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Analysis of carrageenans by enzymic degradation, gel filtration and ^{1}H NMR spectroscopy

Svein H. Knutsen & Hans Grasdalen

Division of Biotechnology, Laboratory for Marine Biochemistry, The Norwegian Institute of Technology, University of Trondheim, N-7034 NTH Trondheim, Norway

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Carrageenans from *Furcellaria lumbricalis* and *Eucheuma gelatinae* were degraded by κ-carrageenase from *Pseudomonas carrageenovora*. Higher and lower molecular weight fractions were separated by ethanol precipitation and further fractionated by GPC on serially connected Sephacryl S-100 and S-200, and BioGel P4, respectively, and investigated by 500 MHz ^{1}H NMR spectroscopy. Except for the lowest molecular weight oligosaccharide fractions from *E. gelatinae*, which contained di- and tetrasaccharides of κ-carrageenan, the lower molecular weight fractions of the carrageenans contained mixtures of neocarrabiose oligosaccharides with different content of D-galactose-4-sulphate. Both neocarrabiose and neocarrabiose-4-sulphate were detected on the non-reducing end. Varying with the source, the higher molecular weight fractions contained methyl, sulphate and other substitutions masking the general repeating backbone of neocarrabiose and neocarrabiose-4-sulphate.

INTRODUCTION

Carrageenan is the name of a family of sulphated galactans extracted from marine red algae consisting of alternating 3-linked β-D-galactopyranose (G-units) and 4-linked α-D-galactopyranose (D-units) or 4-linked 3,6-anhydro-D-galactose (A-units). A Greek letter nomenclature was introduced (Rees, 1969) and expanded (Zablackis & Santos, 1986) to identify the different repeating sequences found. However, this system has limitations (Myslabodski; 1990, Craigie, 1990), especially when naming oligosaccharides with a hybrid structure (see 'nomenclature for carrageenan oligosaccharides' below).

Depolymerizing enzymes with alginate lyase, agarase and carrageenase activity, have been isolated from marine organisms like marine molluscs and different marine bacteria. In a bacterial fermentation with carrageenan as the sole carbon source, a selected bacterium like *Pseudomonas carrageenovora* can be forced to produce a hydrolase specific to the carrageenan included in the growth medium (Bellion *et al.*, 1982).

A κ-carrageenase is an enzyme capable of hydrolysing the carrageenan polymers in sequences composed of neocarrabiose-4-sulphate (A-G4S). The products are A-G4S-oligosaccharides together with an enzyme-resistant fraction (ERF) enriched in the ι (A2S-G4S), μ (D6S-G4S), ν (D2,6S-G4S) and other structural characters or combinations, depending on the algal source. Characterization of ERF with ^{13}C NMR spectroscopy (Bellion *et al.*, 1983) has been very important in confirming the hybrid nature of carrageenans, and to detect 6-sulphated chemical precursors to A units and other substituents. Pioneer work (Yaphe & Baxter, 1955; Yaphe, 1959), based on measuring the liberation of reducing sugar from different κ-carrageenase treated red algal extracts, gave an estimation of the κ-carrageenan content in different sources. For the carrageenans from *Furcellaria lumbricalis* and *Endocladia muricata*, with some of the A-G4S (κ-) sequences replaced by non-sulphated A-G (β-) carrageenan, a reduced degradation related to the total content of G4S was found. κ-carrageenase degradation studies of *Furcellaria* carrageenan by GPC (Bellion, 1979) and HPLC (Knutsen, 1991*a*) confirmed that an enzyme-catalysed depolymerization occurs. Production of low molecular weight oligosaccharides of neocarrabiose were detected by TLC (Bellion, 1979), and found not only to consist of $(A\text{-}G4S)_n$ type sequences (Knutsen, 1991*a*). In the present work attempts were made to fractionate different *Furcellaria* preparations and a related *Eucheuma gelatinae* carrageenan (Greer &

Carbohydrate Polymers 0144-8617/92/$05.00

Yaphe, 1984) after an enzymic treatment. To the authors' knowledge, no work has been published regarding fractionating and characterization of both the ethanol-insoluble and -soluble part of a carrageenase digest. Suspecting the liberation of neocarrabiose oligosaccharides with a reduced hydrodynamic volume due to a lower content of 4-sulphate groups, a gel matrix with higher resolution in the low molecular region, i.e. BioGel P4 (Skjåk-Bræk *et al.*, 1985), compared to the traditionally used BioGel P6 (Rochas & Heyraud, 1981), was selected. The lack of a real separation of the higher molecular fraction on CL-Sepharose series encouraged the authors to use combinations of Sephacryl-100 and -200 as an alternative.

EXPERIMENTAL

Enzyme source

Pseudomonas carrageenovora (originating from the Yaphe collection) was grown in a 10-litre Chemap fermenter (Knutsen, 1991*b*). A high cell density was obtained by adding lactose (20 g/litre) to a slightly modified WY-medium (Weigl & Yaphe, 1966), using κ-carrageenan (Litex Lot 171505) (1·5 g/litre) to induce carrageenase production. After concentration with a 10 000 D membrane filter, κ-carrageenase was purified from the cell-free supernatant by the method of McLean and Williamson (1979).

Extraction and carrageenan source

Furcellaria lumbricalis was harvested in the Trondheimsfjord (27 June 1986). Dried and milled seaweed was pre-extracted with acid (Knutsen *et al.*, 1990) and extracted in distilled water with the pH adjusted to 7·5. The cold-water extract (25 °C) and the combined hot-water extracts (90 °C) were treated by thermostable amylase at 80 °C (Knutsen & Grasdalen, 1987). After precipitation with isopropanol, the carrageenan was fractionated into a soluble and insoluble part by leaching in a 0·1 M KCl solution (Stancioff & Stanley, 1969; Knutsen *et al.*, 1990).

A commercial sample of *Eucheuma gelatinae* carrageenan (EG) was obtained as an ethanol precipitate in KCl. The carrageenan had been produced at Hainan Island, China, by boiling the algae with water after a previous treatment with hot alkaline solution. This carrageenan sample was purified by milling, washing with hot 70% ethanol saturated with NaCl, hot 70% ethanol, dialysis against neutral water and freeze drying. Analysis with ^{1}H NMR showed a G4S to A ratio of ~0·29, little methyl and no signals from precursors or starch. No cold-water extract was used from this algae.

Additional commercial carrageenans from *Furcellaria* (Litex AP641) (AP) and *Eucheuma cottonii* (Litex Lot 171505) (EC) were used after ethanol-washing as above.

Enzymic degradation

For the preparative work only the two presumably most structural dissimilar fractions of the *Furcellaria* extracts, i.e. the hot-water extracted, KCl-insoluble (FLHI) and the cold-water extracted, KCl-soluble (FLCS) (Knutsen *et al.*, 1990) together with EG and AP were used. Samples were solubilized in 0·1 M NaCl containing 5 mM $NaHCO_3$ (Rochas & Heyraud, 1981), excess amounts of κ-carrageenase were added on days 1 and 3 and the reaction mixture was left for one week at 37 °C.

After this enzymic treatment, and prior to gel filtration, the 4 different samples were fractionated by adding 3 volumes of ethanol. The precipitates, representing the higher molecular weight or enzyme resistant (ERF) fractions, were recovered by centrifugation and re-solubilized in water, dialysed against distilled water and lyophilized. The remaining alcohol-soluble supernatants gave the ethanol-soluble oligosaccharide fractions after evaporation of the solvent and freeze drying. The yields of the ethanol-insoluble fractions were; 51, 53, 65 and 70% for FLHI, AP, FLCS and EG, respectively.

