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Comparison of the structures of hybrid κ-/β-carrageenans extracted from *Furcellaria lumbricalis* and *Tichocarpus crinitus*

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ABSTRACT

κ-/β-Carrageenans extracted from *Furcellaria lumbricalis* and *Tichocarpus crinitus* are hybrid polysaccharides that have roughly similar κ- and β-carrabiose contents. The distributions of these carrabiose moieties were compared using gel permeation chromatography to analyse the end-products obtained after degradation with *Pseudoalteromonas carrageenovora* κ-carrageenase. Three fractions were obtained: standard oligo-κ-carrageenans, hybrid oligo-κ-/β-carrageenans and an enzyme-resistant fraction. The structure of the most abundant hybrid oligo-κ-/β-carrageenans and the composition of the κ-/β-fractions resistant to degradation were determined by ^{1}H NMR. Altogether, the results suggest that the κ- and β-carrabiose moieties in *F. lumbricalis* are more randomly distributed than in *T. crinitus* carrageenan, which has a more regular block-type distribution.

1. Introduction

Carrageenans represent a family of highly sulphated galactans that occur in the cell walls of red algae (Rhodophyta). They have a primary backbone structure based on alternately 3-linked α-D-galactose (G unit) and 4-linked, β-D-linked galactose (D unit). The repetition moieties of carrageenan are disaccharides (carrabiose units) and are classified according to the number and the position of ester sulphate groups (S) and by the occurrence of 3,6-anhydro bridges (A unit) in the α-linked galactose residues (Fig. 1). Carrageenans are hybrid polysaccharides – *i.e.* copolymers – made of several carrabiose moieties. Therefore, the terms κ- and ι-carrageenans refer to polysaccharides made of mainly κ- (G4S-DA) and ι-carrabiose moieties (G4S-DA2S), respectively. The great number of possible arrays of the different carrabiose moieties leads to a wide range of possible carrageenan structures (Bixler, 1996; Greer & Yaphe, 1984a, 1984b; Guibet et al., 2008).

Analysis of enzymatic degradation products makes it possible to address not only the composition, but also the distribution of the carrabiose units in hybrid carrageenans (Bellion, Brigand, Prome, Welti, & Bociek, 1983; Greer & Yaphe, 1984a, 1984b). The amount and the structure of hybrid oligosaccharides obtained after incubation with specific enzymes as well as the composition of the enzyme-resistant fraction give insight into the structural characteristics of hybrid carrageenans. The structure of various hybrid κ-/ι-carrageenans obtained after alkaline extraction and the corresponding κ-/μ-/ι-/ν-carrageenans obtained by water extraction have been analysed in depth using recombinant κ- and ι-carrageenases from *Pseudoaltermonas carrageenovora* and *Alteromonas fortis*, respectively (Guibet et al., 2008; Jouanneau, Boulenguer, Mazoyer, & Helbert, 2011). Through complete structure analysis of standard and hybrid oligosaccharides combined with chromatography analysis, it is possible to determine the composition (*e.g.* kappa-rich and iota-rich fractions) and the patterns of distribution of the carrabiose moieties along the carrageenan chains (*i.e.* block, random and non-random).

In addition to κ-/ι-carrageenans, several other types of hybrid carrageenans have also been described. The tetrasprophyte *Gigartina skottsbergii* λ-carrageenan contains ideal λ-carrabiose moieties (G2S-D2S,6S), but also the over-sulphated moiety G2S,6S-D2S,6S (Guibet, Kervarec, Génicot, Chevolot, & Helbert, 2006). Hybrid κ-/β-carrageenan has been reported in *Furcellaria lumbricalis* (also called furcellaran), *Eucheuma gelatinae*, *E. speciosa*, *Endocladia muricata* (Renn et al., 1993) and *Tichocarpus crinitus* (Anastyuk et al., 2011; Barabanova et al., 2005). The enzymatic degradation of *F. lumbricalis* and *E. gelatinae* carrageenan shows that both have co-occurring κ- and β-carrabiose units, but with different distributions. The gelling properties of *T. crinitus* are very different to those of *F. lumbricalis*, suggesting different carrabiose distributions (Yermak, Kim, Titlynov, Isakov, & Solov'eva, 1999). We therefore analysed the composition and

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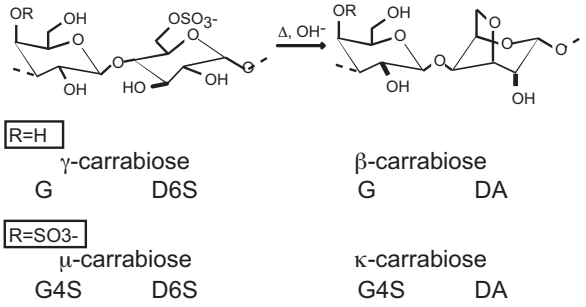

Fig. 1. Structure of κ- and β-carrabiose and their respective biosynthetic precursors: γ- and μ-carrabiose. The cyclisation of the anhydro ring is obtained *in vitro* by hot alkaline treatment (Δ OH^-).

distribution of carrabiose moieties in these two hybrid κ-/β-carrageenans.

