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Structure and properties of carrageenan-like polysaccharide from the red alga *Tichocarpus crinitus* (Gmel.) Rupr. (Rhodophyta, Tichocarpaceae)

A. O. Barabanova · A. S. Shashkov · V. P. Glazunov · V. V. Isakov · T. B. Nebylovskaya · W. Helbert · T. F. Solov'eva · I. M. Yermak

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Abstract Sulfated polysaccharides occurring in the red algae *Tichocarpus crinitus* cell wall were fractionated and purified. NMR and FT-IR spectroscopy analyses revealed that the non-gelling fraction contained a sulfated galactans having a new carrageenan-like structure. It is built with alternatively linked 1,3-linked β-D-galactopyranosyl-2,4-disulphates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues. Minor amounts of its biosynthetic precursor were detected in a water-extracted specimen. Brief analysis of rheological and biological properties of the non-gelling fraction was carried out. The carrageenan-like polysaccharide from *T. crinitus* displayed the properties of "random coil" polymer at high temperature, and possesses high anticoagulant activity at low concentration.

Keywords Anticoagulant activity · Carrageenan · NMR · FT-IR · *Tichocarpus crinitus*

A. O. Barabanova (✉) · V. P. Glazunov · V. V. Isakov · T. B. Nebylovskaya · T. F. Solov'eva · I. M. Yermak
Pacific Institute of Bioorganic Chemistry, Far-East Branch of Russian Academy of Sciences,
Vladivostok 690022, Russia
e-mail: anuta@piboc.dvo.ru

A. S. Shashkov
N.D. Zelinsky Institute of Organic Chemistry of Russian Academy of Sciences,
Moscow 117913, Russia

W. Helbert
Marine Plants and Biomolecules, UMR 7139 University Pierre and Marie Curie, Paris VI-CNRS,
Station Biologique,
Roscoff, France

Introduction

Carrageenans are complex families of water-soluble, linear, sulfated galactans. They are composed of alternating 3-linked β-D–galactopyranose and 4-linked α-D-galactopyranose or 4-linked 3,6–anhydro-α-D-galactopyranose, forming the disaccharide repeating unit of carrageenans. The sulfated galactans are classified according to the presence of the 3,6-anhydro-bridge on the 4-linked galactose residue and the position and number of sulfate group. Native carrageenan always present complex hybrid structures and are generally a mixture of galactans composed of different carrabiose types, the proportions and structures of which vary with species, ecophysiological and development conditions.

Tichocarpus crinitus is a carrageenophyte red alga which is the unique member of the Tichocarpaceae. It is widely distributed in Far East seas including Japan and Ohotskoe Seas, as well as along the Pacific coast of Honshu and Hokaido, Japan (Perestenko 1994). Because of its rapid growth, large size and some development particularities (i.e., the long time period in vegetative state), *T. crinitus* can be considered as a promising species for industrial production of polysaccharides and for introduction into mariculture (Yakovleva et al. 2001). In contrast to other carrageenophyte algae such as Gigartinales, only very few data on cell wall polysaccharides are available. The first data concerning content and composition of *T. crinitus* cell wall polysaccharides were reported in 1969 (Usov et al. 1969). Chemical analysis and ^{13}C-NMR-spectroscopy have revealed that the gel forming polysaccharide in the presence of potassium chloride corresponded to incompletely sulphated κ-carrageenan (DA-G4S) (Yarotsky et al. 1978; Usov and Arkhipova 1981). The soluble fraction in the presence

of potassium chloride exhibited the usual backbone of λ-carrageenan (D2S6S-G2S), built with alternating α-1,3 and β-1,4 linked D-galactopyranose residues (Usov et al. 1970).

We have recently undertaken investigations on physico-chemical properties of carrageenans from *T. crinitus* and shown that the variations in carrageenan yield and composition were dependent on the environmental conditions, such as temperature of water and photon irradiance (Yermak et al. 1999; Yakovleva et al. 2001). Comparative studies on the structure and properties of carrageenans isolated from the vegetative and reproductive forms of *T. crinitus* were performed. The gelling polysaccharide fractions from both forms were found to be κ/β-carrageenans type (DA-G4S/DA-G), the amount of κ-carrabiose unit being superior in the vegetative (80%) compared with the reproductive form (Barabanova et al. 2005).

