partially degraded samples also display bimodal *MWD*s, and the amount of low-M_w chains increases with degradation time (Table 1). Qualitatively similar results have been observed for double-stranded xanthan (Christensen et al., 1993, 1996a), and

Table 1
Molecular weights and mass distributions of undegraded and partially degraded scleroglucan samples

Degradation time (h)	M_w	Mass distribution (%)		
		High-M_w fraction	Low-M_w fraction	Dialysable fraction
0	8 200 000	100	0	0
6	4 600 000	75	21	4
16	3 200 000	78	20	2
32	2 000 000	81	16	3
48	830 000	79	14	7
64	300 000	74	15	11
80	120 000	71	18	11
96	54 000	61	24	15
112	28 000	43	28	29
128	8 400	25	30	45
144	5 200	12	27	61

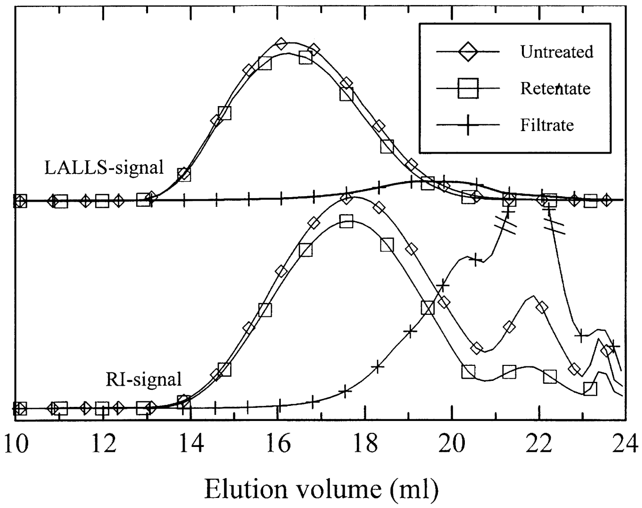

Fig. 4. SEC–LALLS profiles of an untreated (partially degraded) scleroglucan sample, and of the retentate and the filtrate after ultrafiltration.

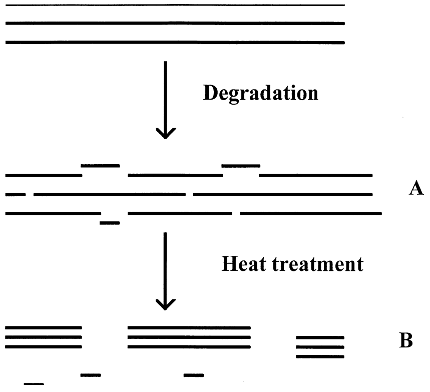

Fig. 6. Illustration of the degradation of a triple-stranded polymer. Heat treatment of a partially degraded sample with hidden chain cleavages (A) may result in the formation of more perfectly matched triple-stranded chains with significantly lower molecular weights (B).

κ-carrageenan and ι-carrageenan (Hjerde et al., 1997) in their ordered conformations.

The calibration curves (Fig. 3) for the least degraded samples are almost horizontal and show that there is very poor separation on the columns, due to the extremely high M_w. The more degraded samples ($M_w < 2\,000\,000$) seem to be homologous in terms of hydrodynamic volume and molecular shape, and the calibration curves are linear over a range of almost three orders of magnitude. The weight-average molecular weights calculated from the elution profiles and calibration plot are presented in Table 1. The loss of mass in the dialysis step (< tetramers (Knutsen et al., 1993)) has been accounted for in the M_w determinations (Table 1) by including the dialysable oligomers in the concentration term. The corrected M_w (Table 1) for the 144 h sample will thus be about 60% less than M_w measured directly by SEC–LALLS. For the least degraded samples, this will have little influence.