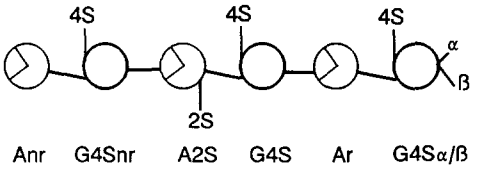
Gel filtration of enzyme treated samples

The ethanol-soluble oligosaccharide samples were fractionated on two serially connected BioGel P4 columns, each 26 mm × 850 mm. For analytical runs 15 mg, and for semipreparative runs ⩽200 mg carrageenan, were applied at room temperature with 0·05 M Na_2SO_4 as the eluent at a constant rate of 25 ml/h (Knutsen, 1991*a*). The degraded samples were typically solubilized in 10 ml Milli-Q water. Stirring in water with moderate heating to avoid destruction of the oligosaccharides, assured solubilization and prevented aggregation.

A second set of serially connected columns with Sephacryl S100 (900 mm) followed by Sephacryl 200 (1200 mm), operated under similar conditions, was used for a further fractionation of the ethanol-insoluble fractions. The system for naming the different fractions is S or P4, for the Sephacryl and the BioGel P4 system, respectively, with '*n*' denoting the fraction number obtained from the enzymic treated carrageenans, giving FLHI,S:*n* or AP,P4:*n*, etc. For desalting, dialysis against water or a similar column system filled with BiGel P2, eluted with Milli-Q water, was used depending on the molecular weight.

Nuclear magnetic resonance spectroscopy (NMR)

The 500 MHz ^{1}H NMR spectra were recorded at 90 °C with a Bruker WM-500 spectrometer using 32K data points. Sweep width was 6024 Hz and the spectra were



Scheme 1.

recorded with a pulse recycling time of 4·7 s. Chemical shifts (ppm) are referred to external Me_4Si via internal sodium 3-(trimethylsilyl) propionate-d_4 (TSP) (Knutsen & Grasdalen, 1992).

Nomenclature for carrageenan oligosaccharides

The nomenclature used (Knutsen & Grasdalen, 1992) (see Scheme 1) to designate the different residues and the positions of the sulphate groups in the oligosaccharides, is modified after Rochas *et al.* (1986). Where appropriate, a subscript indicating the particular proton is included. When the identity of the neighbour(s) in the chain do(es) not result in an observable shift at the field strength used, the nomenclature is 'R'' towards the reducing end and 'R' towards the non-reducing end. A question mark (?) replacing R or R' is used to stress that the identity of the neighbour is unknown. In most cases, when no possibility of misunderstanding is present, the added 'r' and 'nr' of Ar and Anr (or Gnr) are omitted.

RESULTS

Basic ^{1}H NMR assignments

The ^{1}H NMR assignments are based on 500 MHz spectra of neocarrabiose oligosaccharides recorded at room temperature (Knutsen & Grasdalen, 1992) and carrageenan spectra recorded at 80 °C (Welti, 1977). At 90 °C compared to 25 °C the resonance from residual water is shifted up-field from ~4·8 to 4·2 ppm, which is from the regions of H4 of G4S units to H6 of A units, respectively. The most down-field resonance (5·32 ppm, $J_{1,2}$ 3·9 Hz (where J is the coupling constant)) in the spectra arises from reducing end, i.e. R-G4S$_{H\text{-}1\alpha}$ residues. R-G4S$_{H\text{-}1\beta}$ and G4S$_{H\text{-}1}$ resonate at ~4·63 ppm ($J_{1,2}$ 7·9 Hz). Removal of the 4-sulphate group shifts the resonances of R-G$_{H\text{-}1}\alpha$ and R-G-R' up-field to 5·30 ppm and 4·60 ppm, respectively (Knutsen & Grasdalen, 1992). Other characteristics of the spectra (recorded at both 90 °C and 25 °C) from neocarrabiose-type oligosaccharides are the two sets of resonances from Anr$_{H\text{-}5}$-G4S-R' and Anr$_{H\text{-}3}$-G4S-R'. At 90 °C these protons resonate at 4·41 and 4·34 ppm, respectively. The removal of a 4-sulphate group of the neighbour residue introduces a down-field shift of approximately 0·02 ppm. This made it possible to use their resonance areas for detection and estimation of Gnr and G4Snr units in an oligosaccharide sample (see Fig. 1). Other characteristic signals are from H-1 of anhydrogalactose in A-G4S or A-G sequences at 5·11 and 5·09 ppm, respectively, and H4 of the G4S units at ~4·83 ppm. The complete assignments of the 4·3–5·4 ppm region of the ^{1}H NMR spectrum of a sample containing mixtures of oligosaccharides with a reduced content of 4-sulphate groups are given in Fig. 1.

Spectra of carrageenan molecules of a higher molecular weight look less complex since the resonances from end residues are decreased in their intensities. In Fig. 2(A) a typical spectrum of a carrageenan with a reduced content of 4-sulphate groups is shown. The signal at ~5·18 ppm has previously been tentatively assigned to ?-D6S$_{H\text{-}1}$-G-? whereas the broader signal at ~5·27 ppm is assigned to a ?-D6S$_{H\text{-}1}$-G4S-? sequence. It should be noted that the resonances occurring at 5·27 ppm in general are much broader than the other precursor H-1 signal at 5·19 ppm. This could be explained by another signal, from a yet undefined structure, being present in that region. The resonance from the precursor (D2,6S$_{H\text{-}1}$-G4S), to ι-carrageenan, would be expected to be found down-field from A2S$_{H\text{-}1}$, i.e. at ~5·5 ppm. Resonances in that region are in general not observed. The broad signal at 4·86–4·87 ppm, previously identified as an alkali labile shoulder in the 400-MHz spectra (Knutsen *et al.*, 1990) and assigned to

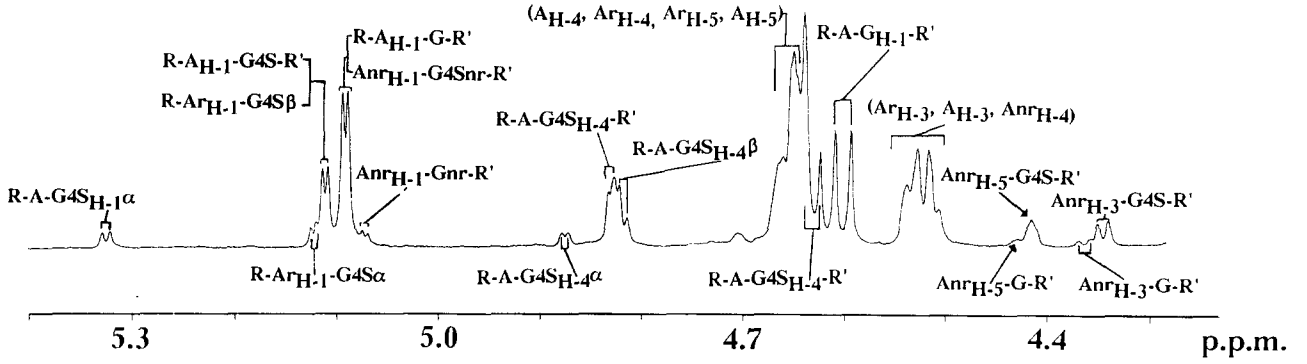


Fig. 1. Region of the 500 MHz ^{1}H-NMR spectrum of neocarrabiose-type oligosaccharides from *Eucheuma gelatinae* with a mixed A-G4S and A-G structure recorded at 90 °C.