In order to complete the characterization of cell wall polysaccharide of *T. crinitus*, we have investigated the structure and properties of the non-gelling fraction extracted from the vegetative form. The sulphated galactans we observed have a new carrageenan structure, the rheological and biological properties of which are different to that previously reported.

Materials and methods

Tichocarpus crinitus (Gmel.) Rupr. specimens were harvested in Sivuchya Bay (Sea of Japan) in October at a depth of 3–5 m. The selected seaweeds were in the vegetative form lacking any reproductive organs. The algae were washed with tap water in order to remove excess salt. Bleaching of the seaweed was achieved by maintaining the specimen in pure acetone for 3 days prior to being dried in air.

Polysaccharide extraction

Dried algae (10 g) were cut into small pieces and immersed in distilled water (300 mL). The suspension of algal fragments was heated at 90°C for 3 h in a water bath and, after cooling to room temperature, the preparation was centrifuged (2,500 *g*, 20 min, 20°C). The supernatant was collected and the pellet consisting of insoluble algal residues was re-extracted twice as previously. The supernatants were pooled and concentrated by rotary evaporation to reach a volume of about 100 mL. The polysaccharides were separated into (1) the gelling (KCl-insoluble) and (2) the non-gelling (KCl-soluble) fractions as described previously (Yermak et al. 1999). The supernatant obtained after KCl precipitation was concentrated and $CaCl_2$ was added in small portions to reach a final concentration of 2%. The suspensions were left at 4°C for 12 h and then centrifuged (25,000 *g*, 30 min, 4°C). The pellet was suspended in hot water, dialyzed against 0.15 mol L^{-1} NaCl and then against water for 4 days at 4°C. The polysaccharide was precipitated by 3 volumes of 95% ethanol, washed with ethanol and dried. The polysaccharide was designed as the KCl-soluble fraction. Carrageenan yields were calculated as a percentage of algal dry matter.

General and analytical methods

Total content of carbohydrate was determined by the phenol-sulfuric acid method using D-galactose as standard (Dubois et al. 1956). Neutral monosaccharides were determined as alditol acetates derivatives by gas-liquid chromatography using an Agilent 6850 chromatograph equipped with a capillary HP-5MS column (30×0.25 mm) with 5% Phenol Methyl Siloxane and a flame ionization detector. The analyses were carried out at temperature programming from 175 to 225°C with 3°C min^{-1} (Englyst and Cumming 1984). The content of the 3,6-anhydrogalactose was determined by complete reducing hydrolysis (Usov and Elashvili 1991). The sulfate ester content of polysaccharide was determined according to the method Lahaye and Axelos by HPLC equipped (conductivity detector Waters 431) with an IC-Pack an Anion column (50×4.6 mm 10 m, Waters), eluted by 2 mM borate/gluconate eluent (flow rate: 1.0 mL min^{-1}) (Lahaye and Axelos 1993).

Measurement of shear viscosity

Solution of non-gelling polysaccharide (1% w/v) was obtained by heating the polysaccharide in water at 70°C for 30 min. Shear viscosity was measured for 2 min on 9-mL samples using a HAAKE RV 20 Rotovisco viscometer (UK), equipped with a NV sensor system (cylindrical type) at shear-rates ranging between 1 and 1,000 s^{-1}. The measurements were performed at 20, 35, 50 and 65°C.

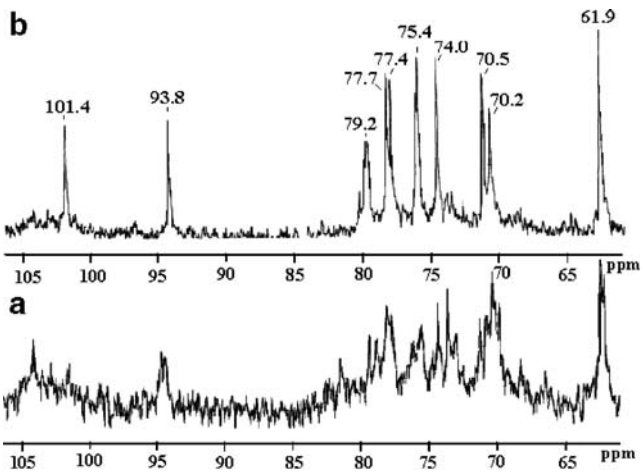

Fig. 1 ^{13}C-NMR-spectra of native (**a**) and alkali-treated (**b**) non-gelling polysaccharide from *T. crinitus*