2. Experimental

2.1. Hybrid κ-/β-carrageenan

F. lumbricalis was collected in Kassari Bay (Baltic Sea, Estonia) and carrageenan was extracted from the vegetative thallus following Tuvikene et al. (2010). *T. crinitus* was harvested in the Peter the Great Bay (Sea of Japan, Russia) and preparation of the carrageenan from the vegetative thallus is given in Barabanova et al. (2010).

2.2. Enzymatic degradation kinetics

Enzymatic degradations were carried out using recombinant *P. carrageenovora* κ-carrageenase which was over-expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography according to Michel et al. (2001). Incubations were conducted as described in Guibet et al. (2008) and Jouanneau et al. (2010). Briefly, carrageenans (0.5%, w/v, in 0.1 M $NaNO_3$ pH 7.5) were incubated with an aliquot of κ-carrageenase (0.3 μg mL^{-1}) for 24 h at 40 °C. These incubation conditions led to the complete degradation of κ-carrageenan, which was characterised by the absence of a signal corresponding to neo-κ-carrahexaose in high-performance anion-exchange chromatography (HPAEC) or gel permeation chromatography.

The amount of reducing sugars produced during enzymatic incubation was determined using the reducing sugar method adapted from Kidby and Davidson (1973). Aliquots (100 μL) of the reaction medium were mixed with 900 μL of ferricyanide solution (300 mg potassium hexacyanoferrate III, 24 g Na_2CO_3, 1 mL NaOH 5 M, completed to 1 L). The mixture was maintained in boiling water for 10 min, cooled to room temperature and absorbance was read at 420 nm.

2.3. Gel permeation chromatography

Analyses of degradation products were carried out using analytical Superdex 200 (GE Healthcare) and Superdex (GE Healthcare) columns connected in series. Filtered (0.22 μm) samples of 200 μL were eluted in 0.1 M $LiNO_3$ at 20 °C using an isocratic Dionex Ultimate 3000 pump working at a flow rate of 0.3 mL min^{-1}. Chromatography experiments were monitored using a Wyatt Optilab Rex refractive index detector.

Purification of hybrid oligo-κ-/β-carrageenans was performed according to Jouanneau et al. (2010) using three GE Healthcare Pharmacia Superdex 30 preparative grade columns (600 mm × 26 mm i.d.) mounted in series. 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^{-1}. Oligosaccharides were detected by differential refractometry (Spectra System RI-50, Thermo Separation products) and fractions were collected with a Gilson 215 Liquid Handler.

2.4. Preparation and enzymatic hydrolysis of fluorescent oligosaccharides

Fluorescent oligosaccharides were prepared by grafting 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) to the reducing ends using a protocol adapted from Goubet, Jackson, Deery, and Dupree (2002). Hybrid oligosaccharides, pure or in mixture (about 50 μg), were mixed with 2 μL of 0.15 M ANTS in acetic acid:water (3:17, v:v) and 1 M $NaCNBH_3$ in DMSO. After the reaction was conducted overnight at 37 °C, samples were freeze-dried and stored at 4 °C in the dark. Fluorescent oligosaccharides were electrophoresed on a 27% (w/v) carbohydrate polyacrylamide gel (C-PAGE) running at 20 mA for 30 min. The migration front of the fluorescent oligosaccharides was visualised using a UV Transilluminator Biovision+ 1000/26 M (Vilber Lourmat).

2.5. ^1H NMR analyses

^1H NMR spectra were recorded with a BRUKER Advance DRX 500 spectrometer equipped with an indirect 5 mm gradient probehead 1H/13C/31P. Samples were exchanged twice in D_2O, and re-dissolved at a concentration of about 5 mg mL^{-1} in 99.97% atom D_2O. Chemical shifts are expressed in ppm in reference to an external standard (trimethylsilylpropionic acid, van de Velde, Pereira, & Rollema, 2004).

