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Complete assignment of ^{1}H and ^{13}C NMR spectra of *Gigartina skottsbergii* λ-carrageenan using carrabiose oligosaccharides prepared by enzymatic hydrolysis

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Abstract—λ-Carrageenan extracted from *Gigartina skottsbergii* tetrasporophyte was completely digested by a purified *Pseudoalteromonas carrageenovora* λ-carrageenase. The main digestion products were fractionated and analysed by ^{1}H and ^{13}C NMR spectroscopy. All the oligosaccharides observed belong to the neo-carrabiose oligosaccharide series indicating that the λ-carrageenase cleaves the β-(1→4) glycosidic bonds. ^{1}H and ^{13}C NMR spectra recorded on oligomers from DP 2 to DP 8 were fully interpreted allowing unambiguous assignment of the λ-carrageenan spectra. Besides the typical oligo-λ-carrageenans, we have also characterised a heptasulfated tetrasaccharide which demonstrates the random over-sulfation along the chain of *G. skottsbergii* λ-carrageenan.
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Keywords: λ-Carrageenan; Carrageenase; *Gigartina skottsbergii*; Hybrid structure

1. Introduction

Carrageenans are sulfated galactans occurring in the cell wall of marine red seaweeds (Rhodophyta). They are major components of the matrix involved in the construction of the cell-wall architecture.[1,2] The exceptional physico-chemical properties of these polysaccharides are widely exploited as thickening and gelling agents in various structural and functional applications.[3–5] This large family of hydrocolloids shares the same backbone structure, which consists of linear chains of 3-linked β-D-galactose (G-units) and 4-linked α-D-galactose (D-units). Carrageenans are classified according to the number and position of sulfated (S) groups, and by the occurrence of 3,6-anhydro-bridges in the α-linked residues (DA-units) found in gelling carrageenans.[6–8] For example, the three most commercially exploited carrageenans, namely kappa- (κ, DA–G4S), iota- (ι, DA2S–G4S) and lambda- (λ, D2S,6S–G2S) carrageenans, differ by the presence of one, two and three sulfate groups per repeating disaccharide unit, respectively.

It is now well accepted that carrageenans have very heterogeneous chemical structures, depending on the algal sources, the life stages (i.e., gametophyte vs tetrasporophyte) and the extraction procedures of the polysaccharides. This structural complexity is ascribed to the occurrence of a mixture of carrageenans in extracts as well as the combination of typical carrabiose units distributed along the polysaccharide chains giving rise to hybrid carrageenans.[1,8,9] Consequently, the terms κ- and ι-carrageenan usually describe carrageenans composed essentially, but not only, of κ- and ι-carrabiose patterns. The amount and the chemical structure of carrabiose variants inserted in κ- and ι-carrageenan chains modulate their physico-chemical behaviour. One can mention, for example, that the occurrence of biosynthetic precursors, mu- (μ, D6S–G4S) and nu- (ν,

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D2S,6S–G4S) carrabiose units in κ- and ι-carrageenan chain, respectively, hinders the gelification.[10,11] Other carrabiose associations have been also recently highlighted such as the κ/ι-(DA–G4S/DA2S–G4S) carrageenan hybrids (or κ2-carrageenan) in several species of the Gigartinaceae[12–14] and the κ/β-(DA–G4S/DA–G) carrageenan copolymer found in *Furcellaria* sp. and *Euchema gelatinae*.[15,16]

Unlike κ- and ι-carrageenans, fine structural analysis and, as a consequence, studies dealing with the relationship between structure and functional properties of the non-gelling λ-carrageenan are poorly documented. Nevertheless, it is assumed that λ-carrageenan also contains hybrid structures made of combinations of additional carrabiose units such as the ξ-, π- or θ-carrabioses.[17–19] The difficulties encountered during chemical as well as spectroscopic investigations of this carrageenan are usually due to the high viscosity of the macromolecule. The state of the art concerning the analysis of carrageenan by NMR spectroscopy has been recently reviewed.[20] To summarize, for λ-carrageenan, only the chemical shift of the α-anomeric proton of the D2S,6S and the attribution of the ^{13}C NMR spectra, without using 2D experiments, are currently available.[20–22]

Carrageenases, the enzymes which cleave specifically the glycosidic linkage of carrageenans, are powerful tools for structural investigation of carrageenan; because: (i) they reduce drastically the viscosity of a concentrated solution of carrageenan and; (ii) the integrity of the native chemical structure is well preserved.[9,23] Carrageenases κ- and ι- have been cloned and the pure recombinant enzymes are available in large amounts for structural investigations.[24–26] λ-Carrageenase activity has also been identified and purified from the marine bacterium *Pseudoalteromonas carrageenovora*.[27,28] Though a large amount of recombinant λ-carrageenase is not yet available,[29] the native pure enzyme can be prepared in amounts allowing fine biochemical investigations as well as structural analysis of λ-carrageenan.

In an attempt to assign precisely ^{1}H and ^{13}C NMR spectra of λ-carrageenan, we have prepared λ-carrabiose oligosaccharides by digesting *Gigartina skottsbergii* λ-carrageenan with purified *P. carrageenovora* λ-carrageenase. The complete characterisation of oligo-λ-carrageenans allowed us to unambiguously assign all the ^{1}H and ^{13}C NMR signals of the polymer and to demonstrate the hybrid nature of this λ-carrageenan.

2. Experimental

2.1. Production and purification of oligosaccharides

The λ-carrageenase was extracted from *P. carrageenovora* (strain ATCC 43555) and was purified according to a modified protocol of Greer.[27] Briefly, the bacterium was grown (20 °C, 48 h) in the Y-2 modified medium (5 L) containing λ-carrageenan (86%, CP-Kelco, GENU® 7055, 10 g) according to Weigl and Yaphe.[30] After centrifugation, the cell-free supernatant was concentrated five times by tangential ultrafiltration (Pellicon system, 10 kDa, Millipore). The proteins were then fractionated by ammonium sulfate precipitation. The λ-carrageenase activity was recovered between 30% and 70% ammonium sulfate precipitate by centrifugation. Pure fractions of λ-carrageenase were obtained after elution of the sample on a Phenyl Sepharose 6 Fast Flow column (200 mL, Amersham) using a linear decreasing gradient of ammonium sulfate (30→0% during 440 min at 1 mL/min rate). Pure fractions as seen from SDS PAGE (98 kDa) were pooled and stored at 4 °C.

