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Short communication

Preparation and detailed NMR analyses of a series of oligo-α-carrageenans

Aurélie Préchoux[a], William Helbert[a,b,*]

[a] Centre National de la Recherche Scientifique, Université Pierre et Marie Curie-Paris 6, Unité Mixte de Recherche 7139 "Marine Plants and Biomolecules", Station Biologique, F-29682 Roscoff Cedex, France
[b] Centre de Recherches sur les Macromolécules Végétales (CERMAV, UPR-CNRS 5301), Affiliated with the Université Joseph Fourier (UJF) and Member of the Institut de Chimie Moléculaire de Grenoble (ICMG, FR-CNRS 2607), BP53, 38041 Grenoble Cedex 9, France

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ABSTRACT

Standard oligo-ι-carrageenans were produced with ι-carrageenases isolated from the marine bacteria *Alteromonas fortis* and *Pseudolalteromonas atlantica*. The carrageenans were then desulfated using a 4S-carrageenan-sulfatase purified from *P. atlantica*. Using chromatography and NMR analyses, we characterized a series of new standard neo-α-carrageenan oligosaccharides.

1. Introduction

Carrageenans are a class of sulfated galactans found in certain red algae (Rhodophyta). These polysaccharides share a common backbone of D-galactose alternately linked by α(1,3) and β(1,4) glycosidic linkages. The disaccharide repetition moieties, called carrabiose units, are classified according to the number and the position of the sulfate ester groups and the occurrence of a 3,6 anhydro-ring in the α-linked galactose (Knutsen, Myslabodski, Larsen, & Usov, 1994; Usov, 2011). Carrageenans are not homopolymers; they are heteropolymers, usually made up of several different types of carrabiose units. For example, the industrially important ι (iota)-carrageenan from *Eucheuma denticulatum* is composed mainly of ι-carrabiose units, but also contains its biosynthetic precursor ν (nu)-carrabiose as well as some κ (kappa)-carrabiose units (van de Velde et al., 2002; Jouanneau, Boulenguer, Mazoyer, & Helbert, 2010a). Many hybrid carrageenans—ranging from those rich in ι-carrabiose to those rich in κ-carrabiose, with many combinations in between—have been identified, highlighting the complexity and the diversity of carrageenan structures (Bixler, Johndro, & Falshaw, 2001). Because κ-, ι- and λ (lambda)-carrageenans are important for the agrifood industry due to their gelling and texturizing properties as well as their capacity to bind proteins, they have been the subject of many structural and rheological studies.

Structural analyses of carrageenans have been facilitated by using carrageenan-degrading enzymes, called carrageenases, which specifically cleave glycosidic linkages (Michel, Nyvall-Collen, Barbeyron, Czjzek, & Helbert, 2006). The resulting oligosaccharides can be then purified by chromatography and precisely analyzed using NMR and mass spectrometry (Greer & Yaphe, 1984; Guibet et al., 2008; Knutsen & Grasdalen, 1992). These investigations have revealed that the diversity of carrageenan depends on the composition, as well as the distribution, of the carrabiose repetition units along the carrageenan chain. Studies on the hybridity of carrageenans have focused essentially on those of industrial interest, namely ι-rich, κ-rich and, to a lesser extent, λ-rich carrageenans. However, there are 15 Greek letters that are used to describe different carrabiose moieties (Knutsen et al., 1994) and 42 theoretical repeating structures have been proposed by Stortz and Cerezo (1992), suggesting a very wide range of yet-unexplored hybrid structures. One endo-carrageenan sulfatases has been described recently and they convert ι-carrabiose into α-carrabiose (Fig. 1, Préchoux, Genicot, Rogniaux, & Helbert, 2013). Using these sulfatases, it is now possible to prepare a series of hybrid ι-/α-carrageenans and "standard" α-carrageenans. Hybrid ι-/α-carrageenan was first reported in *Catenella* sp. and its structural characterization was elucidated using NMR (Zablackis & Santos,

* Corresponding author at: Centre de Recherches sur les Macromolécules Végétales (CERMAV, UPR-CNRS 5301), Affiliated with the Université Joseph Fourier (UJF) and Member of the Institut de Chimie Moléculaire de Grenoble (ICMG, FR-CNRS 2607), BP53, 38041 Grenoble Cedex 9, France. Tel.: +33 04 76 03 76 61; fax: +33 04 76 54 72 03.
E-mail addresses: helbert@cermav.cnrs.fr, william.helbert@cermav.cnrs.fr (W. Helbert).