3.3. Separation of high- and low-M_w fractions

The high- and low-M_w fractions from a sample degraded for 72 h were separated by ultrafiltration, and the filtrate and retentate were analysed by SEC–LALLS (Fig. 4). The elution profiles of the untreated and retentate samples are very similar, and the calculated molecular weights are almost equal ($M_w \sim 170\,000$). The amount of low-M_w molecules has clearly decreased, and is recovered in the filtrate. The filtrate also consists of some longer, ordered chains (about 15% of the total area), which can be observed in the chromatogram as a small front peak (shoulder). M_w for the whole fraction was 36 000 ($DP_w \sim 56$). This value is close to $DP_w \sim 60$, which has been reported as the lowest DP required for the formation of triplex structures (DP_c) for

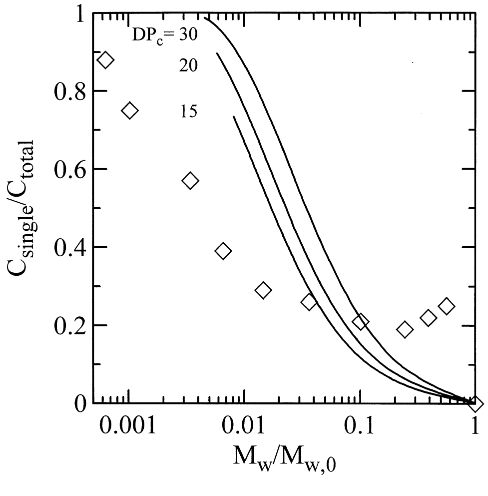

Fig. 5. Relative amount of disordered single-stranded fragments (C_{single}/C_{total}) as a function of the degree of depolymerisation ($M_w/M_{w,0}$) presented as experimental data (◇) and data from Monte Carlo simulations where DP_c was 30, 20 and 15.

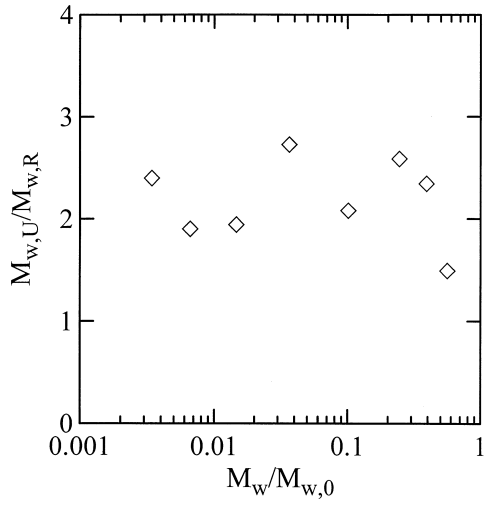

Fig. 7. The ratio between M_w of partially degraded samples before and after heat treatment at 100°C (15 mins) ($M_{w,U}/M_{w,R}$) as a function of the degree of degradation ($M_w/M_{w,0}$).