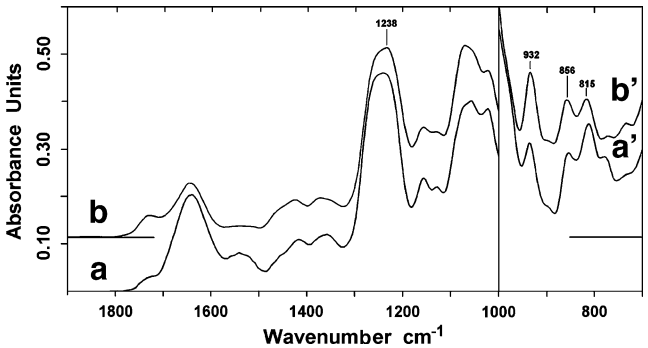

Fig. 2 Infrared spectra of native *line a* and alkali-treated *line b* non-gelling polysaccharide from *T. crinitus*. *a'* and *b'* are the twice-magnified regions of (*a*) and (*b*)

Alkaline modification

Non-gelling polysaccharide fraction (100 mg) was dissolved in distilled water (20 mL). $NaBH_4$ (20 mg) was added to the sample and kept at 24°C for 12 h. After adding $NaBH_4$ (60 mg) and NaOH (800 mg), the mixture was heated at 80°C for 6 h in water, both with constant stirring. The solution was allowed to cool to room temperature and was neutralized to pH 5.5 with acetic acid. The alkali-modified carrageenan was dialyzed against distilled water (3 days) and lyophilized.

Partial reductive hydrolysis

Alkali-treated polysaccharide (5 mg) were placed in a test tube, and borane-4-methyl-morpholine (50 mg) (Aldrich, USA) and aqueous TFA (2 M, 1 mL) was added to the polysaccharide. The solution was heated at 65°C for 8 h. The solvent was evaporated and two portions of 5 mL of ethanol were added to the hydrolysate and evaporated to remove traces of TFA. Acetic anhydride (1 mL) and TFA (0.5 mL) were added and the tube was heated at 100°C for 30 min. Toluene (5 mL) was added twice and the solvent was evaporated to dryness. Then CH_3Cl (5 mL) and water (5 mL) were added and, after shaking, the chloroform layer was separated and evaporated to almost dryness. Product of partial reductive hydrolysis was analyzed by gas-liquid chromatography using a Hewlett-Packard 5890 chromatograph equipped with a capillary HP-5MS column Ultra-I