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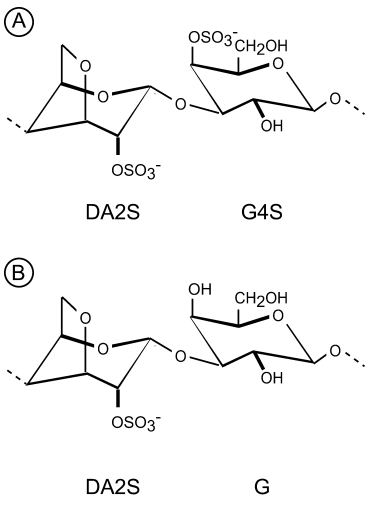

Fig. 1. Chemical structures of neo-ι- (A) and neo-α-carrabiose (B) moieties.

1986; Falshaw et al., 1996). Incubation of hybrid ι-/κ-carrageenan with endo-sulfatase leads to hybrid ι-/κ-/α-carrageenan and finally to κ-/α-carrageenan. These latter carrageenan compositions have no known equivalents in nature and open new biotechnological perspectives for carrageenans and their potential applications (Préchoux et al., 2013).

Structural analyses of standard oligo-carrageenans using NMR (Guibet, Kervarec, Génicot, Chevolot, & Helbert, 2006; Jouanneau, Boulenguer, Mazoyer, & Helbert, 2010b; Knutsen & Grasdalen, 1992) and mass spectrometry (Antonopoulos, Favetta, Lafosse, & Helbert, 2004; Antonopoulos, Favetta, Helbert, & Lafosse, 2005; Yu et al., 2006) is required prior to in-depth characterization of hybrid oligo-saccharides, such as hybrid carrageenans. In contrast to studies on κ-, ι- and λ-carrageenan structures, which benefitted from available κ-, ι- and λ-carrageenases, there are no described α-carrageenases that can produce standard α-carrageenan oligosaccharides by specific cleavage of α-carrageenan. However, we found that the 4S-carrageenan-sulfatase completely converts series of oligo-ι-carrageenans into standard oligo-α-carrageenans. Here we report the preparation of standard oligo-α-carrageenans and their in-depth NMR characterization.

2. Experimental

2.1. 4S-ι-carrageenan sulfatase and ι-carrageenases

Recombinant *Alteromonas fortis* ι-carrageenase was over-expressed in *Escherichia coli* BL21(DE3) and purified by affinity chromatography according to Michel, Helbert, Kahn, Dideberg, and Kloareg (2003). The *Pseudoalteromonas atlantica* ι-carrageenase was partially purified from strain T6c (ATCC-BAA-1087). The marine bacterium was grown (48 h, 20 °C, 180 rpm) in 1 L ZoBell medium (ZoBell, 1941) containing ι-carrageenan (1 g/L). After centrifugation, an anti-protease tablet (Complete, EDTA-free) was added to the cell-free supernatant. The supernatant was concentrated five times by ultrafiltration (Amicon Bioseparation, Millipore) on a 10 kDa MWCO membrane and under nitrogen pressure (1 bar). The extract was dialyzed overnight (Spectra/Por MWCO 3500 Da) against Tris–HCl buffer 50 mM pH 7.5 at 4 °C prior to fractionation on a DEAE-Sepharose column (Hiprep 20 mL, GE Healthcare) with an NaCl gradient of 0 to 1 M. Fractions