2.6. Oligosaccharide nomenclature

We used the nomenclature established by Knutsen, Myslabodski, Larsen, and Usov (1994) for carrageenans and oligo-carrageenans. The 4-linked α-D-galactopyranosyl unit is designated as a D unit and the 3-linked β-D-galactopyranosyl unit as a G unit. The disaccharide repetition unit of κ-carrageenan and β-carrageenan are DA-G4S and DA-G, respectively. For oligosaccharides of the neo-carrabiose series, the internal κ- and β-carrabiose units are written as DA-G4S and DA-G without additional indices. When a κ-carrabiose moiety (DA-G4S) is positioned at a reducing end, it is referred to as DAr′-G4Srα or DAr′-G4Srβ, according to its anomeric configuration. At the non-reducing end, it is designated as DAnr-G4Snr′. Similar rules apply to β-carrabiose units.

3. Results and discussion

κ- and β-carrageenan were distinguished in the anomeric region of ^1H NMR spectra by a signal at 4.85 ppm attributed to the G4S-H4 proton observed in κ-carrageenan spectra (Fig. 2A, top), but absent in β-carrageenan spectra (Fig. 2A, bottom). They could also be differentiated by the chemical shift of the anomeric signal of the α-linked galactose (DA-H1), which was measured at 5.12 ppm and 5.10 ppm for κ- and β-carrageenan, respectively. This very slight difference in chemical shift made the integration of DA-H1 signals in κ-/β-carrabiose mixtures difficult due to the highly overlapping peaks. Therefore, as suggested by Welti (1977), the G4S-H4 signal was used as a reference for κ-carrabiose content. Accordingly, the calculated κ:β-carrabiose molar ratio was about 61:39 (mol:mol) and 57:42 (mol:mol) in *T. crinitus* and *F. lumbricalis*, respectively.

As illustrated in Fig. 2B and C, the κ- and β-carrabiose content of *F. lumbricalis* and *T. crinitus* carrageenan samples could be also estimated after deconvoluting the overlapping signals

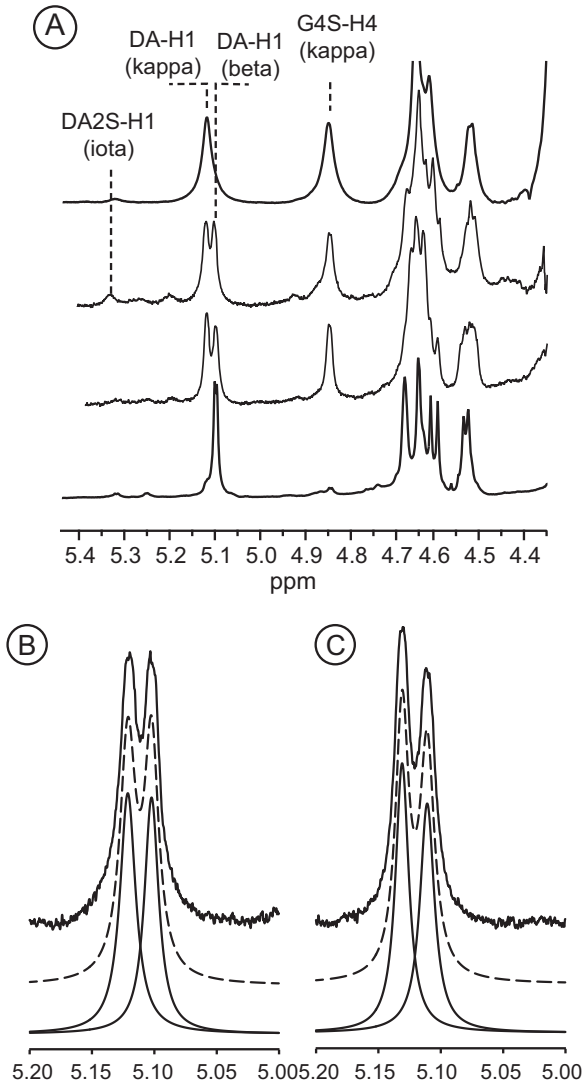

Fig. 2. (A) ^{1}H NMR (500 MHz) spectra of, from top to bottom, *Kappaphycus alvarezii* κ-carrageenan, *Tichocarpus crinitus* κ-/β-carrageenan, *Furcellaria lumbricalis* κ-/β-carrageenan and β-carrageenan prepared chemically. The characteristic anomeric protons of the κ- and β-carrabiose moieties are indicated. (B) and (C) Fitted α-anomeric signals (dashed lines) corresponding to κ- and β-carrabiose in *T. crinitus* and *F. lumbricalis*, respectively.