λ-Carrageenan (0.5% (w/v) in $NaNO_3$ 0.1 M, pH 7.5), purified from the tetrasporophytic plants of *G. skottsbergii* (86%, CP-Kelco, GENU®7055) was incubated at 30 °C with pure λ-carrageenase (17 μL/mL substrate). The extent of degradation was monitored by high performance anion exchange chromatography (HPAEC Dionex) as illustrated in Figure 1. The hydrolysate (20 μL) was injected on an AS11 analytic column (4 × 250 mm Ion Pac® Dionex) coupled with an AS11 guard column. The elution was conducted at a flow rate of 0.5 mL/min with a NaOH (280 mM) step gradient (0–5 min: 3–5%, 5–6.5 min: 5–30%, 6.5–15 min: 30–57.5%, 15–26 min: 57.5–100%) monitored by a GP40 Gradient Pump (Dionex). Oligosaccharide elution was detected by conductivity with an ED40 (Dionex) equipment after a 300 mA current suppression with an ASRS ultra II-4 mm (Dionex) equipment. A chromeleon-Peak Net software was used for data acquisitions. Complete degradation was reached after 24 h (unchanged chromatogram after new addition of enzyme).

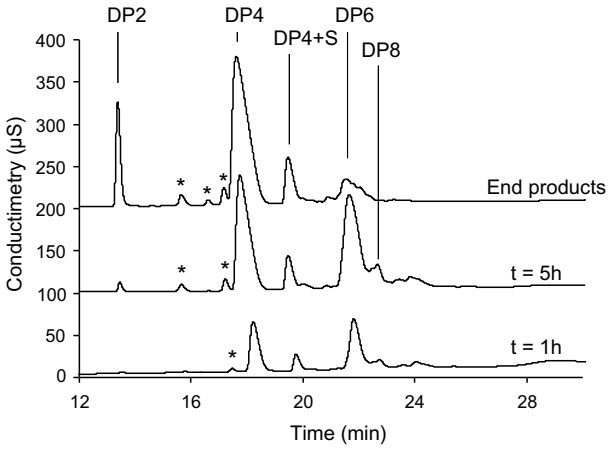

Figure 1. Time course extent of degradation of λ-carrageenan from *G. skottsbergii* incubated with the *P. carrageenovora* λ-carrageenase followed by HPAEC. Peaks marked with an asterisk correspond to oligosaccharides not investigated in this study.

DP 2 and DP 4 oligosaccharides were obtained after 24 h incubation, while in the case of DP 6 and DP 8, which are intermediate products of the λ-carrageenase digestion, the degradation was stopped after 5 h by boiling the medium (Fig. 1). The oligo-λ-carrageenans were size-fractionated by low-pressure gel-permeation chromatography. The digestion mixtures (80 mg/2 mL) were injected into a Pharmacia Superdex 30 prep grade (600 × 26 mm i.d.) column and eluted with 50 mM NH_4HCO_3 at a flow rate of 102 mL/h (pump P-500, Amersham Biosciences) at 18 °C. Elution was followed with a refractive index detector (Spectra System RI-50, Thermo Separation products). Fractionated samples were concentrated by rotary-evaporation, freeze dried and stored at 4 °C. Because the ammonium carbonate salt was removed during the freeze-drying, no further desalting experiments were found necessary at this stage.

The DP 2 fraction was pure after a single gel-permeation fractionation. The DP 4, DP 6 and DP 8 oligosaccharides were co-eluted with minor amounts of oligosaccharides having probably the same DP and/or different sulfation patterns. The oligosaccharides were then successfully separated at semi-preparative scale by HPAEC as follows: The oligosaccharide fraction (10–50 mg/mL; 50 μL) was injected on an AS11 semi-preparative column (9 × 250 mm, Ion Pac®, Dionex) coupled with an AS11 guard column. The sample was eluted at a flow rate of 1.5 mL/min with a NaOH (280 mM) step gradient monitored by a GP40 Gradient Pump (Dionex). For the DP 4 purification, the gradient was 0–11 min: 50–71.2%, 11–12 min: 71.2–100%. The DP 4 and the DP 4 + S were observed at a retention time of 9 and 11.5 min which corresponds to about 190 and 210 mM NaOH, respectively. In the case of the DP 6, the NaOH gradient used was: 0–11 min: 50–78%, 11–20 min: 78–91.1%, 20–21 min: 91.1–100%. The DP 6 fraction was eluted after 14 min corresponding to a NaOH concentration of 230 mM. For DP 8, the gradient was: 0–4 min: 50–70%, 4–7.9 min: 70–75%, 7.9–10 min: 75–79%, 10–15.5 min: 79–85%, 15.5–16 min: 85%, 16–16.5 min: 85–100%. DP 8 was observed at 15.5 min, equivalent to 240 mM NaOH. During the collection, the samples were neutralised with AcOH (0.4 M), then dialysed (Spectra/Por® CE, MWCO 500), concentrated and freeze dried.

2.2. NMR spectroscopy

Prior to NMR analysis, the molecular weight of the polymer was reduced by grinding 1.5 g of λ-carrageenan for 24 h at 40% of the maximal speed with a MM200 ball miller (RETSCH). Polysaccharide and purified oligosaccharide samples were exchanged twice in D_2O, and redissolved in D_2O (~99.7%) at a 10 mg/mL concentration.

^1H NMR spectra were recorded with a BRUKER Avance DRX 500 spectrometer equipped with an indirect 5 mm gradient probehead TXI $^1H/^{13}C/^{31}P$, at a probe temperature of 25 and 70 °C. Chemical shifts are expressed in ppm with reference to the external standard trimethylsilylpropionic acid (TSP). By comparison to the DSS reference, recommended by IUPAC, TSP has a chemical shift of δ −0.18 (^{13}C) and δ −0.017 (^1H) which allows the use of TSP as an alternative for DSS. Use of TSP or DSS instead of tetramethylsilane (TMS), results in chemical shifts of 2.5 ppm larger for all common carrageenan types as reported by van de Velde et al.[20]

Non-exchangeable proton and carbon assignments and sugar sequences of oligosaccharides were obtained from 1D (1H, *J* mode) and 2D experiments (COSY-DQF, ^1H–^{13}C HMQC, ^1H–^{13}C HMBC, *J* delta) using the conventional pulse program provided by Bruker. ^1H NMR (500 MHz) spectra of the polymer and the oligomers of λ-carrageenan were recorded at 70 and 25 °C. At 70 °C (Figs. 2 and 3), spectra were recorded using 32 K data points and the parameters were as follows: pulse angle, 30°; sweep width, 10,330 Hz; acquisition