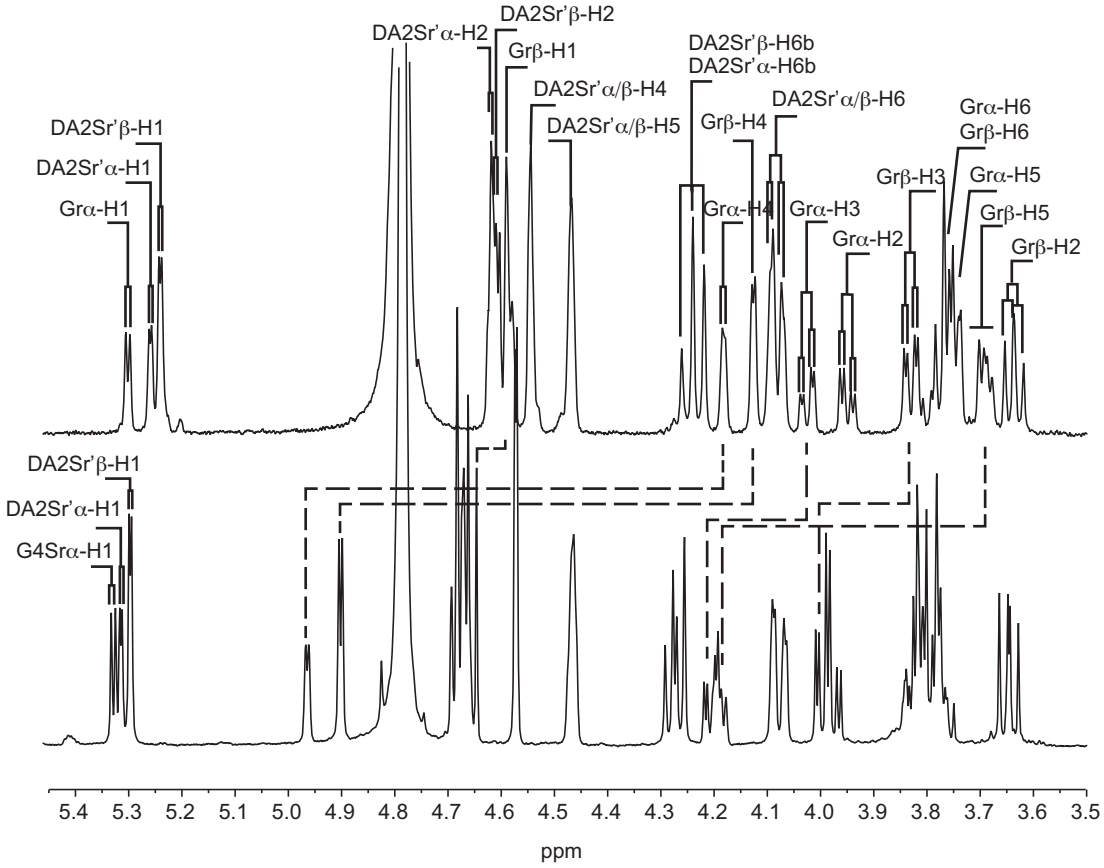

Fig. 2. ^{1}H NMR (500 MHz) spectra of neo-α-carrabiose (top) and neo-ι-carrabiose (bottom). Attributions of the signal are indicated on the spectra, highlighting the shift of the proton signal at position 4 and its neighboring protons.

Table 1
^{1}H NMR data (δ ppm) of neo-ι-carrabiose (DA2S-G4S), neo-α-carrabiose (DA2S-G), neo-α-carratetraose $(DA2S\text{-}G)_2$, neo-α-carrahexaose $(DA2S\text{-}G)_3$ and α-carrageenan polymer $(DA2S\text{-}G)_n$.

		DA2S-G4S			DA2S-G	$(DA2S\text{-}G)_2$	$(DA2S\text{-}G)_6$	$(DA2S\text{-}G)_n$
G4Srα	H-1	5.329	Grα	H-1	5.301	5.298	5.299	
G4Srβ	H-1	4.654	Grβ	H-1	4.596	4.626	4.623	
G4S	H-1		G	H-1			4.623	4.640 (4.61)
G4Snr′	H-1		Gnr′	H-1		4.568	4.568	
G4Srα	H-2	3.974	Grα	H-2	3.948	3.948	3.950	
G4Srβ	H-2	3.645	Grβ	H-2	3.636	3.635	3.638	
G4S	H-2		G	H-2			4.634	3.683 (3.62)
G4Snr′	H-2		Gnr′	H-2		3.634	4.634	
G4Srα	H-3	4.206	Grα	H-3	4.025	4.029	4.031	
G4Srβ	H-3	3.997	Grβ	H-3	3.829	3.852	3.872	
G4S	H-3		G	H-3			3.870	3.868 (3.82)
G4Snr′	H-3		Gnr′	H-3		3.852	3.870	
G4Srα	H-4	3.964	Grα	H-4	4.182	4.184	4.184	
G4Srβ	H-4	4.903	Grβ	H-4	4.125	4.130	4.132	
G4S	H-4		G	H-4			4.132	4.156 (4.10)
G4Snr′	H-4		Gnr′	H-4		4.130	4.132	
G4Srα	H-5	3.754	Grα	H-5	3.698	3.730	3.729	
G4Srβ	H-5	3.780	Grβ	H-5	3.681	3.687	3.690	
G4S	H-5		G	H-5			3.690	3.750 (3.65)
G4Snr′	H-5		Gnr′	H-5		3.687	3.690	
G4Srα	H-6	3.806	Grα	H-6	3.757	3.788	3.759	
G4Srβ	H-6	3.806	Grβ	H-6	3.757	3.765	3.768	
G4S	H-6		G	H-6			3.768	3.816 (*n.d.*)
G4Snr′	H-6		Gnr′	H-6		3.765	3.768	
DA2Sr′α	H-1	5.313	DA2Sr′α	H-1	5.257	5.272	5.273	
DA2Sr′β	H-1	5.297	DA2Sr′β	H-1	5.238	5.253	5.256	
DA2S	H-1		DA2S	H-1			5.256	5.274 (5.22)
DA2Snr	H-1		DA2Snr	H-1		5.241	5.240	
DA2Sr′α	H-2	4.685	DA2Sr′α	H-2	4.611	4.653	4.646	
DA2Sr′β	H-2	4.673	DA2Sr′β	H-2	4.609	4.642	4.632	
DA2S	H-2		DA2S	H-2			4.632	4.656 (4.61)
DA2Snr	H-2		DA2Snr	H-2		4.608	4.603	
DA2Sr′α	H-3	4.684	DA2Sr′α	H-3	4.589	4.761	4.768	
DA2Sr′β	H-3	4.684	DA2Sr′β	H-3	4.589	4.759	4.748	
DA2S	H-3		DA2S	H-3			4.748	4.782 (4.73)
DA2Snr	H-3		DA2Snr	H-3		n.d.	n.d.	
DA2Sr′α	H-4	4.572	DA2Sr′α	H-4	4.543	4.708	4.709	
DA2Sr′β	H-4	4.572	DA2Sr′β	H-4	4.543	4.708	4.709	
DA2S	H-4		DA2S	H-4			4.709	4.724 (4.65)
DA2Snr	H-4		DA2Snr	H-4		4.543	4.543	
DA2Sr′α	H-5	4.465	DA2Sr′α	H-5	4.467	4.465	4.465	
DA2Sr′β	H-5	4.465	DA2Sr′β	H-5	4.467	4.466	4.465	
DA2S	H-5		DA2S	H-5			4.565	4.697 (4.67)
DA2Snr	H-5		DA2Snr	H-5		4.470	4.668	
DA2Sr′α	H-6a	4.274	DA2Sr′α	H-6a	4.240	4.095	4.093	
DA2Sr′β	H-6a	4.274	DA2Sr′β	H-6a	4.240	4.095	4.093	
DA2S	H-6a		DA2S	H-6a			4.093	4.139 (4.09)
DA2Snr	H-6a		DA2Snr	H-6a		4.077	4.074	
DA2Sr′α	H-6b	4.077	DA2Sr′α	H-6b	4.079	4.244	4.242	
DA2Sr′β	H-6b	4.077	DA2Sr′β	H-6b	4.079	4.244	4.242	
DA2S	H-6b		DA2S	H-6b			4.242	4.257 (4.20)
DA2Snr	H-6b		DA2Snr	H-6b		4.225	4.224	