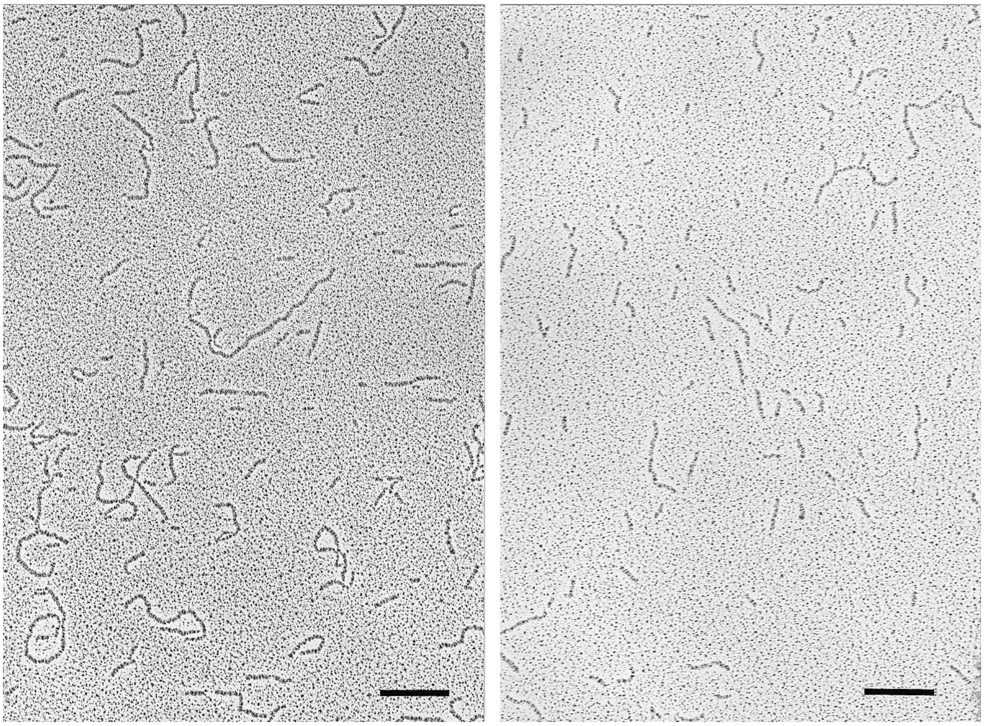

Fig. 8. Electron micrographs of a partially degraded scleroglucan sample (48 h) before (left) and after (right) the heat treatment (scale bar = 200 nm).

schizophyllan (Yanaki et al., 1983). Our result, on the other hand, implies that DP_c is lower, due to the appearance of triple-stranded chains in the filtrate.

We suggest that the low-M_w fraction consists of short, single-stranded chains, as previously shown for xanthan (Christensen et al., 1993, 1996a), κ- and ι-carrageenan (Hjerde et al., 1997). The molecules have a degree of branching of < 0.25, as shown by ^{13}C-NMR, and may possibly be potent biological response modifiers (Bohn and BeMiller, 1995).

3.4. Monte Carlo simulations

The critical DP for triplex formation (DP_c) can also be estimated by analysing the relative amount of single-stranded fragments (C_{single}/C_{total}) as a function of the degree of depolymerisation, in this case expressed as the ratio between M_w at a given degradation time and for the undegraded sample ($M_w/M_{w,0}$). The experimental data are then compared with those obtained from a Monte Carlo simulation of the degradation of a triple-stranded polymer with a set of values for DP_c (Christensen et al., 1993). The results are shown in Fig. 5 and suggest that $DP_c < 15$, which is lower than the results suggested by the SEC experiments. C_{single}/C_{total} appears to level off at about 0.2 at high M_w, except for the undegraded sample. The early release of low-M_w fragments is not readily explained by the release of randomly placed fragments with $DP \leq DP_c$. Despite this uncertainty, the value 0.2 appears to be a type of ‘‘background’’ which should be subtracted from the experimental data when estimating DP_c from C_{single}/C_{total}.

3.5. Heat treatment of partially degraded samples

Partially degraded scleroglucan samples may contain hidden cleavages in the triplex structures. A heat treatment above the melting temperature (T_m) may reveal these cleavages and result in a significant reduction in M_w after strand separation and subsequent renaturation (Fig. 6). Triplex structures with hidden chain cleavages are thermodynamically unstable (Stokke et al., 1993; Christensen et al., 1996b) and, by keeping the temperature just below T_m, we may expect partial chain separation followed by rearrangements and a concomitant decrease in M_w.

Fig. 7 shows the ratios between the molecular weight before and after the heat treatment at 100°C (15 min) as a function of the degree of degradation (expressed as the ratio between M_w and M_w of the initial sample). The reduction in M_w is of the order of 2 to 2.5 for all samples.

Electron micrographs of the samples before and after the heat treatment (Fig. 8) show that the average chain length has decreased, in agreement with the SEC–LALLS experiments. The absence of larger aggregates and macrocycles as observed after a denaturation/renaturation cycle (Stokke et al., 1993; McIntire et al., 1995; Kitamura et al., 1996) indicate that, by keeping the temperature lower than T_m during the heat treatment, one can produce triple-stranded molecules with more perfectly matched strands from a partially degraded sample.

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