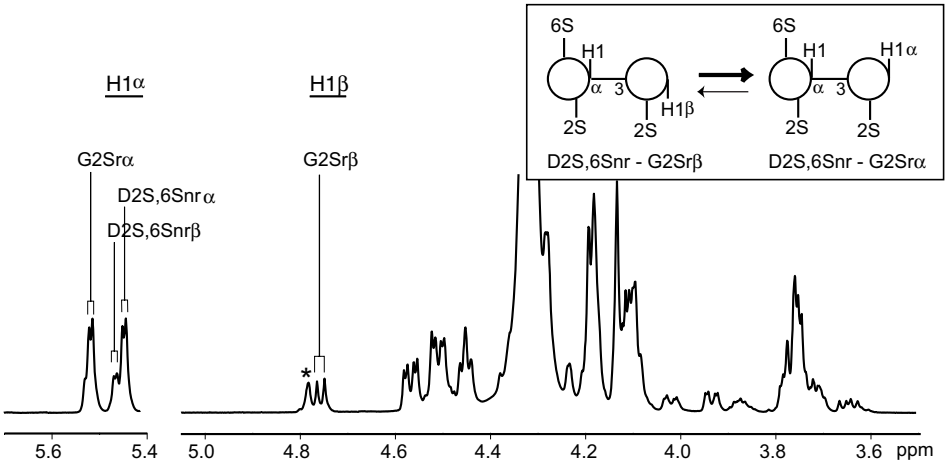

Figure 2. ^1H NMR spectra of neo-λ-carrabiose recorded at 70 °C. As inset: the schematic structure of neo-λ-carrabiose in its α- and β-form.

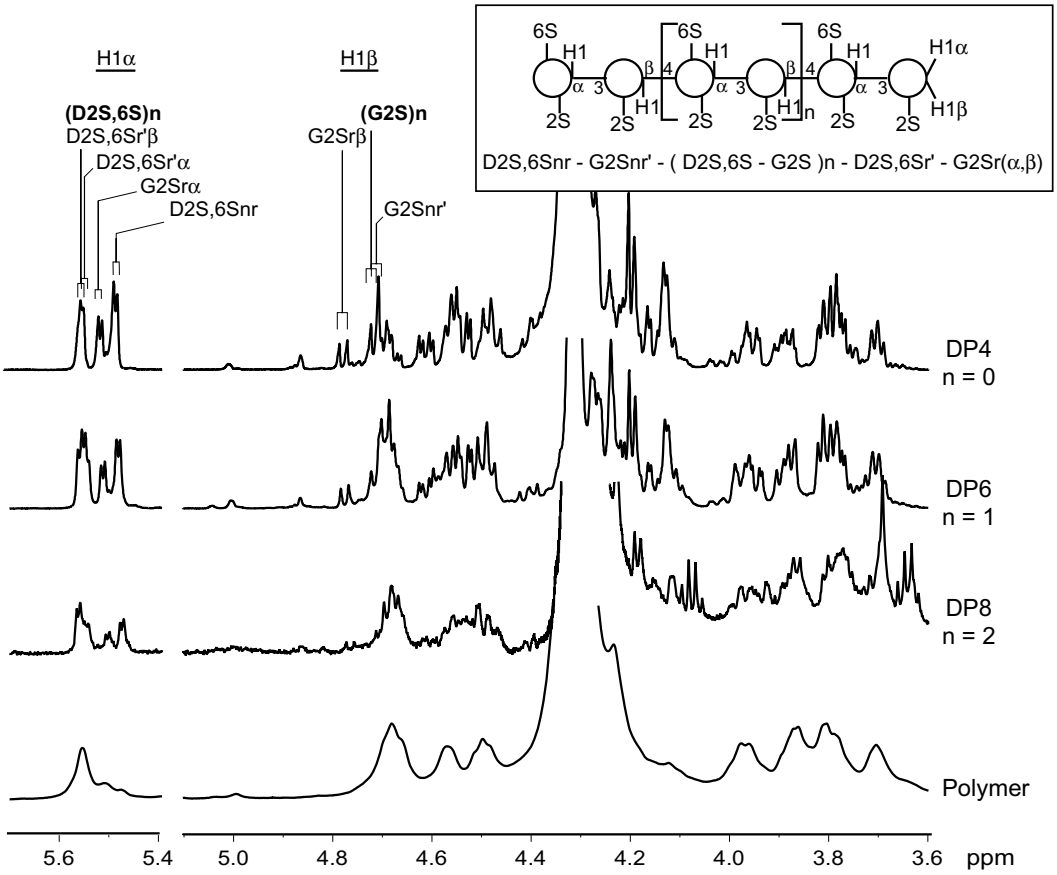

Figure 3. ^{1}H NMR (500 MHz) spectra of the lambda polymer and the neo-λ-carrabiose oligosaccharides (DP 4, DP 6, DP 8) recorded at 70 °C. As inset: schematic structure of the neo-λ-carrageenan oligosaccharides series showing H-1 protons.

time, 1.58 s; relaxation delay, 2 s. The number of scans was 256 for DP 8, 64 for the polymer and DP 6 and 32 for DP 4 and DP 2, digital resolution being 0.31 Hz/point.

At 25 °C (Fig. 6), spectra were recorded using 64 K data points and the parameters were: pulse angle, 30°; sweep width, 7485 Hz; acquisition time, 4.38 s; relaxation delay, 2 s; number of scans, 16; digital resolution 0.11 Hz/point.

^{1}H COSY (500 MHz) experiments were achieved at 25 °C. A total of 120 fid's, each consisting of 64 scans per fid with relaxation delay of 2 s and data acquisition time of 0.18 s, were collected. The sweep width in both dimensions was 2841 Hz. The data matrix was completed to a 1024 * 1024 real matrix with a digital resolution of 2.77 Hz/point. Heteronuclear (500 MHz) ^{1}H–^{13}C chemical shift correlated spectra of the oligo-carrageenans were recorded also at 25 °C. A total of 128 fid's, each consisting of 96 scans per fid with relaxation delay of 2 s and data acquisition time of 0.18 s, were collected. The sweep width in the F2 and F1 dimensions were, respectively, 2841 and 22,646 Hz. The data matrix was completed to a 1024 * 1024 real matrix with a digital resolution of 2.77 Hz/point in F2 and 22.12 Hz/point in F1. A *J* mode NMR spectrum of the neo-λ-carrahexaose (additional material) was recorded at 25 °C using 32 K data points. The parameters were as follows: sweep width, 31,446 Hz; acquisition time, 0.52 s; relaxation delay, 2 s; number of scans, 21,642; digital resolution 0.95 Hz/point. Zero filling of the fid was used.

Estimation of the over-sulfation of the λ-carrageenan was achieved at 70 °C on digested λ-carrageenan. The molar fraction of over-sulfated carrabiose diads was determined by dividing the integrations corresponding to the G2S,4S H-4 (4.99 ppm) and the D2S,6S H-1 (reducing, non-reducing and central) signals resonating between 5.5 and 5.6 ppm. We assumed that the sum of the three D2S,6S H-1 signal intensities is equal to the sum of G2S,4S H-4 and G2S H-4 peaks.