containing ι-carrageenase activity were eluted at about 0.3 M NaCl.

The 4S-ι-carrageenan-sulfatase was purified from *P. atlantica* extracts according to Préchoux et al. (2013). Briefly, the bacterium was grown in 1 L of ZoBell medium (20 °C, 48 h) containing 0.1% (w/v) of ι-carrageenan. After centrifugation, the bacterial pellet was lysed in a French press, and the resulting soluble protein fraction was purified on a DEAE-Sepharose column followed by a Q-Sepharose column using NaCl gradients. The purified protein was stored at 4 °C and was stable for several weeks.

2.2. Preparation of neo-α-carrabiose oligosaccharides

2.2.1. Production of oligo-ι-carrageenans

ι-Carrageenan (0.5% w/v, 50 mM Tris–HCl pH 8.5 buffer) was incubated at 40 °C with aliquots of purified *A. fortis* ι-carrageenase for the preparation of tetra- and hexasaccharide end-products. Aliquots of carrageenase were added repeatedly to obtain complete degradation of the polysaccharides. Similarly, di- and tetrasaccharide end-products were obtained after incubation of ι-carrageenan at 35 °C (0.5% w/v, 50 mM Tris–HCl pH 8.5) with repeated addition of *P. atlantica* ι-carrageenase aliquots.

2.2.2. Desulfation experiments

Mixtures of purified oligo-ι-carrageenans (0.5% w/v, 50 mM Tris–HCl pH 7.5) were incubated with 4S-carrageenan sulfatase at 34 °C for 48 h. The conversion of ι-oligosaccharides into α-oligosaccharides was followed by size-exclusion chromatography. Samples, filtered on a 0.22 μm membrane, were injected on a Superdex 200 column (10/300, GE Healthcare) coupled to a Superdex peptide column and eluted in isocratic conditions with 50 mM $LiNO_3$.

2.2.3. Purification of oligo-carrageenans

Oligo-α- and ι-carrageenans were purified by size-exclusion chromatography using a Pharmacia Superdex 30 prep grade column (600 mm × 26 mm i.d.). The elution was conducted in 50 mM $(NH_4)_2CO_3$ at 20 °C using an isocratic Gilson 306 pump working at a flow rate of 1.7 mL/min. Oligosaccharides were detected by differential refractometry (Spectra System RI-50,Thermo Separation Products) and fractions were collected with a Gilson 215 Liquid Handler. To improve NMR analyses, samples were desalted according to the chromatography protocol except that elution was conducted in distilled water.