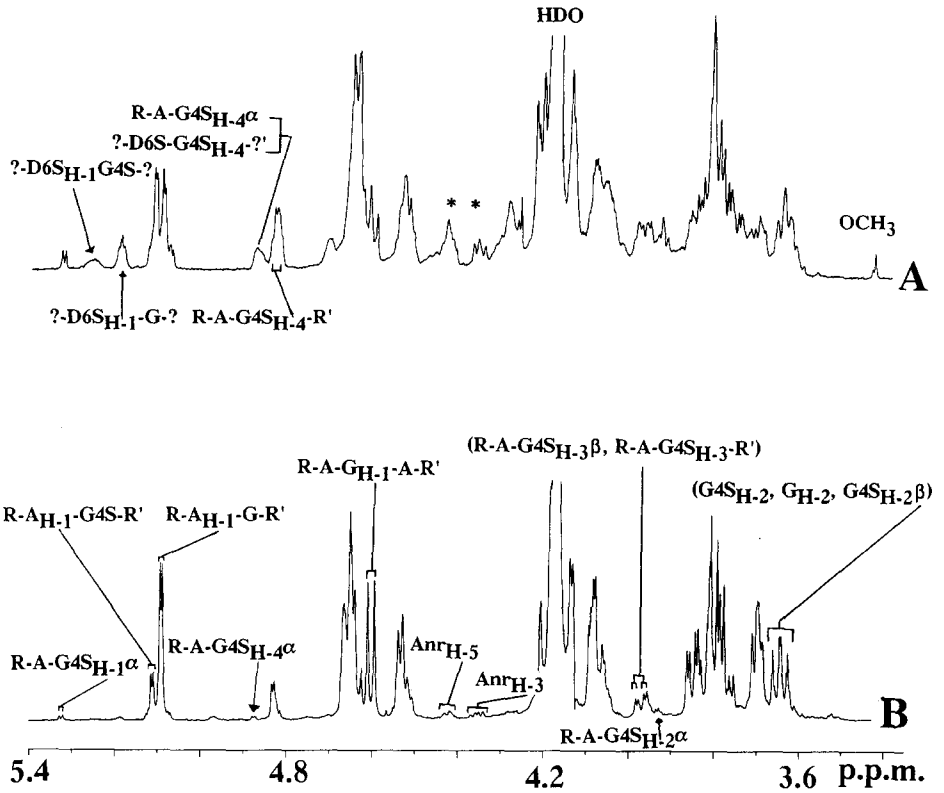


Fig. 2. Assignments for the characteristic resonances in the 500 MHz ^{1}H NMR spectra of κ-ase degraded carrageenans recorded at 90°C: (A) *Furcellaria lumbricalis* (FLHI,P4:2) and (B) *Eucheuma gelatinae* (EG,S:5). Asterisks refer to resonances from D6S units and arrows refer to spinning side bands from residual water.

?-D6S-G4S$_{H-4}$-? completely overlaps with the signal from R-A-G4S$_{H4}\alpha$, i.e. when estimating the quantity of ?-D6S-G4S-? type precursors in oligosaccharide samples, the integrated resonance intensity from R-A-G4Sα_{H-1} at 5·32 ppm should be subtracted.

The presence of methyl groups is normally detected by a sharp signal at ~3·45 ppm (Rochas *et al.*, 1989). However, for some special fractions, especially those containing a low content of 3,6-anhydrogalactose and a corresponding high content of 6-sulphate, the 3·45 ppm region may contain additional and complex signal patterns. These are probably reflecting non-regular sequences of residues with several substitutions.

Finally, some signals additional to those shown in Figs 1 and 2 are found in some fractions from both the hot- and cold-water extracts of *Furcellaria*. A signal found at ~5·0 ppm has a characteristic splitting of ~3·8 Hz. This is close to the value for $J_{H-1,2}$ galactose in the α-configuration, i.e. 3·9 Hz (Knutsen & Grasdalen, 1992), and may be tentatively assigned to unsubstituted 4-linked α-galactose (D units).

Estimation based on internal ratios

The calculation of the degree of polymerization (d.p.) is based on the ratio between the integrated areas of the resonances from all H-1 protons of 3,6-anhydrogalactose occurring in the 5·07–5·12 ppm region (totA) divided by the average area of the H-3 and H-5 of the non-reducing end 3,6-anhydrogalactose (Anr) (Knutsen & Grasdalen, 1992). By multiplying by 2, to correct for the fact that A units represent 50% of the units in the oligosaccharides, the formula for d.p. estimation is (totA/Anr) × 2. When extra signals occur in the actual regions, an estimate of d.p. can be made via the resonance of H-1 reducing ends at 5·32 ppm, taking into account the anomer-equilibrium.

The fraction of α-3,6-anhydro-D-galactose (f_A) of all α(1–4) units is calculated from totA divided by the sum of totA and the areas for other H-1 values of α-linked galactose units in the down-field region from 5·0 to 5·35 ppm. Based on this, the value of f_A in a pure κ-carrageenan molecule is 1·0. As noted previously, some extra signals, possibly due to D type units, occur at 5·0 ppm. The fraction of these residues (f_D) is estimated similarly by the intensity of the resonance at 5·0 ppm relative to all signals in the 5·0–5·35 ppm region. Since D6S precursor residues theoretically are the only remaining possible residues, except from starch which in general is removed, their corresponding fractions (f_{prec}) can be estimated by the formula: $1 - (f_A + f_D)$.

The characteristic resonances for H4 of D-galactose-4-sulphate, i.e. R-G4S$_{H-4}\alpha$, R-G4S$_{H-4}\beta$ and R-G4S-A-R' (totG4S), in the 4·8–4·9 ppm region can be used to determine the ratio between totG4S and totA. When the carrageenan samples contain molecules with a repeating

backbone of A and G4S (or G) units only, corresponding to $f_A = 1$, the ratio totG4S/totA gives an estimate of the true A-G4S : A-G ratio or the κ-carrageenan content. However, when the molecules contain D6S precursor units, replacing the A units, some corrections have to be made, since resonances of the sequence type ?-D6S-$G4S_{H\text{-}4}$-? occur together with R-A-$G4S_{H\text{-}4}\alpha$ at ~4.87 ppm (see Fig. 2, spectrum A). The signals from true κ-sequences, i.e. R-A-$G4S_{H\text{-}4}$-A-R′ and R-A-$G4S_{H\text{-}4}\beta$ type sequences occur up-field at 4·83 ppm. Taking this into account, the content of A in A-G4S sequences, i.e. the content of real κ-sequences compared to A-G (β-carrageenan) can also be estimated in these fractions. The area from R-G4Sα becomes important when the d.p. is low, and the contribution from the α-anomer can be obtained from R-A-$G4S_{H\text{-}1}\alpha$ at 5·32 ppm. It should be noted that several difficulties are met with when the degree of 6-sulphate substitution is higher than for 3,6-anhydrogalactose.

The content of 6-*O*-methyl (G6M) relative to total 3,6-anhydrogalactose is estimated by dividing one-third of the 6-*O*-methyl signal at 3·45 ppm by the totA, i.e. CH_3/A. The backbone of carrageenans belonging to the gelling families is considered to comprise a masked repeating structure of 3-linked galactose and 4-linked 3,6-anhydrogalactose. Therefore, by taking into account both 4-linked (D6S, A and the anomer signal assigned to D units) *and* 3-linked units (G4S or G), a more comprehensive picture of the total degree of methylation along the polymeric chains is obtained. These values for methyl (% CH_3) are calculated relative to all residues on a molar basis and given on the far right of Tables 1–8.

In Tables 1–4 the composition data for ethanol-soluble, κ-ase treated carrageenan fractionated on BioGel P4. Fraction numbers (identified in the chromatograms Fig. 3(a) and 3(b)) are given with the corresponding yields (weight %) of total carrageenan before enzymic treatment. F_κ were only determined for the highest molecular weight fractions from ethanol-soluble *Furcellaria* carrageenan. + refers to weak methyl signals with a CH_3/A ratio in the range 0·001–0·01 and values in parentheses (%CH_3) refer to the molar content of methyl relative to the whole carrageenan molecules.

In Tables 5–8, the compositional data for ethanol-insoluble (ERF), κ-ase treated carrageenans fractionated on Sephacryl 100-200 are shown. Poly refers to fractions with too small end signals for the determination of d.p. by NMR. For detailed explanations of parameters see Tables 1–4.