instead of integrating them. Compared to the calculated molar ratios, the determined κ:β-carrabiose molar ratios indicate a higher number of β-carrabiose units: in *T. crinitus* carrageenan the determined molar ratio was 54:46 (mol:mol) and in *F. lumbricalis* 52:48 (mol:mol). The content of β-carrabiose measured in *F. lumbricalis* carrageenan was similar to previously reported values (Knutsen & Grasdalen, 1992; Laos & Ring, 2005; Tuvikene et al., 2010). In addition to the κ- and β-carrabiose moieties, we observed in *T. crinitus* a non-negligible fraction of ι-carrabiose (DA2S-H1: 5.33 ppm), which represented about 18% of the total amount of carrabiose units. Altogether, the fractions of κ-, ι- and β-carrabioses were of about 43%, 18% and 39% (mol:mol), respectively, in *T. crinitus*.

The similarity in kappa-carrabiose content in *F. lumbricallis* and *T. crinitus* was supported by the degradation kinetics, which revealed that they have similar sensitivity to enzymatic degradation as illustrated in Fig. 3. Accordingly, the shapes of the curves and maximum degradation values were quite comparable (Fig. 3). After complete degradation, the amount of reducing sugars produced was half of what is obtained with *Kappaphycus alvarezii* κ-carrageenan used as standard. This rate of degradation

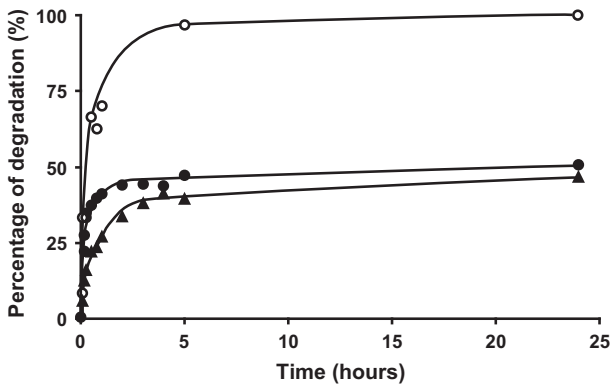

Fig. 3. Degradation kinetics of hybrid κ-/β-carrageenan extracted from *Tichocarpus crinitus* (●) *Furcellaria lumbricalis* (▲) and *Kappaphycus alvarezii* κ-carrageenan (○).

corresponds roughly to the κ-carrabiose content measured in the hybrid κ-/β-carrageenans. As expected, β-carrageenan was not degraded by κ-carrageenase (not shown), indicating that cleavage by κ-carrageenase requires consecutive κ-carrabiose moieties. After complete degradation, the end-products were analysed by gel permeation chromatography (Fig. 4) and compared with degraded *K. alvarezii* κ-carrageenan used as a standard. This gel permeation chromatogram was divided into three parts according to Guibet et al. (2008): a weakly resistant fraction (between 45 and 55 min), a fraction composed of standard oligo-carrageenan (neo-κ-carratetraose: 110 min; neo-κ-carrabiose: 120 min) and a small fraction corresponding to hybrid κ-/ι-oligo-carrageenans (between 90 and 107 min). A cursory examination of the chromatograms of the two degraded κ-/β-carrageenans showed that they were quite different (Fig. 4). In the case of *T. crinitus* carrageenan, the enzyme-resistant fraction was abundant and two peaks were observed eluting like standard oligo-κ-carrageenans. Other oligosaccharides, probably hybrid κ-/β-oligosaccharides, were also detected, but in lower amounts (90 and 107 min). These observations confirmed our previous mass spectrometry analysis, which showed that standard