2.3. Nomenclature

We used the nomenclature established by Knutsen et al.[8] for carrageenans and oligo-carrageenans. The 4-linked α-D-galactopyranosyl unit is designated as D-unit and the 3-linked β-D-galactopyranosyl unit as G-unit. The disaccharide repetitive unit of λ-carrageenan is described as D2S,6S–G2S (Figs. 2 and 3). For oligosaccharides of the neo-carrabiose series, the internal residues are described as D2S,6S–G2S without additional index.

Whereas the D2S,6S–G2S at the non-reducing end are designated as D2S,6Snr–G2Snr′ and as D2S,6Sr′–G2Srα,β then situated at the reducing end. The NMR resonance of the D2S,6Sr′ residue can be affected by the α,β-equilibrium at the neighbouring reducing end G2Sr. These effects are designated by D2S,6Sr′α or D2S,6Sr′β when observed.

3. Results

The undigested tetrasporophyte λ-carrageenan of *G. skottsbergii* was analysed by ^{1}H and ^{13}C NMR spectroscopy. The 500 MHz ^{1}H NMR spectrum presented in Figure 3 is poorly resolved but shares the same features as the spectrum of *Iridaea undulosa* λ-carrageenan[22] with notably the D2S,6S anomeric proton resonating at 5.55 ppm at 70 °C. The ^{13}C NMR spectrum (not shown) exhibited essentially the twelve characteristic signals of λ-carrageenan.[20–22] After prolonged incubation with the purified *P. carrageenovora* λ-carrageenase, the yield of degradation was estimated by gel-permeation chromatography. About 95% of the carrageenan was converted into low molecular weight oligosaccharides. The 5% resistant fraction was analysed by ^{1}H NMR spectroscopy (data not shown) and was found to be composed essentially of ν-, κ- and ι-carrageenans characterised by their α-anomeric protons resonating at 5.51, 5.26 and 5.1 ppm (25 °C), respectively. This indicated that the entire λ-carrageenan polymer has been fragmented and, consequently, the oligosaccharide mixture subsequently analysed by HPAEC (Fig. 1) was representative of the composition and the distribution of carrabiose units within the polysaccharide chain.

The work presented here deals only with the oligosaccharides that could be purified at about 10–15 mg scale required for the complete NMR characterisation. Their purification was successful from DP 2 to DP 8, with a final purity ranging from 90% to 100%. These neo-carrabioses were then fully characterised by ^{1}H and ^{13}C NMR spectroscopy, and their molecular weight determined by mass spectrometry.[31] The main end-products analysed were one disaccharide (DP 2) and two tetrasaccharides (DP 4 and DP 4 + S). For an intermediate time of degradation (*t* 5 h, Fig. 1), the most abundant oligosaccharides eluted after DP 4 were an hexasaccharide (DP 6) and an octasaccharide (DP 8).

The ^{1}H NMR spectra of these purified oligosaccharides were recorded at 25 and 70 °C. The coalescence of the spectra for high temperature was not observed as already mentioned for other carrageenans.[23,33] We found that the 1D ^{1}H NMR spectra were better split at 70 °C than at 25 °C, more particularly for the H-1 signals. Consequently, for more clarity, the spectra recorded at 70 °C are shown in Figures 2 and 3. NMR data of carrageenan usually refer to experiments performed at 25 °C; the complete assignments of the ^{1}H and ^{13}C NMR spectra recorded at 25 °C are reported in Tables 1 and 2, respectively.

3.1. Hydrolytic products are oligosaccharides belonging to the neo-λ-carrabiose series

The disaccharide DP 2 which represents the repeating sequence of λ-carrageenan was first characterised. In accordance with the $^{1}H/^{13}C$ correlations and with integration ratio, all the signals corresponding to the anomeric protons of DP 2 were easily assigned (Fig. 2). The downfield signals observed between 5.4 and 5.6 ppm were assigned to the three anomeric protons in α-anomeric configuration. These signals were doublets corresponding to the typical axial-gauche conformation of H-1–H-2 of D-galactose resulting in a coupling constant of 3 Hz. The most downfield signal (5.508 ppm) was assigned to the α-anomeric proton of the reducing residue (G2Srα H-1). The two other close doublets resonating at 5.455 and 5.436 ppm were assigned to H-1 of the non-reducing galactose unit which is affected by the neighbouring of the reducing residues either in β- or α-configuration, respectively (D2S,6Snr–(G2Srβ) H-1 and D2S,6Snr–(G2Srα) H-1). The doublet at 4.747 ppm (8 Hz) was assigned to H-1 of the reducing residue in β-anomeric configuration (G2Srβ H-1). The intensities recorded for the H-1 signals revealed a displacement of the anomeric equilibrium (α/β 0.7/0.3) towards the α-anomeric configuration. This equilibrium influences the resonance of the six protons of the reducing residue as well as of the six protons of the non-reducing galactose unit with the consequence that the ^{1}H NMR spectra look like the superposition of the spectra of two distinguishable disaccharides (DP 2α and DP 2β) in a 0.7:0.3 ratio.

H-1 in the non-reducing galactose residue of DP 2 had the α-anomeric configuration suggesting that both galactose moieties were linked by an α-(1→3) glycosidic bond. This was supported by the analysis of heteronuclear $^{1}H–^{13}C$ chemical shift correlation (not shown). Indeed, H-3 (and not H-4) of the reducing galactose residue (G2Srα H-3 and G2Srβ H-3) were coupled, respectively, with C-1 of the non-reducing sugar (D2S,6Snr–(G2Srα) C-1 and D2S,6Snr–(G2Srβ) C-1). Inversely the two H-1 doublets of the non reducing galactose unit (D2S,6Snr–(G2Srα/β) H-1) were coupled with C-3 of the reducing galactose (G2Srα/β C-3). The ^{1}H and ^{13}C NMR spectral assignments (see below) of the other λ-oligosaccharides (DP 4–DP 8) allowed to conclude that these oligosaccharides belong to the neo-λ-carrabiose series. Consequently, these results demonstrate that the λ-carrageenase of *P. carrageenovora* cleaves the β-(1→4) linkage, as this has been previously shown for the κ-carrageenase of *P. carrageenavora* and the ι-carrageenase of *Alteromonas fortis*.[32]

Table 1. ^{1}H NMR data (δ ppm) of neo-λ-carrabiose and DP 4 + S oligosaccharides and λ-carrageenan polymer at 25 °C (in brackets, 70 °C)