Gel filtration of oligosaccharides on BioGel P4

Characteristic of all the chromatograms of the 75% ethanol-soluble oligosaccharides (see Fig. 3(a) and (b)) is the broad negative refractive index signal towards the total volume of the column, caused by applying the samples dissolved in distilled water. A typical elution pattern on the BioGel P4 system represented by κ-ase treated *E. cottonii* carrageenan with a major repeating A-G4S structural character, is given in the upper part of Fig. 3(a). For this more or less ideal substrate, the result

Table 1. Alkali-extracted carrageenan from *Eucheuma gelatinae* (EG,P4:*n*)

Fraction no. (%)	d.p.	4S/A	f_A	CH_3/A (%CH_3)
1 (2)	20	0·77	1	0·13 (5)
2 (12)	15	0·52	1	+
3 (3)	10	0·52	1	No CH_3
4 (7)	4·3	0·91	1	No CH_3
5 (6)	2	1·01	1	No CH_3

No resonances from D units were detected and only the largest molecular weight fraction (EGP4:1) contained A2S, giving an A2S/A ratio = 0·27.

Table 2. Alkali-extracted commercial carrageenan from *F. lumbricalis* (AP641) (AP,P4:*n*)

Fraction no. (%)	d.p.	4S/A	f_A	CH_3/A (%CH_3)
1 (20)	12·8	0·79	1	0·04 (1·6)
2 (11)	8·2	0·63	1	0·01 (0·3)
3 (4)	5·9	0·68	1	No CH_3
4 (5)	4·3	0·81	1	No CH_3
5 (4)	3·2	0·70	1	No CH_3
6 (4)	2·3	0·68	1	No CH_3

Only fraction 1 (A2S/A = 0·02) and 2 (traces) had characteristic signals from A2S residues. No D units were detected.

Table 3. KCl-soluble hot-water extracted carrageenans from *F. lumbricalis* (FLHI:P4)

Fraction no. (%)	d.p.	4S/A (f_κ)	f_A	CH_3/A (%CH_3)
1 (12)	25	0·86 (0·54)	0·41	0·2 (4)
2 (10)	14·6	0·63 (0·53)	0·77	0·02 (0·8)
3 (7)	9·1	0·53 (No prec)	0·94	+
4 (5)	8·2	0·51 (No prec)	0·96	+
5 (9)	6·3	0·60 (No prec)	1	+
6 (3)	3·8	0·52 (No prec)	1	No CH_3
7 (3)	2	0·80 (No prec)	1	No CH_3

Only fraction 1 contained D units (f_Δ0·10).

Table 4. KCl-soluble cold-water extracted carrageenans from *F. lumbricalis* (FLCS,P4:*n*)

Fraction	d.p.	4S/A (f_κ)	f_A	f_D	CH_3/A
1 (13)	Poly	0·96 (0·50)	0·30	0·21	?[a]
2 (10)	Poly	0·89 (0·58)	0·54	0·03	?[a]
3 (12)	Poly	0·72 (0·60)	0·64	—	?[a]

[a] ? refers to the lack of identification of the resonances due to small signals and complex spectra in that specific region.

Table 5. Alkali-extracted carrageenan from *Eucheuma gelatinae* (EG,S:*n*)

Fraction no. (%)	d.p.	4S/AG	f_A	f_D	A2S/A	CH_3/A (%CH_3)
1 (13)	Poly	0·18	0·62	0·37	0·14	0·14 (4)
2 (13)	Poly	0·15	0·70	0·25	0·08	0·13 (5)
3 (7)	Poly	0·14	0·80	0·1	0·02	0·05 (2)
4 (15)	47	0·28	0·92	0·05	No A2S	0·02 (1)
5 (17)	28–32	0·28	1	No D	No A25	+
6 (3)	13–18	0·33	1	No D	No A2S	No CH_3
7 (1)	12–14	0·61	1	No D	No A2S	No CH_3

Table 6. Alkali-extracted carrageenan from *F. lumbricalis* (AP641) (AP,S:*n*)

Fraction no. (%)	d.p.	4S/AG	f_A	f_D	A2S/A	CH_3/A (%CH_3)
1 (0·2)	Poly	0·21	0·73 + S	0·27	NS	0·06 (2)
2 (3)	Poly	0·37	0·85 + S	0·14	0·03	0·11 (5)
3 (6)	Poly	0·39	0·89 + S	0·06	0·03	0·10 (5)
4 (12)	86	0·36	0·93 + S	0·03	0·02	0·10 (5)
5 (17)	64	0·33	0·97	NS	NS	0·09 (4)
6 (11)	30	0·37	0·97	NS	NS	0·09 (4)
7 (3)	7	0·42	0·86	0·12	NS	0·05 (2)

Starch is detected in the anomeric region (+S).
NS = not significant.

Table 7. KCl-insoluble hot-water extracted carrageenan from *F. lumbricalis* (FLHI,S:*n*)[a]

Fraction no. (%)	d.p.	4S/A	f_A	f_D	CH_3/A (%CH_3)
1 (12)	Poly	2·3	0·18	0·07	0·33 (3)
2 (9)	Poly	1·6	0·28	0·01	0·25 (4)
3 (13)	Poly	1·02	0·47	NS	0·14 (3)
4 (7)	27–40	0·48	0·78	NS	0·02 (1)
5 (9)	17–20	0·43	0·94	0·03	+
6 (6)	10–12	0·54	1	NS	No CH_3

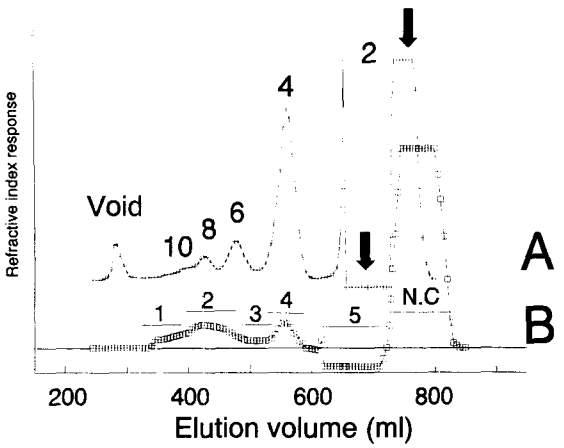
[a]The high content of ?-D6S-? (≫A) gave broad lines and induced difficulties in an accurate determination of the intensity of the ?-A-G4$S_{H\text{-}4}$-? signal (f_κ).

Table 8. KCl-soluble cold-water extracted carrageenan from *F. lumbricalis* (FLCS,S:*n*)