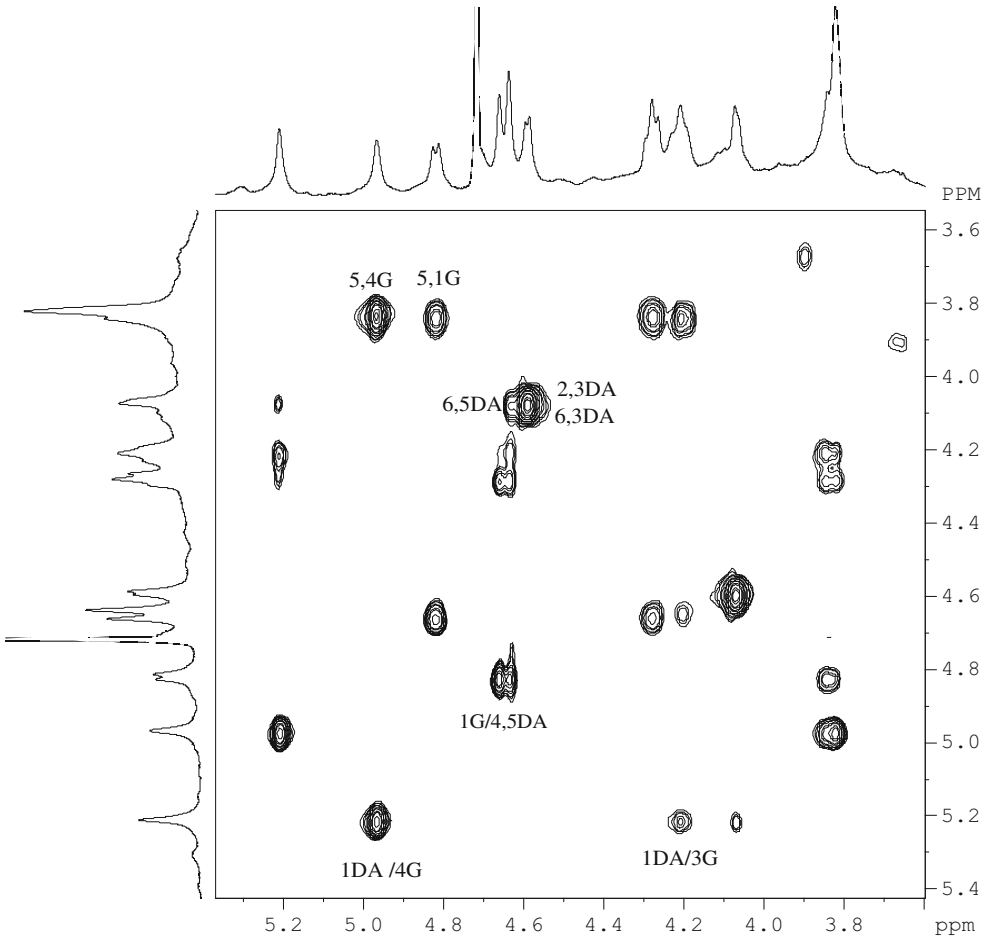

Fig. 3 $^1H/^1H$ ROESY spectrum of the alkali-treated non-gelling polysaccharide fraction of the *T. crinitus*. *Arabic numerals* belong to atoms in the β-D-galactopyranosyl (*G*) and 3,6-anhydro-α-D-galactopyranosyl (*DA*) residues. *Slashes* are used for designation of inter-residue correlation peaks

Table 1 ^{1}H NMR data (δ in ppm) of alkali-treated non-gelling fraction of polysaccharide from *T. crinitus* and known carrageenan types

Carrageenan	Unit	Chemical shift					
		H-1	H-2	H-3	H-4	H-5	H-6
C[a]	G	4.82	4.27	4.20	4.96	3.88	3.81
	DA	5.21	4.06	4.59	4.66	4.84	4.12
α	G	4.61	3.62	3.82	4.10	3.65	3.80
	DA 2S	4.61	4.73	4.65	4.67	4.21	4.10
κ	G 4S	4.61	3.59	3.95	4.81	3.70	3.80
	DA	5.10	4.12	4.50	4.62	4.60	4.10
Found In λ[b]	G 2S4S	4.78	4.46	4.10	5.03	3.83	3.87

[a] Alkali-treated non-gelling fraction of polysaccharide from *T. crinitus*
[b] Chemical shifts relative to DSS as internal standard (Guibet et al. 2006)
G β-D-galactopyranosyl; *DA* 3,6-anhydro-α-D-galactopyranosyl

and a flame ionization detector at 175–290°C; the rate of temperature change was 10°C min^{-1}.

Fourier Transform-Infrared spectroscopy (FT-IR)

Films were obtained by drying 2 mL of polysaccharides (0.25–0.4 % w/v) in aqueous solution in polyethylene molds (about 0.5 cm deep, 2.5 cm diameter) at 37°C. The films were clamped between NaCl windows and FT-IR spectra were recorded on a Vector 22 Fourier transform spectrophotometer (Bruker) with resolution of 4 cm^{-1}. The spectra were normalized by the absorption of the monosaccharide ring at ~1,070 cm^{-1} relatively to local basis line at ~1,500–900 cm^{-1}.

NMR spectroscopy

Samples of polysaccharides were deuterium-exchanged twice from D_2O by freeze-drying prior to being examined in a solution of 99.95% D_2O. ^{13}C-NMR spectra of polysaccharide were recorded with a Bruker DPX-300 spectrometer operating at 60°C and 62.9 MHz. The number of scans was 80,000. Chemical shifts are reported related to internal methanol (δ_C 50.15). The ^{1}H NMR spectrum and 2D COSY, TOCSY, ROESY and HSQC spectra were recorded with a Bruker DRX-500 spectrometer at 30°C using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process the NMR data. Mixing times and spin-