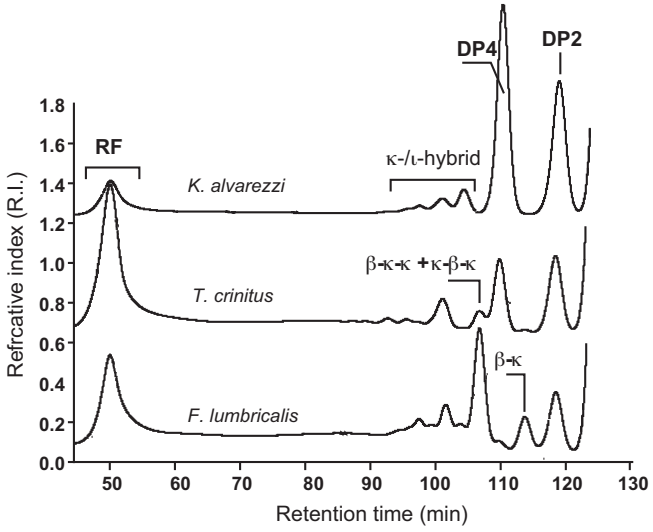

Fig. 4. Gel permeation chromatography of the degradation products obtained after incubation of hybrid κ-/β-carrageenan extracted from *Tichocarpus crinitus* and *Furcellaria lumbricalis* with *Pseudoalteromonas carrageenovora* κ-carrageenase. Degradation products were compared with those obtained after digestion of *Kappaphycus alvarezii* κ-carrageenan. DP2 and DP4 designate neo-κ-carrabiose and neo-κ-carratetraose, respectively. RF: enzyme-resistant fraction. β–κ, β–κ–κ and κ–β–κ correspond to hybrid oligo-κ-/β-carrageenan whose structure has been resolved.

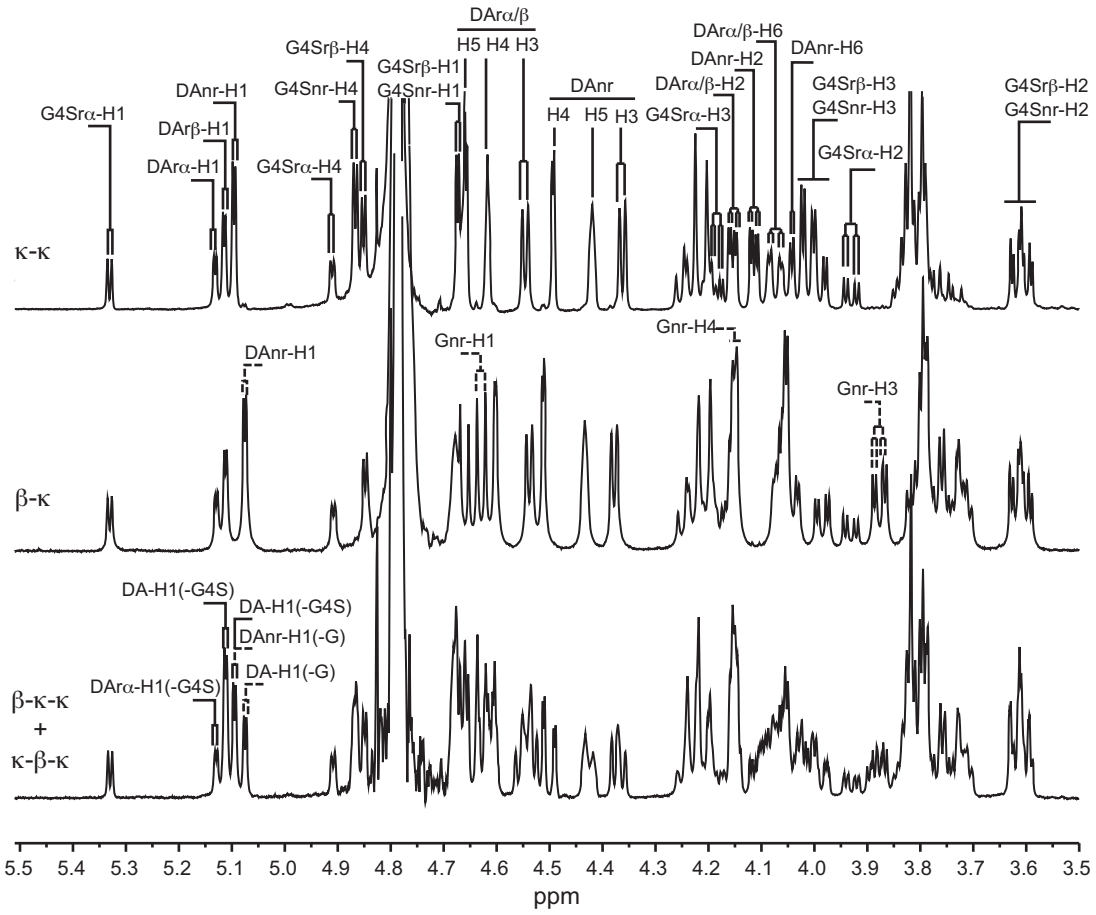

Fig. 5. ^{1}H NMR (500 MHz) of fractionated hybrid oligo-carrageenans recorded at 25 °C including a purified β–κ tetrasaccharide and a mixture of hexasaccahrides (β–κ–κ and κ–β–κ). Dashed lines were used to ascribe the galactose residue (G) of β-carrabiose moieties.

oligo-κ-carrageenans were more abundant than hybrid oligosaccharides (Anastyuk et al., 2011). In contrast, the chromatogram of *F. lumbricalis* revealed a less abundant enzyme-resistant fraction, few oligosaccharides eluting like oligo-κ-carrageenans, but a more abundant fraction of hybrid κ-/β-oligosaccharides.