		λ-Carrageenan	$(D2S,6S–G2S)_3$	$(D2S,6S–G2S)_2$	D2S,6S–G2S,4S–D2S,6S–G2S	D2S,6S–G2S
G2Srα	H-1		5.548 (5.515)	5.518 (5.515)	5.520	5.549 (5.508)
G2Srβ	H-1		4.814 (4.772)	4.803 (4.776)	4.790	4.792 (4.747)
G2S	H-1	4.781* (4.740*)	4.781 (4.740)			
G2Snr′	H-1		4.746 (4.723)	4.760 (4.723)	4.780	
G2Srα	H-2		4.624	4.607	4.603	4.584
G2Srβ	H-2		4.426	4.406	4.408	4.385
G2S	H-2	4.503	4.533			
G2Snr′	H-2		4.502	4.480	4.461	
G2Srα	H-3		4.208	4.195	4.190	4.149
G2Srβ	H-3		4.036	4.025	4.013	3.997
G2S	H-3	3.965	4.036			
G2Snr′	H-3		4.012	4.005	4.102	
G2Srα	H-4		4.354	4.341	4.332	4.314
G2Srβ	H-4		4.304	4.295	4.281	4.272
G2S	H-4	4.319*	4.319			
G2Snr′	H-4		4.304	4.295	5.033	
G2Srα	H-5		4.158	4.147	4.140	4.139
G2Srβ	H-5		3.816	3.800	3.790	3.751
G2S	H-5	3.749	3.757			
G2Snr′	H-5		3.741	3.741	3.826	
G2Srα	H-6a		3.785	3.765	3.770	3.770
G2Srβ	H-6a		3.813	3.805	ND	3.817
G2S	H-6a	3.873	3.826			
G2Snr′	H-6a		3.826	3.807	3.852	
G2Srα	H-6b		3.813	3.795	3.794	3.789
G2Srβ	H-6b		3.898	3.885	ND	3.817
G2S	H-6b	3.822	3.922			
G2Snr′	H-6b		3.922	3.916	3.898	
D2S,6Sr′α	H-1		5.589 (5.543)	5.580 (5.555)	5.571	
D2S,6Sr′β	H-1		5.607 (5.558)	5.585 (5.560)	5.575	
D2S,6S	H-1	5.592 (5.554)	5.607 (5.558)			
D2S,6Snr	H-1		5.537 (5.481)	5.522 (5.483)	5.518	(β) 5.516 (5.455)/(α) 5.492 (5.436)
D2S,6Sr′α	H-2		4.740	4.733	4.702	
D2S,6Sr′β	H-2		4.711	4.705	ND	
D2S,6S	H-2	4.711*	4.711			
D2S,6Snr	H-2		4.550	4.537	4.527	(β/α) 4.522
D2S,6Sr′α	H-3		4.184	4.169	4.150	
D2S,6Sr′β	H-3		4.258	4.247	4.233	
D2S,6S	H-3	4.258*	4.258			
D2S,6Snr	H-3		4.247	4.234	4.392	(β) 4.225/(α) 4.153
D2S,6Sr′α	H-4		4.336	4.325	4.314	
D2S,6Sr′β	H-4		4.293	4.284	4.272	
D2S,6S	H-4	4.293*	4.293			
D2S,6Snr	H-4		4.149	4.136	4.134	(β) 4. 134/(α) 4.153
D2S,6Sr′α	H-5		4.535	4.522	4.497	
D2S,6Sr′β	H-5		4.637	4.601	4.587	
D2S,6S	H-5	4.637*	4.637			
D2S,6Snr	H-5		4.607	4.589	4.429	(β) 4.584/(α) 4.503
D2S,6Sr′α	H-6a		4.349	4.334	4.335	
D2S,6Sr′β	H-6a		4.319	4.313	4.318	
D2S,6S	H-6a	4.367*	4.367			
D2S,6Snr	H-6a		4.223	4.208	4.208	(β) 4.210/(α) 4.210
D2S,6Sr′α	H-6b		4.349	4.334	4.335	
D2S,6Sr′β	H-6b		4.367	4.356	4.358	
D2S,6S	H-6b	4.367*	4.367			
D2S,6Snr	H-6b		4.223	4.208	4.208	(β) 4.210/(α) 4.210

^{1}H NMR chemical shifts ascribed to the polymer marked with an asterisk are deduced from the chemical shifts obtained for the central unit of DP 6.

3.2. Complete assignment of the ^{1}H and ^{13}C NMR spectra of neo-λ-carrabiose oligosaccharide series

The ^{1}H NMR of DP 2 exhibits characteristic features that differ from the NMR spectra recorded on the other λ-carrabiose oligosaccharides (Fig. 3) due to the close proximity of the non-reducing (D2S,6Snr) and the reducing galactose residues (G2Sr).[32,33] For higher molecular weight oligosaccharides, one can clearly distinguish on the NMR spectra, signals belonging to carrabiose units localised at the reducing end (D2S,6Sr′α/β-G2Srα/β), at the non-reducing end (D2S,6Snr–G2Snr′) and, for oligo-λ-carrageenan starting from DP 6, signals belonging to internal carrabiose sequences (D2S,6S–

Table 2. ^{13}C NMR shift data (δ in ppm) of neo-λ-carrabiose and DP 4 + S oligosaccharides and λ-carrageenan polymer at 25 °C

		λ-Carrageenan	$(D2S,6S–G2S)_3$	$(D2S,6S–G2S)_2$	D2S,6S–G2S,4S–D2S,6S–G2S	D2S,6S–G2S
G2Srα	C-1		93.51	93.49	93.50	93.45
G2Srβ	C-1		98.04	98.03	98.00	98.05
G2S	C-1	105.76	105.72			
G2Snr′	C-1		105.79	105.68	105.80	
G2Srα	C-2		76.76	76.74	76.68	76.58
G2Srβ	C-2		80.71	80.69	80.92	80.59
G2S	C-2	79.88	79.85			
G2Snr′	C-2		79.74	79.66	79.83	
G2Srα	C-3		73.17	73.14	72.99	74.27
G2Srβ	C-3		76.23	76.21	76.27	76.89
G2S	C-3	76.51	76.59			
G2Snr′	C-3		77.37	77.34	77.36	
G2Srα	C-4		67.83	67.79	67.66	68.44
G2Srβ	C-4		66.78	66.75	66.56	67.09
G2S	C-4	66.54	66.47			
G2Snr′	C-4		66.85	66.87	76.27	
G2Srα	C-5		72.83	72.81	72.72	72.90
G2Srβ	C-5		77.60	77.59	77.50	77.67
G2S	C-5	77.27	77.20			
G2Snr′	C-5		77.26	77.34	77.09	
G2Srα	C-6		64.01	63.99	63.97	63.93
G2Srβ	C-6		63.82	63.81	63.69	63.75
G2S	C-6	63.55	63.52			
G2Snr′	C-6		63.45	63.45	63.42	
D2S,6Sr′α	C-1		94.53	94.49	94.45	
D2S,6Sr′β	C-1		93.99	93.95	93.97	
D2S,6S	C-1	94.06	93.99			
D2S,6Snr	C-1		94.99	94.98	97.92	(β) 94.85/(α) 95.89
D2S,6Sr′α	C-2		78.11	78.09	77.91	
D2S,6Sr′β	C-2		78.11	78.09	77.91	
D2S,6S	C-2	78.11	78.11			
D2S,6Snr′	C-2		78.11	78.09	78.19	(β/α) 78.07
D2S,6Sr′α	C-3		70.50	70.50	70.26	
D2S,6Sr′β	C-3		70.41	70.42	ND	
D2S,6S	C-3	70.52	70.41			
D2S,6Snr′	C-3		69.62	69.60	69.57	(β/α) 69.67
D2S,6Sr′α	C-4		82.48	82.37	82.97	
D2S,6Sr′β	C-4		82.76	82.66	83.24	
D2S,6S	C-4	82.68	82.76			
D2S,6Snr′	C-4		72.26	72.25	71.97	(β) 72.28/(α) 72.07
D2S,6Sr′α	C-5		71.09	71.04	71.08	
D2S,6Sr′β	C-5		71.03	71.04	71.08	
D2S,6S	C-5	71.12	71.03			
D2S,6Snr′	C-5		71.30	71.30	71.62	(β) 71.36/(α) 71.20
D2S,6Sr′α	C-6		71.67	71.60	71.62	
D2S,6Sr′β	C-6		71.99	72.08	72.17	
D2S,6S	C-6	72.06	71.99			
D2S,6Snr′	C-6		70.23	70.23	69.71	(β) 70.40/(α) 69. 79