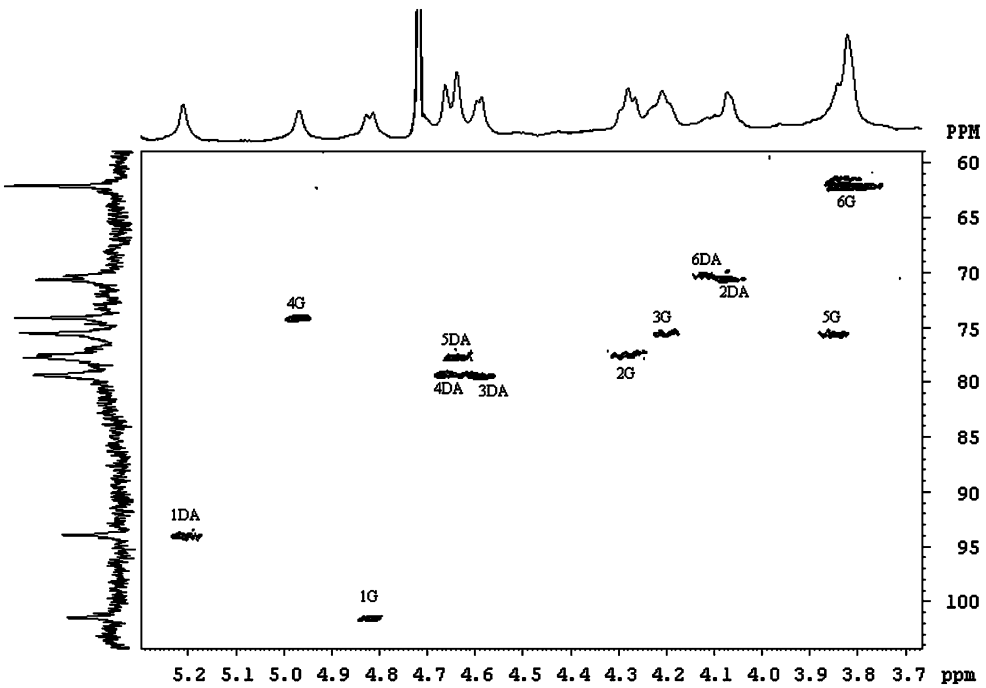

Fig. 4 ^{1}H/^{13}C HSQC spectrum of alkali-treated non-gelling polysaccharide from *T. crinitus*. *Arabic numerals* belong to atoms in the β-D-galactopyranosyl (*G*) and 3,6-anhydro-α-D-galactopyranosyl (*DA*) residues

Table 2 ^{13}C NMR data (δ in ppm) of alkali treated non-gelling fraction of polysaccharide from *T. crinitus* and known carrageenan types

Carrageenan	Unit	Chemical shift					
		C-1	C-2	C-3	C-4	C-5	C-6
C[a]	G	101.4	77.4	75.4	74.0	75.4	61.9
	DA	93.8	70.5	79.2	79.2	77.7	70.2
ω [b]	DA	94.7	70.1	79.5	78.5	76.9	69.4
α [c]	G	102.7	69.6	81.9	66.9	75.3	61.6
	DA 2S	94.7	75.4	78.1	78.3	77.1	70.0
κ [b]	G 4S	102.5	69.6	78.9	74.1	74.8	61.3
	DA	95.3	69.9	79.2	78.3	76.8	69.5
Found in λ [d']	G2S, 4S	105.8	79.8	77.3	76.27	77.0	63.4
θ [e]	G 2S	100.3	77.6	77.2	67.8	74.7	61.1

[a] Alkali treated non-gelling fraction of polysaccharide from *T. crinitus*.
[b, c, e] Chemical shifts are given according to literature. The values refer to internal standard methanol measured at 60°C (50.15 ppm) (Usov and Shashkov 1985; Falshaw et al. 1996; Falshaw and Furneaux 1994)
[d] Chemical shifts relative to DSS as internal standard (Guibet et al. 2006)
G β-D-galactopyranosyl; *DA* 3,6-anhydro-α-D-galactopyranosyl

lock times of 300 ms were used in 2D ROESY and TOCSY experiments, respectively.