2.3. NMR experiments

Purified samples were exchanged twice in D_2O, and redissolved in D_2O (99.7%) at about 5 mg/mL for oligosaccharides and 10 mg/mL for the polysaccharide. NMR spectra were recorded on a Bruker Avance DRX 500 MHz NMR spectrometer at 25 °C for oligosaccharides and 70 °C for the polysaccharide. Chemical shifts are expressed in ppm in reference to an external standard (trimethylsilylpropionic acid). Proton and carbon assignments were determined from 1D (^{1}H and ^{13}C) and 2D experiments (correlation spectroscopy (COSY), heteronuclear ^{1}H/^{13}C chemical shift correlation (HMQC), heteronuclear ^{1}H/^{13}C multiple bond correlation (HMBC)) using Bruker's conventional pulse programs with standard pulse sequences of 2 s of relaxation delay and a 30° angle pulse.

The ^{1}H NMR spectrum of the polymer of pure α-carrageenan was recorded using 64 K data points, a sweep width of 7485 Hz, and 64 scans (digital resolution of 0.11 Hz/point). ^{1}H NMR spectra of oligosaccharides were recorded using 32 K data points. The sweep width was 7507 Hz, and the number of scans was 24 for DP2 and DP6, and 16 for DP4. The digital resolution was 0.23 Hz/point. In contrast, ^{13}C NMR spectra were recorded at 125 MHz, with 100,000 scans for 32 K data points and a sweep width of 31,446 Hz (digital resolution 0.98 Hz/point).

For all 2D experiments, the data matrix consisted of 1024 (F1) × 128 (F2) complex data points, zero filled to 1 K in the F2 dimension prior to Fourier transform, leading to a 1024 × 1024 real matrix. For all degrees of oligosaccharide polymerization, there were 16 scans. ^{1}H/^{1}H COSY experiments were recorded with a sweep width of 1653 Hz in both dimensions for the DP2 oligosaccharide (digital resolution 1.61 Hz/point), 1893 Hz for the DP4 residue (1.85 Hz/point), and 1395 Hz for the DP6 oligosaccharide (1.36 Hz/point). Heteronuclear ^{1}H/^{13}C HMQC spectra of the oligo-α-carrageenans were obtained with a sweep width in the F1 and F2 dimensions of, respectively, 1653 Hz and 25,257 Hz for the DP2 oligosaccharide, 1893 Hz and 25,157 Hz for the DP4 residue, and 1395 Hz and 25,157 Hz for the DP6 oligosaccharide. ^{1}H/^{13}C HMBC experiments were recorded similarly but with a sweep width of 27,681 Hz in the F2 dimension for the three oligosaccharide sizes.

3. Results and discussion

Oligo-ι-carrageenans were obtained after incubation of standard *E. denticulatum* ι-carrageenan with recombinant *A. fortis* ι-carrageenase and *P. atlantica* ι-carrageenase purified from bacterial extracts. The end-products of *A. fortis* ι-carrageenase were neo-ι-carrahexaose and neo-ι-carratetraose whose preparation and complete assignment of ^{1}H and ^{13}C NMR spectra have been reported previously (Jouanneau et al., 2010b). The end-products of *P. atlantica* ι-carrageenase were neo-ι-carrabiose and neo-ι-carratetraose. The ^{1}H NMR spectrum of the neo-ι-carrabiose (Fig. 2B) was assigned completely and the chemical shifts are reported in Table 1. Similar to the characterized oligo-ι-carrageenans, the downfield signals observed between 5.25 and 5.35 ppm were assigned to the three anomeric protons in an α-anomeric configuration (G4Srα-H1: 5.329 ppm; DA2Srʹα-H1: 5.313; DA2Srʹβ: 5.297 ppm). The β-anomeric proton of the G4S residues was measured at 4.654 ppm by a heteronuclear ^{1}H/^{13}C chemical shift correlation. The ring protons of the G4S from H1 to H4 were unequivocally attributed using COSY analysis. H5–H6 correlations were deduced from HMQC analyses and previous ^{1}H NMR data reported on oligo-ι-carrageenans. A similar strategy was used to measure the chemical shifts of the ring protons of the DA2S residue.