G2S) which resonate like the polymer of λ-carrageenan. By a simple comparison of the ^{1}H NMR spectra of DP 2, DP 4, DP 6 and DP 8 the attribution of the anomeric protons was obvious. The most deshielded signal of the DP 6 spectrum resonating at 5.558 ppm was assigned to the α-anomeric proton of the internal D2S,6S sequence. This proton, absent in DP 4, resonates like the internal D2S,6S H-1 of the polymer (5.554 ppm). The D2S,6Sr′β H-1, common to DP 6 and DP 4, resonates also at 5.558 ppm because of its involvement in a α-(1→3) linkage and of a very similar electronic environment. The D2S,6Sr′α H-1 signal is slightly displaced upfield at 5.543 ppm. The last signal corresponding to the D2S,6S α-anomer protons, common to DP 6 and DP 4, was the non-reducing end D2S,6Snr H-1, appearing at 5.481 ppm. The α-anomeric proton of the reducing G2Srα H-1 was measured at 5.515 ppm and its β-anomer counterpart was observed at 4.772 ppm. Finally, the internal G2S H-1 and the non-reducing β-anomer G2Snr′ H-1 were measured upfield at about 4.740 and 4.723 ppm, respectively. One can notice that at 25 °C, the anomeric protons of the G2S residues are hidden by the signal of water.

Assignment of the ring protons of each galactose residue was carried out successfully starting from H-1 to H-4 by using a COSY analysis which shows direct ^{3}J-couplings (vicinal and geminal) (Fig. 4, coupling values Table 3). For example, Figure 4 presents the COSY spectrum of the neo-λ-carrahexaose on which, in contrast to the 1D spectrum, well resolved cross-peaks are observed. The H-1–H-4 correlation system of the protons belonging to the internal carrabiose units D2S,6S and G2S are reported. On the COSY spectrum, it was not possible to visualise the correlation between H-4 and H-5 because of the small coupling constant of the vicinal H-4eq and H-5ax of the galactose moieties[33,34] (Table 3). The H-5–H-6 cross-peaks were visible on the COSY experiment but at this stage of analysis, it was not possible to discriminate H-5 from H-6. Figure 5 displays the heteronuclear ^{1}H/^{13}C chemical shift correlation (HMQC) of DP 6 showing discrete splitting of the cross peaks. The chemical shifts of C-1–C-4 coupled with H-1–H-4 were clearly identified and reported in Table 2. Notably, we observed that the two anomeric carbons belonging to the internal galactose residues, G2S C-1 and D2S,6S C-1, were found at 105.72 and 93.99 ppm which corresponds closely to the previously reported attribution for the λ-carrageenan polymer.[20–22] The final attributions for H-5–H-6 and

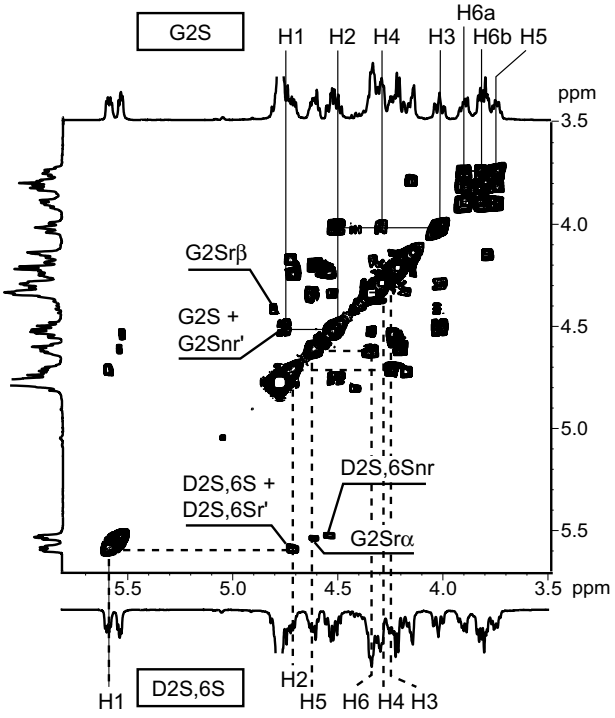

Figure 4. ^{1}H COSY (500 MHz) spectrum of neo-λ-carrahexaose (D2S,6Snr–G2Snr′–D2S,6S–G2S–D2S,6Sr′–G2Sr$_{(α,β)}$) at 25 °C. Correlation system of the protons belonging to the internal G2S (—) and D2S,6S unit (---) of the hexasaccharide are drawn.

Table 3. Coupling constants (*J* Hz) for oligosaccharides of the neo-λ-carrabiose type

	D2S,6Sα (Hz)	G2Sβ (Hz)
$^{3}J_{H1,H2}$	3.8	8
$^{3}J_{H2,H3}$	10.3	9.8
$^{3}J_{H3,H4}$	3.3	3
$^{3}J_{H4,H5}$	2	2
$^{3}J_{H5,H6a}$	NM	5.3
$^{3}J_{H5,H6b}$	NM	7
$^{2}J_{H6a,H6b}$	NM	−11.7

NM stands for not measured.