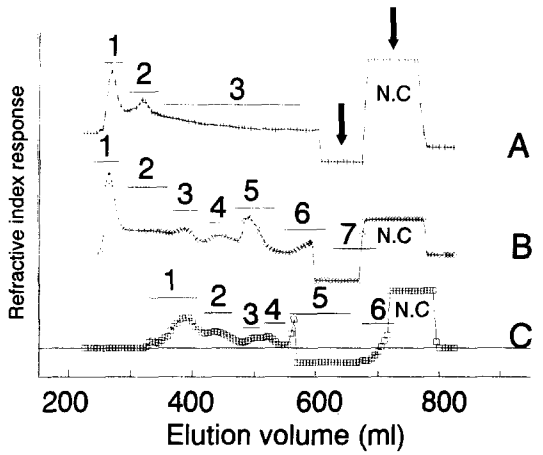
Fraction no. (%)	d.p.	4S/A	f_A	f_D	CH_3/A (%CH_3)
1 (9)	Poly	8·9	0·05	No D	0·56 (1)
2 (19)	Poly	4·8	0·09	No D	0·44 (2)
3 (25)	Poly	3·4	0·11	Trace	0·20 (1)
4 (11)	Poly	0·75	0·34	Trace	0·08 (1)

was a mixture of 4-sulphated neocarrabiose oligosaccharides $(A\text{-}G4S)_n$ with d.p. 2 (n = 1), 4, 6, 8, 10 and 12 (traces) and a small void fraction after enzymic treatment for one week. Similar chromatograms have been obtained from other commercial samples using the more open BioGel P6 support (Rochas & Heyraud, 1981). Fractions representative of the void of the P4 column contained 3,6-anhydro-D-galactose-2-sulphate (A2S-G4S), unidentified carrageenan precursors ?-G6S-? and 6-*O*-methyl. However, small amounts of A2S were also to be found in the oligosaccharide fractions larger than d.p. 4. (Knutsen, 1991*a*; unpublished). The occurrence of small fragments containing A2S or ι-type residues indicates that ι-carrageenan does not exist as separate molecules, but rather inside the κ-carrageenan molecules. This can be explained even with absolute linkage specificity of the κ-carrageenase used. A2S residues can be situated some linkages apart from the G4S-A linkage that is hydrolysed, producing some short chain oligosaccharides of neocarrabiose-4-sulphate containing A2S. In fact, the hexamer A-G4S-A2S-G4S-A-G4S is commercially available (Sigma catalogue) produced by κ-carrageenase treatment of carrageenan from *Chondrus crispus* (McLean, M.W., 1991, pers. comm.).

The yields of the ethanol-soluble fractions obtained from *Furcellaria* and *Eucheuma gelatinae* represent less than 50% of the original (see the Methods section), non-degraded carrageenan samples. These yields are low compared to the *E. cottonii* sample. From the elution profiles it is also evident that the distribution of the oligosaccharides, especially for the neutrally-extracted *Furcellaria* samples, are shifted towards the void, or the higher molecular weight exclusion limit of the column, and that no homologous series of oligosaccharides are produced. Very similar results were obtained by using Li_2SO_4 as the eluent. Substituting with $(NH_4)_2CO_3$ gave very different elution patterns due to aggregation of κ-carrageenan induced by NH_4^+ (Knutsen, unpublished). For the different samples, subsequent runs under identical conditions (i.e. pressure, flow, ionic strength, sample load, temperature, etc.) gave broad fractions/



(a)



(b)

Fig. 3. (a) Elution profiles of κ-ase degraded carrageenans on BioGel P4: A = *Eucheuma cottonii* (EC, whole hydrolysate) and B = ethanol-soluble oligosaccharides (EG-P4:*n*) from *Eucheuma gelatinae*. The y-axis and x-axis represent refractive index and elution volume, respectively, digitalized from the original plots. Fraction numbers (for EG only) are indicated by numbers above horizontal bars. Numbers above chromatogram A refer to d.p. of κ-oligosaccharides. N.C refers to fractions free from carbohydrate and the arrows indicate negative and positive off-scale measurements due to water and NaCl, respectively. (b) Elution profiles of κ-ase degraded *Furcellaria* carrageenans. Ethanol-soluble oligosaccharides are separated on BioGel P4: A = FLCS,P4:*n*, B = FLHI,P4:*n* and C = AP,P4:*n*.

peaks with retention volumes significantly different from that of the pure A-G4S type oligosaccharides. This is explained by the occurrence of *mixtures of* neocarrabiose oligosaccharides, with a different distribution of D-galactose-4-sulphate, within each of the polydisperse fractions (see Tables 1–4). An average d.p. of 5·3 is a result of mixtures of tetrasaccharides and hexasaccharides with a similar hydrodynamic volume as a consequence of different contents of D-galactose-4-sulphate residues. Common features for the oligosaccharide fractions from the different carrageenans with a reduced content of 4-sulphate, are that neocarrabiose-type oligosaccharides with D-galactose-4-sulphate occupying the reducing end (R-Ar-G4Sα/β) were produced. Only one possible exception (i.e. R-Ar-Gr), could be detected with significance (see below). However, with respect to the non-reducing end, both Anr-Gnr-R′ and Anr-G4Snr-R′ were found.

^{1}H NMR analysis of ethanol-soluble oligosaccharides from *E. gelatinae* (EG,P4:*n*)

The fractionation patterns of the ethanol-soluble oligosaccharides had no characteristic void fraction. This does not suggest an even distribution of 4-sulphated neocarrabiose units (A-G4S) as obtained from the *E. cottonii* carrageenan. Signals from -D6S-units were not detected in any fraction, and the spectral region characteristic for α-linked D-galactose is devoid of signals except from anhydrogalactose (f_A = 1).

The most distinct chromatographic peak (EG-P4:4) has a retention volume close to tetrasaccharide in the *E. cottonii* hydrolysate. NMR analysis (Fig. 4, spectrum A) showed a d.p. somewhat larger than 4 (4·3) and a G4S/A ratio smaller than unity (0·9), suggesting this fraction to contain ~90% A-G4S-A-G4S tetrasaccharides mixed with A-G-A-G4S-A-G4S and/or A-G4S-A-G-A-G4S hexasaccharides. The smallest fraction (EG,P4:5) contained Ar-G4Sα/β only.

The small fraction possessing the highest hydrodynamic volume (EG-P4:1, see Fig. 4, spectrum B) has characteristic signals at 5.11 and 5.09 ppm, typical for a carrageenan containing both A-G and A-G4S sequences. Matched with a small end signal (4·3–4·5 ppm), this gives a rough estimate of the d.p. ~20. The resonances at 5·32 and 4·91 ppm and the broad signal at ~4·85 ppm suggest the occurrence of ?-A2S-? residues. The resonance at 5·32 ppm, mainly arising from R-A2S$_{H\text{-}1}$-R′, has a significantly larger area than the resonance from R-A2S-G4S$_{H\text{-}4}$-R′ at 4·91 ppm, suggesting that some of the A2S residues do not occur in pure R-A2S-G4S-R′ (or ι-carrageenan) sequences. The shape of the broad signal in the spectral region typical for A2S$_{H\text{-}1}$ (5·32 ppm) indicates overlapping signals. The down-field shoulder cannot be assigned completely to R-A-G4S$_{H\text{-}1}$α alone. Shift effects induced by desulphation of the reducing-end neighbour in A2S-G4S sequences, i.e. A2S-G, may explain this observation.

^{1}H NMR analysis of ethanol-soluble oligosaccharides from *Furcellaria* (AP,P4:*n*)

The individual oligosaccharide distribution patterns for the three *Furcellaria* carrageenan samples (Fig. 3(b)) are somewhat different. The chromatograms of the ethanol-soluble fractions of the commercial, alkali-treated sample, AP,P4, had no void fraction in contrast to the ethanol-soluble oligosaccharides enzymically