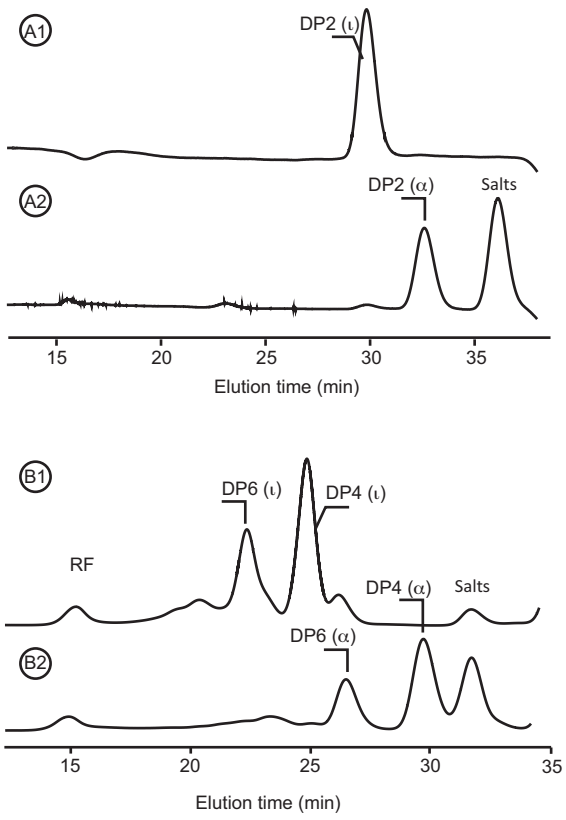

Fig. 3. Gel permeation chromatograms of neo-ι-carrabiose (A1) incubated with 4S-carrageenan sulfatase (A2). The end-products of *A. fortis* ι-carrageenase (B1) were enzymatically desulfated leading to neo-tetra- and neo-hexa-α-carrageenan (B2).

Series of neo-α-carrageenan oligosaccharides were obtained after incubating mixtures of oligo-ι-carrageenans or purified single oligo-ι-carrageenan with carrageenan sulfatase. Desulfation was controlled by size-exclusion chromatography (Fig. 3). After desulfation, a decrease in molecular weight was associated with longer retention times. For example, the neo-ι-carrahexaose eluted at about 22 min whereas the neo-α-carrahexaose was detected at about 26 min. Fractions containing oligo-α-carrageenans were collected and their purity (superior to 95%) was estimated by anion-exchange chromatography.

The ^{1}H NMR spectra of neo-α-carrabiose and neo-ι-carrabiose are presented in Fig. 2 for comparison. The two spectra had some