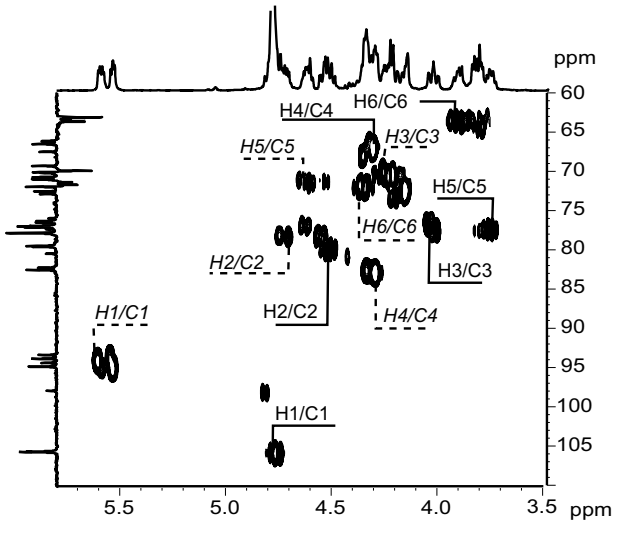

Figure 5. Heteronuclear ^{1}H–^{13}C chemical shift correlated spectrum of neo-λ-carrahexaose (D2S,6Snr–G2Snr′–D2S,6S–G2S–D2S,6Sr′–G2Sr$_{(α,β)}$) at 25 °C. Cross-correlations between protons and carbons of the internal G2S (—) and D2S,6S unit (---) of the hexasaccharide are indicated.

C-5–C-6 were obtained on the basis of long range heteromolecular $^{1}H/^{13}C$ chemical shift correlations (HMBC, not shown). The good quality of the splitting of the HMBC spectrum allowed to well discriminate the H-5/C-1 or H-1/C-5 cross-peak correlations. The attribution was confirmed by a *J* mode experiment (not shown) which allowed to unambiguously distinguish primary (C-6) and secondary (C-5) carbon atoms.

3.3. Evidence for a partial over-sulfation of the *G. skottsbergii* λ-carrageenan

The most abundant oligosaccharides produced after complete digestion of the *G. skottsbergii* λ-carrageenan belong to the neo-λ-carrabiose series. These 'standard' oligo-λ-carrageenans were found in admixture with less abundant fragments of unknown structure but clearly observable by HPAEC (Fig. 1). We successfully purified by semi-preparative HPAEC the minor product which was co-eluted by gel permeation chromatography with DP 4. Its molecular weight measured by mass spectrometry[31] indicated that the structure corresponded to a tetrasaccharide bearing an additional sulfate (DP 4 + S). By analogy with the ^{1}H and ^{13}C NMR spectra of DP 4, the ^{1}H and ^{13}C NMR spectra of DP 4 + S were resolved and the data are summarised in Tables 1 and 2. Figure 6 presents the ^{1}H NMR spectra of the two tetrasaccharides highlighting the differences between the spectra of the DP 4 and the DP 4 + S. The maximum downfield shifting of about 0.76 ppm was observed for H-4 of the non-reducing G2S′nr, the corresponding carbon (G2S′nr C-4) being also shifted downfield by 9.62 ppm. This displacement for the H-4 signal is very comparable, for example, to the 0.72 ppm proton chemical shift observed between G H-4 of β-carrageenan and G4S H-4 of the κ-tetrasaccharide.[33] Concerning the carbon NMR spectrum, we have observed a higher shifting for C-4 when compared to the +7.8 ppm chemical shift between the G and G4S of β- and κ-carrageenan, respectively.[20] The protons and the carbons spatially close to H-4 and C-4 were also affected by the additional sulfate leading to a small downfield chemical shift of their corresponding NMR signals. As previously reported in the case of the β- and κ-carrageenan, we have observed a slight downfield chemical shift of the systems G2S C-3/H-3 and G2S C-5/H-5. The protons of the galactose residue next to the G2S,4Snr′ sequence at the non-reducing end, seem also to be influenced by the supplementary sulfate, as both D2S,6Snr H-3 and H-5 were slightly shifted.

4. Discussion

The study presented here is the first complete characterisation of the λ-oligosaccharide standards from DP 2

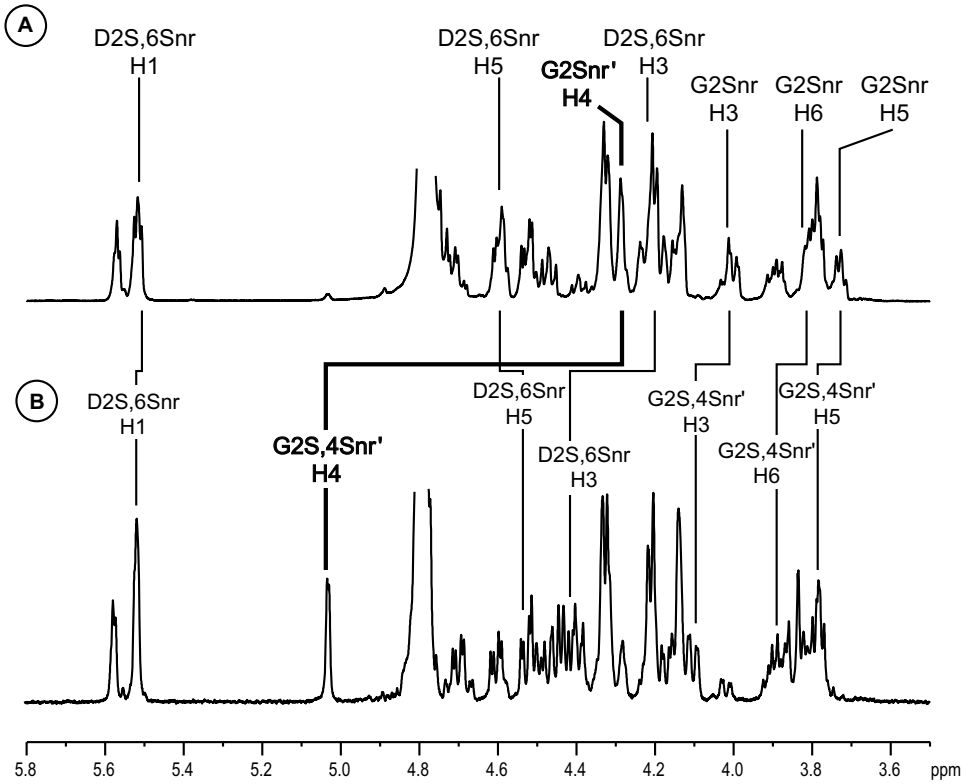

Figure 6. ^{1}H spectrum (500 MHz) of the tetrasaccharides ((A) D2S,6Snr–G2Snr′–D2S,6Sr′–G2Sr$_{(\alpha,\beta)}$) and ((B) D2S,6Snr–G2S,4Snr′–D2S,6Sr′–G2Sr$_{(\alpha,\beta)}$) recorded at 25 °C. The arrows indicate the shifts of the proton signals originating from the additional sulfate at C-4 of the G2Snr′ unit.