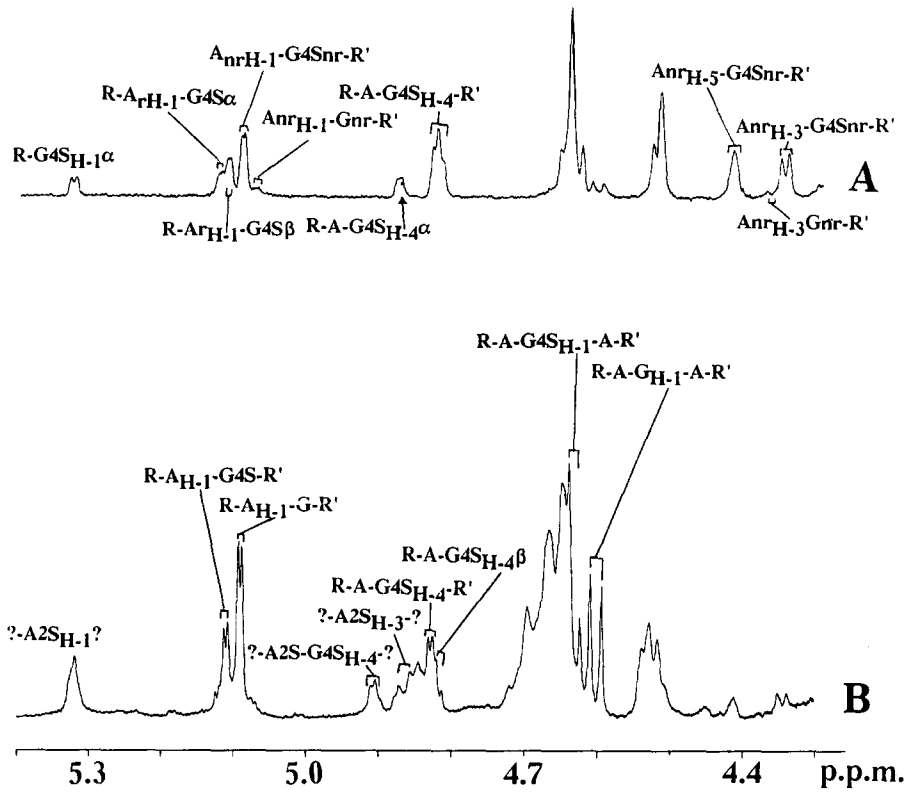


Fig. 4. Region of 500 MHz ^{1}H NMR spectra of neocarrabiose-type oligosaccharides from *Eucheuma gelatinae* recorded at 90 °C, where A is a 9 : 1 mixture (EG,P4:4) of tetrasaccharides (A-G4S-A-G4S) and hexasaccharides with an A-G-A-G4S-A-G4S or A-G4S-A-G-A-G4S structure and B is an oligosaccharide mixture (EG,P4:1) containing R-A-G4S-R', R-A-G-R', ?-A2S-G4S-A2S-? and ?-A2S-G-? sequences.

produced from neutral extracted *Furcellaria* carrageenans with a considerable part eluting in the void. The oligosaccharides from AP had all α-linked galactose as the 3,6-anhydro derivative, in A-G or A-G4S sequences only (f_A = 1). This was also the case for the lower (d.p. ⩽ 8–10) oligosaccharides of the FLHI,P4 fractions. However, the higher molecular weight fractions and all FLCS,P4 fractions contained both precursor (D6S) together with anhydro (A) units. The existence of A2S residues in any of the oligosaccharide fractions from *Furcellaria* is excluded by the lack of ι signals at 5·32, 4·91 and 4·85 ppm.

The G4S/A ratios, reflecting the apparent κ-carrageenan structural character (Welti, 1977) in the different fractions, are in the range 0·5–0·6 for the neutral extracted fractions possessing an f_A close to 1. Deviations are found in the fraction FLHI-P4:7, which consists of dimers mainly represented by Ar-G4Sα/β, and the precursor-rich fractions of FLCS-P4. However, taking into account only the G4S units belonging to A-G4S sequences, the values are approximately in the same range for these samples also. To illustrate the spectral information obtained, and to demonstrate some of the difficulties met, some spectra from different *Furcellaria*-P4-fractions are given in Fig. 5.

Gel filtration of ethanol-insoluble carrageenan on Sephacryl

The elution profile of the sample corresponding to the fully degraded *E. cottonii* carrageenan shown in Fig. 6(a) (same sample as used in Fig. 3(a)) shows that this system has a lower limit for separation corresponding to κ-oligosaccharides above d.p. 12 ± 3. To demonstrate the fractionation range, a partly-degraded sample from *E. cottonii* carrageenan is included in Fig. 6(a). In general, the ^{1}H NMR spectra of the higher oligosaccharide fractions from Sephacryl are considerably more complex than the spectra of the BioGel P4 fractions. The results obtained are summarized in Tables 5–8.

All *E. gelatinae* fractions (Table 5) have very low contents of precursors and starch. The sequence type A-G is dominant, and compared to *Furcellaria* carrageenan, a smaller part of the anhydrogalactose in R-A-G4S-R' sequences. The highest molecular weight fractions contain traces of D units (5·0 ppm) and a low content of A2S residues.

The spectra of the highest molecular weight fractions from the commercial *Furcellaria* sample, have a broad resonance centred at ~5·36 ppm, identified by ^{13}C

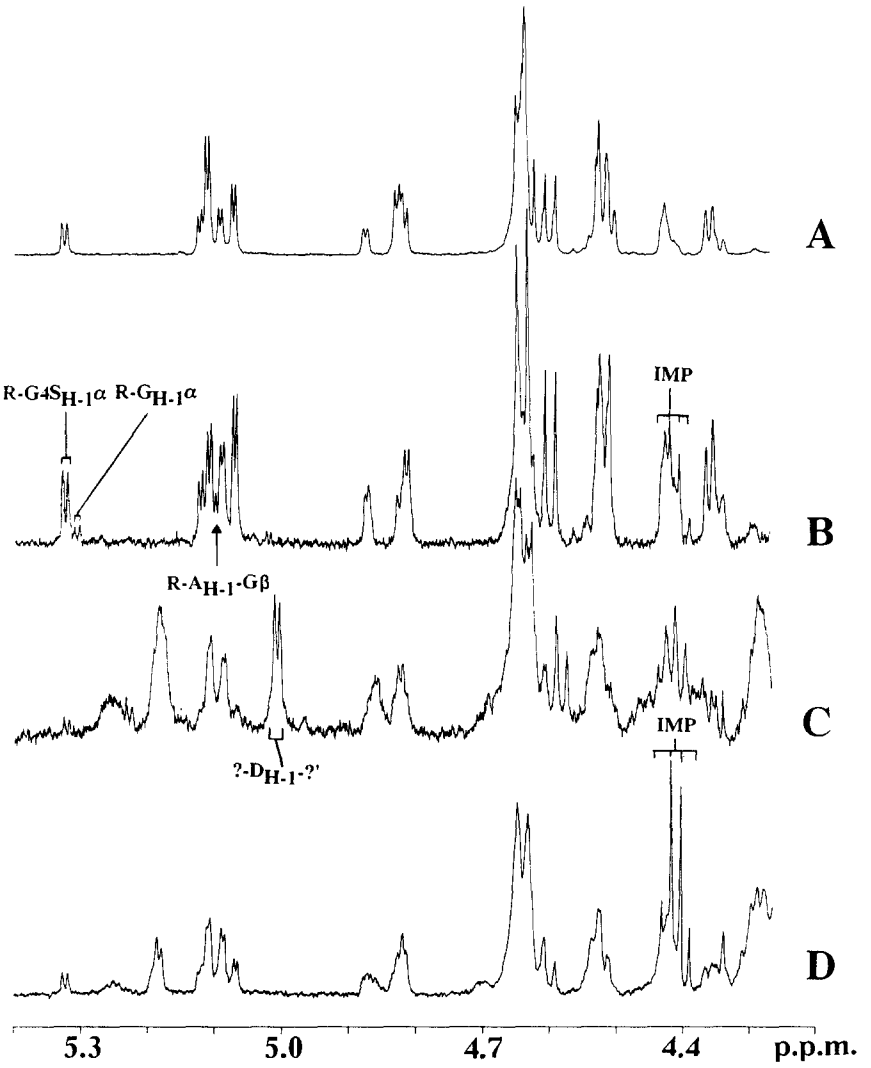


Fig. 5. Region of 500 MHz ^{1}H NMR spectra of oligosaccharides from *Furcellaria* recorded at 90 °C, where A is FLHI,P4:5, B is FLHI,P4:6, C is FLCS,P4:1 and D is FLCS,P4:3. Signals centred at about 4·42 ppm are due to impurities (IMP) from the recovering steps (see discussion).