Anticoagulant activity

The anticoagulant effect of samples was assessed using APTT (activated partial thromboplastin time) assay with citrated plasma sample (1:10 v/v, 3.8% sodium citrate) according to Fox et al. (1993). Coagulation time assays were performed semi-automatically with a blood coagulation analyzer (BC2210, Kyoto-Daiichi Science, Japan). APTT assays were performed with activated Cephaloplastin Reagent (Dade®, Actin®; Dade,USA).

Results

The plants of *Tichocarpus crinitus* (vegetative form) were collected at a single place in the Sea of Japan from the same depth to avoid the influence of the accessory factors, such as the photon irradiance, water motion and salinity on the composition of cell wall polysaccharide. The polysaccharides were extracted from seaweeds by hot water. The extract represents about 21% of the dry weight of the seaweed and is composed of 80% of polysaccharide and contaminated by 10% of proteins. The extract was separated in a gelling fraction (about two-thirds of the extract) and non-gelling fraction (one-third) after 4% KCl precipitation. The insoluble KCl fraction was identified as being κ/β-carrageenan (Barabanova et al. 2005). FT-IR- and ^{13}C NMR spectra (not shown) had the same features as previously reported with, as an example, the anomeric carbon resonating at 104.7 ppm and 104.8 ppm for G4S-C1 and G-C1, respectively.

The KCl-soluble fraction was also subjected to ^{13}C NMR analysis. Although the NMR spectrum presented (Fig. 1a) was not well resolved and difficult to assign, it revealed that the structure is definitely different from κ/β-carrageenan recovered in the KCl-insoluble fraction. We have achieved a brief chemical analysis of this fraction (not shown) and observed the occurrence of three main components: galactose, anhydro-galactose and sulfated ester groups. These preliminary chemical analyses were supported by the FT-IR spectrum. Indeed, in the middle range of the spectrum, one can observe a very intense absorption band at about 1,238 cm^{-1} (Fig. 2: line a) characteristic of the sulfated ester group. This band is associated with lower frequency absorption bands at 815 and 856 cm^{-1} suggesting that the sulfated ester group may

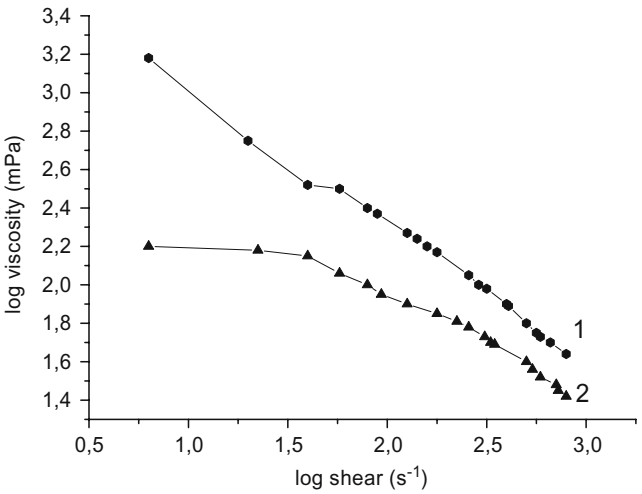

Fig. 5 Double logarithmic plot of viscosity versus shear-rate for non-gelling polysaccharide fraction from *Tichocarpus crinitus*: *1* At 20°C; *2* at 65°C

Table 3 Anticoagulant activity of polysaccharide fractions of *Tichocarpus crinitus*

Sample[a]	Anticoagulant (APTT, s)
Gelling fraction	81.3
Non-gelling fraction	343.0
Control	58.7

[a] Sample concentration 100 μg.mL^{-1}

have a different position along the polysaccharide chain. A characteristic band attributed to the 3,6-anhydro bridge vibrating at 932 cm^{-1} was also clearly identified in the spectrum. Thus, it is likely that the KCl-soluble fraction of *T. crinitus* is a sulfated galactans.

The FT-IR spectrum of the KCl-soluble fraction after alkali treatment was very similar to that of the native polysaccharide (Fig. 2b). This spectrum exhibited similarly an intense absorption band at 1,238 cm^{-1} and medium absorbance at 932 and 815 cm^{-1} (Fig. 2; line b, b'). In parallel, we have performed brief chemical analyses, which again revealed the occurrence of galactose and an increase of anhydro-galactose and sulfated ester groups (not shown). Partial reductive hydrolysis has given rise to carrabiitol derivatives indicating the polysaccharide is likely to belong to the carrageenan family instead of agars.