The most abundant oligosaccharides (Fig. 4; 107 min, 110 min, 113 min) were further purified by gel permeation chromatography using three Superdex columns mounted in series. The low-molecular-weight oligosaccharides were thereby well separated with high purity, as confirmed by HPAEC analysis (results not shown). In these fractionated hybrid oligo-carrageenans then analysed by ^{1}H NMR spectroscopy, the neo-κ-carrabiose and neo-κ-carratetraose were straightforwardly identified based on previous ^{1}H NMR studies (Guibet et al., 2008; Knutsen & Grasdalen, 1992). After integration and chemical shift of the anomeric signals (Fig. 5), the structure of the smallest hybrid oligosaccharide (eluting at 113 min, Fig. 4) was likely a tetrasaccharide with a κ-carrabiose at its reducing end and a β-carrabiose at its non-reducing end. The reducing end was ascribed to a G4S residue (G4Srα-H1, 5.33 ppm; G4Srβ-H1, 4.67 ppm), which is common to all standard oligo-κ-carrageenans. The α-anomeric protons of the anhydro-galactose neighbouring this G4S residue resonated at 5.13 ppm (DAr′α-H1) and 5.11 ppm (DAr′β-H1) and also corresponded to a κ-carrabiose moiety located at the reducing end. The doublet resonating at 5.08 ppm was attributed to anhydro galactose α-linked to a neutral galactose residue.

The second most abundant hybrid κ-/β-oligosaccharide (Fig. 4, 107 min) eluted as a single molecule in HPAEC (not shown), suggesting this oligosaccharide was pure. However, ^{1}H NMR spectra suggested that it was a mixture of hexasaccharides having one β-carrabiose located either at the non-reducing end (5.08 ppm), or within the oligosaccharide (5.10 ppm). This hypothesis was supported by gel electrophoresis of fluorescently labelled degradation products presented in Fig. 6. For *T. crinitus* (Fig. 6B), the bands corresponding to standard κ-carrageenan oligosaccharides were more intense than hybrid oligosaccharides as expected from the chromatography observations. The band migrating between the standard tetra- (κ–κ) and hexa-κ-carrabioses (κ–κ–κ) was attributed to a β–κ oligosaccharide, because it migrated similarly to a chromatographically purified β–κ oligosaccharide. The two bands migrating between the standard hexa- (κ–κ–κ) and octa-κ-carrabioses (κ–κ–κ–κ) corresponded to the single peak observed by gel permeation chromatography (Fig. 4, 107 min) and HPAEC. The very similar migration properties also suggests that these oligosaccharides have very similar structures, in particular the same molecular weight and same charges. In the case of *F. lumbricalis*, the bands corresponding to the hybrid oligosaccharides were the most intense, with a pair of bands migrating between the standard κ–κ–κ and the κ–κ–κ–κ oligosaccharides.

These two hybrid oligosaccharides were separated from other oligosaccharides and were subjected to prolonged incubation with κ-carrageenase. One oligosaccharide was not degraded and the other one was cleaved into two fragments: β–κ and κ oligosaccharides. Because one κ-carrabiose moiety must be at the reducing end, the two possible structures of the hybrid oligosaccharides were κ–β–κ and β–κ–κ. *P. carrageenovora* κ-carrageenase has six subsites and the catalytic cleft is located between the κ-carrabiose at the reducing end and the rest of the oligosaccharide.