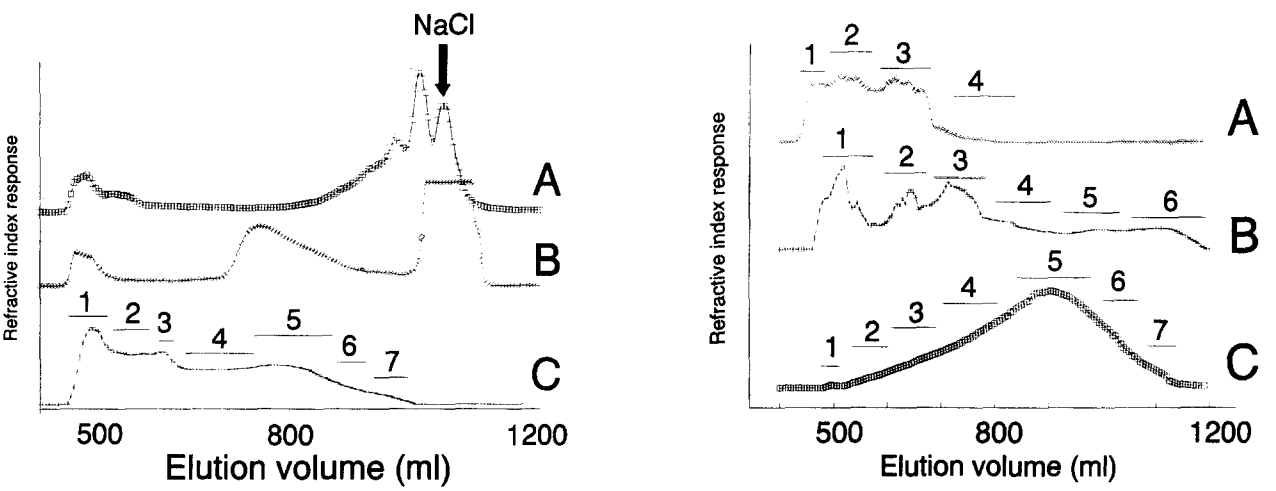


Fig. 6. (a) Elution profiles of κ-ase degraded carrageenans on serially connected Sephacryl-100 and -200, where A is completely degraded EC (same as Fig. 3(a), A), B is short-time degraded EC and C is ethanol-insoluble ERF-fraction of *E. gelatinae* (EG,S:*n*). See legend of Fig. 3(a). (b) Elution profiles of ERF fraction of κ-ase degraded *Furcellaria* carrageenans. Ethanol-insoluble carrageenan separated on serially connected Sephacryl-100 and -200, where A is FLCS,S:*n*, B is FLHI,S:*n* and C is AP,S:*n*.

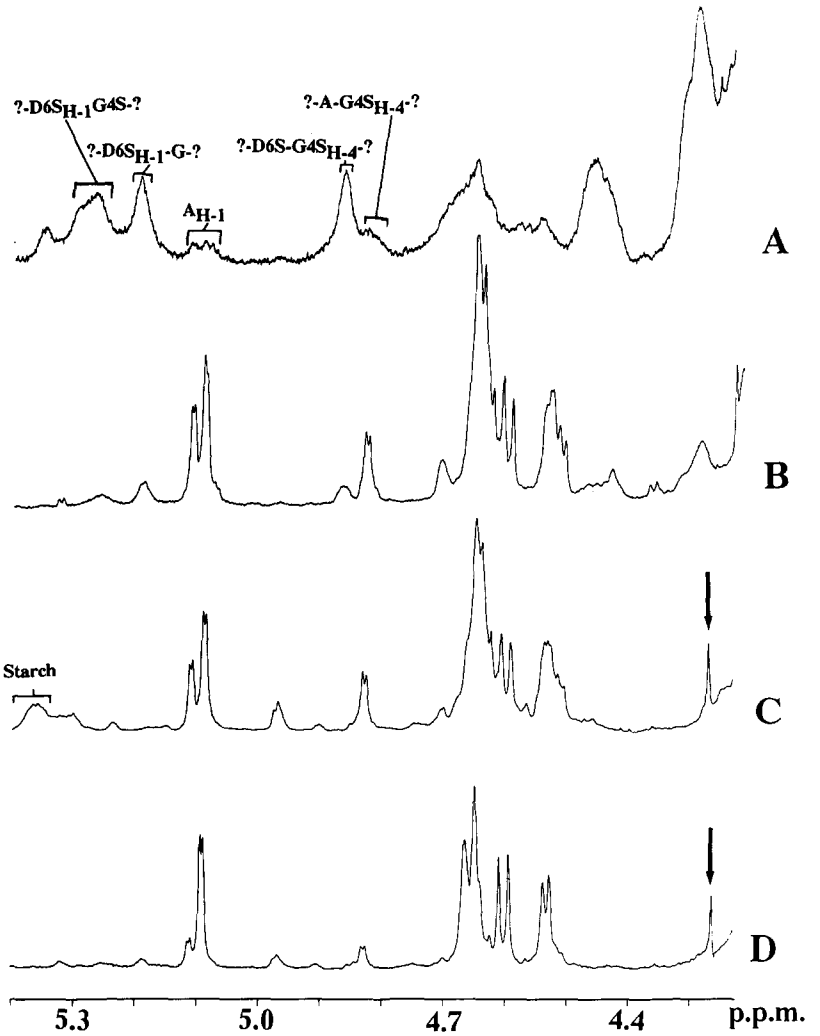


Fig. 7. Region of 500 MHz ^{1}H NMR spectra of high molecular weight carrageenan fragments recorded at 90°C, where A is FLCS,S:2, B is FLHI,S:4, C is AP,S:2 and D is EG,S:3. Arrows indicates spinning side band from residual water.

NMR as starch (data not shown). As for the EG,S the highest molecular weight fractions contained some D units. Additional resonances at 5·24 and 5·15, not exactly coalescing with the 'normal' precursor signals, and resonances from ?-A2S-?, occur slightly above noise level. The content of these 'impurities' can be calculated from the formula: $1 - (f_A + f_{\alpha\text{-gal}})$. However, the main part of all fractions with AP641 origin is predominantly composed of carrageenan with alternating A and G units, with and without sulphate in position 4 (G or G4S).

The highest molecular weight fractions from both the FLHI and all FLCS fractions of *Furcellaria* carrageenan have a high content of precursors and a corresponding low content of A units. Only the lowest molecular weight KCl-insoluble fractions (FLHI-S:5,6) have a structural character that could be described as a repeating backbone of A-G4S or A-G. In the precursor-rich fractions the major part of the G4S units occurs in ?-G6S-G4S-? type sequences, giving very high G4S/A ratios due to the low content of A units. A2S units are definitively not present in the more simple spectra (see FLHI-S:4 in Fig. 7). In these spectra the lack of a ?-A2S$_{H-4}$-G4S-? signal at 4·91 ppm, or even a shoulder on the resonance of ?-D6S-G4S$_{H-4}$-? (4·86–4·87 ppm), may exclude the content of A2S-G4S sequences of the conventional *ι* type. The broad, ill-defined resonance in the 5·34–5·36 ppm region is probably from some *α*-linked galactose or glucose from residual starch. A possible assignment to H-1 of A2S has to take into account that these residues have to be part of ?-A2S-G-? sequences, but this is highly speculative due to the broad peaks.

The content of 6-*O*-methyl

In all fractions the content of methyl is calculated relative to H-1 3,6-anhydrogalactose which in general has signals separated from others in the spectra. A recalculation relative to total carrageenans gave values in the range 0–5% on a molar basis, with the highest content in the highest molecular weight fractions.