Unexpectedly, the ^{13}C NMR spectrum was greatly simplified after alkali treatment and, as illustrated in Fig. 1b, only 12 signals of strong intensity due to 12 carbon atoms were observed. The two signals at 101.4 and 93.8 ppm in the region of resonance of the anomeric carbon atoms indicated the presence of one disaccharide repeating unit. Chemical shifts of the signals were unsuccessfully compared with those of known (idealized) structures of carrageenan. Using ^{1}H/^{13}C HSQC correlation, the anomeric protons were assigned at δ_H 4.82 and 5.21 for G-H1 and DA-H1 residues, respectively. Starting from these values, the ring protons of galactose and anhydro-galactose were ascribed successfully using COSY, TOCSY and ROESY experiments. The ROESY experiment allowed the verification, on the one hand, of bonding between monosaccharide residues and the connection between the H1-H4 spin system and, on the other hand, the H5-H6 spin system (Fig. 3). Correlation peaks between H1 of DA and H-3, H-4 of G (δ_H/δ_H 5.21/4.20 and 5.21/4.96, respectively) and H1 of G and H-4,5 of DA (δ_H/δ_H 4.82/4.66 and 4.82/4.64, respectively) were compatible with α-(1→3) and β-(1→4) linkages in the disaccharide repeating units of the polysaccharide. Additionally, the relationship between H3 and H6 of DA residue indicated the presence of an 3,6-anhydro bridge. Chemical shifts of protons corresponding to the galactose and anhydro-galactose are shown in Table 1. One can see in this table strong similarities between chemical shifts of anhydro-galactose of the alkali-treated sample and the anhydro-galactose of κ-carrageenan, suggesting that the anhydro bridge does not carry a sulfated ester group. The galactose residues had very similar features with that of the G2S4S residue observed in *Gigartina skottsbergii* λ-carrageenan (Guibet et al. 2006).

Attribution of carbons was achieved using ^{1}H/^{13}C HSQC (Fig. 4) and chemical shifts are reported in Table 1. Both the very intense inter-residue correlation peak DA-H1/G-H4 in the ROESY spectrum and the exclusively up-field chemical shift DA-C1 (δ_C 93.8) revealed an α-anomeric configuration for DA at the same (D) absolute configuration of DA and G (Lipkind et al. 1988; Shashkov et al. 1988). The signal of C-2 of the galactose residue at δ_C 77.4 was downfield shifted on 7 ppm in comparison with that in the spectrum of non-sulfated galactose residue (for example, θ-carrageenan, Table 2) indicating the presence of a sulfate group at 2-position of the galactose residue (Van de Velde et al. 2002). Chemical shifts of other signals corresponded to resonance of carrageenan with the sulfate group at C-4 of the galactose residue. Carbon chemical shifts were in agreement with a carrageenan structure having G2S4S-DA as the carrabiose repeat unit.

The 12 signals recorded on the ^{13}C NMR spectra of the alkali-treated sample (Fig. 1b) were also present in the spectra of the native one (Fig. 1a) suggesting that the carrageenan-like structure was already present in the untreated specimen. Additional signals observed in the native sample could correspond to the biosynthetic precursors which were likely converted into the anhydro bridge by alkali treatment. Consequently, the KCl-soluble fraction of *T. crinitus* is probably a hybrid carrageenan built with a G2S4S-DA carrabiose unit associated with their biosynthetic precursor, i.e., G2S4S-D6S carrabiose units.

The viscoelastic behavior of the non-gelling polysaccharide was investigated. The shear-rate dependence of the viscosity of 1% solution of this polysaccharide was observed in the range of 1 to 1,000 s^{-1} at 20, 35, 50 and 65°C. The viscoelastic behavior of the polysaccharide was sensitive to temperature changes. At high shear-rate (800 s^{-1}), the viscosity of polysaccharide decreased from 50 to 28 mPas with increasing temperature from 20 to 65°C

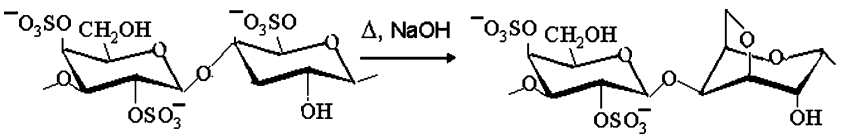

Fig. 6 The proposed structure for the non-gelling polysaccharide from *T. crinitus* and its biosynthetic precursor

(not shown). The classical rheo-thinning behavior of polysaccharide was observed for the solution at 65°C (Fig. 5). The flow curve obtained for polysaccharide at 20°C had a distinct shape: there was no Newtonian plateau at low shear-rates (Morris et al. 1981). The anticoagulant activity of native non-gelling polysaccharide was studied by APTT assay. The polysaccharide posses high anticoagulant activity at low concentration of 100 μg mL^{-1} (Table 3).