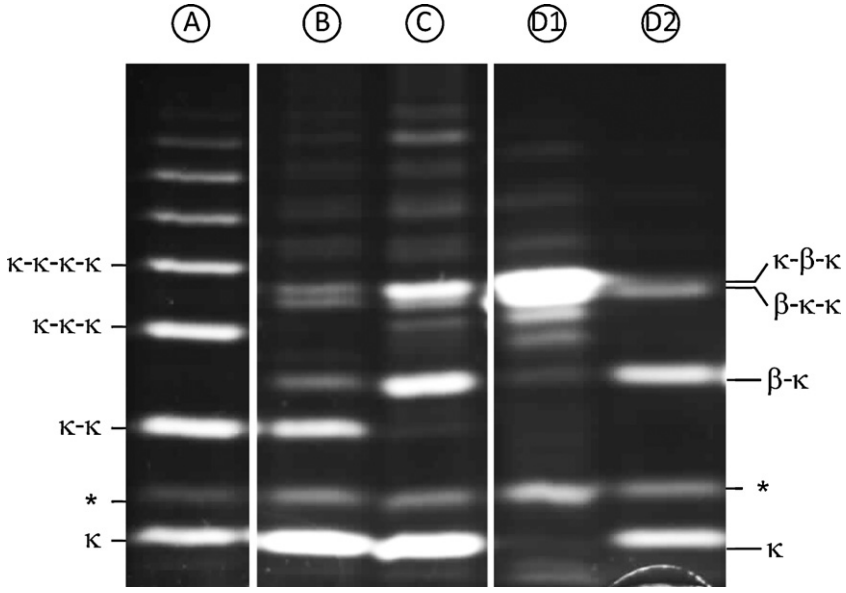

Fig. 6. C-PAGE gel electrophoresis of fluorescently labelled oligosaccharides. (A) Mixture of standard oligo-κ-carrageenan. (B) and (C) Degradation products of *Tichocarpus crinitus* and *Furcellaria lumbricalis* κ-/β-carrageenan. (D) Purified mixture of hexasaccharides (D1) was incubated with the *Pseudoalteromonas carrageenovora* κ-carrageenase (D2). *: fluorescent contaminant.

Consequently, only the β–κ–κ oligosaccharide can be digested by the enzyme leading to β–κ and κ-oligosaccharides. The digestion of κ–β–κ would have produced κ–β and κ; this is not possible due to active site topology and, furthermore, β-carrabiose has never been observed at the reducing end.

Compositions of the resistant fractions were examined by ^{1}H NMR (Fig. 7) and the amount of carrabiose units was estimated by deconvoluting the spectra. In both resistant fractions, κ- (5.12 ppm) and β-carrabioses (5.10 ppm) as well as their biosynthetic precursors, μ- (5.25–5.27 ppm) and γ-carrabioses (5.20 ppm), respectively, were observed. In addition, non-negligible amounts of ι-carrabiose were also detected (5.32 ppm). The shapes of the κ- and β-carrabiose signals in the *T. crinitus* resistant fraction were not as broad (Fig. 7A) as in the *F. lumbricalis* resistant fraction (Fig. 7B), suggesting that the *T. crinitus* carrageenan has a lower molecular weight on average. This is also supported by the clear occurrence of an α-anomeric G4S-H1 signal (5.33 ppm), corresponding to the reducing end of κ-carrabiose. The two resistant fractions could also be distinguished by the κ-:β-carrabiose ratio which was estimated to about 15.6:84.4 (mol:mol) in *T. crinitus* and 34.6:65.4 (mol:mol) in *F. lumbricalis*. Methylation analyses have evidenced the occurrence of ι-carrabioses moieties in *Furcellaria sp.* (Penman & Rees, 1973; Usov & Arkhipova, 1981) which were not reported for *T. crinitus* (Usov & Arkhipova, 1981). Seasonal variations, extraction conditions and other parameters influence likely the amount of carrabiose moieties in carrageenan which could explain some differences between structural analyses.

As previously observed for the enzymatic degradation of various hybrid κ-/ι-carrageenans (Guibet et al., 2008), the degradation products examined by gel permeation chromatography (Fig. 4) showed three main fractions: (1) high molecular weight carrageenan eluted in the void volume, which corresponds to undegraded carrageenan, *i.e.*, the resistant fraction; (2) oligosaccharides belonging to the neo-κ-carrabiose series (DP2 and DP4) and (3) other oligosaccharides which are probably hybrid κ-/β-oligosaccharides. The relative amount of each fraction was very different for the two digested κ-/β-carrageenans. This difference can be attributed to different patterns of carrabiose distribution in the hybrid carrageenan chain. The degradation products of *T. crinitus* carrageenan were composed of low amounts of hybrid