DISCUSSION

It is evident from the chromatograms that the alkali treated *Furcellaria* sample (AP), having the highest content of A-G4S type sequences and $f_A \sim 1$, is the most suitable substrate for the κ-ase enzyme next to *E. cottonii* carrageenan. Both the ethanol-soluble and the ethanol-insoluble ('ERF') part, have virtually no void fraction and the enzyme's mode of action can be described as a partial degradation of the whole molecule population. This may be explained if A-G4S (κ) type sequences are somewhat equally distributed along the alkali-treated polymer. The possibility of the occurrence of long A-G4S repeating blocks or separate molecules possessing A-G4S or A-G structural character, is not likely to exist; no bimodal distribution in the chromatograms or fractions pure, or close to pure, in A-G4S or A-G structural character are found.

Based on the findings that both Anr-Gnr-R′ and Anr-G4S-R′ non-reducing end sequences are found, and that only R-Ar-G4Sr but hardly R-Ar-Gr reducing end types are detected, some information is provided with respect to the mode of the enzymic attack. The κ-ase enzyme catalyses hydrolysis of the central G4S-A linkage of the R-A-G4S-A-G4S-R′ and the R-A-G4S-A-G-R′ type but not in the R-A-G-A-G4S-R′ or R-A-G-A-G-R′ type sequences. Additional information based on 500 MHz ^{1}H NMR analysis of enzymically-produced oligosaccharides from *Furcellaria* recorded at different temperatures (data not shown), showed that also oligosaccharides of the type Anr-Gnr-A-G-A-G4S-R′ or similar are produced, indicating that sulphation in position 4 of the galactose proceeding the linkage is necessary for the enzymatic hydrolysis. However, to confirm this, better fractionation procedures, not based only on hydrodynamic volume or total charge, have to be developed.

The remaining two *Furcellaria* samples gave more heterogeneous fractions after enzymic treatment and gel filtration. The content of A units varied from almost not detectable ($F_A = 0{\cdot}05$ for FLCS-S:1) to a value ($F_A = 1$) which is representative of a repeating structure of A-G or A-G4S. The larger fragments obtained from the KCl-insoluble samples contain residues from both finished (A) and precursor (G6S) type carrageenan. This indicates that these two characteristics are found in the same molecules as indicated by the broad KCl-precipitation curves obtained with neutral extracted *Furcellaria* carrageenan (Smidsrød *et al.*, 1967).

Even with some integration problems in the ^{1}H NMR spectra due to broad peaks and problems with baseline determination in the precursor-rich fractions, some information about the relative proportions of the two proposed precursor sequences ?-G6S-G4S-? (μ) at 5·27 ppm and ?-G6S-G-? (γ) at 5·19 ppm is obtained (data not shown). The ratio between the intensity of the resonances at 5·27 and 5·19 ppm is in the range of 0·45–0·62 and 0·30–0·37 in the BioGel and the Sephacryl fractions, respectively. The precursor-rich fractions have a ratio between G4S and A units which is substantially higher than the ratios found for completely alkali-treated or non-precursor-containing *Furcellaria* carrageenan. This indicates that sulphation in position 4 of the 3-linked unit occurs at an earlier stage than the sulpho-eliminase step forming 3,6-anhydrogalactose (A) from 6-sulphated, 4-linked precursors (G6S). This hypothesis is supported by the fact that the f_κ, i.e. the ratio between ?-A-G4S$_{H\text{-}4}$-? sequences and the total content of A$_{H\text{-}1}$, in all fractions except for the highest molecular weight fractions (FLCS-S) of the KCL-insoluble sample, are in the range 0·36–0·64. Craigie and co-workers (Wong & Craigie, 1978) proposed that sulphation in position 4 of the 3-linked D-galactose units was one possible requirement for the in-vivo formation of 3,6-anhydrogalactose. Another KCl-soluble fraction of a precursor-rich *Furcellaria* sample harvested on 28 March (same year) (Knutsen *et al.*, 1990), early in the light-induced growth season, had all A units in A-G4S sequences.

Comparing these neutral-extracted samples with the alkali-treated sample, the latter has a lower content of A-G4S sequences in ERF and a higher content of A-G4S sequences in the enzymic-produced oligosaccharides. Discussing this apparent contradiction one should be aware that a treatment with hot alkali has no sequence or substitution specificity with respect to 4-sulphate. Formation of anhydrogalactose could be induced by unspecific conversion of D6S units in regions where the sulpho-eliminase enzyme has no affinity, producing an artificially high content of A-G4S sequences not found in the neutral extracted fractions.

In contrast to the earlier pioneer work (Greer & Yaphe, 1984), the present authors were not able to produce any A-G4S (κ)-free fractions upon κ-ase treatment of *E. gelatinae* carrageenan. In the spectra published (Greer & Yaphe, 1984), resonances above noise level in the 73–75 ppm region of the ^{13}C NMR spectra, and small absorbances in the 840 cm^{-1} in the IR-spectra of the actual ERF-fraction, before and after alkali treatment, may indicate that the fraction was not completely free from A-G4S type sequences. Finally, it should be noted that the origin of the two carrageenan samples is different and that ^{1}H NMR is much more sensitive than ^{13}C NMR. Greer and Yaphe (1984) found on a qualitative basis, without extensive analysis, that the ethanol-soluble fraction, which represented 34% of the original carrageenan sample, contained oligosaccharides of the A-G4S and A-G4S-A-G4S type. Indeed, this is also the case in the present study for the smallest fractions, but the higher oligosaccharides definitively contain molecules with a mixed A-G and A-G4S structure. The occurrence of κ-tetrasaccharides and the findings that only G4S occupy the reducing ends implies that at least some of the A-G4S sequences occur with minimum

block length equivalent to 6 sugars. In *Furcellaria* carrageenan these two sequences are probably less separated but not occurring in strictly alternating sequences. Some further answers will be obtained by ^{13}C NMR (data not shown), which is more sensitive to shifts induced through bindings.

The occurrence of A2S residues in some *E. gelatinae* fractions supports the earlier indications (Greer & Yaphe, 1984) that this residue was present in their samples. However, speculation based on a commercial grade sample should be avoided. This should also be considered when discussing the small content of A2S units found in some of the *Furcellaria* carrageenan fractions originating from the commercial AP sample. Furthermore, the uncommon signal encountered in the 5·0 ppm region, assigned to α-linked galactose, demonstrates that the carrageenans may have some irregularities opposed to the idealized repeating structure. The actual chemical shift occurs in the fractions representative of the highest molecular weight fractions and may be caused by some special sequences combined with another uncommon residue or substituent. However, strictly speaking, these D-type residues have not been proven to be part of the carrageenan molecules. Furthermore, even though it was not detected here by ^1H NMR, 3-linked 6-sulphated β-linked galactose units (G6S) have been found in carrageenan fractions from *Furcellaria* of different origins (Usov & Arkhipova, 1981).

Some other signals also occur in the spectra. These are not related to the signal at 5·0 ppm in intensity and even with two-dimensional techniques (COSY and ^{13}C-^1H NMR correlated spectroscopy) the authors have not been able to assign them to residues or substituents belonging to the carrageenan structure. They are not present in the spectra of the starting carrageenan material, and are rather impurities from the recovering steps than completely new, uncommon products produced during the enzymic treatment. These impurity signals, represented by at least 3 independent sets of corresponding resonances, become significant when the amount of carbohydrate is lowered and are finally dominant in fractions from the column with no significant amounts of carrageenan. The quartet ($J_{2,3}$ = 7·1 Hz) centred at ~4·42 ppm (see Fig. 7), linked in intensity to a possible triplet at ~1·36 ppm (spectral region not shown), and the doublet at ~1·31 ppm are possibly due to silicone and some deoxy sugar component, respectively. The latter, and definitively the resonance at 3·71 ppm, may originate from softening-additives in the dialysis bags used for desalting. The magnitude of the impurity signals was reduced when introducing several washings of the dialysis bags before using, and in most cases completely removed when desalting was based on gel-filtration with BioGel P2.

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