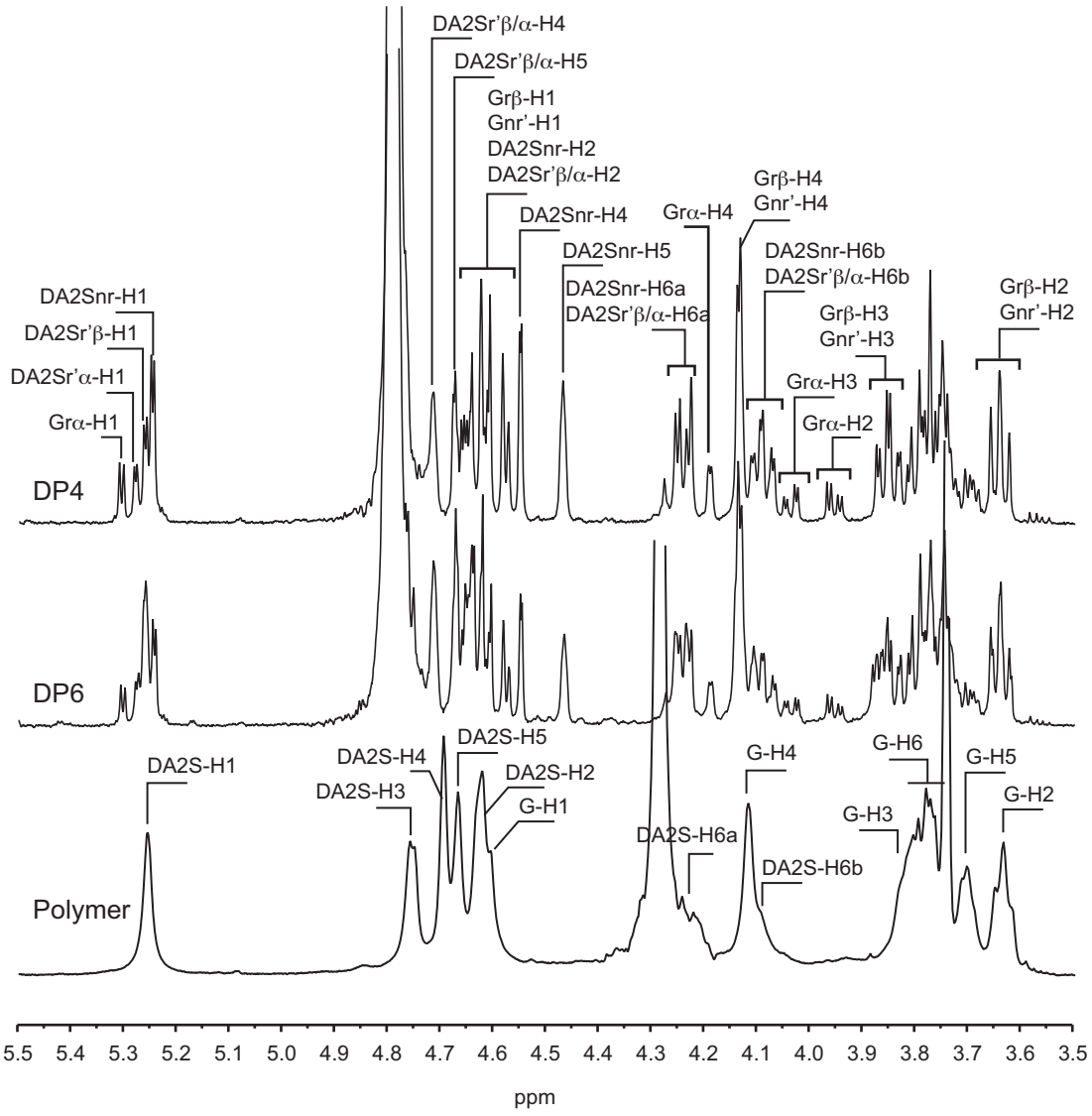

Fig. 4. ^{1}H NMR (500 MHz) spectra of neo-α-carratetraose (DP4), neo-α-carrahexaose (DP6) and the α-carrageenan polymer.

similar features, such as the three downfield signals corresponding to the α-anomeric protons. The signal of the α-anomeric proton of the neutral galactose (Grα-H1, in the neo-α-carrabiose) was measured at 5.301 ppm and the signals of the anomeric protons of the sulfated anhydro-galactose were found at 5.257 ppm (DA2Sr′α) and 5.238 ppm (DA2Sr′β). The β-anomeric proton located at the reducing end was attributed at 4.596 ppm (Grβ) using HMQC analyses and confirmed by H1–H2 correlations observed during COSY experiments. The ring protons of the α- and β-galactose were assigned using COSY analyses from H1 to H4. As expected, the chemical shift of the proton at position 4 (Grα-H4: 4.182 ppm; Grβ-H4: 4.125 ppm) was strongly shifted high-field compared to the corresponding signal of the neo-ι-carrabiose (G4Srα-H4: 4.903 ppm; G4Srβ-H4: 4.964 ppm). HMQC spectra made it possible to ascribe H6 protons and their correlating H5. The protons neighboring the G4Srα/β-H4 position, such as the G4Srα/β-H3 and G4Srα/β-H5 positions, were also shifted high-field as indicated in Fig. 2. Desulfation of position 4 in the galactose also affected the chemical shift of the protons of the anhydro-galactose although to a lesser extent. The assignment of the neo-ι-carrabiose spectra greatly facilitated the interpretation of COSY and HMQC spectra and chemical shifts of the protons of DA2Sr′α/β residues of the α-carrabiose are reported in Table 1.

As for oligo-κ- (Knutsen & Grasdalen, 1992), oligo-ι- (b, Jouanneau et al., 2010b, and this study) and oligo-λ-carrageenans (Guibet et al., 2006), the ^{1}H NMR of disaccharides showed characteristic features resulting from the close proximity of reducing and non-reducing ends. For tetra- and hexasaccharides (Fig. 4), moieties located at the reducing (DA2Sr′α/β-Grα/β) and non-reducing ends (DA2Snr-Gnr′) had characteristic chemical shifts. For the hexasaccharide and higher-molecular-weight oligosaccharides, there were additional moieties (DA2S-G) located internally whose chemical shifts were typical of polymeric carrageenan. The four signals between 5.2 and 5.35 ppm corresponded to the α-anomeric protons (Grα-H1: 5.299 ppm, DA2Sr′α-H1: 5.273 ppm, DA2Sr′β-H1: 5.256 ppm, DA2Snr-H1: 5.240 ppm). The signal of the internal α-anomeric proton DA2S-H1 was only present when the polymer had the same chemical shift as the DA2Sr′β-H1 in tetra- and hexasaccharides. The attributions of the ring protons were carried out using the same strategy as for the neo-α-carrabiose and the oligo-ι-carrageenans. Fig. 5 shows the ^{1}H–^{1}H correlation system of the internal α-carrabiose of the hexasaccharide