Discussion

We have found that the KCl-soluble fraction of *Tichocarpus crinitus* is a carrageenan unrelated to any previously observed structure. We have determined the structure of this fraction based on two lines of evidence. First, chemical analyses have revealed the occurrence of galactose, anhydro galactose and sulphated ester groups. Second, we have solved, by ^{1}H and ^{13}C NMR, the structure of the alkali treated polysaccharide whose structure consists of a unique carrabiose unit: G2S4S-DA. This structure (Fig. 6) is consistent with the chemical shift previously recorded for the DA unit of κ- and ω-carrageenan, and for the G2S4S unit observed in a fraction of *G. skottsbergii* λ-carrageenan (Guibet et al. 2006).

Consequently, we have concluded that the non-gelling fraction extracted from *T. crinitus* vegetative plants represents a novel type carrageenan-like polysaccharide. The backbone of this polysaccharide is composed of alternating 1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues (Fig. 6). Furthermore, it seems that a large amount of the biosynthetic precursor of this polysaccharide is also present in the native fraction.

The mechanical spectrum of non-gelling polysaccharide from *T. crinitus* at 65°C had a broad range of shear-rate indicating that this system had rheo-thinning behavior. The double logarithmic plot of shear viscosity against shear-rate of polysaccharide was essentially identical to those of the conformationally disordered "random coil" polysaccharide in concentrated solutions (Morris 1988). This plot showed a horizontal Newtonian plateau at low shear-rates and then a drastic reduction in viscosity from this maximum value at high shear-rates. This behavior was consistent with the formation of a dynamic entangled structure in concentrated solutions of a "random coil" polymer. At low shear-rates, the disruption of polysaccharide chain entanglement, by the imposed deformation and the formation of new interaction between different chains, equilibrated, and thus no reduction of viscosity was observed (Newtonian plateau). The beginning of shear-thinning occurred at high shear-rates when the rate of disruption of existing entanglements became greater than the rate of formation of new ones, and thus the cross-line density of the network was depleted and the viscosity was reduced (Morris 1988).

At 20°C, this polysaccharide demonstrated a behavior that, we suggest, is of polysaccharide forming weak gels in solution, although the shear-thinning of this polysaccharide in solution may begin at lower shear-rates than those we used. Such behavior of the non-gelling polysaccharide may be due to the presence of 3,6-anhydro-galactose.

The structure and behavior of polysaccharide in a solution influences its biological activity. It is known that λ-carrageenan has a greater antithrombic activity than other types of carrageenan, probably due to to its higher sulfate content (Shanmugam and Mody 2000). Indeed, in our case, the non-gelling carrageenan-like polysaccharide from *T. crinitus* possesses high anticoagulant activity, while the gelling (κ/β-carrageenan) shows low activity. However, we have not previously found a correlation between anticoagulation activity of carrageenans isolated from *Chondrus pinnulatus* and the sulfate content (Yermak et al. 2006). As was shown by Pereira et al. (2005), the presence of 2,3–disulfate galactose units in the sulfated galactan from the marine alga *Botryocladia occidentalis* has an amplifying effect on the anticoagulant activity, but the proportion and/or the distribution of these units along the polysaccharide chain may be a critical motif to possess its activity. According to our current data, non-gelling polysaccharide from *T. crinitus* contains not only repeating 3-linked β-D-galactopyranosyl-2,4-disulfates residues, but also 4-linked 3,6-anhydro-α-D-galactopyranosyl residues. It seems that the anticoagulant activity depends on the monosaccharide composition, sites of sulfation and/or the distribution of sulfated units along the galactan chain, and also the molecular weight of the polysaccharide as has been shown previously (Yermak et al. 2006; Zúñiga et al. 2006).

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