to DP 8, allowing to assign unambiguously the ^{1}H NMR spectrum and to correct the previous tentative attribution of the ^{13}C NMR spectrum of λ-carrageenan. The main difficulty encountered for complete attribution of the spectra lies in the positioning of the galactose moieties occurring in the same chair conformation along the oligosaccharide fragments. Nevertheless, because of the good resolution of the homo- and heteronuclear 2D chemical shift correlation spectra, we were able to identify each galactose moiety and assign its surrounding and linkages. The λ-carrageenan NMR spectra showed strong differences with those reported for κ- and ι-carrageenan because anhydro-bridges are not found in ideal λ-carrageenan chains and because of its higher degree of sulfation. However, the protons assignment of the G2S unit of λ-carrageenan exhibits some similarities with those of the G4S of κ-carrageenan. Except for H-2 and H-4 signals of the λ- and κ-carrageenan, respectively, which are shifted downfield, the values reported for the other protons support our analysis.[33]

Because we have worked with purified oligosaccharides and combined both ^{1}H and ^{13}C NMR data, we were able to more accurately interpret our results. The attribution of the ^{13}C NMR spectra of oligosaccharides allowed us to extrapolate our analysis to the λ-carrageenan polymer and to reconsider the previously proposed assignments of λ-carrageenan.[20–22] Indeed, we have shown that the three very close signals of the ^{13}C NMR spectra appearing at 78.11, 77.27 and 76.51 ppm should be assigned to D2S,6S C-2, G2S C-5 and G2S C-3, respectively. Also, we have observed that the chemical shift previously assigned to D2S,6S C-3 and D2S,6S C-6 have to be exchanged.

λ-Carrageenase is a glycoside hydrolase which catalyses the cleavage of the glycosidic bond without modifying otherwise the chemical structure of the carrageenan. We have observed that *G. skottsbergii* λ-carrageenan was completely depolymerised by the *P. carrageenovora* λ-carrageenase while the chemical structure of the substrate is known to contain non-λ-carrabiose motives.[35] This indicates that the enzyme is able to accommodate in its active site substrates with a modified carrageenan structure (Guibet et al., in preparation). The plasticity of the recognition by λ-carrageenase has been already reported for other carrageenases and probably reflects the adaptation of these enzymes involved in the bioconversion of naturally complex substrates.[13,15,36] The HPAEC chromatogram of the oligosaccharide mixtures presented in Figure 1 could be seen as representative of the heterogeneity of the polysaccharide, the neo-λ-carrabiose oligosaccharides presenting the typical repetitive unit of λ-carrageenan and the other oligosaccharides being seen as deviations from this polysaccharide framework. Consequently, to go further into the fine structure *G. skottsbergii* λ-carrageenan by such an approach, it seems essential to correlate a chemical structure to each individual peak of the chromatogram. In an attempt to partially solve the chromatogram, we have analysed and purified the most abundant oligosaccharide carrying a non-ideal λ-carrageenan structure (DP 4 + S). We have shown that the most abundant structure irregularity found in the λ-carrageenan of *G. skottsbergii* is the addition of one sulfate group leading to a four times sulfated carrabiose unit: D2S,6S–G2S,4S. We have estimated the abundance of this over-sulfated pattern by integrating the characteristic G2S,4Snr′ H-4 signal appearing at 5.033 ppm in the ^{1}H NMR spectra recorded on completely digested λ-carrageenan. We found that the polysaccharide contains about 8% of D2S,6S–G2S,4S diads. In spite of careful inspection of the ^{1}H NMR spectra of partly fractionated oligosaccharides and oligosaccharide mixtures, no other sulfation features or chemical modifications (i.e., pyruvate, methyl) were identified. Interestingly, the occurrence of G2S,4S (3-linked 2,4 disulfated galactose) and/or G2S,6S was suggested from chemical analysis of *G. skottsbergii* and *I. undulosa*.[22,35,37] In *G. skottsbergii*, the amount of G2S,6S residue was measured as ∼4.9%[35] or 3%[37] which represents 9.8% or 6% of D2S,6S–G2S,6S diads. Similarly, the concentration of G2S,4S residue was reported to be 5.9%[35] and 3%[37] (equivalent to 11.8% and 6% D2S,6S–G2S,4S diads). Our observations confirm the presence of non-negligible amounts of G2S,4S in a very similar ratio as previously reported, but, even after close inspection of the NMR spectra, we were not able to highlight a relevant over-sulfation at C-6. Nevertheless, the chromatogram in Figure 1 shows some less intense peaks marked by an asterisk that contain very low amounts of structures that might contain D2S,6S–G2S,6S diads. This supposes that the enzyme could accommodate both the G2S,4S and the G2S,6S residues in its active site. The non-negligible amount of undigested carrageenan (5%) may also contain a fraction of 3-linked unit of 2,6-disulfated galactose. Because we did not observe 4-linked units of 2,6-disulfated galactose in the undigested fraction, we infer that all the λ-type carrageenans were enzymatically depolymerised. Consequently, if the G2S,6S residue remains in the undigested fraction, it is probably linked within the carrageenan chain to an α-D-galactose residue that differs from the D2S,6S repetitive unit of the λ-carrageenan structure.

The structure and the amount of the end-product of the whole enzymatic digestion of the *G. skottsbergii* λ-carrageenan strongly suggests that this polysaccharide possess a hybrid structure built mainly of the characteristic λ-carrabiose D2S,6S–G2S pattern and 8% of the D2S,6S–G2S,4S disaccharide unit. The distribution of D2S,6S–G2S,4S along the chain is probably random since two contiguous over-sulfated residues were not found in the most abundant components. The occurrence of two neighbouring D2S,6S–G2S,4S units cannot be excluded, but according to our observations this

combination should represent only a very minor fraction of the digest.

5. Conclusion

The enzymatic digestion of λ-carrageenan allowed to recover low molecular weight oligosaccharides holding the chemical structure of the native substrate. Because *G. skottsbergii* λ-carrageenan was completely hydrolyzed, the enzymatic products are representative of the overall structure of the polymer. ^{1}H and ^{13}C NMR analysis of the oligosaccharides demonstrate that the *P. carrageenovora* λ-carrageenase cleaves the β-(1→4) linkage giving rise to the neo-λ-carrabiose series. We observed that *G. skottsbergii* λ-carrageenan is mainly composed of the typical λ-carrabiose unit, D2S,6S–G2S, and a non-negligible amount of a tetrasulfated carrabiose, D2S,6S–G2S,4S.

Similarly to the gelling κ- and ι-carrageenans, NMR analysis combined with chromatographic analysis of enzymatically digested λ-carrageenan should give a better insight of the λ-carrageenan structure itself and its diversity among algae. Finally, the complete understanding of the λ-carrageenan implies also the analysis of the minor oligosaccharide fractions not investigated in this study because of their very low concentration. In this context, strategies allowing the characterization of minute amounts of oligosaccharides (such as mass spectrometry) should be encouraged.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.04.018.

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