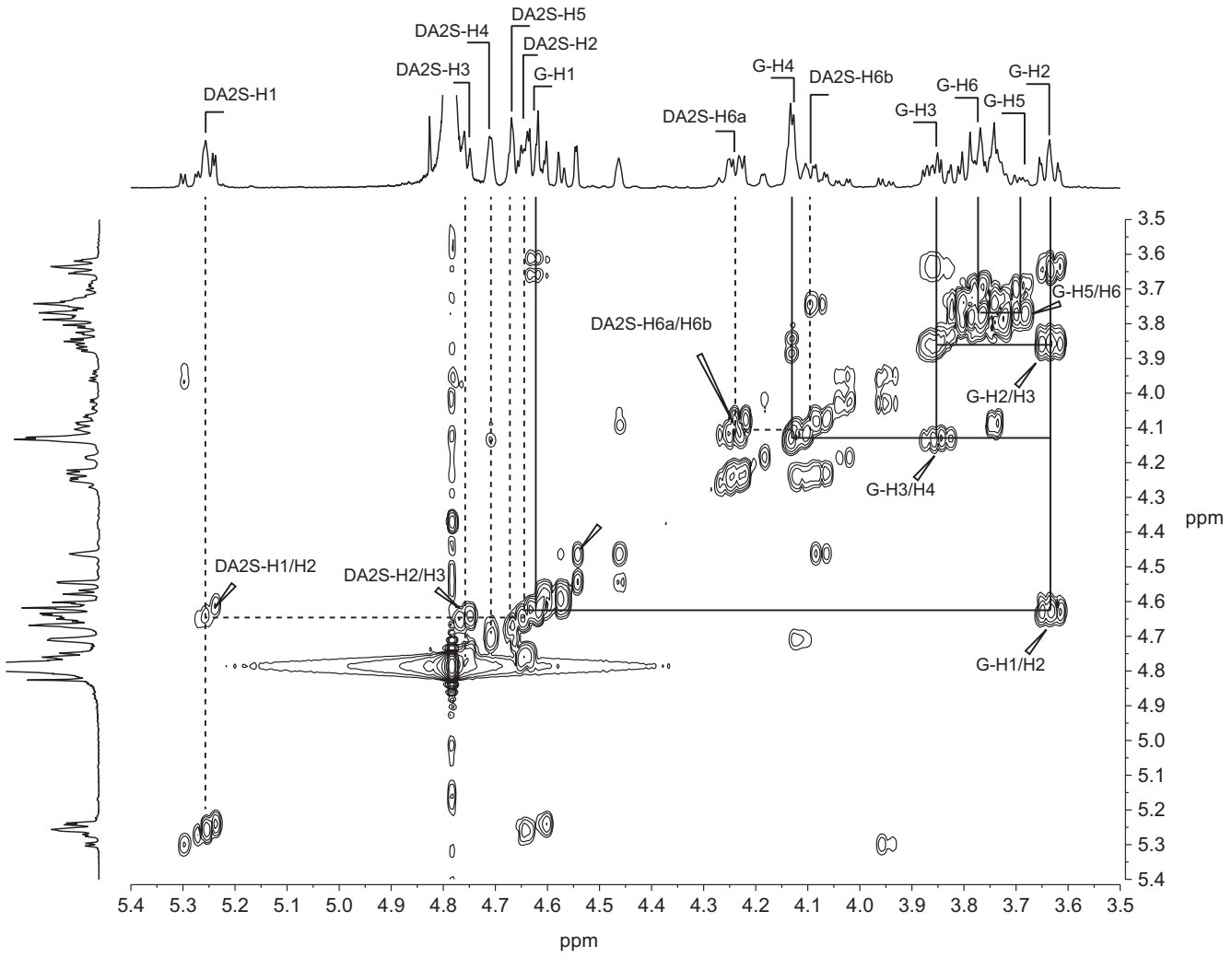

Fig. 5. ^{1}H COSY (500 MHz) spectrum of the neo-α-carrahexaose recorded at 25 °C. The ^{1}H–^{1}H correlation system of the internal carrabiose moiety is indicated.

whose chemical shifts corresponded to the proton of the polymer.

4. Conclusion

The preparation of a series of neo-α-carrageenans demonstrated that new series of oligo-carrageenans can be prepared using the combined action of carrageenases and carrageenan-sulfatases. This allow envisioning the enzymatic production of a wide range of standard and hybrid oligo-carrageenans having, therefore, a controlled distribution of sulfate ester groups. Potential applications, i.e. biological activities, of these new series of sulfated oligosaccharides need to be explored. The purified oligo-α-carrageenans were characterized by NMR and led us to unambiguously ascribed α-carrageenan spectra. These data are expected to be very helpful for characterizing hybrid carrageenan structures.

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