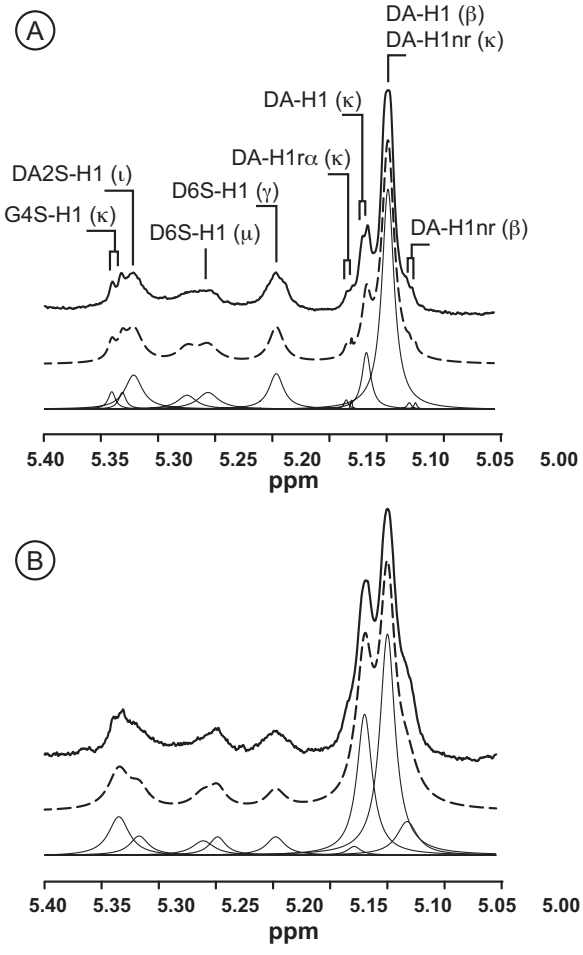

Fig. 7. Anomeric region of ^{1}H NMR (500 MHz) spectra of the enzyme-resistant fractions of (A) *Trichocarpus crinitus* and (B) *Furcellaria lumbricalis*. Simulated spectra (dashed lines) calculated from signal fits (thin solid lines) are presented below the corresponding recorded spectra (thick solid lines). The characteristic α-anomeric protons of the κ-, ι-, μ-, γ and β-carrabiose moieties are indicated.

oligo-κ-/β-carrageenan, suggesting that κ- and β-carrabioses are distributed in blocks. The fraction of standard oligo-κ-carrabiose probably represents κ-blocks and the resistant fraction strongly enriched with β-carrabiose moieties probably represents β-blocks. The blockwise distribution deduced for *T. crinitus* resembles the distribution that has been suggested for *Eucheuma gelatinae* carrageenan. The distribution profile in *F. lumbricalis* carrageenan was different to that of *T. crinitus*. The main oligosaccharide fraction was composed of hybrid κ-/β-oligosaccharides and the resistant fraction was not enriched in β-carrabiose as in *T. crinitus*. This indicated a less blockwise distribution of κ- and β-carrabioses; the two moieties appear to be distributed more randomly than in *T. crinitus* carrageenan.

4. Conclusion

Structure analysis of purified hybrid κ-/β-oligosaccharides (β–κ, β–κ–κ and κ–β–κ) help reveal the hybrid structure of the two κ-/β-carrageenans. Our investigations confirmed the distribution of carrabiose in *F. lumbricalis* previously suggested by Greer and Yaphe (1984a) based on their characterisation of the mixture of oligosaccharides obtained by enzymatic degradation. The co-occurrence of κ- and β-carrabiose has also been suggested after investigation of physico-chemical properties of *F. lumbricalis* carrageenan (Zhang, Piculell, & Nilsson, 1991; Zhang, Piculell, Nilsson, & Knutsen, 1994). We demonstrated that *T. crinitus* has a more blockwise distribution as in the case of *E. gelatinae* carrageenan (Greer & Yaphe, 1984a). *T. crinitus* carrageenan makes weak gels in KCl in contrast to the strong gels observed with *F. lumbricalis* carrageenan (Yermak et al., 1999). Our analysis highlighted some composition differences between the two hybrid κ-/β-carrageenans, especially the occurrence of ι-carrabiose and a slightly higher amount of β-carrabiose in *T. crinitus* compared to *F. lumbricalis*. However, in addition to differences in composition, the different distributions of the κ- and β-carrabioses along the polysaccharide chain explains the very different rheological properties of *T. crinitus* and *F. lumbricalis* carrageenans. The random distribution of κ- and β-carrabiose, which leads to a more regular distribution of charges, appears to maintain good rheological properties, whereas the blockwise distribution strongly affects the carrageenan gelling properties.

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